‘Ecdysterone Use and Misuse in Sports: Diagnostic Markers in Human Specimen and Determination in Supplements’

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy
of Freie Universität Berlin

by
Gabriella Ambrosio

December 2019

Research of the present study was conducted from 2015 till 2019 under supervision of Prof. Dr. Maria Kristina Parr at the Institute of Pharmacy of the Freie Universität Berlin in collaboration with the Anti-Doping Laboratory of Rome

1st Reviewer: Prof. Dr. Maria Kristina Parr
2nd Reviewer: Prof. Dr. Francesco Botrè

date of defense: 7th February 2020

“Life isn’t about finding yourself. Life is about creating yourself”

George Bernard Shaw

Dedicated to my mother

Acknowledgements

A special thank to Prof. Dr. Maria Kristina Parr for the support she gave me in every moment and to Prof. Dr. Francesco Botrè for believing in me and being always so human. I will be always deeply thankful to both of you for giving me the possibility to discover this “new world” and consequently, myself.

I would like to thank Dr. Monica Mazzarino for her deep sensitivity and for being a special person.

I would like to thank Mr. Bernhard Wüst for his professional help in lot of moments and for his friendliness.

Thanks to Dr. Jan Felix Joseph for being a friend and a great colleague, always helpful and available.

I would like to thank Annette and Jaber for making this journey more genuine.

I would like to thank Dr. Peter Witte and Anna for their kind help when needed.

A special thanks to Ginevra, a new beautiful discovery in my life and for her honesty.

I would like to thank Renée, for being in my life, for her understanding and for giving me a lot of strength.

I would like to thank my friends, one of the most beautiful thing in my life.

I would like to thank the music, my fuel and precious passion.

I would like to thank my family, for being a safe place in spite of everything and I would like to deeply thank my mother, for being my guide, for supporting me in my life and in this experience, despite the impossibility of having me closer.

I would like to thank who is not “here” anymore, but still so much present in my sweetest thoughts.

Table of Contents

Abbreviations

AAF	Adverse analytical finding
AAS	Anabolic-androgenic steroids
ADRV	Anti-doping rules violations
AOAC	Association of Official Agricultural Chemist
AR	Androgen receptor
cGMP	current Good Manufacturing Practices
COG	Control-group
DHT	Dihydrotestosterone
dMRM	Dynamic multiple reaction monitoring
DSHEA	Dietary Supplement Health Education Act
E2	Estradiol
EAAS	Endogenous anabolic androgenic steroids
Ec1	Ecdysterone1 group
Ec2	Ecdysterone2 group
EFSA	European Food Safety Authority
EI	Electron ionization
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ERbeta	Estrogen receptor beta
ESI	Electrospray ionization
ESI+	Positive electrospray ionization
EU	European Union
FDA	Food and Drug Administration
FID	Flame ionization detection
FMSI	Federazione Medico Sportiva Italiana
FTC	Federal Trade Commission
GC	Gas chromatography
GTFCh	Gesellschaft für Toxikologische und Forensische Chemie
HMPs	Herbal medicinal products
HRMS	High resolution mass spectrometry
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

IGF-1	Insulin like growth factor 1
IOC	International Olympic Committee
ISL	International Standard for Laboratories
ITP	Initial testing procedures
LC	Liquid chromatography
LLE	Liquid-liquid extraction
MS(/MS)	(Tandem) mass spectrometry
PLG	Placebo-group
PPT	Protein precipitation
QTOF	Quadrupole time-of-flight
SPE	Solid-phase extraction
T	Testosterone
T4	Thyroxin
TD	Technical Document
TMIS	Trimethyliodsilane
UHPLC	Ultra-high performance liquid chromatography
US	United States of America
USP	United States Pharmacopeia
WADA	World Anti-Doping Agency
WADC	World Anti-Doping Code

1 Introduction and Aim of the Project

The practice of sport is predominant in our society and it plays an important international role in the development not only of physical aspects but also of psychological skills.

Unfortunately, some disregard the ideal of fair play and the principle of winning at all costs became dominant. The demand for ever higher physical performances and intensive training and competition, drive athletes to optimize nutrition, including the use of dietary supplements, in the belief of obtaining an advantage in competition by maximizing their performances while remaining healthy. Unfortunately, the lack of analytical control on dietary supplements can be the cause, in the worse but not only case, of possible risks for the health of the athletes. Indeed, were reported several cases of contamination of supplements with banned substances and presence of substances not listed or incorrectly listed on the label [1-17]. Furthermore, athletes' interest in a ‘natural alternative’ to improve muscular growth and, consequently, to enhance performances, is progressively increasing [18]. In this scenario, ecdysterone, a steroid hormone naturally occurring in plants and invertebrates, has been investigated since a large number of products containing ecdysterone are easily available via internet and there are many rumors on ecdysterone misuse by athletes. Ecdysterone is promoted as a ‘natural steroid’ capable of increasing strength and muscle mass as well as having anabolic effects.

Consequently, this suggests that supplements containing ecdysterone may be used by athletes during training and competition to circumvent the anti-doping test since they are not yet included in the World Anti-Doping Agency’s (WADA) list of banned substances and methods. Only a few scientific studies are available to demonstrate the performance-enhancing effect of ecdysterone and little is known about its metabolism.

Thus, this project aims to investigate the effects of a long-term administration of ecdysterone containing dietary supplement in humans, to evaluate its efficacy in enhancement of sports performance during resistance training.

Furthermore, this study is designed to develop sensitive and specific analytical methods for the unambiguous identification and quantitation of ecdysterone in natural preparations and in human biological fluids (urine and serum). A complete anti-doping screening examination of supplements and urines was carried out to exclude underlying effects from potential cross contamination.

To trace back an administration of ecdysterone from urine samples, its analytical properties have been investigated in order to assess its integration into initial testing procedures (ITP) in doping control laboratories for monitoring the prevalence in elite sports.

2 Background

2.1 Ecdysteroids: Characteristics, Occurrence in plants, Pharmacological and Anabolic effects

Firstly reported in the mid-1960s studies of ecdysteroids began. A Japanese group, while researching anticancer substances, isolated ecdysteroids from a plant source [19, 20]. Ecdysteroids belong to a group of polyhydroxylated steroids, natural hormones of arthropods, responsible for moulting and metamorphosis, as the term ecdysis from Ancient Greek ἐκδύω (ekduo, “take it off”), indicates [19]. In plants, they operate as protective agents against predatory insects [21].

The structure of the majority of ecdysteroids is based on C27 cholest-7-en-6-one backbone and cholesterol is considered the direct precursor. Indeed, following steps of their biosynthesis include conversion of cholesterol, (dehydrogenation to 7-dehydrocholesterol), further molecular modifications, and, especially, hydroxylation at various carbon atoms [22]. The most widely distributed ecdysteroid is ecdysterone (**Figure 1**) (also called β-ecdysone or 20-hydroxyecdysone) and is thought to be present in 147 plant species [19].

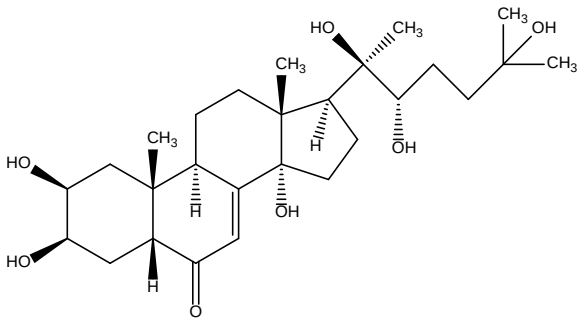


Figure 1 Chemical structure of ecdysterone (2β,3β,14α,20β,22R,25-hexahydroxy-5β-cholest-7-en-6-one)**.**

One of the well-known sources of ecdysterone is Suma (common name: Brazilian Ginseng, *Pfaffia glomerata*) [21], in which it is mostly accumulated in roots in a percentage variation of 0.42 to 0.66% [23]. Spinach is another source of ecdysterone, but it contains only ca. 0.005-0.08% of ecdysteroids (mostly ecdysterone) per fresh weight, depending on the time of harvest [24, 25]. That means that even after a high consumption of spinach would be

hard to reach more than a hundred milligrams of ecdysterone per day [24]. *Cyanotis arachnoidea*, a plant native in China, is the richest source of ecdysterone (4-5%) [24].
A wide range of effects such as stimulation of protein synthesis, changes in carbohydrate and lipid metabolism, increased cell immunity, adaptogenic, anti-diabetic, hepatoprotective and perhaps even anti-tumor effects [26-28] as well as growth-promoting and anabolic effects of ecdysterone in various animal species and in humans, have been reported [28-45].
The anabolic effect of ecdysterone is mediated by activation of estrogen receptor beta (ERbeta) [29, 46, 47] and different studies reported the effect of ecdysterone to be even stronger than that of anabolic androgenic steroids (AAS) without sharing any of their classical side effects, such as virilization [28, 48, 49].

2.2 Dietary Supplements Containing Performance Enhancer

2.2.1 Definition and regulation of dietary supplements

The system of regulation of dietary supplements is not universal, and countries differ in their approach and practice [50].

United States of America

Dietary supplements are defined in the United States of America (US) under the Dietary Supplement Health Education Act (DSHEA) of 1994, final version of which was published in 1997 [51], as certain products (other than tobacco) intended to supplement the diet that are not represented as conventional food. A dietary supplement can contain vitamins, minerals, amino acids, dietary substances, metabolites, constituents, herbs or other botanicals and their extracts or concentrates. Furthermore, it must be intended for ingestion in pill, capsule, tablet, powder or liquid form not represented for use as the sole item of a meal or diet and labelled as a ´dietary supplement' [50, 52].
In the US, while the Food and Drug Administration (FDA) supervises the quality, the Federal Trade Commission (FTC) supervises the marketing and advertising of dietary supplements [53, 54]. According to the 1994 DSHEA [55], dietary supplements sold in the US, that are not intended to diagnose, treat, cure, or prevent any disease, do not need to be evaluated by the FDA before the product enters the market, unless they contain a new dietary ingredient [56]. A new dietary ingredient is defined as a dietary ingredient that was not marketed in the US before October 15th, 1994 [55-57]. In case of new ingredients, the

manufacturer is obliged to submit a 75-day pre-market notification to FDA, providing some evidence of the safety of the new ingredient but not being responsible for proving that a new ingredient is safe [58]. The FDA has, on the other hand, the authority to remove a product from the market if it exposes people to a risk of illness or injury [56, 57, 59].

If a manufacturer of a dietary supplement is making a claim about his product, before of the marketing of the product, the company is obliged to submit the claim to FDA within 30 days for approval [60].

The 1994 version of the DSHEA contains a provision requiring the FDA to establish and enforce current Good Manufacturing Practices (cGMP) for dietary supplements [60]. It was not before June 2010 that new FDA rules were able to ensure compliance of dietary supplements with cGMP in order to manufacture them with "controls that result in a consistent product free of contamination, with accurate labelling" [50, 60]. Whereas the implementation of GMP requirements are specific for each manufacturer, it occurs a lack of uniformity across the industry. Even if FDA expressed explicitly, in the preamble of the GMP, that validated methods may be used for the uniformity in product quality (such as from Association of Official Agricultural Chemists (AOAC) International or United States Pharmacopeia (USP)), neither the validation of analytical methods is required nor the use of validated methods [56]. Consequently, supplements and food ingredients are similarly regulated and therefore they do not have strict labelling requirements and are not subject to the regulations applied to the pharmaceutical industry [59].

European Union and other countries

In the European Union (EU), the Food Supplements Directive 2002/46/EC [61] defines food supplements as foodstuffs intended to supplement a normal diet and in which are concentrated sources of nutrients (vitamins and minerals) or other substances with a nutritional or physiological effect, alone or in combination. They are marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities.

The Directive 2002/46/EC establishes a list, also called "positive list", of allowable vitamins and minerals (including requirements to the maximal and minimal amounts that can be used) and sets labeling requirement [50, 62]. However, the Directive 2002/46/EC only establishes rules for vitamins and minerals and not on the use of botanicals in food supplements [63]. In absence of centralized authorization procedure, the European Commission has stated that the use of botanicals and derived preparation in food supplements is requested to comply with the requirements for food established by the EU General Food Law as well as requirements for novel food [63]. Those requirements ban

products than are injurious to health and require authorization procedure, which includes safety assessment before they can be commercialized [63].

Behind this Directive, there is the European Food Safety Authority (EFSA), established from the European Parliament in the Regulation 2002/178/EC [64]. This Directive regulates principles and requirements of the Food Law, and scientific expert opinions are developed and interchanged, based on scientific evidence concerning the safety for human health of food supplements [62]. The EFSA introduced the term “novel food” which includes all food that has not been consumed to any significant degree in the EU before 15 May 1997 (when the first novel food legislation entered into force) [65]. Novel food can be innovative food produced with or without involving new technologies or production processes and cannot be included in the “positive list” before a pre-market authorization [65]. The EFSA is also responsible of performing analysis to approve possible health claims. Indeed, as it exists a positive list of substances, there is also a positive list of health claims permitted for use, as guarantee of reliability for consumers [50, 62].

Anyway, botanical extracts are often used either in food supplements or in herbal medicinal products (HMPs), which are, unlike food supplements, under pre-market regulatory pathways established from the European Medicines Agency (EMA) [63]. Consequently, some products are marketed as food in certain EU Member States and as medicinal products in others, due to the lack of a harmonized authorization procedure [63].

In other countries botanical supplements are regulated either as a special category of food (New Zealand, Japan, China and India) or as medicinal products (Canada, Australia [63]).

In **Table 1** is reported a summary of the relevant legislative instruments and information about the pre-market authorization of botanical food, provided and adapted from [63].

Table 1 Summary of relevant legislative instruments and requirements for premarketing authorization of botanical supplements in Australia, Canada, China, India, the European Union, New Zealand, Japan, and the USA, reported and adapted from [63].

Jurisdiction	Legislative instrument	Pre-marketing authorization required
Australia	Therapeutic Goods Act	Yes
Canada	Natural Health Products Regulations	Yes
China	Food Safety Law of the People's Republic of China and Measures for the Administration of Registration and Recording of Health Food	Yes
India	Food Safety and Standards (Health Supplements, Nutraceuticals, Food for Special Dietary Use, Food for Special Medical Purpose, Functional Food and Novel Food) Regulations, 2016	No
European Union	Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements	No, botanical ingredients meeting the definition of "novel food" must be authorized first
Japan	Food Sanitation Act (Act No. 233,1947)	No – "Foods in general" Yes – "Foods with health claims"
New Zealand	Dietary Supplements Regulations 1985 Food Act 2014	No
USA	Dietary Supplement Health Education Act (DSHEA) 1994	No

2.2.2 Use of dietary supplements in sport

In the global market, the use of dietary supplements is estimated to reach almost $180 billion by 2020 [66]. Their use within athletes is reported to range from 40 to 88% [50, 67]. Only in the US dietary supplements are used by 80% of the population [68]. The factors supporting the growth of this market are a higher interest of consumers to implement their diet, a high consumption by athletes, promotion strategies promoted by manufacturers and distributors, a liberal legal framework and a liberal distribution and sales regime [62]. Studies suggest that the use of supplements varies between different sports, increases with level of training or performance and with age, is strongly influenced by cultural customs and is higher in men than in women [59]. Female athletes are more likely to take supplements for health reasons or to implement their diet, while men to improve agility, strength and power, and to gain weight or muscles [50]. A study reported that the primary source of nutrition information are athletic trainers (40%), strength and conditioning coaches (24%) and dietitians (14%) [50, 69]. When a high commitment to sport training among young people is associated to certain psychological disposition, such as insecurity and low self-

esteem, it is more likely that they could benefit of the use of nutritional supplements [50, 70].

Within the sport context, the interest to 'alternative natural approaches' has grown in the last decades and among those 'natural' supplements, ecdysteroids are definitely highly attractive together with phytoestrogens and vegetal sterols [18].

Borrione et al. reported that for athletes, the possibility of increasing physical performances using natural products (erroneously considered harmless) is undoubtedly more attractive than having to rely on pharmacological approaches. Furthermore, the use of those natural therapeutic variants, not only is considered not doping but is also considered side-effect free [18].

Ecdysteroids are widely marketed to athletes as dietary supplements advertising to increase strength and muscle mass during resistance training, to reduce fatigue and to ease recovery [48]. Ecdysterone has also been reported in the list of herbs most commonly used, nowadays, to enhance physical performances by providing or mimicking anabolic effects of testosterone (T) in humans because of their similarity of chemical structure [52].

What most of the users do not know, is that for most of the supplements, the advertised ergogenic effects are not based on scientific evidence and, subsequently, they cannot be sure if their mid-term or long term consumption may constitute a risk for health [18]. **Figure 2** provides a hierarchical model of the suggested strength of the evidence provided and modified by Burke et al. [71].

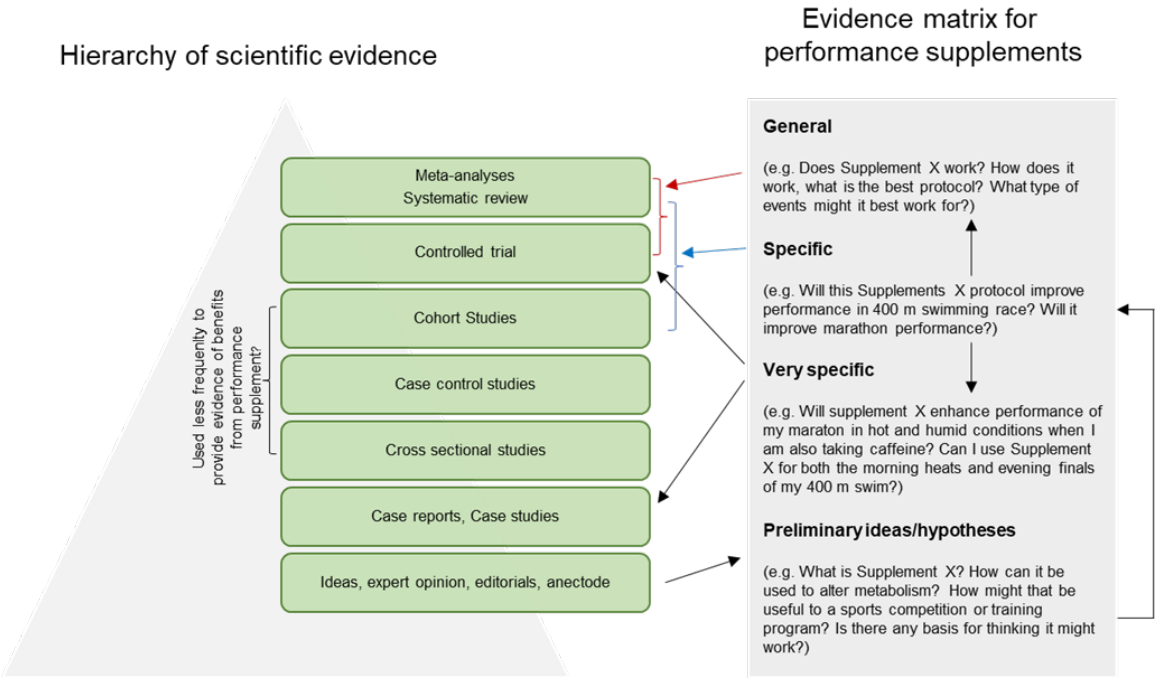


Figure 2 Different types of evidence that might be applied to decisions made about the efficacy of use of performance supplements modified from [71].

2.2.3 Risks related to the use of dietary supplements in sport

The online expansion of the advertising and marketing of dietary supplements, due to the free sale of products, the lack of a uniform and adequate regulation and the manufacturer reluctance to adopt the FDA cGMP guidelines, are the causes of several risks for consumers and start to be the reason of a public health problem [53, 54]. Indeed, the exposition to those risks can cause toxicity and side effects, including allergic reactions, overexposure and poisoning due to contaminants [50]. Cases of supplements containing herbal extracts that caused liver, kidney and heart toxicity, have been reported [72, 73]. During an inspection, the presence of one or more contaminants such as lead, arsenic, mercury, cadmium, or pesticide trace amounts in 93% of the supplements analyzed, has been reported [53, 74]. “In Fiscal Year 2017, it was reported that approximately 23.48% of the FDA’s 656 total cGMP inspections resulted in citations for failing to establish specifications for the identity, purity, strength, and composition of dietary supplements. Further, 18.47% were cited for failing to establish and/or follow written procedures for quality control operations” [60].

The consumption of contaminated supplements can affect not only health, but can be also the cause of social-moral damages and penalties. This may occur when supplements are contaminated with substances prohibited in sport [54].

In this regard, presence of several cases of substances not listed on label and banned by the (WADA) were reported either due to cross contamination or intentional contamination and the most frequently reported components in these supplements were anabolic steroids or stimulants [1-7, 10, 13-17]. It is not possible to guarantee complete safety of the products, this is why athletes are guided to make systematic and informed decision about the supplements they want to use and be able to assess the pros (benefits) and cons (harm) [75] (in **Figure 3** is reported a guide to make an informed decision modified from [59]).

However, this is not the only problem derived from the consumption of supplements. Another issue is the lack of precision, in terms of quantity, in the labeling of the products. Indeed, cases of substances added in smaller or even larger amounts than the one declared or moreover, cases in which the active ingredients of dietary supplements may be omitted or in which the indication on the label is very generic (preventing a correct qualitative interpretation of the composition of a product), are reported [4, 6-9, 11, 12, 15, 16]. Other studies reported that contamination levels are observed within a single production batch and even within a single packaging and additional active compounds possibly present in the product, are often unknown [76].

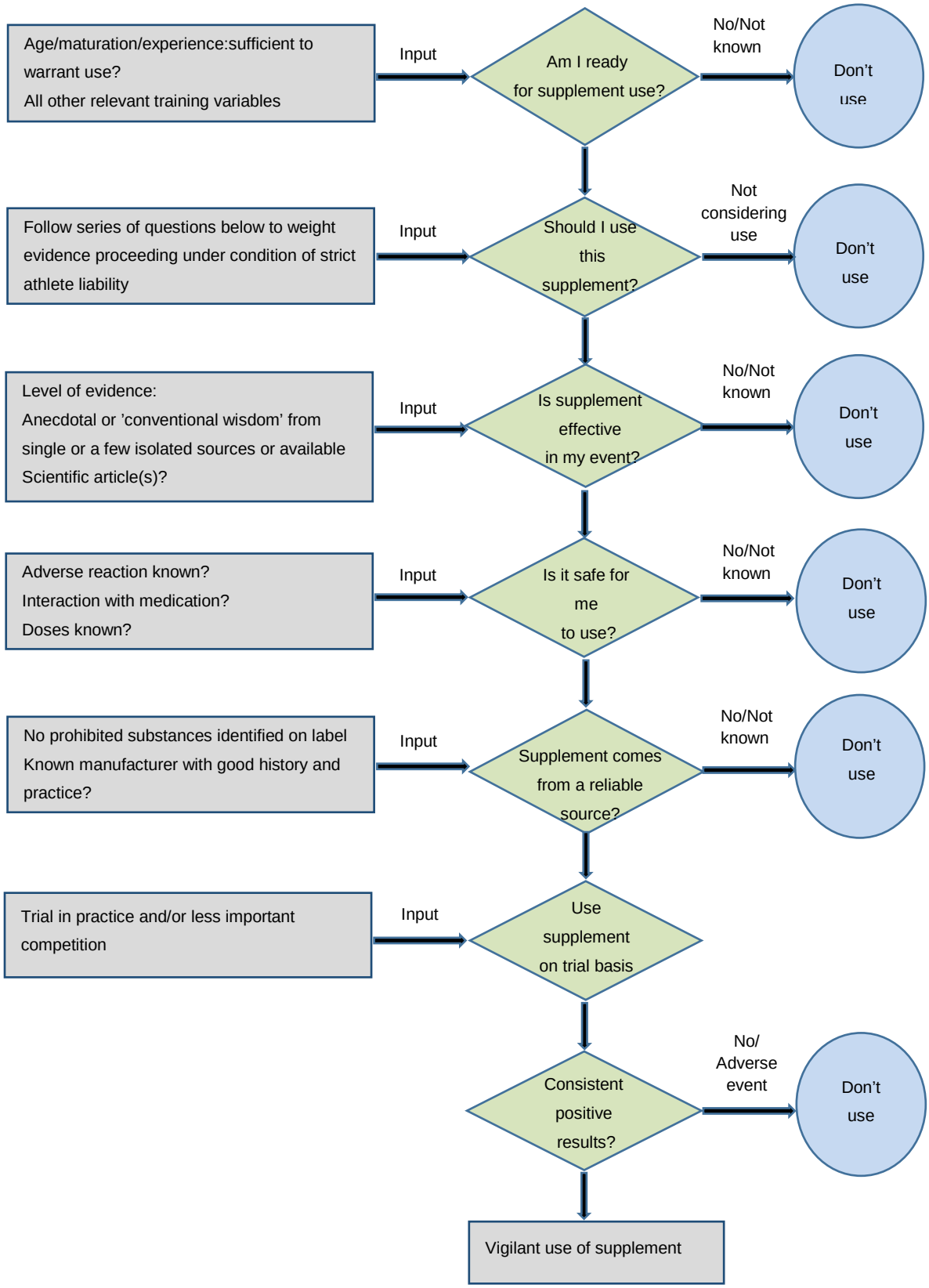


Figure 3 Guide informed decision making and reducing risk of Anti-Doping Rules Violations (ADRV) during ergogenic supplement use modified from flow chart [59].

2.3 Doping in Sport

The term “doping” is used to describe the administration or use of a substance foreign to the body or any physiological substance taken in an abnormal quantity by a competing athlete with the intention of increasing in an artificial and unfair manner his or her performance in competition [77, 78]. Doping is used to illicitly increase short-term acute responses and long-term adaptation, in order to raise the level that athletes could reach by training alone [79, 80]. The prevalence of different drugs and doping procedures will differ accordingly to the specific demands of each sport [79]. Historically, the control of doping in sport dates back to the early 1960s, when the International Olympic Committee (IOC) constituted its Medical Commission, and, later on the Subcommission “Doping and Biochemistry in sport” (reviewed in [81, 82]). The Subcommission ruled all anti-doping activities worldwide until the constitution of the WADA.

2.3.1 World Anti-Doping Agency

The WADA was established in 1999 as an independent, international agency aiming to create an environment in competitive sports that is doping-free and to support high-quality research in order to stay update and one step ahead of athletes and organizations who attempt to illegally enhance performances [83]. Sports are guided by rules and codes and the most well-known of these is the “World Anti-Doping Code” (WADC) [84]. The document is based on five international standards and provides consistency of anti-doping programs across sports and across international borders with the aim to bring consistency among anti-doping organizations [83]. It covers testing and investigations, laboratories, therapeutic use exemptions, the list of prohibited substances and methods, and protection of privacy and personal information. The Code requires frequent updating to adapt to changing knowledge [83].

The Prohibited List

Prohibited substances and methods may be included in the WADA prohibited list of substances and methods [85] if they meet two of the following criteria:

- The substance or method has the potential to enhance, or does enhance, performance in sport.
- The substance or methods have the potential to risk the athlete’s health.
- WADA has determined that the substance or methods violates the spirit of sport.

The WADA list clusters its doping categories into: ‘Substances and Methods Prohibited at All Times (in- and out of competition)’, ‘Substances and Methods Prohibited In-Competition’, and ‘Substances Prohibited in Particular Sports’ [83].

The Monitoring Program and other substances

Not all ergogenics are banned from sport. Some substances in fact, while not prohibited, are monitored to assess their use and to guide future changes to the list. An example is caffeine, which has strong research evidence for its efficacy [86-88].

For this reason, in addition to the list of prohibited substances, there is also a list of monitored substances. WADA, after consultation with signatories and governments, monitors those substances in order to detect patterns of misuse in sport. WADA publishes, before any testing, the substances that will be monitored [89]. After that, WADA accredited Doping Control Laboratory will report to WADA possible cases in which some of the monitored substances are detected in-competition or out-competition periodically, on an aggregate basis, by sport [89]. The reported use or detected presence of a monitored substance does not constitute an anti-doping rule violation [89].

Furthermore, some substances which demonstrated to have enhancing promoting effects are currently neither included on the prohibited list nor on the monitoring list [90].

2.4 Doping Control Analysis

Anti-doping analysis exists to detect the use of prohibited substances with established and validated screening and confirmation methods. Even if anti-doping analysis may be considered as an area of forensic toxicology, it constitutes a specific field, thus, exclusive application and requirements are needed. In order to have uniform, harmonized results reported by all laboratories and to ensure the validity of tests results and evidentiary data, the International Standard for Laboratories (ISL) was established [91]. The ISL includes several Technical Documents (TD) and it consists in a set of specific rules applicable to anti-doping analysis combining the knowledge of international experts in different analytical fields and it is regularly updated [92].

The analyses are performed at an International level by the anti-doping laboratories accredited by WADA, which as of December 1st 2019 are a total of 26, distributed worldwide [93]. The majority of these analyses are performed using urine specimens as the matrix rather than blood. Urine, indeed, allows for non-invasive collection of even relatively large volumes. Nevertheless, blood testing is recently increasing [94, 95]. Blood is more suitable

than urine to establish dose/effect response of a substance [95, 96]. Furthermore, when evaluating specific markers of an athlete's steroidal profile in urine, different factors can negatively influence the analysis (resulting in a high rates of false negatives), which could be avoided using blood as matrix [95, 97-105]. For doping control purpose, samples are collected in two separated aliquots (A-and B-sample) of the same sample and are labelled with a code number to preserve the anonymity of the athlete. Analyses include a first screening applied to all samples, which should be fast, selective and sensitive to avoid false-negative results. This is followed by confirmatory analysis in case of suspicious screening test samples results in order to avoid false positives. These analyses are performed during training and competition periods, by targeting the identified compound or/and its metabolites. In case of an adverse analytical finding (AAF), before sanctioning, the supervised analysis of the B-sample can be requested [94, 106, 107].

2.4.1 Historical development of doping control analysis and analytical challenges

The evolution of pharmacology in recent years has opened the access to athletes of a large number of drugs more difficult to detect [92].

In order to understand the analytical challenges that have occurred over the last 40 years, it is necessary to pay attention to different aspects that influence the practices used today.

The first official anti-doping testing at Olympic Games was in 1972 at the Summer Olympic Games in Munich. On this occasion, only stimulants, narcotics and drugs of abuse were included in the list of prohibited substances and gas chromatography utilizing flame ionization detection (FID) and mass spectrometry (MS) was the only reliable method [108-110] .

At that time all the abused substances were xenobiotics (from the Greek words ξένος (xenos) = foreigner, stranger and βίος (bios) = life), meaning not naturally present in the body, and their pharmacokinetic profile was satisfactorily known. In addition to that, to obtain an enhancing effect in competition, the administered doses of those substances were high enough to result in high concentrations of drug and metabolites in urines. Furthermore, their intake was followed only for several hours [111].

In 1976, when AAS have been included in the list of prohibited substances of the IOC, the traditional methods used for the other doping agents and straightforward sample pretreatment were ineffective if applied to steroids [110, 111].

Indeed, in contrast to stimulants and narcotics, the efficacy of steroids is maximum if their intake takes place during training (starting months before), stimulating muscle growth,

strength and power. As consequence, if the collection of urines for the anti-doping analysis is performed at the competition, the concentration of steroids can be really low, resulting in a (false) negative response [111]. Additionally, steroids are extensively metabolized and excreted in the urine as glucuronides or sulfates. Therefore they need a sample preparation involving hydrolysis or direct targeting of the conjugates. An extraction of the deconjugated steroid using liquid-liquid extraction (LLE) or in some cases, solid phase extraction (SPE), for the concentration of the analytes is requested before analysis [106, 107, 111, 112].

With the introduction of MS coupled with gas chromatography (GC-MS) into routine screening, further followed by GC-high resolution mass spectrometry (HRMS), among the anti-doping analysis, the detection of AAS in urine became not only possible but even more, their detection window was up to several weeks from the time of the last administration [111].

GC-MS based techniques, which involve derivatization for sample preparation, became the primary analytical method used in laboratories for the detection and quantitation of most of the steroids and other doping substances.

With the inclusion of new classes of low molecular weight, hydrophilic, thermolabile, nonvolatile substances in the Prohibited List, the use of MS coupled with liquid chromatography (LC-MS) took hold and new methods were designed, developed and validated. Those techniques have made possible the detection of those new prohibited drugs, that were partly or completely undetectable with GC-MS, reducing the extensive and time-consuming sample preparation procedures, the derivatization steps, improving the detection limits and allowing direct analysis of conjugated metabolites [113, 114].

2.4.2 Analytical methodologies currently used in anti-doping analysis

Even though the continuing advance of LC coupled with MS or (tandem) MS (MS/MS) in anti-doping analysis, GC-MS(/MS) with electron ionization (EI) is still undeniable. Particularly concerning anabolic agents, it is still an analytical technique used in sport drug testing to monitor prohibited substances based on targeted approaches [114, 115].

LC-MS(/MS) with electrospray ionization (ESI) and more recently ultra-high performance liquid chromatography (UHPLC)-ESI-MS(/MS) based procedures, are able to screen for a considerable number of different analytes or classes of analytes in a single chromatographic run and to characterize phase I and phase II metabolites.

LC-MS/MS systems equipped with new generations of mass spectrometers are used for the detection of a wide range of known or unknown substances. Indeed, relying on targeted

approaches, it is possible to identify compounds already characterized, whether operating in full scan mode allows to detect unknown targets [113].

Currently, Quadrupole-Time-of-Flight (QTOF) mass analyzers are the commonly used for high-resolution MS in doping control analysis (LC-HRMS or GC-HRMS).

3 Steps on the Investigation of Ecdysterone

3.1 Analytical Profiling of Supplements Labelled to contain Ecdysterone

Twelve commercial dietary supplements (all in the dosage form of capsules) available on internet and labelled to contain ecdysterone have been purchased, with the aim to determine their actual ecdysterone content. For this purpose, different extraction procedures have been evaluated, using one of the products as model supplement (Peak Ecdysone). An analytical method, which allowed the quantitative determination of ecdysterone in the supplements with reasonable accuracy and precision and not time consuming, had to be developed and fully validated according to the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), USP 42/41 and AOAC guidelines. For the determination of ecdysterone content in the supplements, two different quantitative analysis approaches have been considered and compared: standard addition method and external calibration. A study to evaluate the influence of the matrix effect has been performed. Analyses were carried out by UHPLC-MS/MS and the mass spectrometer was operated in dynamic multiple reaction monitoring (dMRM) acquisition mode (manuscript 1).

3.2 Effects on Sports Performance and Diagnostic Markers

In order to investigate the effects of a long-term administration of an ecdysterone containing product on human sport exercise, a ten-week intervention study of strength training of 46 healthy male subjects has been carried out. With this purpose, different doses of the dietary supplement “Peak Ecdysone”, labelled to contain 100 mg of ecdysterone per capsule from spinach extract, have been administered to the subjects. The study was conducted in a double blind, placebo controlled design.

Volunteers were divided as followed: two training groups, ecdysterone1 group (Ec1) and ecdysterone2 group (Ec2), which took two or eight capsules per day respectively; one non-training control group (COG), which took two capsules per day as Ec1, and one placebo group (PLG), which was training without taking any ecdysterone containing supplement. After ten weeks of treatment, anthropometrical (body weight, muscle mass, fat mass and totally body water) and performance parameters (power and strength performance) have

been evaluated. C2C12 cells, a myoblast cell line derived from murine satellite cells, have been used as *in vitro* model, to study muscle hypertrophy through ecdysterone.

The supplement administered has been analyzed to determine its amount of ecdysterone by standard addition method, using UHPLC-MS/MS (manuscript 1). The analysis of the supplement for confirmation of the absence of anabolic steroid cross-contamination in the product was adapted from already reported methods for steroids and was carried out using GC-QTOF-MS and LC-QTOF-MS.

Serum and urine specimen have been collected at different times of the study: pre-test (t1), after five weeks of training period (thalf) and after ten weeks of training and supplement administration period (t2).

Blood serum samples have been analyzed to investigate potential effects of ecdysterone, training and combinations thereof on the endocrine system using specific enzyme-linked immunosorbent assay (ELISAs). Therefore, analyses of serum concentrations of the biomarkers of liver and kidney toxicity were performed by the accredited medical analytics laboratory Dr. Wisplinghoff of Cologne, Germany. Finally, all the serum samples were analyzed by LC-MS/MS to establish their concentration of ecdysterone. Two different sample preparation procedures, namely protein precipitation (PPT) and SPE, have been evaluated and optimized. The validation of the method has been carried out according to the guidelines “Bioanalytical Method Validation” published by the US-FDA and to the “Gesellschaft für Toxikologische und Forensische Chemie” (GTFCh).

Additionally, urine samples have been analyzed with LC-MS/MS according to anti-doping screening routine analysis, in order to exclude intentional or unintentional co-administration of substances prohibited in sports. Furthermore, the study of the urinary profile of endogenous anabolic androgenic steroids (EAAS), in agreement with the WADA TD 2016 EAAS “Markers of urinary steroid profile” [116], has been carried out to provide evidence on any biological influences induced by the administration of ecdysterone, using GC-MS/MS. All the analyses on urine samples were performed in accordance with the procedures used in the WADA accredited Anti-Doping Laboratory of Rome, Italy (Laboratorio Antidoping Federazione Medico Sportiva Italiana, (FMSI)) (manuscript 2-3).

3.3 Investigation of the Urinary Metabolic Profile of Ecdysterone

Analytical properties of ecdysterone have been investigated after administration of a single dose (51.5 mg) of pure ecdysterone to one healthy volunteer. Post-administration urine samples have been collected for 33 hours, and spot urines in the following 3 days.

Extraction procedures have been evaluated using ecdysterone reference as model compound. They included LLE, at different pH values and with different solvents, or SPE, with different cartridges. The recovery of ecdysterone has been evaluated performing analysis with LC-MS/MS.

The sample preparation of post-administration urines have included enzymatic hydrolysis with β-glucuronidase or a mixture of β-glucuronidase and arylsulfatase for the evaluation of a potential phase II-metabolism or simply dilution of urines with water (1:4) before injection. The presence of ecdysterone in post- administration urines has been confirmed by comparison with reference material. The evaluation of the presence of a desoxy metabolite in post-administration urines has been carried out through the comparison with reference standard material of 20-desoxy-ecdysterone (ecdysone or α-ecdysone) and 25-desoxy-ecdysterone (ponasterone), and with in-house synthetized 14-desoxy-ecdysterone (synthesis according to Kumpun et al. [31]). Analyses of the mass spectrometric properties of the reference materials have been performed with LC-ESI-QTOF-MS and GC-EI-QTOF-MS. The investigation of ecdysterone metabolites in post-administration urine samples was performed using LC-QTOF-MS in MS1 mode, with LC-QTOF-MS/MS experiments for confirmation. The resulting excretion profiles of ecdysterone and possible metabolites have been evaluated (manuscript 4). **Figure 4** graphically summarizes the different steps of the investigation on ecdysterone.

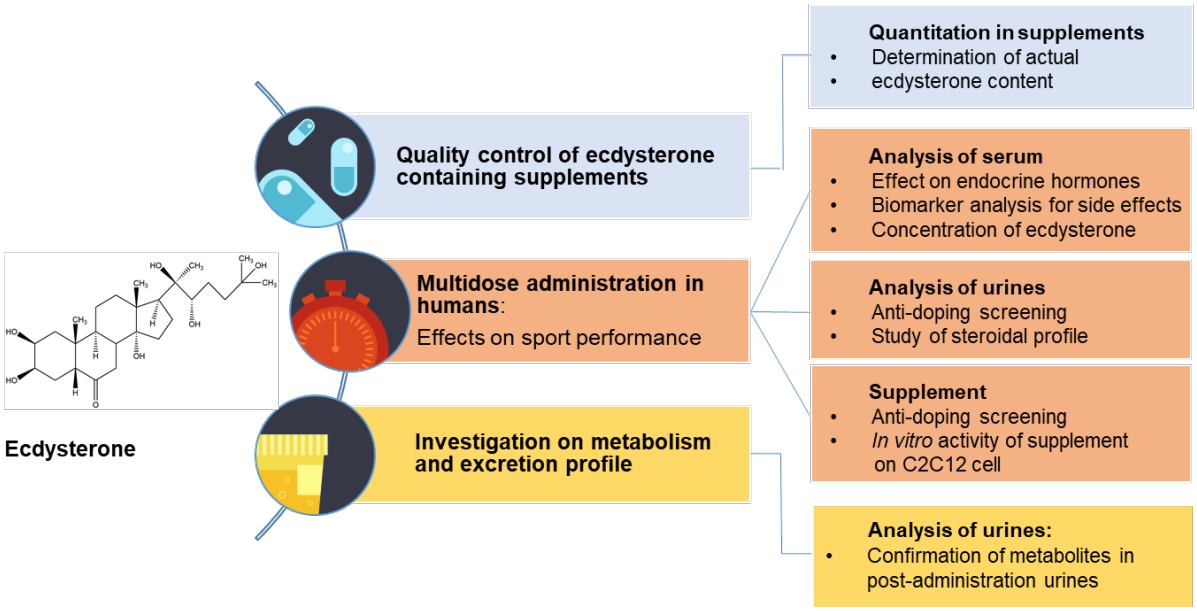


Figure 4 Different steps of the investigation on ecdysterone.

4 Publications

4.1 Manuscript No. 1

“**How reliable is dietary supplement labelling? - Experiences from the analysis of ecdysterone supplements”**

Gabriella Ambrosio, Dave Wirth, Jan Felix Joseph, Monica Mazzarino, Xavier de la Torre, Francesco Botrè, Maria Kristina Parr

Journal of Pharmaceutical and Biomedical Analysis 177 (2019) in press
https://doi.org/10.1016/j.jpba.2019.112877

The present study aimed to design, develop, and optimize an analytical procedure to perform the quantitative determination of ecdysterone in commercially available dietary supplements. The newly developed procedure is based on the extraction of ecdysterone from the supplements and the subsequent analysis by an optimized UHPLC-MS/MS method. Chromatographic separation was performed on an Agilent Eclipse Plus C18 column (2.1 mm x 100 mm, particle size 1.8 µm). The mass spectrometer was operated in positive ionization mode (ESI+) with acquisition in dynamic multiple reaction monitoring (dMRM) mode. Using the protonated molecular ion $[M+H]^+$ ecdysterone (target) and cortisol (internal reference) were detected at m/z 481 and 363, respectively. The assay was fully validated according to ICH guidelines and the method resulted to be fit for purpose in terms of accuracy and precision (CV% and RE% <15). Time-different intermediate precision was found within the reported range according to AOAC guideline for dietary supplements and botanicals. Quantitation has been performed using an external calibration considering the minimal matrix influences, preliminarily assessed following a cross comparison with an elaborate and time consuming standard addition method. The method was successfully applied to12 different dietary supplements labelled to contain ecdysterone, showing an actual content generally much lower than the labelled one.

4.2 Manuscript No. 2

"**Ecdysteroids as non-conventional anabolic agent: performance enhancement by ecdysterone supplementation in humans"**

Eduard Isenmann, Gabriella Ambrosio, Jan Felix Joseph, Monica Mazzarino, Xavier de la Torre, Philipp Zimmer, Rymantas Kazlauskas, Catricn Goebel, Francesco Botrè, Patrick Diel, Maria Kristina Parr

Archives of Toxicology 93 (2019) 1807-1816
https://doi.org/10.1007/s00204-019-02490-x

Recent studies suggest that the anabolic effect of ecdysterone, a naturally occurring steroid hormone claimed to enhance physical performance, is mediated by estrogen receptor (ER) binding. In comparison to the prohibited anabolic agents (e.g. metandienone and others) ecdysterone revealed to be even more effective in a recent study performed in rats. However, scientific studies in humans are very rarely accessible. Thus, our project aimed at investigating the effects of ecdysterone containing products on human sport exercise. A ten-week intervention study of strength training of young men (n=46) was carried out. Different doses of ecdysterone containing supplements have been administered during the study to evaluate the performance enhancing effect. Analysis of blood and urine samples for ecdysterone and potential biomarkers of performance enhancement have been conducted. In order to ensure the specificity of the effects measured, a comprehensive screening for prohibited performance enhancing substances was also carried out. Furthermore, the administered supplement has been tested for the absence of anabolic steroid contaminations prior to administration. Significantly higher increases in muscle mass were observed in those participants that were dosed with ecdysterone. The same hypertrophic effects were also detected in vitro in C2C12 myotubes. Even more relevant with respect to sports performance, significantly more pronounced increases in one-repetition bench press performance were observed. No increase in biomarkers for liver or kidney toxicity were noticed. These data underline the effectivity of an ecdysterone supplementation with respect to sports performance. Our results strongly suggest the inclusion of ecdysterone in the list of prohibited substances and methods in sports in class S1.2 "other anabolic agents".

4.3 Manuscript No. 3

"**Detection and quantitation of ecdysterone in human serum by liquid chromatography coupled to tandem mass spectrometry"**

Gabriella Ambrosio, Jan Felix Joseph, Bernhard Wüst, Monica Mazzarino, Xavier de la Torre, Patrick Diel, Francesco Botrè, Maria Kristina Parr

Steroids
Revisions submitted

The polyhydroxylated phytosteroid ecdysterone is present in various plants (e.g. spinach). It is widely marketed as the active component of dietary supplements, due to its reported health and performance promoting effects. For evaluation of its actual bioavailability, a fast and sensitive method was developed, optimized and validated for human serum. Instrumental analysis was performed utilizing liquid chromatography-tandem mass spectrometry with positive electrospray ionization and acquisition in multiple reaction mode. Solid phase extraction and dilute-and-inject (following protein precipitation) were tested to isolate ecdysterone from human serum. Both methods were compared in the light of the preset analytical target profile. The limit of detection (LOD) and quantification (LOQ) for ecdysterone in human serum after SPE extraction corresponded to 0.06 ng/mL and 0.14 ng/mL, respectively, meeting the requested sensitivity of the method. The assay was linear over the range from 0.10 ng/mL to 20.83 ng/mL. As expected, the sensitivity of the SPE method was better than that of the dilute-and-inject procedure, which did not allow for quantification of all post administration serum samples.
Accuracy (relative error; %) and precision (coefficient of variation; %), were both within acceptance criteria (<15%).
The developed method was successfully applied to a ten week intervention study conducted in young men performing regular resistance training. Different doses of supplements containing ecdysterone from spinach extract have been administered during the study and the quantitation of ecdysterone in serum samples has been successfully conducted. Ecdysterone could be quantified in all post-administration samples using solid phase extraction (SPE) for sample pretreatment.

Detection and quantitation of ecdysterone in human serum by liquid chromatography coupled to tandem mass spectrometry

Gabriella Ambrosio[a], Jan Felix Joseph[a,b], Bernhard Wuest[c], Monica Mazzarino[d], Xavier de la Torre[d], Patrick Diel[e], Francesco Botrè[d,f], Maria Kristina Parr[a]

[a] Institute of Pharmacy, Pharmaceutical and Medicinal Chemistry (Pharmaceutical Analysis), Freie Universitaet Berlin, Berlin, Germany

[b] Core Facility BioSupraMol, Department of Biology, Chemistry, Pharmacy, Freie Universitaet Berlin, Berlin, Germany

[c] Agilent Technologies, Santa Clara CA, USA

[d] Laboratorio Antidoping FMSI, Rome, Italy

[e] Department for Molecular and Cellular Sports Medicine, Institute for Cardiovascular Research and Sports Medicine, German Sport University Cologne, Cologne, Germany

[f] Department of Experimental Medicine, "Sapienza" University of Rome, Rome, Italy

Corresponding author:
Prof. Dr. Maria Kristina Parr
Freie Universitaet Berlin
Institute of Pharmacy
Koenigin-Luise-Str. 2+4
14195 Berlin
Germany
Phone +49 30 838 57 686
Fax +49 30 838 457 686
Email maria.parr@fu-berlin.de

Highlights

- An UHPLC/MS/MS assay to quantify ecdysterone in human serum has been developed.
- The method was applied to a ten week intervention study conducted in young men.
- For sample pretreatment solid phase extraction (SPE) has been used.
- Ecdysterone could be quantified in all post-administration samples.

Abstract

The polyhydroxylated phytosteroid ecdysterone is present in various plants (e.g. spinach). It is widely marketed as the active component of dietary supplements, due to its reported health and performance promoting effects. For evaluation of its actual bioavailability, a fast and sensitive method was developed, optimized and validated for human serum. Instrumental analysis was performed utilizing liquid chromatography-tandem mass spectrometry with positive electrospray ionization and acquisition in multiple reaction mode. Solid phase extraction and dilute-and-inject (following protein precipitation) were tested to isolate ecdysterone from human serum. Both methods were compared in the light of the preset analytical target profile. The limit of detection (LOD) and quantification (LOQ) for ecdysterone in human serum after SPE extraction corresponded to 0.06 ng/mL and 0.14 ng/mL, respectively, meeting the requested sensitivity of the method. The assay was linear over the range from 0.10 ng/mL to 20.83 ng/mL. As expected, the sensitivity of the SPE method was better than that of the dilute-and-inject procedure, which did not allow for quantification of all post administration serum samples.

Accuracy (relative error; %) and precision (coefficient of variation; %), were both within acceptance criteria (<15%).

The developed method was successfully applied to a ten week intervention study conducted in young men performing regular resistance training. Different doses of supplements containing ecdysterone from spinach extract have been administered during the study and the quantitation of ecdysterone in serum samples has been successfully conducted. Ecdysterone could be quantified in all post-administration samples using solid phase extraction (SPE) for sample pretreatment.

Keywords

Ecdysterone, Ecdysteroids, LC-MS/MS, nutritional supplements, spinach extract, serum analysis

1. Introduction

Ecdysteroids are polar, polyhydroxylated steroids involved in the molting of insects and crustaceans. They are also present in a variety of plants such as spinach (*Spinacia oleracea* L.) and are among the main bioactive components of herbs. In spinach, the most abundant ecdysteroid is ecdysterone (chemical structure **Fig.1a**) which, depending on the growth rate of the plant, is present in a concentration range between 50 and 800 µg/g [1, 2].

Several studies conducted *in-vitro* and in animals have reported a variety of pharmacological effects of ecdysterone: an increase in carbohydrate and fatty acid metabolism, the stimulation of immune response, and an enhancement in protein synthesis, and physical power [3, 4]. Ecdysterone is advertised to increase strength and muscle mass during resistance training, to reduce fatigue, to ease recovery, and to induce anabolic effects, without any of the classical side effects of anabolic androgenic steroids, such as virilization [2, 5, 6]. Also for these reasons, ecdysterone has been marketed as dietary supplement for athletes.

Extensive investigations on the possible growth-promoting effects of ecdysterone in various animal species (rats, mice, Japanese quail and cattle) with a few studies recently performed also in humans, have been reported [2, 6-20]. There are several anecdotal reports on ecdysterone misuse by athletes, but only few scientific studies are available to demonstrate its potential performance enhancing effects.

Mechanistically, ecdysterone has been characterized as devoid of binding ability to either androgen receptor (AR), estrogen receptor (ER, where ERalpha was targeted), or glucocorticoid receptor [17, 21]. Only recently, binding of ecdysterone to the ERbeta could be shown *in-vitro* and *in-silico* [2, 22, 23].

In this study, we report the development and validation of a method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the identification and quantitation of ecdysterone in human serum. As proof of concept this method was applied to serum samples obtained from a ten week intervention study conducted in young men after the oral administration of a dietary supplement containing ecdysterone from spinach extract.

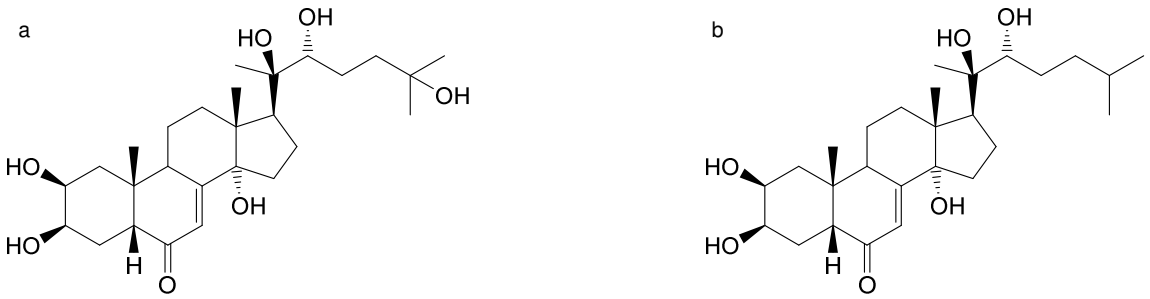


Fig. 1. Chemical structure of (a) ecdysterone and (b) ponasterone (used as internal standard).

2. Experimental

2.1. Chemicals and materials

Ecdysterone (2β,3β,14α,20β,22R,25-hexahydroxy-5β-cholest-7-en-6-one, purity>95%) and ecdysone (2β,3β,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one, used for selectivity testing of the method) were purchased from Steraloids (Newport, USA). Ponasterone (2β,3β,14α,20β,22R-pentahydroxy-5β-cholest-7-en-6-one), used as internal standard (ISTD), was obtained from Cayman Chemical Company (Ann Arbor, Michigan USA). Methanol, LC-MS grade, was purchased from J.T. Baker, acetonitrile, LC-MS grade, from Fisher Scientific GmbH (Schwerte, Germany), and formic acid, LC-MS grade, from Honeywell International Inc. (Bucharest, Romania). A Milli-Q water purification system LaboStar 2-DI/UV to obtain purified water for the analysis was obtained from SG Wasseraufbereitung und Regenerierstation GmbH (Barsbüttel, Germany). Blank human serum (male, USA) was obtained from Sigma Aldrich (Taufkirchen, Germany) and stored at -18 °C until use.
Oasis HLB 3cc Vac cartridges (60 mg sorbent per cartridge, 30 µm particle size) were purchased from Waters GmbH (Eschborn, Germany) and were used for the extraction of the target ecdysterone from the serum.

2.2. Instrumentation

The instrumental analyses were carried out using an ultrahigh performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) system (Agilent 1290 Infinity II UHPLC coupled to an Agilent 6495 triple quadrupole tandem MS system, Agilent Technologies GmbH, Waldbronn, Germany) utilizing an Agilent Jet Stream ESI source and Ion Funnel.
Chromatographic separation was achieved on an Agilent Eclipse C18 column (2.1 mm x 100 mm, particle size 1.8 µm). A linear gradient (10% B for 2 min, linear increase to 90% in 4 min, 1 min hold, in 0.5 min back to 10% B for reequilibration) was used with aqueous formic acid (H_2O:formic acid, 99.9:0.1, v:v, eluent A) and acetonitrile:formic acid (99.9:0.1, v:v, eluent B) as mobile phase constituents at a flow rate of 0.5 mL/min. The total run time was 7.5 min and the column equilibration time after each run was 2.5 minutes. The sample injection volume was 5 µL. The temperature of the autosampler was maintained at 5°C.

Table 1

Mass spectrometry parameters for MRM transitions in positive ion monitoring for ecdysterone and ponasterone (ISTD)

Analytes	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy	Cell accelerator voltage	Polarity
ecdysterone						
quantifier	3.78	481	371	12	4	positive
qualifier	3.78	481	445	12	5	positive
qualifier	3.78	481	165	28	5	positive
ponasterone						
quantifier	4.5	465	109	32	6	positive
qualifier	4.5	465	173	24	6	positive

The mass spectrometer was operated in positive electrospray ionization (ESI+). A capillary voltage of 3.500 V, a nozzle voltage of 300 V, a drying gas flow of 15 L/min (nitrogen) at 150°C, sheath gas flow of 12 L/min (nitrogen) at 375°C and a nebulizer pressure of 25 psi (nitrogen) were used. The protonated molecular ion $[M+H]^+$ for ecdysterone and ponasterone (ISTD) were detected at m/z 481 or 465, respectively. Ecdysterone was detected by the ion transition 481→371 (quantifier), 481→445 (qualifier 1) and 481→165 (qualifier 2). For the detection of the internal standard the ion transition 465→109 (quantifier) and 465→173 (qualifier), were used. **Fig. 2** shows the corresponding chromatograms. The MS parameters were fine-tuned for maximum sensitivity, and ion transitions were selected to obtain the best response (for the spectrum analysis). Details of MRM transitions, retention time, collision energy, cell accelerator voltage and polarity applied for ecdysterone and ISTD are shown in **Table 1**. A diverter valve of the LC system was used to minimize the influence of the matrix interfering substances on MS analysis (though source contamination) [24]. This valve was open during the first 3.5 minutes of the chromatographic run and after 5 minutes, avoiding contamination of the ESI source considering that the elution time of ecdysterone and its internal standard was between 3.5 and 5 minutes. Mass Hunter software from Agilent was used for data acquisition and processing.

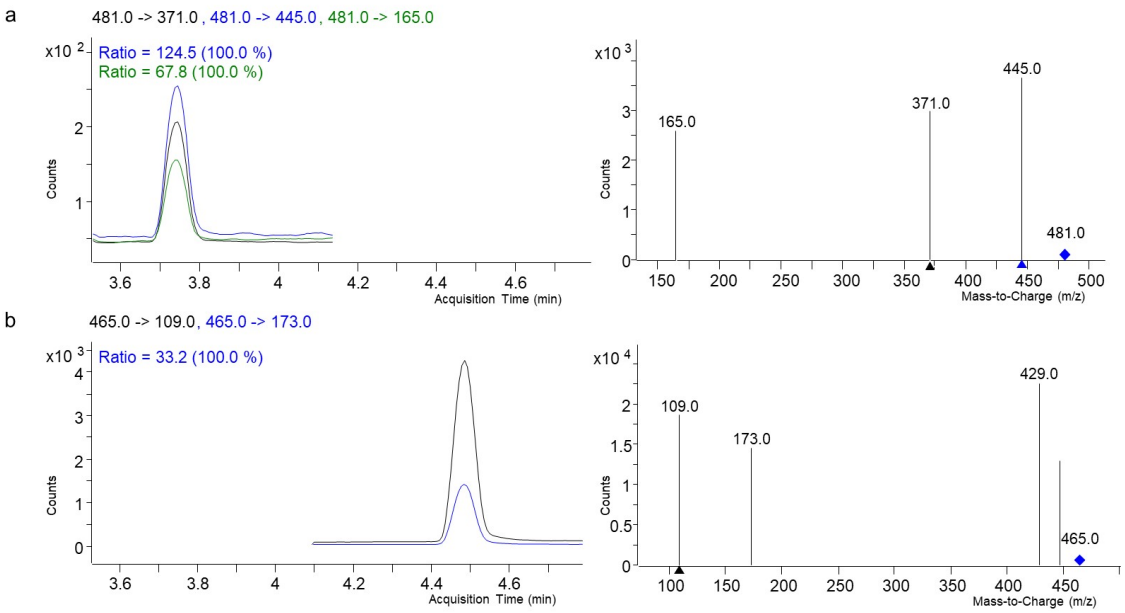


Fig. 2. MRM chromatograms and product ions of ecdysterone (a) and ponasterone (b) (ISTD). Chromatograms displayed inhere represent the LQC sample, i.e. c(ecdysterone)= 0.21 ng/mL, c(ponasterone)= 6.25 ng/mL.

2.3. Preparation of standard solutions and quality control samples

Stock solutions of ecdysterone and ponasterone (ISTD) were prepared in methanol at a concentration of 1 mg/mL and stored at -18°C. Working standard solutions of ecdysterone and ISTD were prepared by dilution of stock solutions in methanol. The working solutions of ecdysterone were diluted by methanol before preparation to obtain 0.5, 2.5, 5, 25, 50, 100, 200, 300, 400 and 500 ng/mL reference solutions, that were used for spiking and for preparation of the calibrants. The final concentrations of ecdysterone in the standard calibration plasma samples were 0.02, 0.10, 0.21, 1.04, 2.08, 4.16, 8.33, 12.5, 16.66 and 20.83 ng/mL (matrix matched standards).

The quality control samples (QC) were independently prepared at three levels of concentration for ecdysterone: 0.21 ng/mL (low concentration QC, LQC), 8.33 ng/mL (medium concentration QC, MQC), and 16.66 ng/mL (high concentration QC, HQC) spiked in blank plasma.

2.4. Evaluation of pre-analytical sample processing

2.4.1. Protein precipitation (PPT)

The efficiency of the protein precipitation step was optimized by evaluating different solvents, i.e. acetonitrile and methanol, in different proportions with the serum. The most suitable solvent for the PPT was cold methanol. Therefore the final preparation was performed adding to 50 µL of human serum, 40 µL of ice cold HPLC-MS grade methanol and 5 µL of diluted standard methanolic solutions of both ecdysterone and ISTD. A subsequent centrifugation for 15 minutes, at a relative centrifugal force (RCF) =2490*g and 20°C to separate the precipitated proteins followed. Aliquots of 5 µL of the supernatants were injected in the UHPLC-MS/MS system.

2.4.2. Solid phase extraction (SPE)

Due to the unsuitability of a liquid-liquid extraction (LLE) which was carried out using t-butyl methyl ether and ethyl acetate, resulting in recoveries of ecdysterone lower than 5 and 10%, respectively, a solid phase extraction (SPE) has been performed.

For this purpose, 480 µL of human serum were diluted with 480 µL of ultra-purified water and 20 µL of ecdysterone reference solution (working solutions of 0.5, 2.5, 5, 25, 50, 100, 200, 300, 400, and 500 ng/mL), and 20 µL of ISTD (150 ng/mL). The samples were vortexed and loaded onto HLB OASIS cartridges. Before sample application, the SPE column was conditioned by 1 mL of methanol and re-equilibrated using 1 mL of ultra-purified water. After a washing step with 1 mL of water the samples were finally eluted using 1 mL of methanol and concentrated under stream of nitrogen. The residue was reconstituted in 100 µL of methanol. Aliquots of 5 µL were injected into the UHPLC-MS/MS system for analysis.

2.5. Pretreatment of study samples

The serum samples (480 µL) were diluted with 480 µL of ultra-purified water and 40 µL of ISTD (working solution 75 ng/mL) were added. To isolate ecdysterone and the ISTD from the human plasma, the samples were vortex mixed for 5 seconds and then loaded onto the HLB OASIS cartridges.

Loading was performed after previous conditioning and re-equilibration of the cartridges with a flow of 1 mL/min to which a washing step using 1 mL of ultra-purified water was followed.

The analytes were eluted with 1 mL of methanol and reconstituted with 100 µL of methanol, after eluates were evaporated under stream of nitrogen and 5 µL of the samples were finally injected in the UHPLC-MS/MS system for the analysis.

2.6. Validation of the final analytical procedure

The analytical procedure was validated in terms of selectivity, limits of detection (LOD) and quantification (LOQ), linearity, accuracy, recovery, ion suppression/enhancement, intra-day and inter-day precision. Validation was performed in accordance to the guidelines "Bioanalytical Method Validation" published by the US-Food and Drug Administration (FDA, [25]) and to the "Guidelines for quality assurance in forensic-toxicological analyses requirements for the validation of analytical methods" [26].

2.6.1. Selectivity

The selectivity was studied by comparing the LC-MS/MS signals, acquired in MRM mode, of blank serum with those of serum samples spiked with ecdysterone ($C_{27}H_{44}O_7$), ponasterone and ecdysone (both $C_{27}H_{44}O_6$) at LQC (c=0.21 ng/mL, MQC (c=8.33 ng/mL) and HQC (c=16.66 ng/mL) levels.

2.6.2. Response function, LOD and LOQ

For the response function different aliquots of blank serum spiked with ecdysterone at ten concentration levels from 0.02 ng/mL to 20.83 ng/mL were prepared in duplicate. Calibration curves were constructed by least-squares linear regression of the peak area ratio of ecdysterone to ISTD (y-axis) and the nominal standard concentration (x-axis), described as $y = ax + b$. Testing the homogeneity of variance was performed according to DIN 38402 T51 including the test for outliers by using the Grubbs test. In order to verify the homogeneity of variances (n=10) standard samples of each of the lowest and highest concentration of the preliminary range have been analyzed. The variances of both series of measurements have been checked for homogeneity using the F-test.

Back calculations were performed to determine the concentration of ecdysterone in each calibration standard and the resulted calculated parameters were used to quantify the concentration of analytes in QCs or unknown samples using Mass Hunter Quant Software from Agilent. The Grubbs test for outliers was performed according to the GTFCh "Guidelines for quality assurance in forensic-toxicological analyses requirements for the validation of analytical methods". A correlation coefficient (R^2)>0.990 was considered satisfactory. The linearity test according to Mandel was assessed by a linear regression using appropriate weighting. The LOD and the LOQ were determined based on the uncertainty of the calibration according to DIN 32645. Representative chromatograms of authentic study samples with concentrations close to the calculated LOQ are available as supplemental material.

2.6.3. Accuracy and precision

The intra-day accuracy and precision were evaluated with 10 replicates of QC samples at three different concentrations (low, medium, high). Calculation of the concentration was performed from the ratios of the areas of ecdysterone and its ISTD using Mass Hunter Quant software and matrix matched calibrants. Accuracy was expressed as the percent relative error (RE%) and the assay precision was calculated by the percent coefficient of variation ($CV\%_{conc}$). The acceptance values used for validation of RE% and $CV\%_{conc}$ were within 15%, except at LOQ (there within 20%). Inter-day

precision was evaluated running analysis of variance (ANOVA), by injecting 5 individually prepared replicates on 3 different days of the serum spiked with ecdysterone and its ISTD at concentration of 8.33 ng/mL (MQC).

2.6.4. Recovery and matrix effect

The recovery percent of ecdysterone was determined by comparing the ratios of the areas of ecdysterone and the ISTD of extracted LQC, and HQC (0.21 and 16.66 ng/mL) with post-extraction spiked samples. The matrix effect of ecdysterone was determined by comparing the ratios of the areas of ecdysterone and its ISTD in post-extraction spiked sample in blank human serum to those obtained from the standard solutions in methanol at the HQC. Analyses were performed in six replicates.

2.6.5. Retention time repeatability, carry over, auto sampler stability

The retention time of characteristic ion transition repeatability was evaluated by injecting 5 times on 3 different days the serum samples spiked with ecdysterone and its ISTD at a concentration of 8.33 ng/mL (MQC). Carry over was tested by running pure methanol injections after each series of calibrators. The injection was carried out twice or three times after the highest calibrant (c=20.83 ng/mL), to ensure no ecdysterone nor ISTD was detected in the blank samples. All the samples were freshly prepared before analysis. After injection of the freshly prepared samples, the stability was evaluated by leaving the treated samples in the auto sampler for 72 h at 5°C and reinjecting them again.

2.7. Controlled administration study

The performance of the validated method has been assessed to quantify ecdysterone concentrations in serum of human male subjects.

The human intervention trial [20] was approved by the local ethical committee of German Sport University Cologne, Germany (No. 067/2016). Forty-six healthy male subjects successfully completed the study that was carried out in double blind design. The participants were assigned to four different groups: Placebo-group (PLG, n=12), Spinach1-group (SP1G, n=12), Spinach2-group (SP2G, n=10) and Control-group (COG, n=12). The PLG took two placebo capsules each day over a period of 10 weeks, the SP1G and COG took two capsules of “Peak Ecdysone” a day. The SP2G took a high dosage of ecdysterone, eight capsules of “Peak Ecdysone” each day. All participants were informed regarding potential risks related to the study and signed a declaration of no objection. Serum samples were collected from all participants prior to first administration (T1), after 5 weeks of intervention (T2) and after 10 weeks of intervention (T3). Samples were stored at -18 °C until analyses and gently thawed at +4 °C for sample preparation.

3. Results

Analytical target profile

Prior to method development the analytical target profile was established as prerequisite for rational method development [27]. Criteria for the developed method are its ability to separate, detect, identify and quantify ecdysterone in human serum using UHPLC/MS-MS. Thus, the procedure must be able to differentiate and quantify ecdysterone and its ISTD in the presence of other components in the serum samples. Due to the lack of stable isotope labelled ecdysterone, ponasterone (25-desoxyecdysterone) was chosen as ISTD. The total run time should not exceed 10 minutes to allow reasonable sample throughput. Acceptance values of RE% for the accuracy and $CV\%_{conc}$ for the precision, have to be within 15% (except at the LOQ where 20% are considered acceptable), indicating that ecdysterone can be determined in human serum samples with reasonable precision and accuracy. The calibration (standard) curve should cover the expected study sample concentration range. The range considered for the calibration has to follow linear correlation and the LOQ has to be as low as the concentration present in the post administration samples considered in this study as proof of concept. Recovery of the analytes should be consistent, precise, and reproducible.

3.1. Comparison of extraction procedures

The increasing interest on high throughput sample analysis has led to the common practice of preparing samples by the simplest and fastest possible method, which often means using PPT only. However, the specific organic solvent used in PPT may have dramatic effects on the overall cleanliness of the final extract [28].

In this study the LOQ of ecdysterone in blank serum after preprocessing using PPT and SPE were compared. Using PPT LOQ_{PPT}=9.92 ng/mL was found while SPE allowed for LOQ_{SPE}=0.14 ng/mL, respectively. According to Chemspider database, the analytes show a relatively high polarity, expressed by their reported logP values (logP(ecdysterone)=0.5, logP(ponasterone)=1.55, logP(ecdysone)=0.87, and for comparison logP(cortisol)=1.43, predicted values ACDLabs Percepta). Thus, liquid-liquid extraction was found unsuitable and recoveries of ecdysterone were found to be less than 5 and 10%, respectively.

As expected, the procedure including the solid phase extraction produced better results considering the range of serum concentrations of ecdysterone in the controlled administration study. While analytes are diluted in protein precipitation (1:1.v:v), a concentration factor of 4.8 was achieved in SPE, concomitant with additional purification of the analytes. The Oasis HLB cartridges were selected as suitable cartridges as they contain a universal polymeric reversed-phase sorbent that was developed for the extraction of a wide range of acidic, basic, and neutral compounds from various matrices using a simple, generic protocol. Moreover, since the Oasis HLB sorbent is water wettable, it maintains its capability for higher retention and excellent recoveries even if the sorbent runs dry. This means there is no need to take extraordinary steps to keep the sorbent beds from drying out during the critical steps prior to sample loading.

3.2. Selectivity

No interfering compounds at the retention time of ecdysterone, ponasterone, or ecdysone have been detected. Furthermore, ecdysterone, ponasterone, and ecdysone were clearly separated by retention time.

3.3. Response function, LOD and LOQ

The linear range for the ecdysterone calibration curve was 0.10-20.83 ng/mL. The best fit was indicated by a correlation coefficient of ≥ 0.995. A linear regression, which did not result into significantly better description than the quadratic regression (p value ≤ 0.05), was determined according to DIN 38402 T51 (ISO 8466). It was used to produce the best fit for the analyte concentration-detector response relationship using 1/x least square weighting, after testing the homogeneity of variance according to DIN 38402 T51. The test of homogeneity of variance showed a significant difference between the variances, i.e., TV > F (f_1=9, f_2=9, P=99%). As consequence the weighted factor 1/x has been applied. LOD and LOQ were calculated according to DIN 32645 and corresponded to 0.06 ng/L and 0.14 ng/mL, respectively.

3.4. Accuracy and precision

The accuracy and intra-day precision were determined at the LQC (0.21 ng/mL), MQC (8.33 ng/mL) and HQC (16.66 ng/mL), respectively. Results are shown in (**Table 2**). The criteria for intra-day precision (CV < 15%) and accuracy (RE < 15%), indicate that ecdysterone concentrations can be determined in human serum samples with reasonable precision and accuracy and that the method is suitable for use in the administration study. Inter-day precision was performed running the analysis of variance (ANOVA) using Excel according to GTFCh "Guidelines for quality assurance in forensic-toxicological analyses requirements for the validation of analytical methods", after injection of the MQC on three different days. No significant differences (p ≤ 0.05) were detected.

Table 2

Intra-day accuracy and precision of ecdysterone (n=30)

Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	CV (%)	RE (%)
0.21	0.24 ± 0.05	7.78	14.28
8.33	7.75 ± 0.51	6.27	-6.96
16.66	17.02 ± 0.59	5.36	2.16

SD standard deviation, CV coefficient of variation, RE relative error

3.5. Recovery and matrix effect

Respective recoveries of ecdysterone are shown in **Table 3**. For QC level of 0.21 (LQC) it corresponded to 100.7% with CV of 12.8%. At QC level of 16.66 ng/mL (HQC) the recovery was 94.4% with CV of 14.8%. The CV % at the LQC and HQC < 15% indicate that the method produced good and reproducible recovery for ecdysterone from human serum after extraction. The matrix factor was tested at the QC level of 16.66 ng/mL (HQC) and corresponding to 109.9% with $CV\%_{conc}$=14.5. Finally, matrix matched calibrants were used to compensate for both effects.

Table 3

Assessment of the recovery and matrix effect of ecdysterone in human serum

Compound	Nominal concentration (ng/mL)	Recovery (%, n=6)	CV (%)	Matrix effect (%, n=6)	CV (%)
ecdysterone	0.21	100.7	12.8	-	-
	16.66	94.4	14.8	109.9	14.5

Each value is presented as mean. CV, coefficient of variation

3.6. Retention time stability, carry over, and auto sampler stability

Good repeatability of the relative retention time was tested and CV%RT < 0.05% was obtained even over several days. No signal different from the base line was observed injecting pure methanol after a highly concentrated sample or calibrant. Thus, carry-over can be considered irrelevant. No variation higher than analytical imprecision (CV < 10 %) were observed during this study.

3.7. Controlled administration study

The application of the developed and validated LC-MS/MS method utilizing SPE demonstrated that it is suitable for the quantification of ecdysterone in human serum. It allows for fast analysis times and sensitive quantitation of even trace amounts of ecdysterone. In serum samples collected before the intervention period (T1) after 5 weeks of intervention (T2) and after 10 weeks of intervention (T3), serum concentrations of ecdysterone were determined. Serum concentrations of ecdysterone are displayed as boxplots **(Fig.3)** grouped for 2 capsule/day, 8 capsule/day and baseline (no administration of supplement). All serum concentrations in the post administration samples could be quantified (i.e. LOQ<concentration in the samples).

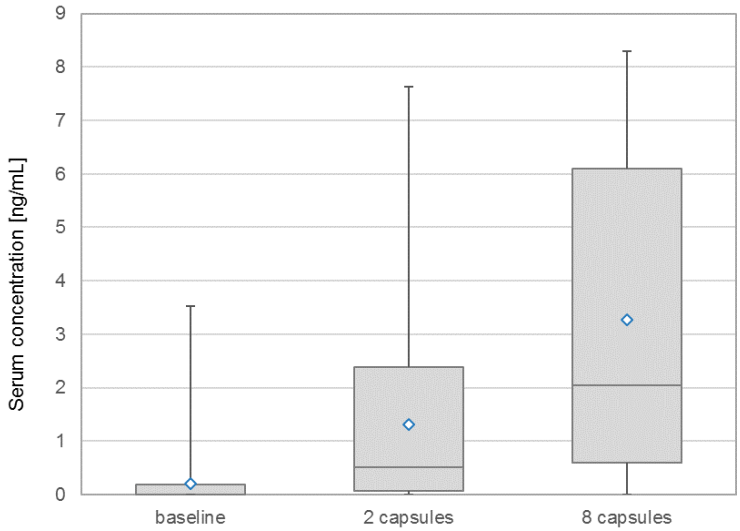


Fig. 3. Serum concentration of ecdysterone after administration of 2 capsules/day, 8 capsules/day (duration 5 or 10 weeks respectively), or at baseline (no administration of supplement).

4. Discussion

A liquid chromatographic-mass spectrometric method for the quantification of ecdysterone in human serum was developed, optimized and validated. All parameters of the analytical target profile were met. Working range with linear correlation was shown for 0.10-20.8 ng/mL in serum. Appropriate accuracy and precision were confirmed together with a good recovery for human serum. The validated method was successfully applied to samples from an intervention study for the determination of ecdysterone in serum as proof of concept. Concentrations determined in the samples collected post administration ranged between 0.18 and 8.2 ng/mL of ecdysterone in serum. Clear dose-dependent levels of ecdysterone in serum were detected. The details are reported in Isenmann et al. [20]. Also reported therein, significantly higher increases in muscle mass as well as in sports performance in one-repetition bench-press were after oral ecdysterone.

Conflict of Interest

All authors declare no conflict of interest.

Acknowledgments

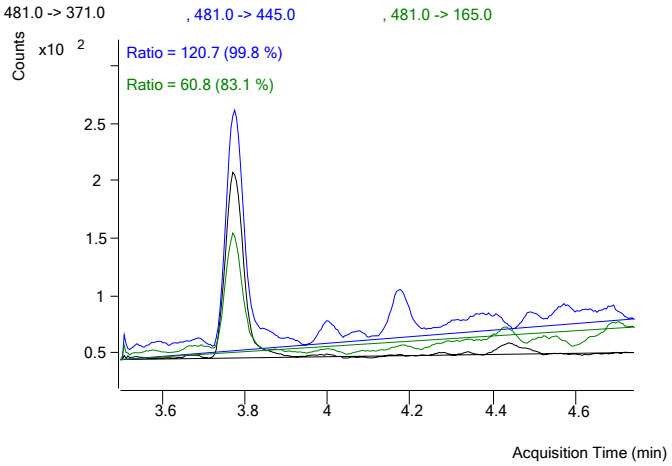
The authors acknowledge the financial support from the World-Anti Doping Agency (grant no. WADA 15C18MP). Ms. Ginevra Giacomello, Mrs. Maxi Wenzel, and Mr. Dave Wirth, all Institute of Pharmacy, Freie Universitaet Berlin, Germany, are acknowledged for technical assistance.

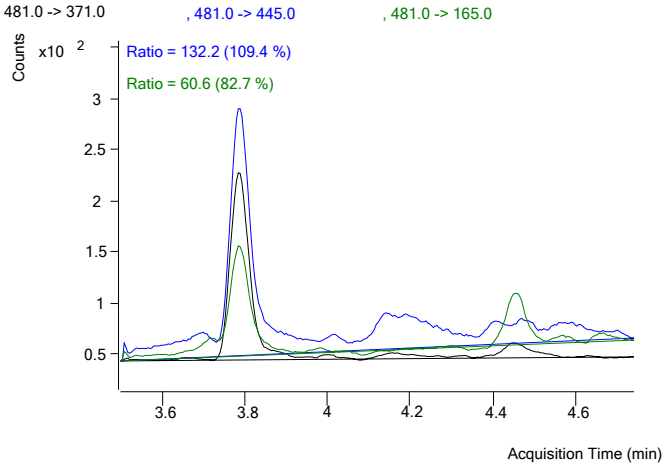
References

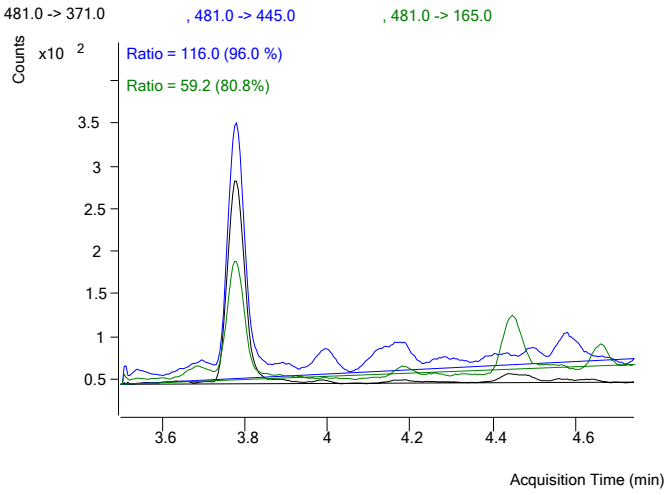
[1] Grebenok RJ, Ripa PV, Adler JH. Occurrence and levels of ecdysteroids in spinach. Lipids. 1991;26:666-8.
[2] Parr MK, Zhao P, Haupt O, Ngueu ST, Hengevoss J, Fritzemeier KH, et al. Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone. Molecular nutrition & food research. 2014;58:1861-72.
[3] Slama K, Lafont R. Insect hormones-ecdysteroids: Their presence and actions in vertebrates. European Journal of Entomology. 1995;92:355-77.
[4] Burdette W, Coda R. Effect of ecdysone on the incorporation of 14C-Leucine into hepatic protein in vitro. Proceedings of the Society for Experimental Biology and Medicine. 1963;112:216-7.
[5] Parr MK, Botre F, Nass A, Hengevoss J, Diel P, Wolber G. Ecdysteroids: A novel class of anabolic agents? Biol Sport. 2015;32:169-73.
[6] Dinan L. The Karlson Lecture. Phytoecdysteroids: what use are they? Archives of insect biochemistry and physiology. 2009;72:126-41.
[7] Haupt O, Tchoukouegno Ngueu S, Diel P, Parr M. Anabolic effect of ecdysterone results in hypertrophy of C2C12 myotubes by an estrogen receptor mediated pathway. In: Schänzer W, Geyer H, Gotzmann A, Mareck U, editors. Recent Advances in Dope Analysis (20). Cologne: Sport und Buch Strauß; 2012.
[8] Greenwood M, Rasmussen Chris J, Kerksick C, Campbell Bill I, Taylor Lemuel W, Wilborn Colin D, et al. Effects of Methoxyisoflavone, Ecdysterone, and Sulfo-Polysaccharide Supplementation on Training Adaptations in Resistance-Trained Males. Journal of the International Society of Sports Nutrition. 2006;3:19-27.
[9] Dinan L, Lafont R. Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. The Journal of endocrinology. 2006;191:1-8.
[10] Lafont R, Dinan L. Practical uses for ecdysteroids in mammals including humans: an update. Journal of insect science. 2003;3:7.
[11] Courtheyn D, Le Bizec B, Brambilla G, De Brabander HF, Cobbaert E, de Wiele AV, et al. Recent developments in the use and abuse of growth promoters. Anal Chim Acta. 2002;473:71-82.
[12] Gorelick-Feldman J, Maclean D, Ilic N, Poulev A, Lila MA, Cheng D, et al. Phytoecdysteroids increase protein synthesis in skeletal muscle cells. J Agric Food Chem. 2008;56:3532-7.
[13] Toth N, Szabo A, Kacsala P, Heger J, Zador E. 20-Hydroxyecdysone increases fiber size in a muscle-specific fashion in rat. Phytomedicine. 2008;15:691-8.
[14] Slama K, Koudela K, Tenora J, Mathova A. Insect hormones in vertebrates: anabolic effects of 20-hydroxyecdysone in Japanese quail. Experientia. 1996;52:702-6.
[15] Slama K, Kodkoua M. Insect hormones and bioanalogues: their effect on respiratory metabolism in Dermestes vulpinus L. (Coleoptera). Biol Bull. 1975;148:320-32.
[16] Zwetsloot KA, Shanely AR, Merritt EK, McBride JM. Phytoecdysteroids: a novel, non-androgenic alternative for muscle health and performance. J Steroids Horm Sci. 2013; s12: 10-12.
[17] Bathori M, Toth N, Hunyadi A, Marki A, Zador E. Phytoecdysteroids and anabolic-androgenic steroids--structure and effects on humans. Current medicinal chemistry. 2008;15:75-91.
[18] Okui S, Otaka T, Uchiyama M, Takemoto T, Hikino H. Stimulation of protein synthesis in mouse liver by insect-moulting steroids. Chemical & pharmaceutical bulletin. 1968;16:384-7.
[19] Arking R, Shaaya E. Effect of ecdysone on protein synthesis in the larval fat body of Calliphora. J Insect Physiol. 1969;15:287-96.
[20] Isenmann E, Ambrosio G, Joseph JF, Mazzarino M, de la Torre X, Zimmer P, et al. Ecdysteroids as non-conventional anabolic agent: performance enhancement by ecdysterone supplementation in humans. Archives of toxicology. 2019;in press.

[21] Seidlova-Wuttke D, Ehrhardt C, Wuttke W. Metabolic effects of 20-OH-ecdysone in ovariectomized rats. The Journal of steroid biochemistry and molecular biology. 2010;119:121-6.
[22] Parr MK, Haupt O, Ngueu ST, Fritzemeier K-H, Muhn P, Diel PR. Estrogen Receptor Beta Mediated Anabolic Effects - Insights from Mechanistic Studies on the Phytoecdysteroid Ecdysterone and Selective Ligands. Endocrine reviews. 2013:SAT-340-SAT-.
[23] Parr MK, Wolber G, Naß A, Ambrosio G, Botrè F, Diel PR. ER-Beta Mediated Action of Dietary Supplement Ingredient Edcysterone Confirmed By Docking Experiments. Endocrine reviews. 2015:FRI-270.
[24] Seraglio SKT, Valese AC, Daguer H, Bergamo G, Azevedo MS, Gonzaga LV, et al. Development and validation of a LC-ESI-MS/MS method for the determination of phenolic compounds in honeydew honeys with the diluted-and-shoot approach. Food Res Int. 2016;87:60-7.
[25] U.S. Food and Drug Administration. Draft Guidance for Industry - Bioanalytical Method Validation. US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research, Center for Veterinary Medicine; 2013.
[26] Peters FT, Drummer OH, Musshoff F. Validation of new methods. Forensic Sci Int. 2007;165:216-24.
[27] Parr MK, Schmidt AH. Life cycle management of analytical methods. J Pharm Biomed Anal. 2018;147:506-17.
[28] Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. J Chromatogr B. 2007;852:22-34.

Supplement 1: Chromatograms representing ion transitions of ecdysterone in authenic study samples
concentrations of ecdysterone calculated as
c(upper)=0.20 ng/mL, c(middle)=0.20 ng/mL, c(lower)=0.25 ng/mL







4.4 Manuscript No. 4

“Targeting the administration of ecdysterone in doping control samples”

Maria Kristina Parr, Gabriella Ambrosio, Bernhard Wüst, Monica Mazzarino, Xavier de la Torre, Francesca Sibilia, Jan Felix Joseph, Patrick Diel, Francesco Botrè

Forensic Toxicology 2019
https://doi.org/10.1007/s11419-019-00504-y

Purpose The phytosteroid ecdysterone was recently reported to enhance performance in sports and may thus be considered as a substance of relevance in anti-doping control. To trace back an administration of ecdysterone from urine samples, analytical properties have been investigated to assess its integration into initial testing procedures (ITP) in doping control laboratories.

Methods Analytical properties of ecdysterone were evaluated using gas chromatography-quadrupole/time-of-fight-mass spectrometry (GC–QTOF–MS) and liquid chromatography (LC)–QTOF–MS. Its metabolism and elimination in human were studied using urines collected after administration.

Results The detectability of ecdysterone by GC–MS (after derivatization) and/or LC–MS(/MS) has been demonstrated and sample preparation methods were evaluated. Dilute-and-inject for LC–MS(/MS) or solid phase extraction using Oasis HLB for GC–MS or LC–MS were found most suitable, while liquid–liquid extraction was hampered by the high polarity of ecdysteroids. Most abundantly, ecdysterone was detected in the post administration urines as parent compound besides the metabolite desoxy-ecdysterone. Additionally, desoxy-poststerone was tentatively assigned as minor metabolite, however, further investigations are needed.

Conclusion An administration of ecdysterone can be targeted using existing procedures of anti-doping laboratories. Ecdysterone and desoxy-ecdysterone appeared as suitable candidates for integration in ITP. Using dilute-and-inject a detection of the parent compound was possible for more than 2 days after the administration of a single dose of ~ 50 mg.

5 Discussion

This project had to go through different stages to demonstrate the performance-enhancing effects deriving from a long-term administration of ecdysterone containing supplements and it was supported by evidences from previous studies, which reported growth promoting and anabolic effects of ecdysterone in various animal species and in humans [28-44], mediated by its binding to ERbeta receptors [29, 46, 47]. A few studies investigated on ecdysterone's metabolism, most of them in mice and in rats [31, 117, 118], while those in humans differ for structure assignment [119, 120].

In this project, analytical methods were developed and validated to provide concrete and precise data, using different biological specimens or supplements. In parallel, an intervention study in humans has been carried out.

The developed and validated UHPLC-MS/MS method allowed for determination of the amount of ecdysterone in dietary supplements with reasonable accuracy and precision in a reasonable time. All supplements have been quantified using an external calibration, because the matrix effect has been considered within the acceptable limits. The developed method allowed to evaluate the actual reliability of the information reported on the labelling of 12 dietary supplements. Unfortunately, the concern about consistency between their actual content and label is confirmed. In 67% of the supplements, the content of ecdysterone is much lower than indicated on the label. For some of them, the indication on the label is very generic, preventing a correct qualitative and quantitative interpretation of the composition. In addition to that, quantitative differences are identified between distinct batches belonging to the same dietary supplement. This shows that it is necessary to develop analytical methods in order to characterize the chemical composition of products and to avoid undesired misdosage by users, which increases the risk of side effects. Also, this allows to correctly correlate the effects obtained in any intervention study, with the real qualitative and quantitative composition of a product.

The supplement "Peak Ecdysone" was chosen for the administration trial, due to the results obtained from purity control analysis and easy availability of a large number of capsules from the same lot. However, in the "Peak Ecdysone" supplement only 6% of the labelled content was found, corresponding to 6 mg/capsule. This results in an administration equivalent to 0.15 mg/kg and 0.6 mg/kg per day in an 80-kg volunteer if administered as reported in the human administration trial. Contamination of the supplements administered to volunteers with other performance enhancing drugs and their presence in urines, due to co-administration with other prohibited substances included in the anti-doping screening, have been excluded. Nevertheless, after ten weeks of treatment (training and/or supplement administration), positive time effects of ecdysterone on anthropometric (body

weight and muscle mass) and on strength performance (upper body strength) were observed. A dose-dependent effect is detected by the enhancement of the average training and from the fact that both ecdysterone groups (Ec1 and Ec2) increased their performance significantly compared to the PLG. Thus, a tendency for an enhanced performance by supplementation with ecdysterone was detected. These findings are corroborated by in-vitro experiments. A hypertrophy in C2C12 myotubes after 48 hour of treatment with supplement extract were detected. Hypertrophy was found similar to that obtained by dihydrotestosterone (DHT) estradiol (E2), or pure ecdysterone reference, which were used as controls. On that basis, further *in-vitro* experiments will be performed to identify potential metabolites, which may be included in targeted screening methods.

To evaluate the bioavailability of ecdysterone and to correlate serum concentrations with biological effects, a method for quantitation of ecdysterone in human serum has been successfully established and validated. As expected, the sample preparation utilizing SPE (Oasis HLB cartridges, 96% recovery of ecdysterone) resulted in much lower limit of quantification than simple PPT or LLE, considering the range of serum concentration of ecdysterone in the controlled administration study. Indeed, PPT was not suitable maybe due to non-elimination of matrix compounds, and LLE neither, due to the relative polarity of ecdysterone. The newly validated UHPLC-MS/MS method allowed for fast analysis and sensitive quantitation of even trace amounts of ecdysterone. A clear dose-dependent increment of ecdysterone in serum samples is detected. Variances of concentration in all groups most likely result from different individual pharmacokinetic parameters and slightly different time intervals of sample collection since the last supplements' administration.

No clinically relevant side effects attributable to ecdysterone supplementation were observed in the administration trial: serum concentration of the biomarkers of liver and kidney toxicity did not change significantly after 10 weeks. Between the endocrine hormones considered, changes can be observed for insulin like growth factor (IGF-1) and for thyroxin (T4). From the results obtained, training without supplementation of ecdysterone (PLG) resulted in a decrease of IGF-1. This can be explained with the fact that as a result of intensive training, cytokines, in particular tumor necrosis factor and interleukin 1, increase in the muscle and this effect decreases the level of IGF-1 and prevent it by acting on its receptors. Therefore, the intake of ecdysterone, in the case of Ec1 and Ec2, can antagonize this effect, resulting in an increase of IGF-1. Decreased concentrations of T4 have been observed in the three training groups (PLG, Ec1 and Ec2). No differences in T4 between the ecdysterone groups compared to the placebo have been reported. This shows that an intensive training, and not the administration of ecdysterone, may have an influence on T4. High intensive training may stimulate accelerated metabolism and stimulate changes in the in the body-weight regulatory hormones [121]. Variations in thyroid hormones'

concentrations can be correlated to intensive training [122]. Anyway further investigations are necessary to assess the influence of ecdysterone or of intensive training on hormone expression.

From the results obtained, no significant variation of the steroidal module parameters are detected in the post-administration urines, so it seems not possible to detect an ecdysterone intake from athletes due to an alteration of the steroidal profile. This suggests that the anabolic effect of ecdysterone is based on a mechanism different from other “traditional” AAS, which is in line with activation of ERbeta instead than AR receptors.

The optimization of the extraction procedure showed that either extraction using SPE (Oasis HLB cartridges) or dilution and injection of post-administration urine sample are the most suitable methods for sample pretreatment. Anyway, since no differences in abundance of ecdysterone were found after enzymatic hydrolysis (with β-glucuronidase or β-glucuronidase/ arylsulfatase) and neither ecdysterone glucuronide nor ecdysterone sulfate were detected in the non-hydrolyzed samples, we can exclude that ecdysterone undergoes phase II metabolism in the body, at least by conjugation with glucuronic or sulfuric acid. On the other hand, thanks to the successful synthesis of 14-desoxy-ecdysterone, this metabolite has been confirmed in post administration urines by comparison of retention time and relative mass spectra of the reference material, using LC-QTOF-MS in MS1 mode, with MS/MS spectra used for confirmation, after simply diluting and injecting the urine samples. The 20-desoxy- and 25-desoxy-ecdysterone, were excluded as possible metabolites by comparison with the corresponding standard references.

Through this study, the parent compound ecdysterone, which is the most abundant analyte in the post administration urines, and its 14-desoxy metabolite, can be easily integrated in the current initial testing procedures for monitoring the prevalence in elite sport. Even more, through the evaluation of the excretion profiles of ecdysterone and its desoxy metabolite, it is possible to detect the parent compound ecdysterone for more than 2 days using LC-QTOF-MS with no pre-concentration. The maximum concentration was reached in the urine collected at 2.0-3.5 hours after administration. The desoxy metabolite, instead, has a shorter detection window (2 days) with a maximum concentration in the 7.25-9 hour urine. This study showed that targeting the parent compound can be performed either with LC-MS/MS by dilute-and-inject or alternatively using GC-MS after derivatization with trimethyliodsilane (TMIS). Due to the impossibility of using mass spectrometry alone to provide reliable assignment of the exact isomer of metabolites, the synthesis of additional reference material is requested for the correct identification of possible metabolites.

In the future, *in-vitro* experiments should be evaluated in order to investigate metabolic pathways of ecdysterone and controlled administration trial in humans (including men and women) should be performed, to elucidate its metabolism and differences in gender.

In conclusion, due to its effective potential to enhance performance in sport, it is suggested to include ecdysterone in the S1.2 “other anabolic agent” list of prohibited substances and methods. Recently, ecdysterone has been included in the 2020 Monitoring Program [85] under the session “Anabolic agents”, *In-and Out-of-Competition*. Based on the investigation reported herein, methods to trace back an administration of ecdysterone in urine and serum are made available together with a method for the unambiguous identification and quantitation of ecdysterone in natural preparations.

6 Summary

The focus of this study is ecdysterone, a steroid hormone naturally occurring in plants and invertebrates [19, 20]. Ecdysterone is contained in a wide range of dietary supplements available on internet, and there are many rumors on ecdysterone misuse by athletes. Ecdysterone is not yet included in the list of prohibited substances and methods in sports, even if previous studies reported growth promoting and anabolic effects of ecdysterone in various animal species and in humans [28-45], mediated by its interaction with ERbeta [29, 46, 47]. With the exception of few studies [32, 119, 120], the evaluation of the use of ecdysterone supplementation to improve performances and study on its metabolism, have not yet been extensively investigated in humans. As part of this project, an intervention study of strength training of 46 healthy males has been carried out. The evaluation of a long-term administration of an ecdysterone containing dietary supplement has been made, with a special focus on the enhance of the performance during resistance training. Different doses of a supplement labelled to contain 100 mg of ecdysterone per capsule were administered to volunteers for ten weeks. This study included two training groups, Ec1 and Ec2 , which took respectively two and eight capsules per day, one non-training COG, which took two capsules per day (similar to Ec1), and one PLG, which was training taking a supplement not containing any ecdysterone. This study considered and evaluated anthropometrical and performance parameters after ten weeks of administration of ecdysterone containing supplements, and as results, reports an increase in body weight, muscle mass and upper body strength performances. In addition, the *in-vitro* study performed, shows a hypertrophy in C2C12 myotubes after 48 hour of treatment with supplement extract. The liver and kidney toxicity of ecdysterone is excluded by evaluation of specific biomarker in serum; changes in the serum concentration of endocrine hormones IGF-1 and T4 are observed. Even if further investigations will be needed, also considering other endocrine hormones, it seems that ecdysterone can antagonize the decrease in IGF-1 levels and that intensive training may have an influence on T4. In serum samples, a dose dependent increase of ecdysterone is reported after a successful development and validation of an UHPLC-MS/MS method. After a complete anti-doping screening, no other prohibited substance has been detected in the post administration urines as well as in the supplements administered to volunteers, demonstrating that the obtained effects on performance are resulting from consumption of ecdysterone. Following evaluation of the steroidal profile, it is proved that ecdysterone cannot be detected by changes in parameters of the steroidal module. This suggests that the anabolic effect of ecdysterone is based on a different mechanism than other AAS. From the analysis of the supplement administered to volunteers results that only 6% of the labelled content of ecdysterone is present in the

supplement and there is no presence of other performance enhancing substances. After a single dose administration of pure ecdysterone, its metabolism has been preliminary evaluated. Ecdysterone does not undergo phase II metabolism by conjugation with glucuronic or sulfuric acid. The 14-desoxy-metabolite is identified in post-administration urines through analysis with LC-QTOF-MS and confirmed by comparison with the MS/MS spectra of the synthetized 14-desoxy-ecdysterone. As result, ecdysterone (the most abundant analyte detected in post-administration urines) and the 14-desoxy-ecdysterone can be used for initial testing procedures for monitoring of ecdysterone administration in sport. Even more, excretion profiles of both ecdysterone and the 14-desoxy metabolite have been delineated. Finally, since the use of dietary supplements is increasing and their quality is not always guaranteed to consumers, due to the lack of adequate and uniform regulations, an UHPLC-MS/MS method for quantitation of 12 supplements labelled to contain ecdysterone has been developed and validated. The results report that in the 67% of the analyzed supplements, the labelled content of ecdysterone is highly inaccurate and, for some of them, the composition on the label is imprecise.

Thanks to this project, the WADA has included ecdysterone in the 2020 Monitoring Program. Methods to trace back an administration of ecdysterone in urine and serum are made available together with a method for the unambiguous identification and quantitation of ecdysterone in natural preparations.

7 Zusammenfassung

Der Fokus dieser Studie ist Ecdysteron, ein Steroidhormon, welches natürlich in Pflanzen und Wirbellosen vorkommt [19, 20]. Ecdysteron ist in vielen über das Internet erhältlichen Nahrungsergänzungsmitteln enthalten und es gibt viele Spekulationen über den Missbrauch von Ecdysteron bei Sportlern. Ecdysteron ist noch nicht in die Liste der verbotenen Substanzen und Methoden im Sport aufgenommen, selbst wenn frühere Studien Wachstumsförderung und anabole Effekte von Ecdysteron bei vielen Tierarten und beim Menschen nachweisen konnten [28-45]. Dies geschieht hauptsächlich über die Interaktion mit ERbeta [29, 46, 47]. Mit Ausnahme weniger Studien [32, 119, 120] ist der Einsatz von Ecdysteron-Supplementierung zur Leistungssteigerung und der Stoffwechsel beim Menschen noch nicht umfassend untersucht worden. Als Teil dieser Arbeit wurde eine Interventionsstudie zum Krafttraining mit 46 gesunden Männern durchgeführt. Es wurde der Langzeitgebrauch von einem ecdysteronhaltigen Nahrungsergänzungsmittel mit besonderem Fokus auf die Leistungssteigerung während des Krafttrainings untersucht. Unterschiedliche Dosen eines Nahrungsergänzungsmittels, welches laut Deklaration 100 mg Ecdysteron pro Kapsel enthalten sollte, wurden über einen Zeitraum von zehn Wochen von den Probanden eingenommen. Die Studie beinhaltete insgesamt vier Gruppen: zwei Trainingsgruppen, Ec1 und Ec2, nahmen jeweils zwei bzw. acht Kapseln pro Tag ein, eine nicht-trainierende COG, welche zwei Kapseln pro Tag einnahm (vergleichbar mit Gruppe Ec1) und eine PLG, welche bei Einnahme eines Nahrungsergänzungsmittels ohne Ecdysteron trainierte. Diese Studie betrachtete und beurteilte anthropometrische und Leistungsparameter nach zehn Wochen der Einnahme von ecdysteronhaltigen Nahrungsergänzungsmitteln und stellte als Ergebnis eine Zunahme des Körpergewichts, der Muskelmasse und der Oberkörperkraft fest. Darüber hinaus zeigt die durchgeführte *in-vitro* Studie eine Hypertrophie in C2C12 Myotuben nach 48 Stunden der Behandlung mit Nahrungsergänzungsmittel-Extrakt. Die Leber- und Nierentoxizität von Ecdysteron wird durch den Nachweis von spezifischen Biomarkern im Serum ausgeschlossen; Veränderungen in den Serumkonzentrationen der endokrinen Hormone IGF-1 und T-4 wurden beobachtet. Auch wenn noch weitere Untersuchungen unter Berücksichtigung anderer endokriner Hormone erforderlich sein sollten, kann festgestellt werden, dass Ecdysteron der IGF-1 Abnahme entgegenwirken kann und intensives Training einen Einfluss auf T4 haben kann. Nach erfolgreicher Entwicklung und Validierung einer UHPLC-MS/MS-Methode konnte eine dosisabhängige Zunahme von Ecdysteron in Serumproben nachgewiesen werden. Nach einem kompletten Anti-Doping Screening konnten weder in den postadministrativen Urinproben noch in den verabreichten

Nahrungsergänzungsmitteln verbotene Substanzen nachgewiesen werden. Dies zeigt, dass die beobachteten Effekte der Leistungssteigerung von der Ecdysteroneinnahme herrühren. Nach Beurteilung des Steroidprofils konnte gezeigt werden, dass Ecdysteron nicht über Veränderungen von Parametern des Steroidmoduls nachgewiesen werden kann. Das deutet darauf hin, dass der anabole Effekt von Ecdysteron auf einem Mechanismus beruht, der von anderen AAS abweicht. Die Analyse der verabreichten Nahrungsergänzungsmittel zeigte, dass nur 6 % der deklarierten Menge an Ecdysteron tatsächlich in den Kapseln vorhanden war und dass keine weiteren leistungssteigernden Substanzen zugesetzt wurden. Der Metabolismus von Ecdysteron wurde nach einmaliger Applikation der Reinsubstanz vorläufig untersucht. Ecdysteron unterzieht sich keinem Phase II Metabolismus durch Konjugation mit Glucuron- oder Schwefelsäure. Der 14-Desoxy-Metabolit wurde in Postadministrationsurinen mittels Analyse an einem LC-QTOF-MS Instrument detektiert und durch den Vergleich mit dem MS/MS Spektrum der synthetisierten Substanz bestätigt. Folglich können Ecdysteron, der am häufigsten in postadministrativen Urinen detektierte Analyt und 14-Desoxy-Ecdysteron für erste Testverfahren für die Überwachung des Ecdysterongebrauchs im Sport genutzt werden. Des Weiteren wurden Ausscheidungsprofile von Ecdysteron und 14-Desoxy-Ecdystreon erstellt. Da die Verwendung von Nahrungsergänzungsmitteln zunimmt und ihre Qualität den Verbrauchern mangels angemessener und einheitlicher Vorschriften nicht immer garantiert ist, wurde schließlich eine UHPLC-MS/MS-Methode zur Quantifizierung von 12 mit Ecdysteron markierten Nahrungsergänzungsmitteln entwickelt und validiert. Es wurde herausgefunden, dass in 67 % der analysierten Nahrungsergänzungsmittel der gekennzeichnete Gehalt an Ecdysteron sehr ungenau ist und für einige von ihnen die Zusammensetzung unpräzise deklariert ist. Infolge dieser Arbeit hat die WADA Ecdysteron in das Monitoring Programm 2020 aufgenommen. Methoden zur Rückverfolgung einer Ecdysteroneinnahme im Urin und im Serum wurden zusammen mit einer Methode zur eindeutigen Identifikation und Quantifikation von Ecdysteron in natürlichen Zubereitungen bereitgestellt.

8 Declaration of Own Contribution

In the following, the author´s contribution to the individual publications, which are used in this cumulative work, are disclosed:

Manuscript number 1

- Conception and design of the experiments for the quantitation of ecdysterone in dietary supplements
- Execution of experiments, development and validation of the method and analysis in collaboration with co-workers
- Evaluation of the data in cooperation with co-authors
- Manuscript preparation in cooperation with co-authors

Manuscript number 2

- Conception and design of the experiments in collaboration with the co-authors
- Sample preparation and analysis of urine samples for the study of the steroidal profile and anti-doping screening analysis of supplements and urines
- Evaluation of the data in cooperation with co-authors
- Cooperation for the preparation of the manuscript with co-authors

Manuscript number 3

- Conception and design of the experiments for the quantitation of ecdysterone in human serum
- Execution of sample preparation, development and validation of the method and analysis
- Evaluation of the data in collaboration with co-authors
- Manuscript preparation and in cooperation with co-authors

Manuscript number 4

- Collaboration for the design of experiments
- Execution of experiments in collaboration with co-authors
- Evaluation of the data in collaboration with co-authors
- Review of the manuscript

9 References

1. Parr, M.K., et al., *Clenbuterol marketed as dietary supplement.* Biomedical Chromatography, 2008. **22**(3): p. 298-300.
2. Van Poucke, C.D., C.; Van Cauwenberghe, R.; Van Peteghem, C., *Determination of anabolic steroids in dietary supplements by liquid chromatography-tandem mass spectrometry.* Anal. Chim. Acta, 2007. **586**(1-2): p. 35–42.
3. Parr, M.K.F., G.; Schlörer, N.; Opfermann, G.; Geyer, H.; Rodchenkov, G.; Schänzer, W., *Detection of D6-methyltestosterone in a "dietary supplement" and GC-MS/MS investigations on its urinary metabolism.* Toxicol. Lett., 2011. **201**(2): p. 101–104.
4. Geyer, H.K., et al., *Analysis of Non-Hormonal Nutritional Supplements for Anabolic-Androgenic Steroids - Results of an International Study.* International Journal Of Sports Medicine, 2004. **25**(2): p. 124-129.
5. Baume, N., et al., *Research of stimulants and anabolic steroids in dietary supplements.* Scandinavian Journal of Medicine & Science in Sports, 2006. **16**(1): p. 41-48.
6. Martello, S., M. Felli, and M. Chiarotti, *Survey of nutritional supplements for selected illegal anabolic steroids and ephedrine using LC-MS/MS and GC-MS methods, respectively.* Food Additives & Contaminants, 2007. **24**(3): p. 258-265.
7. Kamber, M.B., N.; Saugy, M.; Rivier, L., *Nutritional supplements as a source for positive doping cases?* Int. J. Sport Nutr. Exerc. Metab., 2001. **11**: p. 258–263.
8. Watson, P., et al., *Urinary nandrolone metabolite detection after ingestion of a nandrolone precursor.* 2009. **41**(4).
9. Parr, M.K., et al., *High amounts of 17-methylated anabolic-androgenic steroids in effervescent tablets on the dietary supplement market.* Biomedical Chromatography, 2007. **21**(2): p. 164-168.
10. Thevis, M., et al., *Does the analysis of the enantiomeric composition of clenbuterol in human urine enable the differentiation of illicit clenbuterol administration from food contamination in sports drug testing?* Rapid Communications in Mass Spectrometry, 2013. **27**(4): p. 507-512.
11. Green, A.G., H.D. Catlin, and H.B. Starcevic, *Analysis of Over-the-Counter Dietary Supplements.* Clinical Journal of Sport Medicine, 2001. **11**(4): p. 254-259.
12. Champagne, A.B. and K.V. Emmel, *Rapid screening test for adulteration in raw materials of dietary supplements.* Vibrational Spectroscopy, 2011. **55**(2): p. 216-223.
13. Maughan, R., *Contamination of dietary supplements and positive drug tests in sport.* Journal of Sports Sciences, 2005. **23**(9): p. 883-889.
14. Lisi, A., et al., *Studies of methylhexaneamine in supplements and geranium oil.* Recent Advances in Doping Analysis (19). W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck. Cologne, Sport & Buch Strauss. , 2011. **19**: p. 10-14.
15. Kazlauskas, R., *Supplements & WADA List.* Recent Advances in Doping Analysis (15). W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck. Köln, Sport und Buch Strauß, 2007: p. 31-40.
16. Kazlauskas, R. and N. Hasick, *ASDTL Supplements Project 2010.* Recent Advances in Doping Analysis (19). W. Schänzer, H. Geyer, A. Gotzmann and U. Mareck. Cologne, Sport & Buch Strauss., 2011. **19**: p. 10-14.
17. Geyer, H., et al., *Nutritional supplements cross-contaminated and faked with doping substances*, M. Thevis, Editor. 2008: Chichester, UK. p. 892-902.
18. Borrione, P., et al., *Consumption and biochemical impact of commercially available plant-derived nutritional supplements. An observational pilot-study on recreational athletes.* Journal of the International Society of Sports Nutrition, 2012. **9**.

19. Baltaev, U., *Phytoecdysteroids: Structure, Sources, and Biosynthesis in Plants.* Russian Journal of Bioorganic Chemistry, 2000. **26**(12): p. 799-831.
20. Nakanishi, K., et al., *Insect hormones. The structure of ponasterone A, insect-moulting hormone from the leaves of Podocarpus nakaii Hay.* Chemical Communications (London), 1966(24): p. 915-917.
21. Le Bizec, B., et al., *Ecdysteroids: one potential new anabolic family in breeding animals.* Analytica Chimica Acta, 2002. **473**(1-2): p. 89-97.
22. Thiem, B., et al., *Ecdysteroids: production in plant in vitro cultures.* Phytochem Rev, 2017. **16**(4): p. 603-622.
23. Reginaldo A. Festucci-Buselli, L.A.S.C., Luiz Claudio A. Barbosa, Jeff J. Stuart, Roberto F. Vieira, Wagner C. Otoni, *Level and distribution of 20-hydroxyecdysone during Pfaffia glomerata development.* Braz. J. Plant Physiol., 2009. **20**(4): p. 305-311.
24. Attila, H., et al., *Ecdysteroid-containing food supplements from Cyanotis arachnoidea on the European market: evidence for spinach product counterfeiting.* Scientific Reports, 2016. **6**(1).
25. Grebenok, R.J., P.V. Ripa, and J.H. Adler, *Occurrence and levels of ecdysteroids in spinach.* Lipids, 1991. **26**(8): p. 666-668.
26. Otaka, T., S. Okui, and M. Uchiyama, *Stimulation of protein synthesis in mouse liver by ecdysterone.* Chemical & pharmaceutical bulletin, 1969. **17**(1): p. 75.
27. Slama, K. and R. Lafont, *Insect hormones-ecdysteroids: Their presence and actions in vertebrates.* European Journal of Entomology, 1995. **92**(1): p. 355-377.
28. Dinan, L., *The Karlson Lecture. Phytoecdysteroids: What use are they?* Archives of Insect Biochemistry and Physiology, 2009. **72**(3): p. 126-141.
29. Parr, M.K., et al., *Estrogen receptor beta is involved in skeletal muscle hypertrophy induced by the phytoecdysteroid ecdysterone.* Molecular Nutrition & Food Research, 2014. **58**(9): p. 1861-1872.
30. Tchoukouegno, N.S., *Bioactivity of plants secondary metabolites: Estrogenic, cytotoxic and anabolic effects on estrogen target organs of an extract of Erythrina excelsa and Ecdysterone.* PhD, German Sport University, Cologne, 2013.
31. Kumpun, S., et al., *The metabolism of 20-hydroxyecdysone in mice: relevance to pharmacological effects and gene switch applications of ecdysteroids.* J Steroid Biochem Mol Biol, 2011. **126**(1-2): p. 1-9.
32. Wilborn, C.D., et al., *Effects of methoxyisoflavone, ecdysterone, and sulfo-polysaccharide supplementation on training adaptations in resistance-trained males.* Journal of the International Society of Sports Nutrition, 2006. **3**(2): p. 19-27.
33. M McBride, J., *Phytoecdysteroids: A Novel, Non-Androgenic Alternative for Muscle Health and Performance.* Journal of Steroids & Hormonal Science, 2013. **s12**(01).
34. Gorelick-Feldman, J., et al., *Phytoecdysteroids increase protein synthesis in skeletal muscle cells.* Journal of agricultural and food chemistry, 2008. **56**(10): p. 3532.
35. Dinan, L., *Phytoecdysteroids: biological aspects*. 2001. p. 325-339.
36. Dinan, L. and R. Lafont, *Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals.* Journal of Endocrinology, 2006. **191**(1): p. 1-8.
37. Lafont, R. and L. Dinan, *Practical uses for ecdysteroids in mammals including humans: an update.* Journal of Insect Science, 2003. **3**.
38. Courtheyn, D., et al., *Recent developments in the use and abuse of growth promoters.* Analytica Chimica Acta, 2002. **473**(1-2): p. 71-82.
39. Tóth, N., et al., *20-Hydroxyecdysone increases fiber size in a muscle-specific fashion in rat.* Phytomedicine, 2008. **15**(9): p. 691-698.

40. Bathori, M., et al., *Phytoecdysteroids and anabolic-androgenic steroids--structure and effects on humans.* Curr Med Chem, 2008. **15**(1): p. 75-91.
41. Sláma, K., et al., *Insect hormones in vertebrates: Anabolic effects of 20-hydroxyecdysone in Japanese quail.* Experientia, 1996. **52**(7): p. 702-706.
42. Sláma, K. and M. Hodková, *Insect hormones and bioanalogues: their effect on respiratory metabolism in Dermestes vulpinus L (Coleoptera).* The Biological Bulletin, 1975. **148**(2): p. 320-332.
43. Okui, S., et al., *Stimulation of Protein Synthesis in Mouse Liver by Insect-Moulting Steroids.* Chemical and Pharmaceutical Bulletin, 1968. **16**(2): p. 384-387.
44. Arking, R. and E. Shaaya, *Effect of ecdysone on protein synthesis in the larval fat body of Calliphora.* Journal of Insect Physiology, 1969. **15**(2): p. 287-296.
45. Burdette W, C.R., *Effect of ecdysone on the incorporation of 14C-Leucine into hepatic protein in vitro.* Proc Soc Exp Biol Med, 1963. **12**: p. 216–217.
46. Parr, M.K., et al., *Estrogen Receptor Beta Mediated Anabolic Effects – Insights from Mechanistic Studies on the Phytoecdysteroid Ecdysterone and Selective Ligands. .* Endocrine Reviews, 2013: p. p. SAT-340-SAT-340.
47. Parr MK, W.G., Naß A, Ambrosio G, Botrè F, Diel PR, *ER-beta mediated action of dietary supplement ingredient edcysterone confirmed by docking experiments.* Endocrine Reviews, 2015. **FRI-270**.
48. Parr, M.K., et al., *Ecdysteroids: A novel class of anabolic agents?* Biology of sport, 2015. **32**(2): p. 169-173.
49. Chermnykh, N.S., et al., *The action of methandrostenolone and ecdysterone on the physical endurance of animals and on protein metabolism in the skeletal muscles.* Farmakol Toksikol, 1988. **51**(6): p. 57-60.
50. Molinero, O. and S. Márquez, *Use of nutritional supplements in sports: risks, knowledge, and behavioural-related factors.* Nutricion hospitalaria, 2009. **24**(2): p. 128-134.
51. Department of Health and Human Services, *Food labeling; statement of identity, nutrition labeling and ingredient labeling of dietary supplements; compliance policy guide, revocation.* Fed Regist, 1997. **62**: p. 49826–92.
52. Bucci, L.R., *Selected herbals and human exercise performance.* The American journal of clinical nutrition, 2000. **72**(2 Suppl): p. 624S-36S.
53. Starr, R.R., *Too little, too late: ineffective regulation of dietary supplements in the United States.* American journal of public health, 2015. **105**(3): p. 478-485.
54. Martínez-Sanz, J.M., et al., *Intended or Unintended Doping? A Review of the Presence of Doping Substances in Dietary Supplements Used in Sports.* Nutrients, 2017. **9**(10).
55. US Dietary Supplement Health and Education Act of 1994, *Public Law 103-417.* Available online at https://ods.od.nih.gov/About/DSHEA_Wording.aspx (last accessed December 5th 2019), 1994.
56. Sarma, N., G. Giancaspro, and J. Venema, *Dietary supplements quality analysis tools from the United States Pharmacopeia.* Drug Testing and Analysis, 2016. **8**(3-4): p. 418-423.
57. Nowak, R.E., *DSHEA's failure: why a proactive approach to dietary supplement regulation is needed to effectively protect consumers. (Dietary Supplement Health and Education Act of 1994).* University of Illinois Law Review, 2010. **2010**(3): p. 1045-1081.
58. U.S. Department of Health and Human Services Food and Drug Administration Center for Food Safety and Applied Nutrition, *Dietary Supplements: New Dietary Ingredient Notifications and Related Issues: Guidance for Industry.* Avilable at https://www.fda.gov/media/99538/download (last accessd December 5th 2019), 2016.

59. Maughan, R.J., et al., *IOC Consensus Statement: Dietary Supplements and the High-Performance Athlete.* International Journal of Sport Nutrition and Exercise Metabolism., 2018.
60. Kerksick, C.M., et al., *ISSN exercise & sports nutrition review update: research & recommendations.* Journal of the International Society of Sports Nutrition, 2018. **15**(1): p. 38-38.
61. European Commission, *Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements.* 2002a.
62. Petkova-Gueorguieva Elina, S., et al., *Regulatory Requirements for Food Supplements in the European Union and Bulgaria.* Folia Medica, 2019. **61**(1): p. 41-48.
63. Low, T.Y., et al., *The Regulatory Framework Across International Jurisdictions for Risks Associated with Consumption of Botanical Food Supplements.* Comprehensive Reviews in Food Science and Food Safety, 2017. **16**(5): p. 821-834.
64. European Commission, *Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.* 2002b.
65. European Commission, *Novel food.* Avaiable online at https://ec.europa.eu/food/safety/novel_food_en (last accessed December 14th 2019), 1997.
66. http://globenewswire.com/news-release/2015/04/23/727513/10130416/en/Dietary-Supplements-Market-is-Expected-to-Reach-US-179-8-Billion-Globally-in-2020-Persistence-Market-Research.html.
67. Silver, D.M., *Use of Ergogenic Aids by Athletes.* Journal of the American Academy of Orthopaedic Surgeons, 2001. **9**(1): p. 61-70.
68. Department of Health and Human Services, *Dietary supplements: companies may be difficult to locate in an emergency.* Available online at https://oig.hhs.gov/oei/reports/oei-01-11-00211.pdf (last accessed December 5th 2019), 2012.
69. Braun, H., et al., *Dietary Supplement Use Among Elite Young German Athletes.* International Journal of Sport Nutrition and Exercise Metabolism, 2009. **19**: p. 97-109.
70. Pesce C, D.A., Magri L, Cereatti L, Giampietro M, Monacelli C, Zelli A., *Behavioral and psychological factors related to the use of nutritional ergogenic aids among preadolescents.* Pediat Exerc Sci, 2004. **16**(3): p. 231-249.
71. Burke, L.M. and P. Peeling, *Methodologies for Investigating Performance Changes With Supplement Use.* International Journal of Sport Nutrition and Exercise Metabolism, 2018. **28**(2): p. 159-169.
72. Brown, A.C., *Heart Toxicity Related to Herbs and Dietary Supplements: Online Table of Case Reports. Part 4 of 5.* Journal of Dietary Supplements, 2018. **15**(4): p. 516-555.
73. Brown, A.C., *Kidney toxicity related to herbs and dietary supplements: Online table of case reports. Part 3 of 5 series.* Food Chem Toxicol, 2017. **107**(Pt A): p. 502-519.
74. United States. Government Accountability Office, *Herbal Dietary Supplements: Examples of Deceptive or Questionable Marketing Practices and Potentially Dangerous Advice*. 2010.
75. Burke, L.M., *Supplements for Optimal Sports Performance.* Current Opinion in Physiology, 2019. **10**: p. 156-165.
76. Dwyer, J.T., P.M. Coates, and M.J. Smith, *Dietary Supplements: Regulatory Challenges and Research Resources.* Nutrients, 2018. **10**(1).

77. Bernstein, A., J. Safirstein, and J.E. Rosen, *Athletic ergogenic aids.* Bulletin of the NYU Hospital for Joint Diseases, 2003. **61**(3 4): p. 164.
78. Knopp WD, W.T., Bach Jr BR,, *Ergogenic Drugs in Sports.* Clinics in Sport Medicine, 1997. **16**(3): p. 375-392.
79. Bird, S.R., et al., *Doping in sport and exercise: anabolic, ergogenic, health and clinical issues.* Annals of Clinical Biochemistry, 2016. **53**(2): p. 196-221.
80. Rivera-Brown, A.M. and W.R. Frontera, *Principles of Exercise Physiology: Responses to Acute Exercise and Long-term Adaptations to Training.* PM&R, 2012. **4**(11): p. 797-804.
81. Hemmersbach, P., *History of mass spectrometry at the Olympic Games.* J Mass Spectrom, 2008. **43**(7): p. 839-53.
82. Krieger, J., *Intended. Underrated. Disputed. The IOC Medical Commission's "Subcommission on Doping and Biochemistry in Sport" between 1980 and 1988.* Performance Enhancement & Health, 2016. **4**(3-4): p. 88-93.
83. Hughes, D., *The World Anti-Doping Code in sport: Update for 2015.* Australian prescriber, 2015. **38**(5): p. 167-170.
84. World Anti-doping Agency, *The Code.* Available online at https://www.wada-ama.org/en/what-we-do/the-code (last accessed December 5th 2019), 2015.
85. World Anti-Doping Agency, *The 2020 List of Prohibited Substances and Methods.* Available online at https://www.wada-ama.org/en/media/news/2019-09/wada-publishes-2020-list-of-prohibited-substances-and-methods (last accessed December 1st 2019).
86. Burke, L.M., *Caffeine and sports performance.* Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme, 2008. **33**(6): p. 1319-1334.
87. Ganio, S.M., et al., *Effect of Caffeine on Sport-Specific Endurance Performance: A Systematic Review.* Journal of Strength and Conditioning Research, 2009. **23**(1): p. 315-324.
88. Tarnopolsky, M.A., *Caffeine and Creatine Use in Sport.* Annals of Nutrition and Metabolism, 2011. **57**(2): p. 1-8.
89. World Anti-doping Agency, *Monitoring Program.* Available online at https://www.wada-ama.org/en/what-we-do/the-prohibited-list (last accessed December 1st 2019).
90. Vlad, R.A., et al., *Doping in Sports, a Never-Ending Story?* Advanced pharmaceutical bulletin, 2018. **8**(4): p. 529.
91. World Anti-doping Agency, *International Standard for Laboratories (ISL).* Available online at https://www.wada-ama.org/en/resources/laboratories/international-standard-for-laboratories-isl (last accessed December 1st 2019), 2019.
92. Schamasch, P., *Challenges and perspectives in anti-doping testing.* Bioanalysis, 2012. **4**(13): p. 1691-1701.
93. World Anti-Doping Agency, *List of the Accredited Laboratories.* Available online at https://www.wada-ama.org/en/resources/laboratories/list-of-wada-accredited-laboratories (last accessed December 1st 2019).
94. Protti, M., R. Mandrioli, and L. Mercolini, *Perspectives and strategies for anti-doping analysis.* Bioanalysis, 2019. **11**(3): p. 149.
95. Faiss, R., J. Saugy, and M. Saugy, *Fighting Doping in Elite Sports: Blood for All Tests!* Frontiers in Sports and Active Living, 2019. **1**.
96. Saugy, M., N. Robinson, and C. Saudan, *The fight against doping: back on track with blood.* Drug Testing and Analysis, 2009. **1**(11-12): p. 474-478.

97. Ayotte C., *Detecting the administration of endogenous anabolic androgenic steroids.* Handb Exp Pharmacol., 2010(195): p. 77-98.
98. Van Renterghem, P., et al., *Reference ranges for urinary concentrations and ratios of endogenous steroids, which can be used as markers for steroid misuse, in a Caucasian population of athletes.* Steroids, 2010 **75**(2): p. 154-63.
99. Kotronoulas, A., et al., *Evaluation of markers out of the steroid profile for the screening of testosterone misuse. Part II: Intramuscular administration.* Drug Testing and Analysis, 2018. **10**(5): p. 849-859.
100. Sottas, P.-E., C. Saudan, and M. Saugy, *Doping: a paradigm shift has taken place in testing.* Nature, 2008. **455**(7210): p. 166.
101. Jenny Erkander, M., et al., *The impact of genetics and hormonal contraceptives on the steroid profile in female athletes.* Frontiers in Endocrinology, 2014. **5**.
102. Mullen, J.E., et al., *Urinary steroid profile in females – the impact of menstrual cycle and emergency contraceptives.* Drug Testing and Analysis, 2017. **9**(7): p. 1034-1042.
103. de La Torre, R., et al., *Changes in Androgenic Steroid Profile Due to Urine Contamination by Microorganisms: A Prospective Study in the Context of Doping Control.* Analytical Biochemistry, 2001. **289**(2): p. 116-123.
104. Mazzarino, M., et al., *Urine stability and steroid profile: towards a screening index of urine sample degradation for anti-doping purpose.* Anal Chim Acta, 2011. **683**(2): p. 221-6.
105. Kuuranne, T., M. Saugy, and N. Baume, *Confounding factors and genetic polymorphism in the evaluation of individual steroid profiling.* Br. J. Sports Med, 2014. **48**: p. 848–855.
106. Brun, E.M., R. Puchades, and Á. Maquieira, *Analytical methods for anti-doping control in sport: anabolic steroids with 4,9,11-triene structure in urine.* TrAC Trends in Analytical Chemistry, 2011. **30**(5): p. 771-783.
107. Parr, M.K. and W. Schanzer, *Detection of the misuse of steroids in doping control.* J Steroid Biochem Mol Biol, 2010. **121**(3-5): p. 528-37.
108. M. Thevis, W.S., *Mass Spectrometry in Doping Control Analysis.* Current Organic Chemistry, 2005. **9**: p. 825-848.
109. Botrè, F., *New and old challenges of sports drug testing.* Journal of Mass Spectrometry, 2008. **43**(7): p. 903-907.
110. Stojanović, E. and D. Radovanović, *Historical Development of Analytical Methods for Anti-Doping Control.* Physical Education and Sport Through the Centuries, 2017. **4**(1): p. 15-23.
111. Botrè, F., *Mass spectrometry and illicit drug testing: analytical challenges of the anti-doping laboratories.* Expert Review of Proteomics, 2008. **5**(4): p. 535-539.
112. Trout, G.J. and R. Kazlauskas, *Sports drug testing-an analyst's perspective.* Chemical Society reviews, 2004. **33**(1): p. 1-13.
113. Botrè, F., X. de La Torre, and M. Mazzarino, *Multianalyte LC-MS-based methods in doping control: what are the implications for doping athletes?* Bioanalysis, 2016. **8**(11): p. 1129.
114. Schänzer, W. and M. Thevis, *Human sports drug testing by mass spectrometry.* 2017. **36**: p. 16-46.
115. Thevis, M., et al., *Annual banned-substance review: analytical approaches in human sports drug testing.* Drug testing and analysis, 2011. **3**(1): p. 1-14.
116. World Anti-Doping Agency, *Technical Document TD2016EAAS.* Available online at https://www.wada-ama.org/sites/default/files/resources/files/wada-td2016eaas-eaas-measurement-and-reporting-en (last accessed December 5th 2019), 2016.

117. Lafont, R., et al., *Ecdysteroid metabolism: A comparative study.* Insect Biochemistry, 1986. **16**(1): p. 11-16.
118. Ramazanov, N., Z. Saatov, and B. Syrov, *Study of ecdysterone metabolites isolated from rat urine.* Chemistry of Natural Compounds, 1996. **32**(4): p. 545-549.
119. Tsitsimpikou, C., et al., *Study of excretion of ecdysterone in human urine.* Rapid Communications in Mass Spectrometry, 2001. **15**(19): p. 1796-1801.
120. Brandt, F., *Pharmakokinetik und Metabolismus des 20-Hyroxyecdysons im Menschen.* PhD, Philipps-Universität Marburg, Marburg, 2003.
121. Simsch, C.G., et al., *Training Intensity Influences Leptin and Thyroid Hormones in Highly Trained Rowers.* International Journal Of Sports Medicine, 2002. **23**(6): p. 422-427.
122. Steinacker, J.M., et al., *Thyroid Hormones, Cytokines, Physical Training and Metabolic Control.* Hormone And Metabolic Research, 2005. **37**(9): p. 538-544.

10 Appendix

10.1 List of Figures

10.2 List of Tables

11 List of Peer-Reviewed Publications

Ambrosio G, Wirth D, Joseph JF, Mazzarino M, de la Torre X, Botrè F, Parr MK.
How reliable is dietary supplement labelling? Experiences from the analysis of ecdysterone supplements, Pharmaceut Biomed (2019) in press.
doi: 10.1016/j.jpba.2019.112877

Isenmann E, Ambrosio G, Joseph JF, Mazzarino M, De La Torre X, Zimmer P, Kazlauskas R, Goebel C, Botrè F, Diel P, Parr MK.
Ecdysteroids as non-conventional anabolic agent: performance enhancement by ecdysterone supplementation in humans, Arch Toxicology 93 (2019) 1807-1816.
doi: 10.1007/s00204-019-02490-x

Ambrosio G, Joseph JF, Wüst B, Mazzarino M, de la Torre X, Diel P, Botrè F, Parr MK.
Detection and quantitation of ecdysterone in human serum by liquid chromatography coupled to tandem mass spectrometry, Steroids (2019).
Revision submitted

Parr MK, Ambrosio G, Wüst B, Mazzarino M, De La Torre X, Sibilia F, Joseph JF, Diel P, Botrè F.
Targeting the Administration of Ecdysterone in Doping Control Samples, Forensic Tox (2019) in press.
doi: 10.1007/s11419-019-00504-y

Ambrosio G, de la Torre X, Mazzarino M, Parr MK, Botrè FM,
Effect of non-prohibited drugs on the phase II metabolic profile of morphine. An in vitro investigation for doping control purposes, Drug Test Anal 10 (2018) 984-994.
(doi:1 0.1002/dta.2344)

12 Relevant Presentations and Posters in International Conferences.

Parr MK, Ambrosio G, Wüst B, Mazzarino M, De La Torre X, Sibilia F, Joseph JF, Diel P, Botrè F. Targeting the Administration of Ecdysterone in Doping Control Samples, bioRxiv (2019) 685230. doi: 10.1101/685230

Parr MK, Ambrosio G, Wüst B, Joseph J, Mazzarino M, de la Torre X, Botrè F.
Mass Spectrometry in Metabolite Investigations: Strength and Challenges Exemplified by the Phytosteroid Ecdysterone. Abstract Book “The 40th BMSS ANNUAL MEETING 2019. 76-77

Ambrosio G, Wirth D, Botrè F, Parr MK. Quality assessment of dietary supplements containing ecdysterone. DPhG Conference 2018 “Shaping future pharmaceutical research“, Book of Abstracts (2018)

Parr MK, Wolber G, Naß A, Ambrosio G, Botrè F, Diel P. ER-beta mediated action of dietary supplement ingredient edcysterone confirmed by docking experiments. Endocr Rev (2015), FRI-270

Parr MK, Ambrosio G, Wüst B, Joseph J, Mazzarino M, de la Torre X, Botrè F. Mass Spectrometry in Metabolite Investigations: Phytosteroid Ecdysterone. Annual Meeting of the British Mass Spectrometric Society, 05.09.2019, Manchester, UK

Ambrosio G, de la Torre X, Mazzarino M, Parr MK, Botrè FM. Effects of genetic polymorphism and drug-drug interactions on the detection of psychoactive substances: a model study on morphine metabolites. 5th International Conference on Novel Psychoactive Substances, 23.-24.10.2017, Vienna, Austria

Parr MK, Wolber G, Naß A, Ambrosio G, Botrè F, Diel P. ER-beta mediated action of dietary supplement ingredient edcysterone confirmed by docking experiments. Annual Meeting of the Endocrine Society, 05.-08.03.2015, San Diego, USA

Ambrosio G, Wirth D, Botrè F, Parr MK. Quality assessment of dietary supplements containing ecdysterone. DPhG Conference 2018 “Shaping future pharmaceutical research“, Berlin, 06.07.2018