
# THE GREY ZONE IN DOPING

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## Woord Vooraf

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## List of abbreviations

| | |
|---|---|
| 6-OXO | 4-androsten-3,6,17-trione |
| 7-keto-DHEA | 7-keto-dehydroepiandrosterone |
| ANOVA | Analysis of variances |
| APCI | Atmospheric pressure chemical ionisation |
| ATP | Association de tennis professionnelle |
| CE | Capillary electrophoresis |
| CE | Collision energy |
| CF | Communauté Française |
| CI | Chemical ionisation |
| $c_{max}$ | Maximal concentration |
| COX | Cyclo-oxygenase |
| DHEA | Dehydroepiandrosterone |
| DHT | Dihydrotestosterone |
| DI | Diagnostic ion |
| DoCoNed | Dopingcontrole Nederland |
| DSHEA | Dietary Supplement Health and Education Act |
| ECA-stack | Ephedrine-caffeine-aspirin stack |
| EI | Electron ionisation |

| | |
|---|---|
| FDA | Food and Drug Administration |
| GC | Gas chromatography |
| GC/MS | Gas chromatography – mass spectrometry |
| GC/MS/MS | Gas chromatography – tandem mass spectrometry |
| GPC | Gel permeation chromatography |
| HPLC | High performance liquid chromatography |
| I.D. | Internal diameter |
| IAAF | International Association of Athletics Federations |
| ILAC | International Laboratory Accreditation Cooperation |
| IOC | International Olympic Committee |
| IS | Internal standard |
| ISO | International Organisation for Standardisation |
| KBWB | Koninklijke Belgische Wielrijdersbond (Royal Belgian Cyclist Federation) |
| LC/MS | Liquid chromatography – mass spectrometry |
| LC/MS/MS | Liquid chromatography – tandem mass spectrometry |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| m/z | Mass/charge |
| MAOI | Monoamine oxydase inhibitor |

| | |
|---|---|
| MBTFA | N-methyl-bistrifluotoacetamide |
| MDA | 3,4-methylenedioxyamphetamine |
| MDEA | 3,4-methylenedioxyethylamphetamine |
| MDMA | 3,4-methylenedioxymethamphetamine |
| MeOH | methanol |
| MRPL | Minimum required performance limit |
| MS | mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| MSD | Mass selective detector |
| $MS^n$ | Multiple mass spectrometry |
| MSTFA | N-methyl-N-trimethylsilyltrifluoroacetamide |
| NADO | National doping organisation |
| NARL | National Analytical Reference Laboratory |
| NeCeDo | Nederlands centrum voor dopingvraagstukken |
| NPD | Nitrogen-Phosphorus detector |
| NPN | Natuur- en gezondheidsproducten Nederland |
| NSAID | Non-steroidal anti-inflammatory drug |
| NZVT | The Netherlands Security System Nutritional Supplements |
| ODS | Octadecylsilica |

| | |
|---|---|
| OFN | Oxygen free nitrogen |
| PI | Precursor ion |
| RRT | Relative retention time |
| RSD | Relative standard deviation |
| $RSD_{max}$ | Maximal relative standard deviation |
| RT | Retention time |
| SD | Standard deviation |
| SIM | Selected ion monitoring |
| SSRI | Selective seretonine re-uptake inhibitor |
| T/E | Testosterone-epitestosterone ratio |
| TCA | Tricyclic antidepressant |
| TIC | Total ion chromatogram |
| TLC | Thin Layer Chromatography |
| TMS | Trimethylsilyl |
| TSP | Thermo Separation Products |
| TUE | Therapeutic use exemption |
| U.S. | United States |
| UCI | Union Cycliste International |
| UEFA | Union of European Football Associations |
| UK | United Kingdom |

| | |
|---|---|
| UV | Ultra-violet |
| VlGem | Vlaamse Gemeenschap |
| WADA | World Anti-Doping Agency |

## Outline of the study

Anti-doping programs seek to preserve what is intrinsically valuable about sport. This intrinsic value is often referred to as "the spirit of sport", which is the celebration of the human spirit, body and mind and is characterised by values such as ethics, fair play, honesty, health, fun, team work, courage and many others. Doping, which is not only the detection of prohibited substances but also its possession and use, failing or tampering a doping test or refusing a control, is considered as fundamentally against the spirit of sport.

As a result of this definition, lists of prohibited substances and methods have been developed to keep the spirit of sport as pure as possible. Since the introduction of the first doping test in the early 1960's and the development of anti-doping rules by the UCI in the same period, doping rules have been extensively revised by the IOC and currently by WADA. These rules are very extensive and include numerous classes defined as doping agents, such as anabolic steroids, stimulants, diuretics and EPO, and also prohibited methods as blood doping.

Doping control generally operates a strict liability principle. In other words, the presence of a banned substance, or its metabolite, in a urine or body fluid constitutes and doping offence. In a refinement of this principle, several substances now have a requirement for laboratories to report their presence only above a specified urinary concentration. Some of these substances are regulated by a urinary threshold level because they are, or may be, endogenous. Examples of those substances are testosterone and 19-norandrosterone, the major metabolite of nandrolone. Other substances can be present as a constituent in the daily diet, such as caffeine and ephedrine, and are only restricted when large urinary concentrations indicate the deliberate intake of this substance to enhance performance.

In an attempt to run faster, jump higher or throw further athletes are always seeking for means to accomplish this goal. Since the mid 1990's the use of diverse types of nutritional supplements has become very popular in an attempt to optimise performance. The most important factor behind the explosive growth of the supplement market was the passage of the Dietary Supplement Health and Education Act (DSHEA) which makes it possible to market numerous products as nutritional supplements as long as no statements are made that these

products prevent, treat or cure a specific disease. Other factors behind the growth of this market is the easy access through the internet and the ever-improving results and record performances of top-level athletes who are promoting the use of supplements.

However, the uncounselled use of these dietary supplements by athletes is not always without risk as some cases have proven that positive doping tests can be obtained as a result of the use of (deliberately) contaminated nutritional supplements. Seven professional tennis players for instance, tested positive for the nandrolone metabolite norandrosterone. This positive doping test was attributed to the use of a nutritional supplement which was distributed among those players by ATP-accredited physicians. As a result, all seven athletes were acquitted from a doping offence. Similar stories can be found in athletics where a female marathon runner tested positive for anorexic agents after the use of a contaminated nutritional supplement and in cycling where an improperly prepared supplement seemed to contain methadone.

Following these facts, it can be concluded that a zone can be situated between uncontested doping products, such as injectable anabolic steroids, diuretics and growth hormone, and products with doubtful effects on the performance or products not listed on the WADA list of prohibited substances. This area is defined as "the grey zone" in this thesis. The substances situated in this "grey zone" are in most cases substances with a urinary threshold level, or compounds that are allowed if a proper medical cause justifies their use, such as corticosteroids. Nutritional supplements (un)intentionally contaminated with doping substances are also considered as part of this area if the supplement label does not specifically mentions the presence of the contested agent.

The aim of this work is an evaluation of the "grey" area in doping control. In this thesis, some substances that are situated at the borderline, i.e. substances with urinary threshold levels, will be discussed as well as the complex situation that arises from contaminated nutritional supplements and their influence on the outcome of a doping test. In addition, the declared use of medication, i.e. substances allowed as a result of a proper medical cause, on the doping control forms filled out during a doping control will also be considered.

The introduction part of this thesis focuses on the validation and identification criteria used in doping control to allow the unequivocal determination of substances. These criteria, which are

in accordance with WADA identification criteria set for accredited doping control laboratories, are applied throughout this thesis.

The second part of this thesis focuses on two doping substances with a urinary threshold level. The first is caffeine, a substance present in numerous products incorporated in our daily diet such as coffee, tea and chocolate. In addition, caffeine has proven to enhance performance at doses between 3 and 6 mg/kg body weight. As a result of the reported misuse of high doses, the use of caffeine was considered as a doping offence in the past when its urinary concentration exceeded 12 µg/ml. However, this substance has been removed from the WADA list of prohibited substances from January 1$^{st}$ 2004, because the effect of caffeine on performance was said to be small. Because caffeine is frequently promoted as an ergogenic aid and as a tool to accomplish weight reduction, the removal could have resulted in an increased use of this drug. This study aimed to find out if differences in urinary caffeine concentration could be observed before and after its removal from the list of prohibited substances. Therefore, a statistical evaluation was made between concentration obtained during doping control in the period 1993-2002 and results monitored in 2004.

A second substance with a urinary threshold level according to WADA criteria is morphine. Morphine was isolated from opium, the dried juice obtained from the green capsules of the plant *Papaver somniferum*, as the active compound that is capable of alleviating pain. Nowadays, morphine is used as pain relief in cancer treatment or to treat post-surgery pain. Because of those painkilling effects morphine can be misused in sport and is therefore classified as a doping agent in class S7 of the WADA list of prohibited substances with a urinary threshold level of 1 µg/ml. However, several of its possible precursors, such as codeine and ethylmorphine, are not prohibited. In addition, investigations have shown that the use of poppy seed, originating from the plant *Papaver somniferum*, might result in the detection of morphine. However, results shown in those studies are depending on the origin of the investigated poppy seeds. Because no data was available on the use of these poppy seeds and other parts of the plant *Papaver somniferum* in relation to doping control an excretion study was set up to investigate if doping positive results for morphine could be obtained after the use of herbal products in the daily diet. These products are a cake containing high amounts of poppy seeds and two commercially available teas containing parts of the plant *Papaver somniferum*.

Besides substances with a urinary threshold level, this thesis mainly focuses on nutritional supplements. Nutritional supplements are defined as food supplying in one or more nutrients in a concentrated form, such as minerals and vitamins, which are theoretically present in the daily diet. Although the use of these supplements is strongly discouraged by several official authorities, studies dealing with the prevalence of use have shown that supplement intake increases year after year. Especially athletes, ranging from amateur to elite level tend to use these supplements to compensate for an inadequate diet or to improve their performance. Nutritional supplements are not listed on the WADA list of prohibited substances. Nevertheless, the use of these substances is strongly discouraged because of the possible contamination of these supplements with components prohibited by national and international sports authorities.

In a first chapter of the investigation on nutritional supplements an extensive review of the literature was made dealing with the use of dietary supplements and the problems associated with mislabelling of these products, with special attention to substances that are prohibited according to the WADA anti-doping rules.

Following the results obtained from the review of the literature it seemed necessary to develop methods capable of detecting (un)intentional contamination with products listed on the WADA list of prohibited substances in nutritional supplements. The second and third chapter in the part on nutritional supplements therefore aimed at the development of sensitive methods for the detection of both anabolising agents and stimulants as these are the substances that are most frequently reported in the literature. As no validated methods where described before, an additional goal was to validate these methods according to quality criteria as set in an ISO 17025 accredited laboratory.

Chapter 4 on nutritional supplements describes four supplements in detail that have found to be contaminated with one or more prohibited substances. In addition, the influence of these contaminated nutritional supplements on the outcome of a doping test was investigated. This was done by establishing urinary detection times for the doping substances not mentioned on the product label. This chapter also summarises the overall results obtained using the developed methods during 4 years of supplement testing in the Ghent Doping Control Laboratory.

Similar to known anabolic agents frequently promoted in the U.S. numerous designer steroids are also frequently released on the supplement market. As routine screening methods for anabolic steroids in doping control traditionally rely on GC/MS in the SIM mode the detection of these new substances in unlikely to occur. One of these new so called nutritional supplements is 6-OXO, an aromatase inhibitor with steroidal structure, which is described in detail in chapter 5 of Part III. The main goal of the research on this supplement was to elucidate its metabolism and to determine markers for misuse of this substance in doping control analysis. This was done by setting up an excretion study using this supplement.

A final part of this work focuses on the declared use of medication on the doping control forms filled out at a doping control. These substances are in most cases substances allowed in sport or substances allowed if a proper medical cause justifies their use. The latter can be considered as part of the "Grey zone" in doping and are of major interest in this work. A statistical evaluation of the results obtained from the doping control forms filled out at a doping control in the period 2002 –2005 should allow for a general view on the medication used by athletes, and more specifically should point out if different kinds of medication are used in different areas of sport. Special attention was given to the use of β-agonists, used to treat asthma, and corticosteroids, frequently used to deal with inflammation, as these substances require a therapeutic use exemption.

# Part I: Introduction: Quality criteria in doping analysis

# 1. Introduction

The ultimate objective of method validation is to provide evidence that the analytical method does what it is intended to do. It is essential to use well defined and fully validated analytical methods to obtain reliable results that can be satisfactorily interpreted. All analytical methods described in this thesis were validated. A distinction can be made between quantitative and qualitative methods. This chapter describes all parameters tested during validation procedures before analysing routine samples.

Besides the criteria set for validation, criteria for the identification of substances have to be specified. According to ILAC-G7, the identification of a prohibited substance must result from the direct comparison with a reference material analysed in parallel or series with the test sample using a mass spectrometric technique [1]. Similar criteria are required for WADA-accredited laboratories [2]. In addition to the validation parameters, a brief description of the identification criteria used for the identification of substances detected throughout this thesis, in compliance with WADA requirements, is also described.

# 2. Method Validation

## *2.1. Quantitative methods*

### 2.1.1. Calibration curve

Calibration curves are established in blank material previously tested for the presence of the substance. Threshold levels should be within the range of the calibration curve. Each calibration curve consists of at least 5 levels (calibrator), including a zero value. The detection range of the calibration curve should at least be from 50 % to 200 % of the threshold level.

Mathematically, linear calibration curves can be described as:

$$y = ax + b$$

with: y = ratio area under the curve of analyte/internal standard
x = ratio of concentration analyte/internal standard
a = slope
b = intercept

If analytes are naturally occurring in the negative matrix (e.g. caffeine in urine; cfr: Part II Chapter 1) calibration curves can be established in water providing the slopes of the calibration curve in water and in the matrix do not significantly differ

For validation purposes, each calibrator should be analysed in triplicate. The coefficient of correlation r of the calibration curve should exceed 0.98.

### 2.1.2. Trueness

Trueness can be defined as the similarity between the measured and the theoretical value. Trueness is measured by the analysis of spiked blank samples. At least 3 samples (≥ 6 is recommended) have to be analysed on the lowest and highest calibrator level as well as on the threshold. Trueness can be expressed as a percentual deviation of the average measured value from the true value. Maximal tolerances are +/- 15 % [3].

### 2.1.3. Precision

Precision can be divided in repeatability and reproducibility. Precision is expressed by the relative standard deviation (RSD) calculated from the determination of a number (n ≥ 6) of analyses of an identical sample.

#### *2.1.3.1 Repeatability*

The repeatability is defined as the similarity between successive measurements obtained under identical circumstances (same operator, same method, same equipment, same time of analysis). Therefore at least 3 (6 is recommended) negative samples are spiked with the

components of interest at the level of lowest and highest calibrator level as well as on the threshold. The repeatability may not exceed the limit value of 2/3 $RSD_{max}$ with $RSD_{max}$ calculated from the Horwitz equation:

$$RSD_{\max} = 2^{(1-0.5\log C)}$$

*2.1.3.2 Reproducibility*

The reproducibility is the level of correspondence between results obtained with the same analytical method but analysed by a different analyst and spread in time. Therefore, samples spiked at the same levels as for the repeatability are analysed by different persons. This allows for the determination of the between-day RSD. The obtained value may not exceed $RSD_{max}$.

An analytical method is robust if small variations in analytical conditions do not result in significant differences. The robustness of an analytical method is determined via the reproducibility.

### 2.1.4. Limit of quantification

The limit of quantification (LOQ) of an analytical method is the lowest measured level, different from zero, which can be determined in a reproducible way. For quantitative methods this concentration is equal to the lowest point of the calibration curve and may not exceed half of the threshold level.

Sensitivity is a term often misused instead of LOQ. A method is said to be sensitive if small variations in concentrations cause large variations in response and is in fact equal to the slope of the calibration curve .

### 2.1.5. Limit of detection

The limit of detection (LOD) of an analyte is the lowest concentration, different from zero, which can be detected, but not necessarily quantified according to criteria of trueness and precision. In this theses, the LOD is arbitrarily set at half of the LOQ.

### 2.1.6. Selectivity and specificity

The selectivity and specificity of an analytical method describes the ability to distinguish the analyte from other substances present.

An analytical method is specific if it is able to determine the analyte without interference of endogenous substances or other interferences. Therefore, negative matrices ($n \geq 6$) are analysed and no interferences should occur neither at the retention time of the analyte(s) nor at the retention time of the internal standard.

An analytical method is selective if it is capable of distinguishing the analyte of interest from other (spiked) components belonging to the same class of substances. Therefore, negative matrices are spiked with these substances. Based upon relative retention time and/or MS ion ratios, the positive identification of the analyte should not be hampered by these related substances.

## *2.2. Qualitative methods*

Qualitative methods are validated according to the procedure described by Verwaal *et al.* [5]. This validation procedure includes the determination of the LOD and tests for selectivity and specificity.

### 2.2.1. Limit of detection

The determination of the LOD is described by Eurachem [6]. According to these guidelines, the LOD is the lowest concentration that can be determined with a statistical certainty ≥ 95% ($n \geq 10$). Therefore, negative samples are spiked at different levels and a response curve is established. The LOD is the lowest concentration with a score ≥ 95%. The tested levels should at least be: 2 x LOD, LOD, ½ x LOD and negative samples.

2.2.2. Selectivity and specificity

Similar to quantitative methods selectivity and specificity have to be determined. The procedure for qualitative methods is described by Verwaal *et al.* [5]. The minimum concentration of related substances spiked in a negative matrix should at least be twice the LOD in testing for selectivity.

Specificity is determined by the analysis of negative matrices used to establish the LOD of the analytical method.

# 3. Identification criteria

## *3.1. Retention time criteria*

The retention time (RT) is characteristic for a substance. Hence, retention times of the analyte in a sample and of the reference compound should be comparable, if analysed under the same conditions. The use of the relative retention time (RRT), calculated by dividing the retention time of a substance by the retention time of an internal standard, can be helpful to differentiate between substances with similar mass spectra. Nowadays, capillary GC and HPLC are the most frequently used chromatographic techniques. Table 2.2.1 summarises the criteria set up by WADA for these chromatographic techniques [2].

**Table 3.1.1 Maximum tolerance for deviations in retention time (RT) and relative retention time (RRT) between an analyte in a sample and the reference compound.**

| | Maximum allowed deviation | |
|---|---|---|
| GC | 1 % or 12 s for RT | whichever is smaller |
| | 1 % for RRT | |
| HPLC | 2 % or 24 s for RT | whichever is smaller |
| | 2 % for RRT | |

Less stringent criteria are applied for HPLC because capillary gas chromatography (GC) has shown to provide a higher reproducibility in retention times than HPLC.

## *3.2. Mass spectral criteria*

Mass spectrometry (MS) is a detection technique based upon the fragmentation of a molecule in an electric field, generating a fragmentation pattern of the molecule with can be considered as a fingerprint.

Although a mass spectrum can be regarded as unique for a substance, different substances can have the same mass spectrum [7]. The mass spectrum of an analyte can slightly differ depending on instrumental parameters, matrix and concentration. Therefore, strict criteria have to be set for several parameters allowing the unequivocal identification of a substance. As for the retention time, WADA has set criteria for mass spectrometric detection [2].

The mass spectrometer can be operated in scan mode, detecting all masses within a set interval, or in SIM mode. In the latter, only a limited number of ions are monitored. Mass spectra acquired in the scan mode are preferred over SIM-spectra. In both scan and SIM mode, at least three diagnostic ions are needed for the unequivocal determination of a substance. WADA describes diagnostic ions as molecular ion or fragment ions whose presence and abundance are characteristic of the substance and thereby may assist in its identification [2]. A second ion belonging to the same isotopic cluster may also be used as diagnostic only when the peculiarity of the atomic composition of the fragment so justifies (e.g. presence of Cl, Br or other elements with abundant isotopic ions). When full scan mass spectrometry is used, all diagnostic ions with a relative abundance greater than 10 % in the reference spectrum must be present in the spectrum of the unknown substance. In addition, for both SIM and scan spectra the relative abundance of three diagnostic ions in the analyte may not differ by more than the tolerance windows shown in Table 2.3.2 compared to the relative intensities of the same ions obtained from a spiked urine, a reference collection or a reference material.

**Table 3.2.1 Maximum tolerance windows for relative ion intensities ensuring identification.**

| Relative Abundance (% of base peak) | GC/MS (EI) | GC/MS (CI), $GC/MS^n$, LC/MS , $LC/MS^n$ |
|---|---|---|
| > 50 % | ± 10 % (absolute) | ± 15 % (absolute) |
| 25 % to 50 % | ± 20 % (relative) | ± 25 % (relative) |
| < 25 % | ± 5 % (absolute) | ± 10 % (absolute) |

In some cases, SIM is required to detect substances at the MRPL. The signal to noise ratio of the least abundant ion must be greater than 3:1. The relative abundance of the ion is determined by peak area or height in the integrated selected ion chromatograms. The relative intensities of any of the diagnostic ions should meet the criteria given in Table 2.3.1. An additional diagnostic ion with a relative abundance of less than 5 % in the reference must be present in the unknown.

As it is well known that the relative abundances of diagnostic ions are concentration dependent, the concentration of the substance in a reference sample should be comparable to the concentration of the analyte in the sample.

A second derivatisation and/or ionisation technique is required if less than three diagnostic ions are present.

In $MS^n$ a precursor ion is selected and fragmented into product ions. The combination of the selection of the precursor ion and mass selection or scanning of the product ions results in increased specificity. According to WADA criteria, the collision conditions should be selected to ensure the presence of the precursor ion in the MS/MS spectrum [2]. The combination of one product ion with its precursor ion can be sufficient for identification purposes.

However, if more than one product ion is monitored, the relative abundance of these ions shall not differ by more than the percentages in Table 2.3.1 The signal to noise ratio of the least intense diagnostic ion should be greater than 3:1. Similar as for SIM methods, any additional diagnostic ion in the reference spectrum with a relative abundance smaller than 5 % should also be present in the spectrum of the analyte. Again, if unique diagnostic product ions are not available, another derivatisation, ionisation or fragmentation technique should be used.

## *3.3. References*

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# Part II: Substances with a threshold level

# CHAPTER 1: CAFFEINE

Adapted from:

- W. Van Thuyne, K. Roels and F.T. Delbeke, *Distribution of Caffeine Levels in Urine in Different Sports in Relation to Doping Control.* Int. J. of Sports Med., 2005. **26**: p. 714-718.

- W. Van Thuyne and F.T. Delbeke, *Distribution of Caffeine Levels in Urine in different Sports in Relation to Doping Control before and after the Removal of Caffeine from the WADA doping List.* Int. J. of Sports Med., in press.

# Chapter 1: Caffeine

## *1.1. Introduction*

Caffeine (1,3,7-trimethylxanthine) is according to pharmacological criteria the most common drug of abuse used in the world today. Besides colas, chocolate and energy drinks, the main source of caffeine is coffee. In addition, caffeine, just as theophylline, is readily available as an over the counter drug in numerous countries in its pure form as "wake-up" pills [1]. Also, multiple nutritional supplements, promoted as having a performance enhancing and stimulating effect, became available the last decade and contain caffeine. Some of these products include, besides caffeine, extracts of guarana (*Paullinia cupana*), a South American plant which has been shown to contain caffeine [2]. According to European regulations, caffeine can be added to sport/energy drinks in levels up to 320 mg/l. A complete list of the caffeine content of numerous drinks including coffee, teas, cola energy drinks and chocolate products has been published [2]. Highest concentrations were found in tea which contains up to 630 mg/l. Cola drinks, very often used by athletes during competition, contain from 33 to 213 mg/l and coffee between 210 and 340 mg/l.

Ingestion of caffeine in order to improve athletic performance is common use among athletes. A survey conducted in Canada revealed that 35% of 11-18 year-old students questioned believed that caffeine could enhance their performance and 25% admitted using caffeine to enhance their athletic performance [3]. There are also reports that top endurance athletes use Coca-cola in the late stage of a race as a source of caffeine for the ergogenic effect (http://www.sportsci.org/news/news9711/martin.html).

### 1.1.1. Performance enhancing effects of caffeine

A brief overview of the history of caffeine in sport sciences is given by Perkins and Williams [4]. They show that current issues regarding the use of caffeine in sports are not new. The last decennia the performance enhancing effects after ingestion of caffeine on endurance sports have been discussed in numerous papers [5-8]. Generally spoken, caffeine tends to have a

positive influence on this type of performance [9-13]. This hypothesis is supported by most, but not all studies [14, 15]. Nevertheless, there seems to be a consensus on caffeine having an ergogenic effect on endurance exercise when fatigue occurs in 30 to 60 minutes. Less and more inconsistent data was found on the impact of caffeine on short term exercise. Collomp *et al.* reported no significant effect of caffeine on maximal and mean power during a 30 s Wingate test [16], so did Paton *et al.* [17]. On the other hand, other investigations showed a performance enhancing effect of caffeine in short term exercises [18-20]. The most obvious reason for this discrepancy is the difficulty to quantify the effect as the potential improvement is small and difficult to measure.

It is not clear whether caffeine improves muscular strength or not. Jacobs *et al.* reported muscular endurance improvement after ingestion of caffeine, but this was in combination with ephedrine [21]. In spite of lacking evidence many strength athletes use caffeine to increase their performance. It is not clear whether an improvement in strength is obtained or the rate of fatigue is diminished.

In almost all investigations caffeine was ingested one hour before the start of the experiment [22, 23]. In this way, the plasma caffeine peak concentration is situated during the exercise. However, it is not known if this is the best timing to maximize the ergogenic effect of caffeine because caffeine is slowly metabolised and individuals maintain a high level for 3 to 4 hours [24]. It has been suggested to wait for 3 hours, when the caffeine induced lipolysis results in the highest free fatty acid level [7].

An ergogenic effect can be obtained after the ingestion of caffeine in doses in the range 3-6 mg/kg. Bruce *et al.* reported that doses of 6 and 9 mg/kg were equally effective in increasing performance [25]. The lowest reported dose found to be ergogenic was 2.1 mg/kg [11]. Simultaneously, it was concluded that doses of 3.2 and 4.5 mg/kg had a greater effect than 2.1 mg/kg. Repeated ingestion seems questionable because a single dose of caffeine elevates the circulating plasma levels of caffeine for several hours [24]. Only two studies investigated the effect of a divided dose of caffeine on performance [12, 26]. The results in both studies suggest that ingesting a divided dose of caffeine during an endurance exercise has no ergogenic effect on performance over a bolus of caffeine given before exercise.

Until now, the mechanism by which caffeine releases its ergogenic effects is not revealed completely. Several parameters including adenosine receptor antagonism, epinephrine release, elevated free fatty acid levels etc. are proposed and have been discussed extensively in the literature [8, 24].

The most popular and also most used form of caffeine is coffee. Caffeine levels in instant coffee vary between 210 and 340 mg/l [2], although higher concentrations (up to 1.6 g) were reported in dripped coffee and espresso [1]. So 1 litre of coffee corresponds with caffeine doses used in the previously mentioned publications investigating the ergogenic effect of caffeine. Surprisingly, Graham et. al [9] showed that when caffeine was ingested together with decaffeinated coffee or as coffee itself, no ergogenic effect was reported although similar plasma methylxanthine concentrations were noticed. So a change in the metabolic pathway can not be responsible for this phenomenon. It appears that some component(s) present in coffee interfere with the ergogenic properties of coffee. More investigations have to be performed.

It is generally accepted that caffeine should not be added to beverages which are designed to stimulate fluid replacement during exercise because of the diuretic properties of caffeine as this can lead to dehydration [27, 28]. However, Wemple *et al.* proved that caffeine does not exhibit its diuretic properties during endurance exercise [29]. The diuretic effect is only observed at rest. So athletes can use caffeine containing beverages in endurance exercise to improve their performance without the concern for diuresis.

In conclusion, caffeine can be a powerful ergogenic aid not only in competition, but also in training sessions.

### 1.1.2. Caffeine and doping

As a result of the reported misuse of high doses, caffeine was listed as a doping agent in class I A "Stimulants" by the IOC with a urinary threshold level of 15 µg/ml in 1984. This threshold level was reduced in 1985 to 12 µg/ml [30]. Starting from January 1$^{st}$ 2004 caffeine was removed from the list of prohibited substances. Dosages of 3-6 mg/kg used in the above mentioned papers did not result in urinary concentrations close to the former threshold level of 12 µg/ml.

Removing caffeine from this list could have resulted in an increased use of caffeine. If the goal of drug testing in sport is to prevent unfair advantage, to encourage ethical behaviour and to protect the health of the athletes, caffeine should remain on the list of prohibited substances. A complete ban of caffeine from sports is not possible due to the presence of the drug in numerous dietary products. Setting a threshold level is more realistic.

Before the removal of caffeine from the doping list no data was available on the distribution of caffeine in urine samples tested for doping control. This study aims at the evaluation of the urinary caffeine concentrations measured before and after the removal of caffeine from the WADA doping list.

## *1.2. Experimental*

### 1.2.1. Chemicals and reagents

Caffeine was a gift from Merck (Darmstadt, Germany). β-OH-ethyltheophylline was purchased from Sigma (St. Louis, MO, USA). All other reagents (dichloromethane, methanol, ammonia, sodium chloride, ammonium chloride and tetrahydrofurane) were of analytical grade.

Ammonia buffer (pH 9.5) was prepared by the addition of ammonia to a saturated ammonium chloride solution until the desired pH was obtained.

### 1.2.2. Chromatographic conditions

The HPLC system consisted of a Model P4000 liquid chromatograph, a Model AS 3000 auto sampler and a Spectra Focus forward optical scanning detector set at 275 nm, all from TSP (Fremont, CA, USA). The column was a Hypersil 5 ODS, 100 x 3 mm I.D., 5 µm (Chrompack, Antwerp, Belgium) with an appropriate precolumn (10 x 2 mm I.D., 40 µm, $C_{18}$). The loop volume was 20 µl. The mobile phase was a mixture of tetrahydrofurane and water (1/100; v/v) at an isocratic flow rate of 1.0 ml/min.

### 1.2.3. Urine analysis

All urine samples were analysed according to the procedure described by Delbeke *et al.* [31]. Briefly, 100-120 mg of sodium chloride, 50 µl of internal standard (β-OH-ethyltheophylline 100 µg/ml, aqua bidest) and 100 µl of ammonium buffer (pH 9.5) were added to 1 ml urine. Extraction was performed by rolling with 5 ml of the dichloromethane-methanol (9/1; v/v) mixture for 20 minutes. After centrifugation, the organic layer was separated and evaporated under oxygen free nitrogen at 40°C. The residue was dissolved in 200 µl mobile phase. 20 µl was injected onto the chromatographic system. The method allowed for the separation of theobromine, theophylline, paraxanthine and caffeine.

### 1.2.4. Statistical analysis

A statistical analysis was made of the caffeine concentrations before and after the removal of caffeine from the WADA doping list.

All data were ranked numerically and divided into intervals of 0.25 µg/ml to create a caffeine distribution, concentrations below the LOQ (before: N = 3002 , after: N = 942) were considered as not detected. To evaluate the difference between sports a log-transformation of the concentrations was necessary. Therefore, all values below the LOQ were excluded. This allowed for the determination of an average concentration with a standard deviation on the concentrations above the LOQ (before: N = 8359 ; after: N = 3691). Comparison of those sports of which more than 200 samples were analysed was done by a one-way ANOVA using the Tukey HSD test comprised in the SPSS software package (SPSS 12.0, SPSS Inc., Chicago, USA).

Comparison of the results obtained before (N = 11361) and after the removal (N = 4633) of caffeine from the WADA doping list was done using an F-test to determine the variances, followed by an appropriate student t-test for the most abundantly tested sports in both periods.

## *1.3. Analytical method validation*

The method was validated. An equally weighted linear calibration curve was constructed in the range from 0-20 µg/ml (0, 4, 8, 12, 16, 20 µg/ml) by plotting the ratio of the peak height

of caffeine to the peak height of the internal standard (β-OH-ethyltheophylline). Therefore aqua bidest samples spiked with the appropriate amounts of caffeine standards were analysed. This matrix was used because of the impossibility to obtain caffeine free urine. A statistical evaluation was performed, using a two sided F-test followed by an appropriate student t-test, showing that the slope of a calibration curve made in aqua bidest does not differ significantly from the slope of a calibration curve made in urine. Each concentration was analysed in triplicate, the averages were used to create the calibration curve. The correlation coefficient of the obtained calibration curve was 0.9981. The deviation of the mean measured concentration from the theoretical concentration (trueness) was far below the maximal value of 15 % [32].

The precision was evaluated by the determination of the repeatability and the reproducibility. To measure the repeatability 6 samples at three different concentrations (1, 5 and 12 µg/ml) were analysed. The relative standard deviation, an indicator of the repeatability, for each of those concentrations never exceeded the limit values derived from the Horwitz equation [33]. Measured concentrations, relative standard deviations and maximal limits for the repeatability are listed in Table 1.3.1.

**Table 1.3.1 Method repeatability.**

| | | | |
|---|---|---|---|
| Theoretical concentration (µg/ml) | 1.00 | 5.00 | 12.00 |
| Average measured concentration (µg/ml) (n=6) | 0.96 | 4.89 | 11.97 |
| SD (µg/ml) | 0.075 | 0.100 | 0.374 |
| RSD (%) | 7.8 | 2.0 | 3.1 |
| 2/3 RSDmax (%) | 10.7 | 8.4 | 7.3 |
| Accuracy (%) | -3.8 | -2.2 | -0.3 |

The reproducibility was examined by analysing 12 samples at 5 µg/ml. This was done by two different analysts, at different times. The results for the reproducibility are summarized in Table 1.3.2. The original limit values calculated by the Horwitz equation are used while for the repeatability the margin was narrowed to two third of the calculated value. For both repeatability and reproducibility the accuracy was measured and is listed in Tables 1.3.1 and 1.3.2. A limit value of 15 % is used.

**Table 1.3.2 Method reproducibility.**

| | |
|---|---|
| Theoretical concentration (µg/ml) | 5.00 |
| Average measured concentration (µg/ml) (n=12) | 5.07 |
| SD (µg/ml) | 0.145 |
| RSD (%) | 2.9 |
| RSDmax (%) | 12.6 |
| Accuracy (%) | 1.4 |

The limit of quantification was defined as the lowest point where caffeine could be detected in a repeatable way. Therefore six samples of aqua bidest spiked with caffeine at 0.05, 0.1 and 0.25 µg/ml were analysed. Table 1.3.3 summarises the repeatability at these low concentrations.

**Table 1.3.3 Method repeatability at low concentrations.**

| | | | |
|---|---|---|---|
| Theoretical concentration (µg/ml) | 0.05 | 0.1 | 0.25 |
| Average measured concentration (µg/ml) (n=6) | / | 0.099 | 0.24 |
| SD (µg/ml) | / | 0.01 | 0.008 |
| RSD (%) | / | 10.22 | 3.39 |
| 2/3 RSDmax (%) | / | 15.08 | 13.14 |
| Accuracy (%) | / | -0.58 | -2.14 |

Caffeine was not detected in 3 out of the 6 samples spiked at 0.05 µg/ml. On the other hand, the obtained values for accuracy and the relative standard deviation at a concentration of 0.1 µg/ml are lower than the limit values of 15 % and 15.08 % respectively, resulting in an LOQ of 0.1 µg/ml.

Selectivity and specificity were tested by the analysis of a system blank ($H_2O$), a quality control sample ($H_2O$) spiked at 12 µg/ml and 10 different urines. Stock solutions of both caffeine and β-OH-ethyltheopylline were also tested. A peak purity analysis showed that the

UV-spectra of both internal standard and caffeine did not change significantly over the chromatographic peak in all analysed urine samples showing that no co-elution of matrix components occurred. In addition, match values of the average UV-spectra of the chromatographic peaks higher than 95% were obtained when compared with the reference spectrum. Analysis of the reference solutions showed no interferences of other substances. Hence the method is selective and specific.

## *1.4. Results*

### 1.4.1. 1993-2002

Caffeine concentrations were determined in 11361 urine samples, originating from in-competition doping tests by the Flemish Community and several sport federations including IAAF, UCI and UEFA. Figure 1.4.1 shows the distribution (intervals: 0.25 µg/ml) of caffeine in athletes' urine specimens. Caffeine was below the LOQ in 3002 (26.42%) samples. Urinary caffeine concentrations ranged from 0 to 29.94 µg/ml.

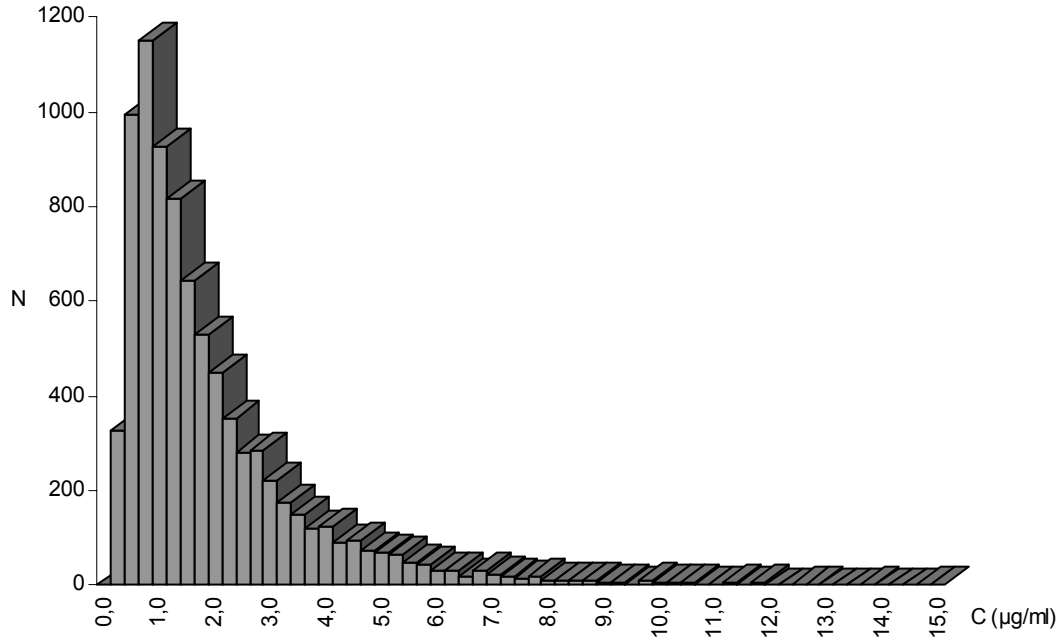


**Figure 1.4.1 Distribution of caffeine levels in the urine of 11361 athletes tested for doping control during the period 1993-2002. Caffeine concentrations <LOQ of 0.1 µg/ml and >15 µg/ml are not shown.**

The histogram of the logarithmic transformation of the concentrations above the LOQ is represented in Figure 1.4.2. These adjustments allow for the data to adapt into a gaussian form. Based on the transformed data an average concentration of 1.22 µg/ml with a standard deviation of 2.45 µg/ml is obtained.

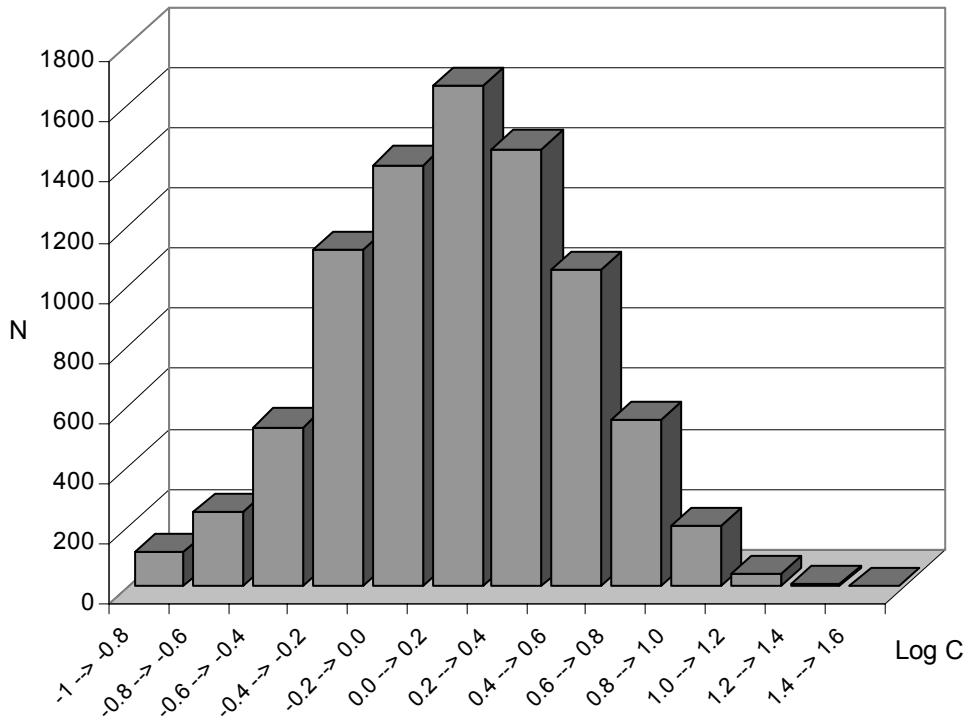


**Figure 1.4.2 Distribution of log-transformed urinary caffeine concentrations in 8359 urine samples exceeding the LOQ of the analytical method.**

Transformed caffeine concentrations are compared using ANOVA for those sports in which more than 200 samples were analysed between 1993 and 2002. The number of samples analysed, the resulting average concentration and standard deviation after transformation, and percentage of values below the LOQ for each of those sports are given in Table 1.4.1.

**Table 1.4.1 Average caffeine concentration and standard deviation after transformation, and percentage of values below LOQ in sports with more than 200 samples analysed (1993-2002).**

| Sport | N | Average concentration (µg/ml) | Standard deviation (µg/ml) | Percentage below LOQ (%) |
|---|---|---|---|---|
| Cycling | 4344 | 1.34 | 2.44 | 22.17 |
| Athletics | 643 | 1.06 | 2.62 | 32.50 |
| Swimming | 215 | 0.90 | 2.37 | 45.12 |
| Soccer | 1295 | 1.05 | 2.23 | 27.80 |
| Basketball | 478 | 1.04 | 2.34 | 32.01 |
| Volleyball | 641 | 1.20 | 2.39 | 20.90 |
| Bodybuilding | 255 | 1.72 | 2.49 | 26.27 |

Results of comparison of these sports using a one-way ANOVA (Tukey HSD post hoc test) are presented in Table 1.4.2.

**Table 1.4.2 Comparison of mean urinary caffeine concentrations between sports with N > 200 before the removal of caffeine from the WADA doping list.** [a]

| | Cycling 1.34 µg/ml | Athletics 1.06 µg/ml | Swimming 0.90 µg/ml | Soccer 1.05 µg/ml | Basketball 1.04 µg/ml | Volleyball 1.20 µg/ml | Bodybuilding 1.72 µg/ml |
|---|---|---|---|---|---|---|---|
| Bodybuilding | # | # | # | # | # | # | |
| Volleyball | = | = | # | = | = | | |
| Basketball | # | = | = | = | | | |
| Soccer | # | = | = | | | | |
| Swimming | # | = | | | | | |
| Athletics | # | | | | | | |
| Cycling | | | | | | | |

[a] post hoc test: Tukey HSD test. = no significant difference ($\alpha = 0.05$). # significant difference ($\alpha = 0.05$).

Sixteen athletes (0.14%) delivered a urine sample with a caffeine concentration exceeding the doping threshold of 12 µg/ml. In 2 out of them the concentration was above 20 µg/ml. The distribution of the positive caffeine results amongst different sports is given in Table 1.4.3.

**Table 1.4.3 Number of positive samples and highest detected urinary caffeine concentration during the period 1993-2002.**

| Sport | Number of positives | Percentage positives | Highest concentration (µg/ml) |
|---|---|---|---|
| Cycling | 4 | 0.09 | 15.0 |
| Bodybuilding | 3 | 1.18 | 29.9 |
| Powerlifting | 3 | 2.05 | 16.9 |
| Volleyball | 2 | 0.31 | 17.7 |
| Athletics | 1 | 0.16 | 14.7 |
| Soccer | 1 | 0.08 | 18.6 |
| Weightlifting | 1 | 2.17 | 12.3 |
| Gymnastics | 1 | 0.81 | 20.1 |

1.4.2. 2004

Similar as for the period 1993-2002, caffeine concentrations were determined in 4633 samples originating from in-competition doping tests by the Flemish and French NADOs in Belgium and The Netherlands and several federations including IAAF, UCI and UEFA.

The distribution (intervals 0.25 µg/ml) of caffeine in athletes' urine specimens is shown in Figure 1.4.3. Caffeine concentrations below the LOQ were found in 942 (20.33%) out of the 4633 samples. Urinary caffeine concentrations ranged from 0 to 20.84 µg/ml.

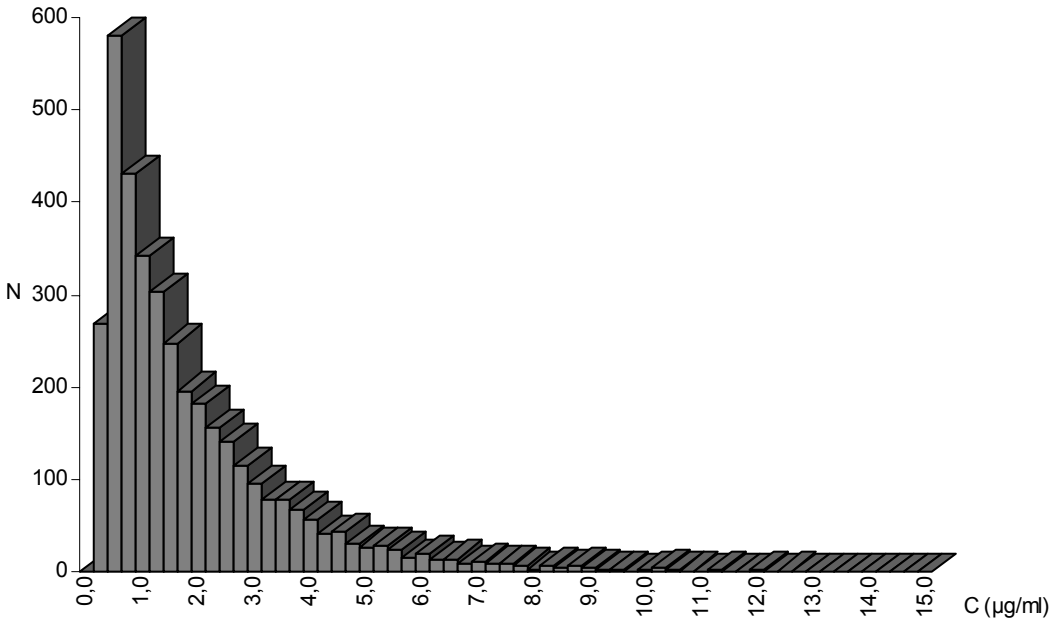


**Figure 1.4.3 Distribution of caffeine levels in the urine of 4633 athletes tested for doping control during 2004. Caffeine concentrations < LOQ of 0.1 µg/ml and > 15 µg/ml are not shown.**

Samples with a urinary caffeine concentration ≥ 4 µg/ml, a caffeine concentration found in urine samples from moderate to frequent coffee drinkers [34], were compared between several sports and between this period (2004) and the previously examined period (1993-2002) (Table 1.4.4).

**Table 1.4.4 Percentage of samples with a urinary caffeine concentration ≥ 4µg/ml in different sports in 1993-2002 and 2004.**

| Sport | 1993-2002 | 2004 |
|---|---|---|
| Overall | 7.01 | 7.55 |
| Cycling | 8.89 | 11.18 |
| Athletics | 6.07 | 4.76 |
| Swimming | 2.79 | 1.33 |
| Soccer | 3.32 | 4.79 |
| Basketball | 5.02 | 3.03 |
| Volleyball | 6.71 | 7.60 |
| Bodybuilding | 13.73 | 13.51 |
| Powerlifting | 19.86 | 18.56 |

A log-transformation allowed the data (concentrations above LOQ) to adapt into a Gaussian form. Based on the transformed data an average concentration of 1.12 µg/ml with a standard deviation of 2.68 µg/ml was obtained.

Transformed caffeine concentrations were compared using ANOVA for the most frequently tested sports in 2004. Major sports in the previously examined period (1993-2002) were included in this comparison. The number of samples analysed, average concentration and standard deviation after transformation and percentage of samples with a urinary caffeine concentration below the LOQ for these sports are given in Table 1.4.5.

**Table 1.4.5 Average caffeine concentration and standard deviation after transformation and percentage of values below the LOQ in frequently tested sports in 2004.**

| Sport | N | Average concentration (µg/ml) | Standard deviation (µg/ml) | Percentage below LOQ (%) |
|---|---|---|---|---|
| Cycling | 1467 | 1.40 | 2.60 | 16.84 |
| Athletics | 357 | 0.95 | 2.74 | 20.73 |
| Swimming | 75 | 0.45 | 2.24 | 48.00 |
| Soccer | 438 | 1.00 | 2.53 | 18.95 |
| Basketball | 165 | 0.83 | 2.41 | 29.70 |
| Volleyball | 171 | 1.03 | 2.75 | 8.77 |
| Bodybuilding | 37 | 1.35 | 2.98 | 27.03 |
| Boxing | 135 | 0.87 | 2.65 | 21.48 |
| Judo | 93 | 0.83 | 2.52 | 35.48 |
| Motor sports | 86 | 1.12 | 2.41 | 26.74 |
| Handball | 98 | 1.00 | 2.68 | 10.20 |
| Gymnastics | 76 | 0.72 | 2.53 | 34.21 |
| Powerlifting | 97 | 1.71 | 2.55 | 9.28 |

Results of the comparison of those sports using a one-way ANOVA test (Tukey HSD post hoc test) are presented in Table 1.4.6.

**Table 1.4.6 Comparison of mean urinary caffeine concentrations in different sports in 2004 [a]**

| | Cycling 1.40 µg/ml | Athletics 0.95 µg/ml | Boxing 0.87 µg/ml | Judo 0.83 µg/ml | Swimming 0.45 µg/ml | Motorsports 1.12 µg/ml | Soccer 1.00 µg/ml | Basketball 0.83 µg/ml | Volleyball 1.03 µg/ml | Handball 1.00 µg/ml | Bodybuilding 1.35 µg/ml | Gymnastics 0.72 µg/ml | Powerlifting 1.71 µg/ml |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Powerlifting | = | ≠ | ≠ | ≠ | ≠ | = | ≠ | ≠ | ≠ | ≠ | = | ≠ | |
| Gymnastics | ≠ | = | = | = | = | = | = | = | = | = | = | | |
| Bodybuilding | = | = | = | = | ≠ | = | = | = | = | = | | | |
| Handball | = | = | = | = | ≠ | = | = | = | = | | | | |
| Volleyball | ≠ | = | = | = | ≠ | = | = | = | | | | | |
| Basketball | ≠ | = | = | = | ≠ | = | = | | | | | | |
| Soccer | ≠ | = | = | = | ≠ | = | | | | | | | |
| Motorsports | = | = | = | = | ≠ | | | | | | | | |
| Swimming | ≠ | ≠ | ≠ | = | | | | | | | | | |
| Judo | ≠ | = | = | | | | | | | | | | |
| Boxing | ≠ | = | | | | | | | | | | | |
| Athletics | ≠ | | | | | | | | | | | | |
| Cycling | | | | | | | | | | | | | |

a post hoc test: Tukey HSD. = no significant difference ($\alpha = 0.05$). ≠ significant difference ($\alpha = 0.05$)

Six athletes (0.13%) delivered a urine sample with a caffeine concentration higher than the former threshold level of 12 µg/ml. Five of them had a urinary caffeine concentration higher than 16 µg/ml.

A comparison was made between the results obtained before and after the removal of caffeine from the WADA doping list of prohibited substances. The resulting caffeine concentrations, percentages of samples with a urinary caffeine concentration below the LOQ and percentages of positive results for the most frequently tested sports are summarised in Table 1.4.7.

**Table 1.4.7 Comparison of the average urinary caffeine concentrations (µg/ml), percentage of samples with a urinary caffeine concentration below the LOQ and percentage of positive results based on the 12 µg/ml threshold level in frequently tested sports in the period 1993-2002 and in 2004.**

| | Average Concentration | | Percentage < LOQ | | Percentage Positives ** | |
|---|---|---|---|---|---|---|
| | 1993-2002 | 2004 | 1993-2002 | 2004 | 1993-2002 | 2004 |
| Cycling | 1.34 | 1.40 | 22.17 | 16.84 | 0.09 | 0.27 |
| Athletics | 1.06 | 0.95 | 32.50 | 20.73 | 0.16 | 0.28 |
| Boxing | 1.01 | 0.87 | 35.23 | 21.48 | 0 | 0 |
| Judo | 0.97 | 0.83 | 43.60 | 35.11 | 0 | 0 |
| Swimming | 0.90 | 0.45* | 45.12 | 48.00 | 0 | 0 |
| Motor Sport | 1.14 | 1.12 | 39.33 | 26.44 | 0 | 0 |
| Soccer | 1.05 | 1.00 | 27.80 | 18.95 | 0.08 | 0 |
| Basketball | 1.04 | 0.83* | 32.01 | 29.70 | 0 | 0 |
| Volleyball | 1.20 | 1.03 | 20.90 | 8.77 | 0.31 | 0 |
| Handball | 1.27 | 1.00 | 18.35 | 11.49 | 0 | 0 |
| Bodybuilding | 1.72 | 1.35 | 26.27 | 27.03 | 1.18 | 2.70 |
| Gymnastics | 0.59 | 0.72 | 68.29 | 34.21 | 0.81 | 0 |
| Powerlifting | 1.74 | 1.71 | 17.12 | 9.28 | 2.05 | 0 |

* Significant difference in average concentration ($\alpha$=0.05) between 1993-2002 and 2004

** Positive according to the former threshold level of 12 µg/ml

## *1.5. Discussion*

Because at low urinary caffeine levels no distinction can be made between caffeine use intended to improve athletic performance and normal caffeine use of coffee and tea, WADA removed caffeine from the list of prohibited substances from January 1st 2004 on. However, some WADA-accredited laboratories (not the Ghent Doping Control Laboratory) were asked

to continue monitoring caffeine levels in order to control if caffeine concentrations above the former threshold level would be detected after the removal of this drug from the WADA-list of prohibited substances. In this study all caffeine concentrations in the period 1993-2002 have been evaluated and were compared to results obtained in 2004.

### 1.5.1. 1993-2002

Of all samples analysed in the Doping Control Laboratory (DoCoLab - UGent) during this period, only in-competition samples were screened for caffeine, resulting in 11361 samples originating from 90 different categories of sport. The distribution of the urinary caffeine concentrations was positively skewed (Figure 1.4.1).

In order to compare the caffeine concentrations amongst different sports the data (concentrations below LOQ were not included) was log-transformed into a gaussian form. This allowed for the determination of an overall average concentration of 1.22 µg/ml with a standard deviation of 2.45 µg/ml resulting in an apparent threshold level of 11.02 µg/ml (average + 4 x SD). Hence, the former caffeine threshold level of 12 µg/ml seemed reasonable.

Only those sports with more than 200 samples analysed over the period 1993-2002 were included in this study. From the results in Table 1.4.1, a large variation in average caffeine concentration between sports (cfr bodybuilding and swimming) can be observed as well as within each sport (cfr standard deviation).

Overall concentrations found in bodybuilding are significantly higher than in other sports including cycling and ball sports (Table 1.4.2). This could be due to the fact that caffeine in combination with ephedrine is advertised as a fat burner. Several studies have demonstrated that ephedrine, particularly in combination with caffeine, is effective in promoting weight loss without adverse effects [35, 36]. Numerous bodybuilding sites on the internet promote the ephedrine – caffeine – aspirin stack (ECA-stack) for its fat burning effect. These stacks are also commercially available on these sites. This could be an explanation for the significantly higher concentrations found in bodybuilding when compared to other sports.

Also caffeine concentrations found in cycling are significantly higher than in other sports, except bodybuilding and volleyball (Table 1.4.2). Use of caffeine by cyclists is popular in the late stage of a race for its a stimulating properties and ergogenic effect on endurance performance [5, 7-9, 11-13].

Most positive cases in the period 1993-2002 were found in cycling (Table 1.4.3), but taking into account the number of samples being analysed this only accounts for a small percentage. Strength sports including bodybuilding, powerlifting and weightlifting are the sports where the highest percentage of positive cases were noticed.

### 1.5.2. 2004

In total 4633 samples originating from in-competition controls tested in the Ghent Doping Control Laboratory in 2004 were screened for caffeine. These samples originate from 56 different sports. Percentages of caffeine values higher than 4 µg/ml, a caffeine concentration found in moderate to frequent coffee drinkers [34] were found to be higher in cycling and strength sports compared to the overall percentage and to values observed in other tested sports, indicating the popularity of caffeine in those particular sports. Compared to the previously examined period, the overall percentage remained approximately the same while the percentage in cycling has increased. The percentages in the other tested sports remained roughly the same after the removal of caffeine from the WADA doping list (Table 1.4.4).

The distribution of the urinary caffeine concentrations in 2004 was positively skewed (Figure 1.4.3). Comparison of the caffeine concentrations amongst different sports in 2004 was achieved by a log-transformation, allowing the data to adapt into a gaussian form. Urinary caffeine concentrations below the LOQ were not included in this transformation. These samples accounted for 20.33% of all samples analysed in 2004, 6 % less than in the period 1993-2002 (26.42%). The transformation resulted in an overall average concentration and a standard deviation of respectively 1.12 µg/ml and 2.68 µg/ml with a calculated apparent threshold level of 11.84 µg/ml (average + 4 x SD). Similar handling of the data in the period 1993-2002 has indicated an average urinary caffeine concentration of 1.22 µg/ml and an apparent threshold level of 11.02 µg/ml. These values approach the former threshold level of

12 µg/ml, indicating that this threshold level was an acceptable criterion to discriminate abuse from use.

Comparison of the caffeine concentrations after transformation was performed between the most frequently tested sports in 2004 (Table 1.4.5). Large variations in average caffeine concentrations between sports (e.g. swimming and powerlifting) as well as within each sport (standard deviation) were observed.

As can be seen in Table 1.4.6, overall concentrations in powerlifting were significantly higher than in almost all other sports including athletics and ball sports. As already mentioned in §1.5.1 the use of caffeine in strength sports, often in combination with ephedrine, is popular among strength athletes as a fat burner. The commercial availability of caffeine on internet sites promoting the ECA-stack could be indicative for the higher concentrations of caffeine found in powerlifting.

Similar to the period 1993-2002 caffeine concentrations in cycling were significantly higher when compared to most other sports. The use of caffeine by cyclists is popular in the late stage of a race for its stimulating and performance enhancing effects [5, 8, 9, 11, 12].

From the comparison of average caffeine concentrations, percentages of samples below the LOQ and percentages of positive results in 2004 and in the previously examined period (Table 1.4.7) it appears that in cycling and gymnastics caffeine concentrations have increased after the removal of caffeine from the doping list although the increase is not significant on the 95% confidence interval. Caffeine concentrations in swimming and basketball however were significantly lower in 2004 when compared to the period 1993-2002.

A trend towards a lower percentage of samples with a urinary caffeine concentration below the LOQ of 0.1 µg/ml was also observed in all sports except swimming and bodybuilding. The higher percentage of concentrations below the LOQ in swimming is reflected in the significant decrease in average caffeine concentration after the removal of caffeine from the WADA doping list. In bodybuilding caffeine concentrations below the LOQ increased and the average concentration decreased although the percentage of positive results increased in 2004. This trend could be due to the low number of samples analysed in bodybuilding in 2004.

Six samples were found to contain a urinary caffeine concentration higher than the former threshold level of 12 µg/ml. Four of them (0.27%) were found in cycling ($C_{max}$: 20.84 µg/ml), one (0.28%) in athletics ($C_{max}$: 16.59 µg/ml) and one (2.70%) in bodybuilding ($C_{max}$: 17.70 µg/ml). The increase in percentage of positive samples in cycling is in accordance with the significantly higher average concentrations found in cycling. No other doping substances were detected in the urine samples exhibiting a caffeine concentration higher than the former threshold level of 12 µg/ml. In the samples with a urinary caffeine concentration higher than 4 µg/ml four samples contained a diuretic agent, 3 of them were found in strength sports. Six samples contained a stimulant, 4 of them were ephedrine. Out of the samples containing the stimulant ephedrine, the maximal caffeine concentration was found in bodybuilding ($C_{caf}$ = 11.78 µg/ml), confirming the previous statement that the combination of both drugs in strength sports seems to be common practice.

## *1.6. Conclusion*

Analysis of 11361 urine samples collected for doping control purposes before the removal of caffeine from the doping list resulted in caffeine concentrations far below the former threshold level of 12 µg/ml. Significantly higher caffeine concentrations were found in bodybuilding and cycling compared to other frequently tested sports.

From the study conducted in 2004 it can be concluded that the overall percentage of samples below the limit of quantification has decreased in 2004. Nevertheless, the overall average caffeine concentration did not significantly differ compared to the period 1993-2002 resulting in an apparent threshold level similar as calculated in the previously examined period. These apparent threshold levels approach the former threshold level of 12 µg/ml.

Caffeine concentrations in individual sports have decreased after the removal of caffeine from the doping list except for cycling and powerlifting. In 1993-2002 caffeine concentrations in those sports were also significantly higher than in other sports highlighting the purported stimulating and ergogenic effects of caffeine in those particular sports.

Generally, caffeine concentrations did not increase after the removal of caffeine from the WADA doping list and consequently the misuse of caffeine did not increase. As the misuse of

caffeine did not increase after the removal from the WADA doping list one could conclude that a threshold level is not necessary. On the other hand, average caffeine concentrations remained far below the former threshold level indicating that dietary caffeine does not result in high urinary caffeine concentrations. Hence, caffeine concentrations higher than 12 µg/ml remain indicative for misuse. If the goal of drug testing is to prevent unfair advantage, to encourage ethical behaviour and to protect the health of the athletes, the position of caffeine with the previous threshold level of 12 µg/ml on the doping list should be reconsidered.

## *1.7. Acknowledgements*

The authors wish to thank the Flemish Ministry of Health for financial support

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# CHAPTER 2: MORPHINE

Adapted from:

- W. Van Thuyne, P. Van Eenoo and F.T. Delbeke, *Urinary concentrations of morphine after the administration of herbal teas containing Papaveris fructus in relation to doping analysis.* J. Chromatogr. B, 2003. **785**: 245-251.

# Chapter 2: Morphine

## *2.1. Introduction*

The knowledge of practices and remedies for alleviating pain dates back to the earliest times. Although Babylon, Egypt and mythological Greece have disclosed rare and scanty evidence of their use, Greece and Rome, on the other hand have given us numerous detailed documents concerning analgesic preparations and their efficacy.

These preparations, as described by Galenus and Dioscorides, were obtained mostly from plants. The most important include mandrake, henbane, certain varieties of hemp and *Papaver somniferum*.

Regarding analgesic properties the opium poppy, originating from *Papaver somniferum*, was certainly the plant with the widest and most justified reputation. Opium and its diverse preparations, entering Europe in the Middle Ages by Arab medicine, have always been identified as the most effective remedies against pain. Opium is the dried juice obtained by making incisions in the green capsules of the *Papaver somniferum*. Varieties of this plant, growing in the Balkans, Asia Minor, Persia, India, China, etc., can be used for the preparation of opium.

This raw product was used in medicine for centuries, in the form of tinctures, elixirs and compound powders, without the knowledge that only one constituent was responsible for the activity and differed in activity according to the origin of the plant.

At the beginning of the nineteenth century, Sertürner in Germany, Derosne and Séguin in France accomplished the isolation of the active constituent of opium [1-3]. These findings passed unnoticed and it was only the second publication of Sertürner in 1817 [4], describing the active principle as “morphine”, that gave rise to a large number of studies.

The discovery of morphine was of great importance because it was for the first time that a basic substance had been isolated from a plant as well as the first indications that the activity of a complex herb was attributed to a clearly identified chemical product. The name

"alkaloid", resembling an alkali, was adopted for this whole group of basic substances. Large numbers of alkaloids were discovered subsequently.

Already in 1803, Derosne had described a precipitate obtained by the action of potassium carbonate on an aqueous extract of opium which he called "salt of opium" [2]. According to Sertürner this salt of opium was acid meconate of morphine [1]. According to Pelletier it was narcotine. This alkaloid was later isolated from opium but, in spite of its name, did not possess any narcotic activity.

Another work of historical importance was Séguin's communication to the Académie des Sciences in 1804, in which he described the isolation and characterisation of morphine. Because this work was not published until 1814 [3], the discovery of morphine was attributed to Sertürner and not to Séguin.

### 2.1.1. Detection of morphine

GC/MS is one of the recommended analytical techniques for the identification, quantification and confirmation of opiates including morphine in urine [5-8]. Hydrolysis is required to obtain unconjugated morphine, as morphine is excreted in urine as 3- and 6-glucuronide (Figure 2.1.1). Enzymatic hydrolysis with β-glucuronidase has proven to give the best result [9].

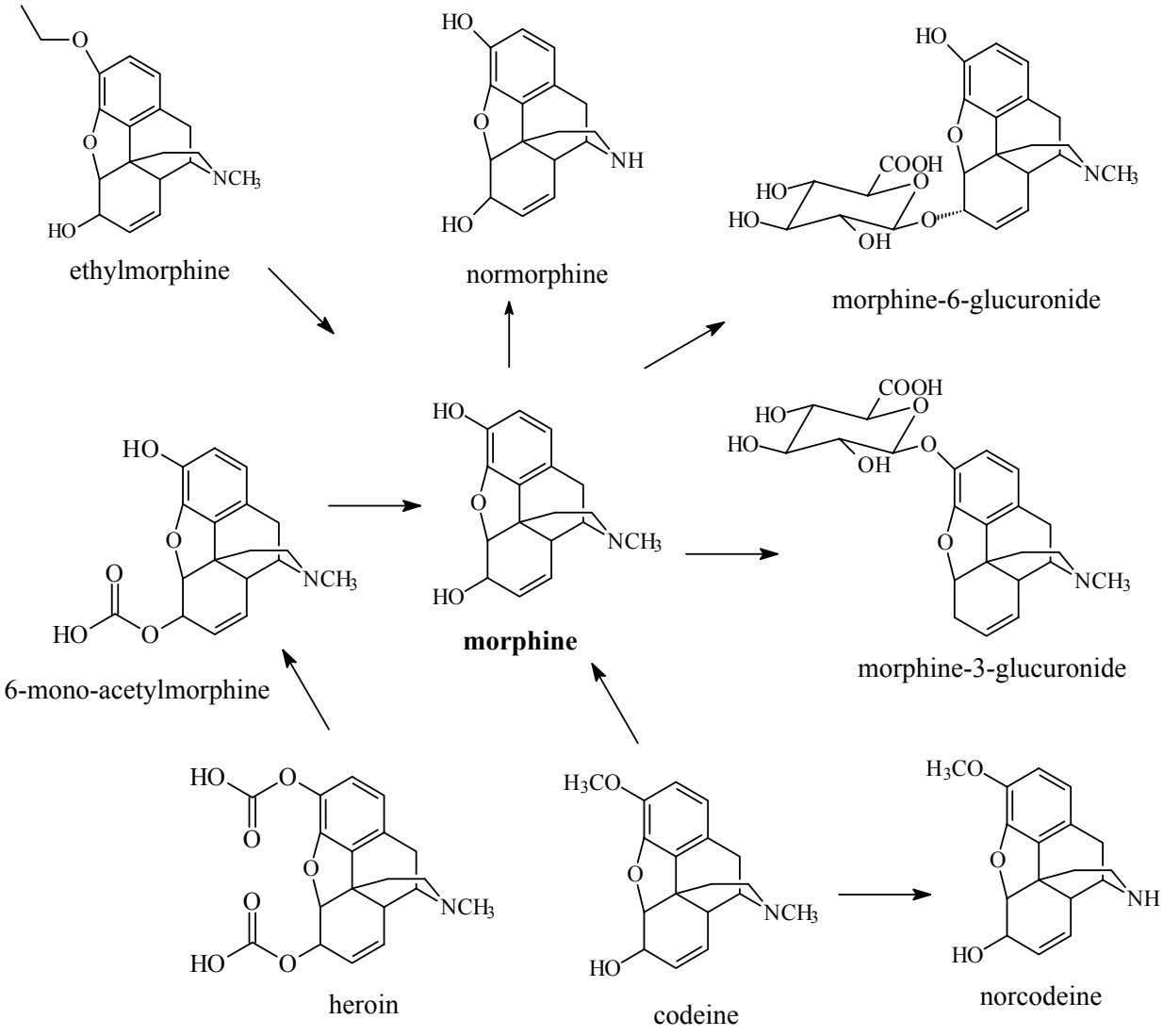


**Figure 2.1.1 Metabolic pathway of morphine and analogues.**

To accomplish GC detection of morphine, the phenolic hydroxy group of morphine must be derivatized before GC separation. Various derivatization procedures, including trimethylsilylation have been described in the literature [10].

### 2.1.2. Morphine and doping

Morphine is a narcotic analgesic commonly used for the short-term treatment of post surgery pain and in long terms for the pain relief of cancer patients [11]. Because of these painkilling effects morphine can be misused by athletes during training or in competition to overcome pain associated with strenuous exercise. This was the major reason why morphine was

included in the IOC list of banned substances [12]. Before 1991, the presence of morphine in human urine, irrespective of its concentration, resulted in a positive doping test. This changed in 1991 because the UCI introduced a 1 μg/ml threshold level for morphine as well as for codeine, one of the precursors of morphine (Figure 2.1.1).

The rules were later on adapted to the rules that are now in force. At present the use of codeine and ethylmorphine, another morphine precursor, is allowed while morphine is forbidden in concentrations higher than 1 μg/ml.

Already in 1991 a study was carried out by Delbeke *et al.* [13] proving that morphine positive urine samples could be delivered six to nine hours after the administration of a therapeutic dose of codeine. A study by Selavka [14] dealt with the possible contribution of poppy seed containing food to positive opiate urinalysis results. This "poppy seed defense" was mainly used by job-applicants controlled by the U.S. Department of Defense or by the National Institute on Drug Abuse [15-17]. Several other experiments were also carried out on the detection of opiates, mainly morphine, after the use of various poppy seed containing food products including cakes and bread rolls [18-22]. The major results from these investigations were that morphine was detected after the use of poppy seed incorporated in food products but the resulting concentrations of morphine in urine were too low to result in a positive urine sample. Another outcome from these studies was that the morphine concentration of poppy seeds substantially differed depending on the origin of the investigated sorts. Although several studies dealt with the detection of morphine after the use of poppy seed containing food [14-18, 20-22], no other related studies were found describing the possible contribution of other parts of the plant *Papaver Somniferum* in positive opiate results.

The objective of the present study was to determine whether doping positive urine samples could be delivered after the administration of "poppy cake" and of herbal teas containing parts from the plant *Papaver Somniferum*. The quantitative GC-MS method was validated according to procedure described in Part I.

## *2.2. Experimental*

### 2.2.1. Reagents

Morphine-3-glucuronide was purchased from Lipomed (Arlesheim, Switzerland), nalorphine.HBr was obtained from Janssen Pharmaceuticals (Beerse, Belgium). The enzyme preparation β-glucuronidase type HP-2 from Helix pomatia (127 300 U/ml β-glucuronidase) was obtained from Sigma (St. Louis, MO). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was bought from Chem. Fabrik. Karl Bucher (Waldstetten, Germany). All other chemicals were of analytical grade.

Grounded poppy seeds (Geriebene Mohn) were bought from Backmit (Vienna, Austria).

Two herbal teas were bought as over-the-counter products at a local chemist. The labeled content of the first tea (**A**) (Tisane Ardennaise, Tilman S.A., Bomal, Belgium) was as follows: Papaveris fructus : 15% - Crataegi folium 12.5% - Passiflorae mexico herba 10% - Viscum album 7.5% - Oleae europaea folium 13.75% - Cynodon dactylon rhizae 12.5% - Triticum rhizae 1.25% - Frangulae cortex 13.75% - Glycyrrhizae radix 13.75%. The recommended dosage was 1-3 cups a day. The content of the second tea (**B**) (Thé de sapin, Laboratoires Colin S.A., Blegny, Belgium) was: Rubi fructicosi folium 31.4% - Carraghene 24% - Papaveris fructus 14.7% - Thymi serpylli herba 7.8% - Eucalypti folium 7.8% - Glycyrrhizae radix 6.9% - Anisi stellati fructus 3% - Hederae terrestris herba 1.2% - Erysimi herba 1.2% - Capilli veneri herba 1% - Salviae folis 1% - Cum pini essent imbuta. A daily dosage of 1 to 3 cups was recommended.

### 2.2.2. Gas chromatographic conditions

The GC/MS analysis was conducted in full scan mode (m/z 50 – 550) on an Agilent 6890 gas chromatograph directly coupled to an Agilent 5973 mass selective detector (Agilent, Palo Alto, USA). The GC column was an Agilent-Ultra 1 (J&W, Folsom, USA), 100 % methylsilicone column with a length of 17 m, an internal diameter of 0.2 mm and a film thickness of 0.11 μm. Helium was used as the carrier gas (linear velocity: 41 cm/s). A total of 0.5 μl was injected splitless. The oven temperature program was as follows: 70°C (0 min) –

70°C/min → 96°C (1 min) – 20°C/min → 320°C (1 min). The electron energy was set at 70 eV and the ion source temperature was 230°C.

### 2.2.3. Urine analysis

One ml of a sodium acetate buffer (pH 5.2, 1 M) and 50 µl β-glucuronidase were added to 3 ml of urine. The mixture was hydrolyzed during 2.5 hours at 56°C. After cooling, 50 µl of the internal standard (nalorphine, 20 µg/ml, MeOH) and 0.5 ml of the ammonium buffer (pH 9.5) were added. Extraction was performed with 5 ml of the mixture $CH_2Cl_2$/MeOH (9/1; v/v) by rolling for 20 minutes. After centrifugation (1200 g, 5 min) the organic layer was separated, dried over anhydrous $Na_2SO_4$ and evaporated under OFN. The residue was derivatized with 100 µl MSTFA at 80°C for 10 min and transferred to an autosampler microvial.

### 2.2.4. Morphine content of tea

0.5 ml of the ammonium buffer (pH 9.5) and 50 µl of the internal standard nalorphine (20 µg/ml, MeOH) were added to 3 ml of the prepared tea. Extraction was performed with 5 ml of $CH_2Cl_2$/MeOH (9/1; v/v) by rolling for 20 minutes. The organic layer was separated after centrifugation (1200 g, 5 min), dried over anhydrous $Na_2SO_4$ and evaporated under OFN. The residue was derivatized with 100 µl MSTFA at 80°C for 10 min. The herbal teas were extracted and analyzed in duplicate by the described method. Tea A was diluted 20 times with distilled water, tea B 30 times.

### 2.2.5. Excretion protocols

A home made cake was prepared containing 200 g of grounded poppy seeds, according to a recipe on the package of the grounded poppy seeds (Geriebene Mohn, Backmit, Austria). Urine samples were collected before (0 h) and quantitatively up to 12 h after the start of the experiment. An additional sample was taken after 24 h.

Twelve table spoons of tea A were infused in 12 cups of boiling water for 10 minutes after which the liquid was sieved. Each volunteer had two cups of this tea of approximately 120

ml/cup. Urine samples were collected before (0h) and quantitatively after 1, 2, 4, 6, 9, 12 h. An additional sample was taken after 24 h.

Tea B was prepared by pouring 1.5 l boiling water over a filter containing 22 table spoons of the tea. Each volunteer drank two cups of tea of each 130 ml. Urine sampling was similar as in the previous experiment except that no sample was taken after 24 h.

## *2.3. Analytical Method Validation*

Under the described chromatographic conditions, morphine as well as the internal standard nalorphine gave sharp peaks with retention times of 9.35 min and 9.84 min respectively (Figure 2.3.1).

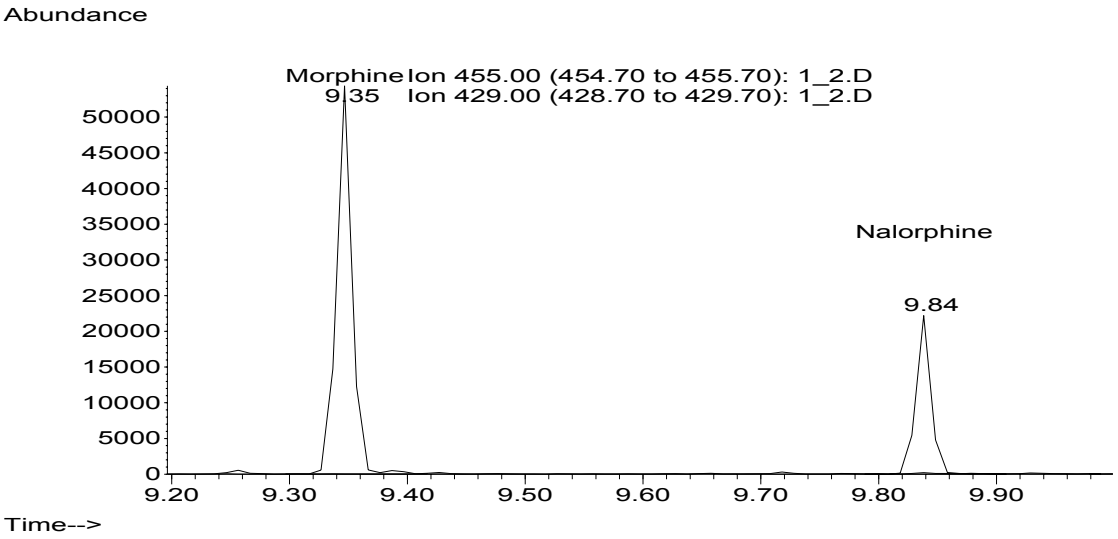


**Figure 2.3.1 Extracted ion chromatogram of morphine-bis-TMS (m/z 429) and nalorphine-bis-TMS (m/z 455) in urine 1h after the administration of herbal tea B.**

Quantification of morphine in the samples was done by the determination of the ratio of abundances of the molecular ion m/z 429 of TMS-derivatized morphine to m/z 455 of the internal standard nalorphine. Unequivocal determination in doping analysis is mostly based on full scan mass spectrometry. The spectrum of TMS-derivatized morphine is shown in Figure 2.3.2.

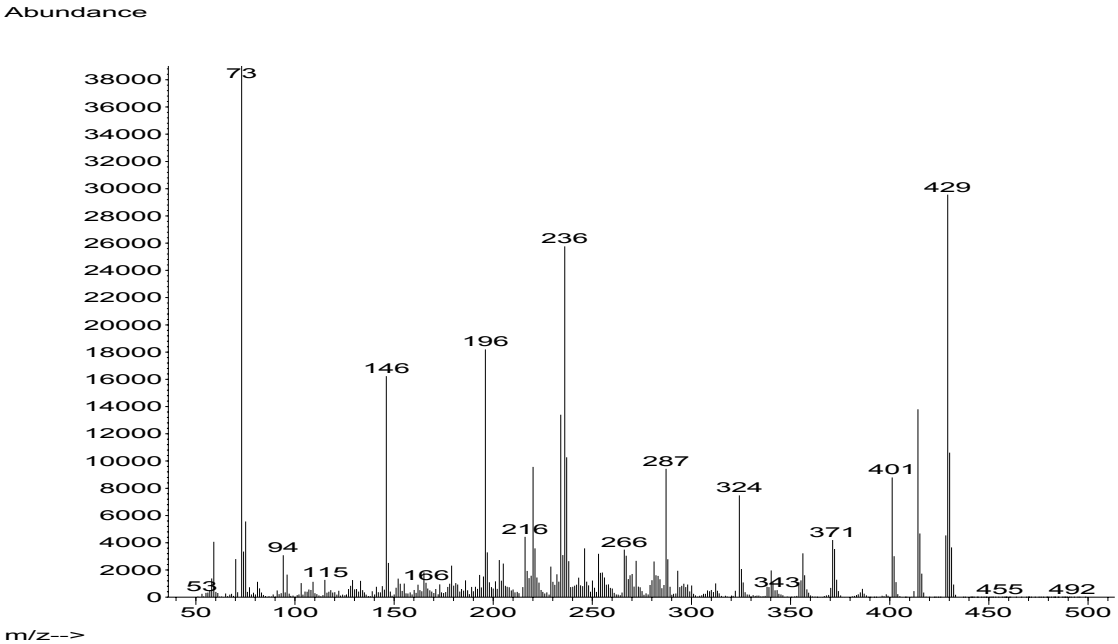


**Figure 2.3.2 Mass spectrum of TMS-derivatized morphine.**

Diagnostic ions besides the molecular ion are the ions with m/z 236, 287, 324, 401 and 414. Based on WADA criteria a maximal relative deviation of 20 % (m/z 287 , 324 and 401) and an absolute deviation of 10 % (m/z 236) in the intensities of these diagnostic ions was used as a qualitative criterion for the relative abundances when compared to a quality control sample spiked with morphine at 1 µg/ml [23]. All samples in the method validation and the excretion studies in which morphine was detected fulfilled this qualitative criterion.

An equally weighted linear calibration curve (not forced through zero) was established in the concentration range from 0 to 2 µg/ml (0, 0.25, 0.50, 1.00, 1.50 and 2.00 µg/ml) by plotting the relative abundances of the ions m/z 429 and m/z 455 for morphine and nalorphine, respectively. Therefore, blank urine, checked for the absence of morphine and nalorphine, spiked with the appropriate amount of morphine standards (100 µg/ml and 10 µg/ml in MeOH), was analysed. Each concentration was analysed in triplicate, the averages were used to construct the calibration curve. The correlation coefficient of the calculated calibration curve was 0.9983. The deviation of the mean measured concentration from the theoretical concentration (trueness) was far below the maximal value of 15 % [24].

The precision was evaluated by the determination of the repeatability and the reproducibility. To measure the repeatability 6 samples at three different concentrations (0.25, 1.00 and 2.00

µg/ml) were analysed. The relative standard deviation, an indicator of the repeatability, for each of the three concentrations (0.25, 1.00 and 2.00 µg/ml) never exceeded the limit values derived from the Horwitz equation [25]. Measured concentrations, relative standard deviations and maximal limits for the repeatability are listed in Table 2.3.1.

**Table 2.3.1 Method repeatability.**

| | | | |
|---|---|---|---|
| Theoretical concentration (µg/ml) | 0.25 | 1.00 | 2.00 |
| Average measured concentration (µg/ml) (n=6) | 0.25 | 0.95 | 2.04 |
| SD (µg/ml) | 0.01 | 0.03 | 0.06 |
| RSD (%) | 3.82 | 3.09 | 2.97 |
| 2/3 $RSD_{max}$ (%) | 13.10 | 10.67 | 9.61 |
| Accuracy (%) | 0.19 | -5.03 | 1.94 |

The reproducibility was examined by analysing 6 samples in triplicate at the same concentrations as for the repeatability. This was done by two different analysts, at different times. The results for the reproducibility are summarized in Table 2.3.2. The original limit values calculated by the Horwitz equation were used while for the repeatability the margin was narrowed to two third of the calculated value. The accuracy was measured for both repeatability and reproducibility and is listed in Table 2.3.1 and Table 2.3.2. A limit value of 15 % is used as for the trueness of the datapoints from the calibration curve.

**Table 2.3.2 Method reproducibility.**

| | | | |
|---|---|---|---|
| Theoretical concentration (µg/ml) | 0.25 | 1.00 | 2.00 |
| Average measured concentration (µg/ml) (n=18) | 0.24 | 0.91 | 1.99 |
| SD (µg/ml) | 0.01 | 0.04 | 0.05 |
| RSD (%) | 2.88 | 4.30 | 2.59 |
| $RSD_{max}$ (%) | 19.65 | 16.01 | 14.42 |
| Accuracy (%) | -2.53 | -9.49 | -0.42 |

Selectivity was tested by the analysis of negative urine spiked with structurally related compounds such as codeine and ethylmorphine and some other narcotic or stimulating compounds including methadone, pholedrine, pethidine, prolintane, etc.

Specificity was tested by analysing 20 different negative urines.

No interference by any of the structurally related or other compounds spiked in negative urine could be found for morphine as well as for the internal standard nalorphine. Hence, the method was selective for morphine as well as for nalorphine. Analysis of 20 different negative urines did not result in the detection of matrix interferences, proving the specificity of the method.

The limit of quantification of the method was 0.25 µg/ml or the lowest point of the calibration curve where morphine could be detected in a reproducible way. The limit of detection was arbitrarily set at 0.125 µg/ml, half the limit of quantification.

## *2.4. Excretion Studies*

All excretion studies were approved by the Ethical Committee, University Hospital (Ghent, Belgium) (EC/2005-81/sdp) and were performed on healthy volunteers. The purpose of the study was explained to each volunteer who where asked not to use morphine, codeine or ethylmorphine containing preparations, nor to eat food containing poppy seeds or parts of the plant *Papaver somniferum* for 1 week before the start of each experiment. Excretion studies

were performed with a poppy seed containing cake and herbal teas containing parts of the plant *Papaver somniferum*.

### 2.4.1. Excretion studies with a poppy seed containing cake

Each of the 5 volunteers ate 1/8 of the cake. Urinary pH, volume and density were measured and all samples were analyzed in duplicate. When appropriate, urine samples were diluted with distilled water before extraction in order to obtain a morphine concentration in the range of the calibration curve.

Urinary excretion profiles after the use of the "poppy-cake" are given in Figure 2.4.1. Morphine could be detected in the urine of all volunteers already 1 h after the start of the experiment and remained detectable in all volunteers until 12 h. In subject 4 the doping threshold level of 1 µg/ml was attained 2 hours after the administration while in subject 2 and 3 positive findings according to the doping threshold level of 1 µg/ml were reached up to 6 h after the start of the experiment. In two other volunteers the current doping threshold level was never attained.

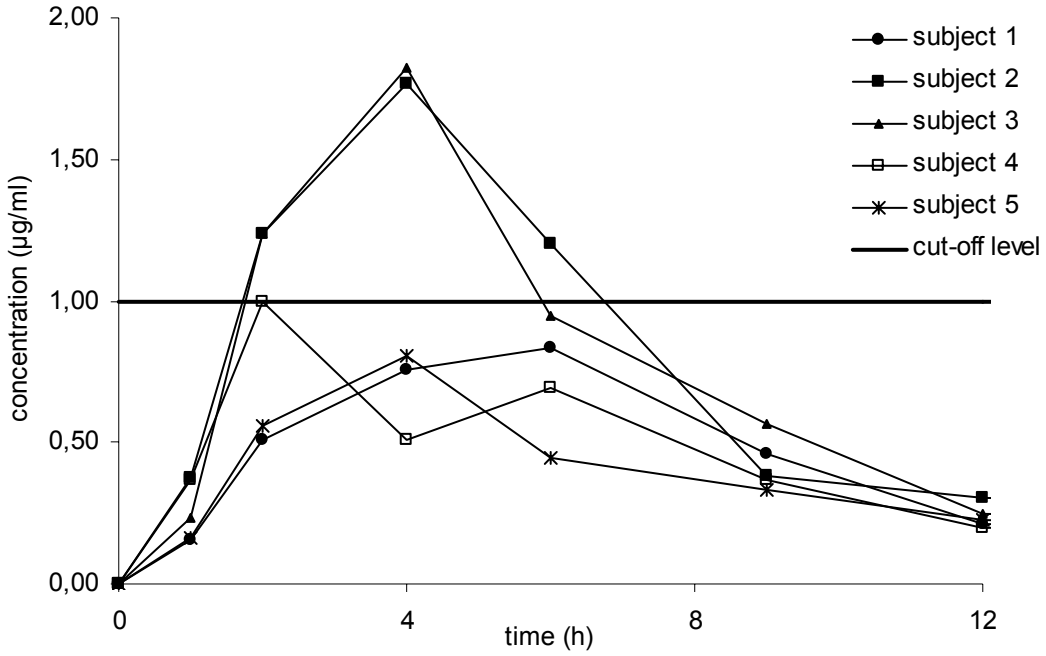


**Figure 2.4.1 Urinary excretion profiles of morphine after the use of poppy-cake.**

This study shows that the use of poppy seeds can result in the detection of morphine as previously described in the literature [14-16] and more importantly to adverse analytical findings for morphine.

### 2.4.2. Excretion studies with herbal teas

Both tea A and B were prepared following the instructions on the package. All urine samples were either analyzed directly or stored deep-frozen for later analysis. Urinary pH, volume and density were measured and all samples were analyzed in duplicate. When appropriate, urine samples were diluted with distilled water before extraction in order to obtain a concentration in the range of the calibration curve.

Analysis of tea A indicated a morphine concentration of 10.4 µg/ml, equivalent to the ingestion of 2.5 mg of morphine when two cups of 120 ml were administered.

The urinary excretion profiles of morphine during the first twelve hours after the administration of herbal tea A are shown in Figure 2.4.2. Morphine could be detected in the urine of all volunteers already after one hour and remained detectable for at least 24 hours.

Subject 1 delivered a doping positive urine sample after 6 hours while morphine in the urine of the other 4 volunteers exceeded the 1 µg/ml doping threshold already one hour after drinking the tea. The maximum morphine concentration found after drinking this herbal tea was 4.34 µg/ml. Morphine positive urine samples were detected up to six hours. All subjects tested doping negative for morphine after 9 h.

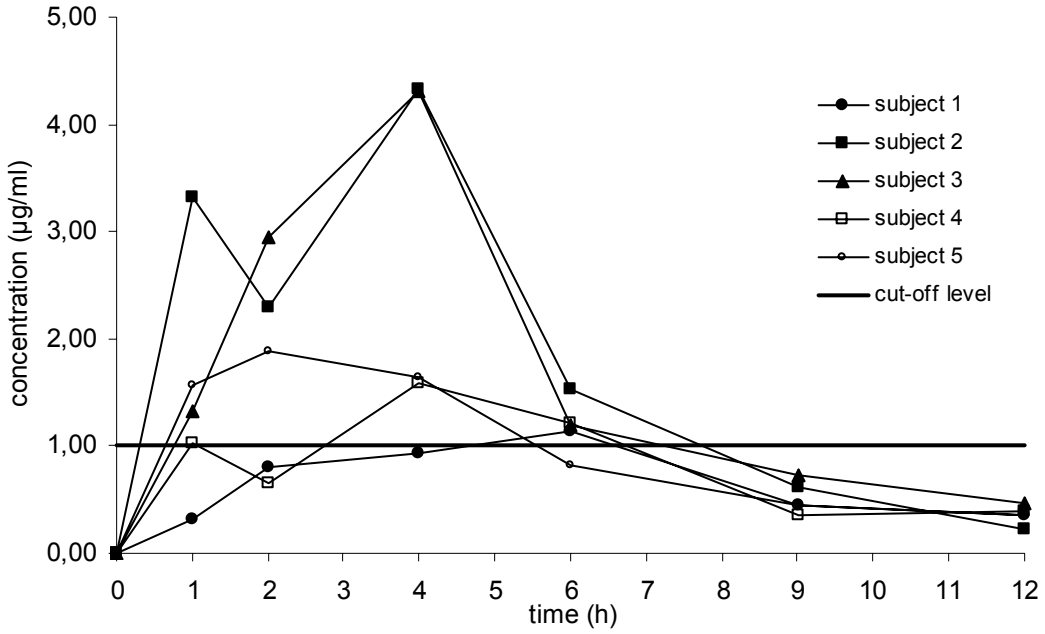


**Figure 2.4.2 Urinary excretion profile after the ingestion of two cups of tea A in 5 subjects.**

Urinary excretion and excretion rates of morphine during the first 12 hours after drinking this tea are given in Table 2.4.1.

**Table 2.4.1 Excreted amounts (mg) and rates (mg/h) of morphine in the urine of 5 subjects after drinking herbal tea A.**

| Time interval | Excreted Amount (mg) | | | | | Excretion Rate (mg/h) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| (h) | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| 0 – 1 | 0.18 | 0.30 | 0.17 | 0.14 | 0.16 | 0.18 | 0.30 | 0.17 | 0.14 | 0.16 |
| 1 – 2 | 0.26 | 0.41 | 0.25 | 0.21 | 0.32 | 0.26 | 0.41 | 0.25 | 0.21 | 0.32 |
| 2 – 4 | 0.36 | 0.43 | 0.28 | 0.25 | 0.40 | 0.18 | 0.22 | 0.14 | 0.12 | 0.20 |
| 4 – 6 | 0.13 | 0.08 | 0.07 | 0.11 | 0.20 | 0.07 | 0.04 | 0.04 | 0.06 | 0.10 |
| 6 – 9 | 0.13 | 0.12 | 0.06 | 0.03 | 0.11 | 0.04 | 0.04 | 0.02 | 0.01 | 0.04 |
| 9 – 12 | 0.15 | 0.21 | 0.07 | 0.07 | 0.09 | 0.05 | 0.07 | 0.02 | 0.02 | 0.03 |
| Total | 1.21 | 1.55 | 0.90 | 0.81 | 1.28 | | | | | |

Maximal excretion was obtained after 2 to 4 h. Inter-individual differences in excretion of morphine were observed. An average amount of 1.15 mg morphine (0.81 mg – 1.55 mg) was excreted during the first 12 h. Based on the ingested amount of 2.5 mg, 46 % (32% - 62%) of the administered dose was excreted during that period.

Similar to the previous experiment, morphine was detected in the urine of all volunteers already 1 hour after drinking tea B and remained detectable after 12 hours. The maximal detected concentration was 7.44 µg/ml (Figure 2.4.3). Three subjects tested doping positive for morphine after 1 h. Similar "positive" results were found until 6 to 9 h after drinking the tea, doping negative results were found after 12 h.

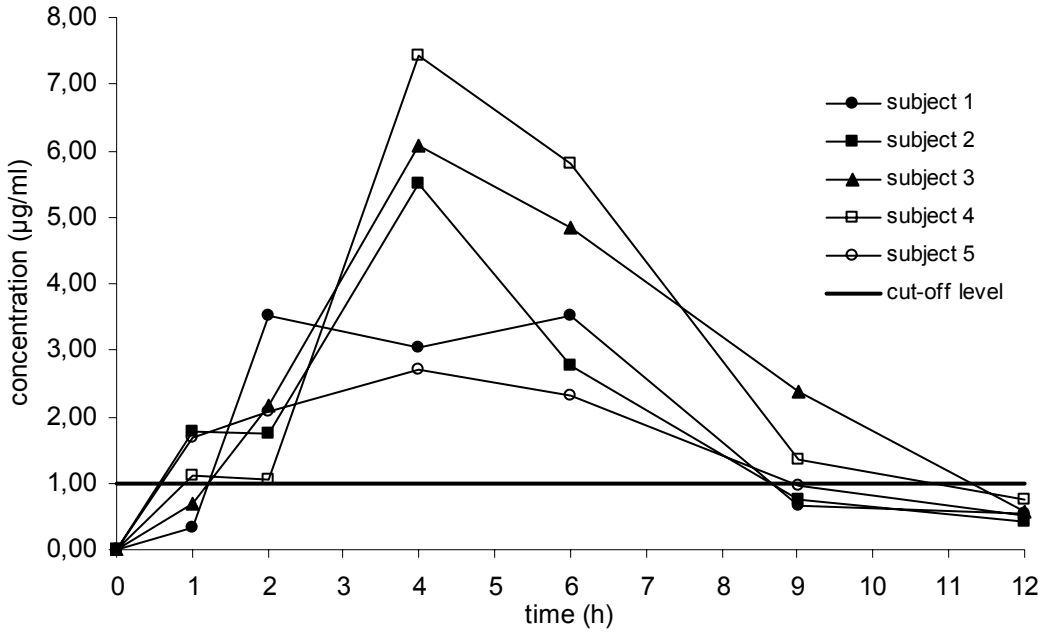


**Figure 2.4.3 Urinary concentration profiles of morphine after the administration of 2 cups of herbal tea B in 5 subjects.**

The excreted amounts and rates of morphine after the use of herbal tea B are listed in Table 2.4.2.

Maximal excretion was obtained after 2 to 4 h. Large inter-individual differences in excretion of morphine were observed. Excreted amounts in volunteer 4 are the lowest, but as a result of the low urine volume they still result in the highest detected concentrations during the excretion study. An average amount of 2.36 mg morphine (1.40 mg – 2.83 mg) was excreted during the first 12 h. Based on the ingested amount of 8.51 mg, 27.8 % (16.6% - 33.1%) of the administered dose was excreted during that period.

**Table 2.4.2 Amounts (mg) and excretion rates of morphine in the urine of 5 volunteers after drinking herbal tea B.**

| Time interval | Excreted amount (mg) | | | | | Excretion rate (mg/h) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| (h) | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| 0 – 1 | 0.22 | 0.36 | 0.44 | 0.18 | 0.22 | 0.22 | 0.36 | 0.44 | 0.18 | 0.22 |
| 1 – 2 | 0.48 | 0.63 | 0.61 | 0.31 | 0.53 | 0.48 | 0.63 | 0.61 | 0.31 | 0.53 |
| 2 – 4 | 0.87 | 0.88 | 0.76 | 0.37 | 0.82 | 0.43 | 0.44 | 0.38 | 0.19 | 0.41 |
| 4 – 6 | 0.40 | 0.44 | 0.36 | 0.29 | 0.39 | 0.20 | 0.22 | 0.18 | 0.15 | 0.20 |
| 6 – 9 | 0.29 | 0.28 | 0.43 | 0.16 | 0.33 | 0.10 | 0.09 | 0.14 | 0.05 | 0.11 |
| 9 – 12 | 0.20 | 0.13 | 0.23 | 0.09 | 0.10 | 0.07 | 0.04 | 0.08 | 0.03 | 0.03 |
| Total | 2.46 | 2.72 | 2.83 | 1.40 | 2.39 | | | | | |

As illustrated in Figures 2.4.2 and 2.4.3 doping positive results were noticed after drinking herbal teas. Unlike tea A, the package of tea B did not mention any sedative or calming effect. An athlete should be aware of the presence and the possible detection of morphine after the use of these kinds of teas.

## *2.5. Conclusion*

Problems with positive doping cases for morphine are frequently connected with the therapeutic use of its precursors codeine and ethylmorphine, substances not longer considered as doping agents. As codeine and ethylmorphine are allowed, the actual doping rules for morphine need to be revised. Hence, the simultaneous presence of morphine and codeine or morphine and ethylmorphine should not constitute a doping offence. To differ between the accidental intake of morphine by poppy seeds [14], food containing poppy seeds or herbal teas and the oral or systemic administration of morphine the actual doping threshold level for morphine in urine of 1 µg/ml should be increased.

Following international doping rules athletes remain responsible for the presence of doping substances in their biofluids, irrespective the origin. Hence, athletes should be warned against the use of food products containing poppy seeds or herbal teas containing parts from the plant *Papaver Somniferum*.

## *2.6. Acknowledgements*

The financial support by the Flemish Ministry of Health is gratefully acknowledged.

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# Part III: Nutritional supplements

# CHAPTER 1: INTRODUCTION

Adapted from:

- W. Van Thuyne and F.T. Delbeke, *Nutritional supplements: prevalence of use and contamination with doping agents*. Nutr. Res. Rev., 2006. **19**: 147-158

# Chapter 1: Introduction

## *1.1. Abstract*

Based upon recent sales numbers, nutritional supplements play a key role in the lifestyle of a substantial fraction of the population. Besides products like vitamins or minerals, also several precursors of anabolic steroids are marketed as nutritional supplements. Another group of commercially available supplements are products for weight loss based upon herbal formulations originating from *Ephedra* species. Apart from supplements indicating the presence of these active compounds, numerous non-hormonal nutritional supplements were found to be contaminated with non-labelled anabolic steroids. Also other stimulating agents besides naturally occurring analogues of ephedrine were detected.

A major group using dietary supplements are sportsmen, ranging from amateur level to elite athletes. Besides the possible health risks associated with the use of dietary supplements, athletes should take care not to violate the rules of WADA because athletes remain responsible for the substances detected in their biofluids, irrespective their origin.

Several analytical methods were developed to evaluate the presence of doping agents as contaminants. This chapter attempts to address the issues concerning the use of nutritional supplements and the detection of doping agents as contaminants in dietary supplements.

## *1.2. Introduction*

The definition of nutritional supplements is an important aspect in the discussion of this group of products. Although no universally accepted definition is available, nutritional supplements can be defined as food supplying in one or more nutrients in a concentrated form, e.g. minerals, vitamins, trace elements and other components that are theoretically present in a normal and balanced diet [1]. The DSHEA, enacted by the U.S. Congress in 1994, went far beyond a logical definition of nutritional supplements such as the definition from Schröder [1] and included, besides vitamins and minerals, also herbs or other botanicals, amino acids, metabolites, constituents, extracts or combinations of any of such ingredients. Although many of these products (particularly herbs) are marketed for their alleged preventive or therapeutic effects, the DSHEA has made it difficult to regulate them as drugs. Since the passage of the DSHEA, even hormones such as DHEA and melatonin are being sold as dietary supplements. Nutritional supplements are frequently offered in an untypical form of food, e.g. tablets, capsules, powders or pills.

The use of nutritional supplements has expanded explosively during the last decade. In 1997 vitamin sales numbers in Germany were estimated at 500 million euro, while the total sales of supplements exceeded 1 billion euro [1]. United States annual sales were estimated at U.S.$12-15 billion in the late 1990's of which U.S.$800 million were spent on sport supplements [2, 3]. In the year 2000 approximately 3.1 million kilogram of creatine was sold while a decade earlier nobody even heard of it [4]. The global market for supplements in 2001 was estimated at U.S.$46 billion, the U.S. supplement market in 2000 at U.S.$16.7 billion [5]. An important factor behind this growth was the passage of the DSHEA allowing U.S. customers free access to supplements [6]. Other factors influencing the growth of the supplement market are the easy access through the internet, which makes it possible for users all over the world to obtain all products freely available in the U.S., and the ever-improving results and record performances of top-level athletes who were promoting the use of supplements.

### 1.2.1. Use of nutritional supplements

Nutritional supplements are used in all layers of the population. General population studies on the use of supplements show a use rate of 40% [7]. Nevertheless it seems that these products are most frequently used by sportsmen ranging from amateur level to elite athletes [8], the category to which they are most aggressively marketed. This high prevalence of use can be seen in a study following the Olympic Games held in Sydney in 2000. In this study the medication use of 2758 athletes selected for doping control was evaluated. Only the reported use the last three days before competition was included. Major findings were that 51% of all tested athletes had used vitamins before competition and a large fraction of the tested athletes used other nutritional supplements. Some athletes even reported up to 26 different supplements on one day [9]. Many surveys dealing with the prevalence of use of nutritional supplements among athletes have been published [10-13]. Sobal and Marquart [14] comprehensively analysed the supplement use among athletes. Totally 10,274 athletes, both male and female, were included in this meta-analysis. Their results show that the mean prevalence of supplement use among athletes was 46%. Approximately the same percentage was found in adolescents and high school athletes [15, 16]. It is not known whether prevalence of use varies widely among gender. Sobal and Marquart [14] found significantly higher percentage of use by female athletes compared to male athletes while a study by Sundgot-Borgen *et al.* [17] did not find a significant difference between both sexes.

Differences can also be observed between sports. Supplement consumption in strength sports like weightlifting, powerlifting and bodybuilding is higher than in other sports [14, 18], confirming the statement that supplement use is most frequent in sports emphasizing on muscle size. However, great care should be taken with results obtained from questionnaires as people tend to underrate their use of nutritional supplements [8]. Also the type of question and the way those questions were put forward to the athletes could significantly influence the outcome. For instance, a higher percentage of runners admitted supplement intake when asked to respond to a frequency questionnaire (69%) than when the supplement use was recorded during a three day period (48%) [19].

Only a few studies have tried to evaluate the frequency of use of nutritional supplements. In a summary of 13 studies using time specified terms [14], it appears that most athletes rather

take their supplements on a daily or routine basis than occasionally or weekly. These supplements are often used more frequently and in higher doses than recommended on the label. The rationale behind this behaviour is the popular belief that the more supplements are taken, the better a performance will be.

However, reasons for use of dietary supplements differ according to the investigated population. Among high school students, boys tend to use supplements more for athletic performance improvement and muscle development than girls do [16]. In general, reasons for supplement use among adolescents are: losing weight and/or keeping weight at a certain level [14, 15], to gain weight and stimulate muscle development or to compensate for an inadequate diet.

Major reasons for use of supplements by athletes include compensation for an inadequate diet, enhancing performance, meeting the abnormal demands during training and/or competition, keeping up with opponents and following recommendations by parents, coaches and team mates [4, 14]. Athletes are mostly influenced by parents, doctors, coaches and friends, with female athletes being more influenced by their parents and male athletes by their coach [16].

It is the position of many official authorities, including the American Dietetic Association, Dieticians of Canada and UK Sport, that the use of supplements to compensate for needs following strenuous exercise is unnecessary when the athlete uses a well balanced diet. Supplement use without a specific need, disease or deficiency is not recommended [20]. If however athletes persist in using supplements for their purported ergogenic effects it is advised to use counselling in product selection and to use them only after careful evaluation of the product for safety, efficacy, potency and legality. In some cases, especially when the manufacturers' recommendations are not followed, the use can be harmful. Schwenk and Costley [21] described several non-anabolic nutritional supplements and their potential risks when used in high doses or without the counselling of a physician. Others have also demonstrated that with excessive use most of the supplements rather harm than improve the condition of the user [22-24].

Again, controversial information can be found on the effectiveness of enhancing sport performances for most marketed supplements. Creatine for instance is promoted for its performance enhancing effect, although several studies are contradicting [25-28].

## *1.3. Nutritional supplements and doping*

Besides the relatively harmless products (when used properly) including vitamins, minerals, etc., other types of ingredients can be present in nutritional supplements. Ingredients from plant origin including ephedrines and caffeine can be present in dietary supplements promoting ergogenic effects. Substances from botanical origin, such as morphine, can be present in the daily diet. In addition, as a consequence of the passage of the DSHEA, prohormones became freely available on the U.S. supplement market. Besides the health risks associated with these products, athletes are also at risk of violating doping rules as ephedrine, morphine and prohormones figure on the WADA list of forbidden substances [29]. An inadvertent doping test resulting from the intake of a supplement can originate from the poor knowledge by the athlete of banned substances indicated on the label, from the fact that the labelled ingredients indeed contain banned substances (e.g. Ma Huang herbal products contain ephedrine) or, more importantly, that the supplement contains a banned substance not indicated on the label.

### 1.3.1. Prohormones and anabolic androgenic steroids

As a result of the passage of the DSHEA several anabolic androgenic steroids including DHEA, androstenedione, androstenediol and 19-nor-steroids became readily available as over-the-counter products in the United States and through the internet in the rest of the world. These steroids can be marketed as nutritional supplements as long as the manufacturers make no claims about the use of a dietary supplement to diagnose, prevent, mitigate, treat or cure a specific disease. On the other hand the DSHEA allowed dietary supplements to bear a "statement of support" that: (a) claims a benefit related to a classical nutrient deficiency disease; (b) describes how ingredients affect the structure or function of the human body; (c) characterises the documented mechanism by which the ingredients act to maintain structure or function and (d) describes general well-being from consumption of the ingredients [6]. In this way, the U.S. FDA rules are circumvented and prohormones, precursors of testosterone (Figure 1.3.1) and nandrolone (Figure 1.3.2), became available on the market. These prohormones are converted in the body into their respective anabolic steroids and consequently act in the same way as the latter do. Supplement manufacturers claim that these products increase serum testosterone concentrations, promote muscle mass and muscle

strength, help reducing body fat and enhance mood and sexual performance. However, many studies do not confirm these claims [30-34]. Although in several studies an increase in serum testosterone levels was measured after prohormone use [35, 36], this does not necessarily results in increased muscle mass, strength or sexual performance [32, 36]. In addition, risks associated with the use of prohormones are similar to those observed with anabolic steroids, e.g. cardiovascular effects, liver tumours, gynaecomastia and aggressive behaviour [37-39].

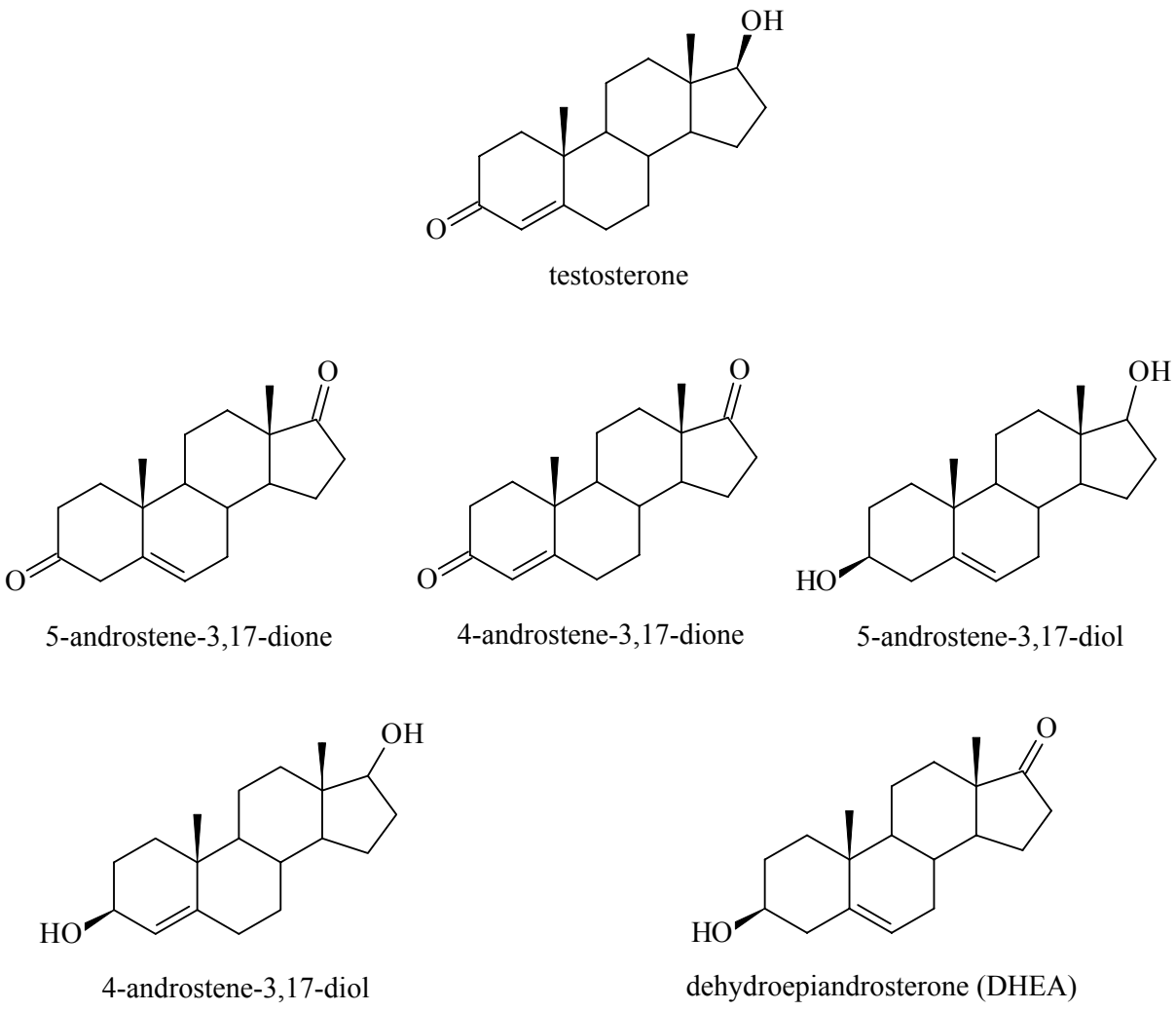



**Figure 1.3.1 Chemical structure of testosterone and some of its precursors.**

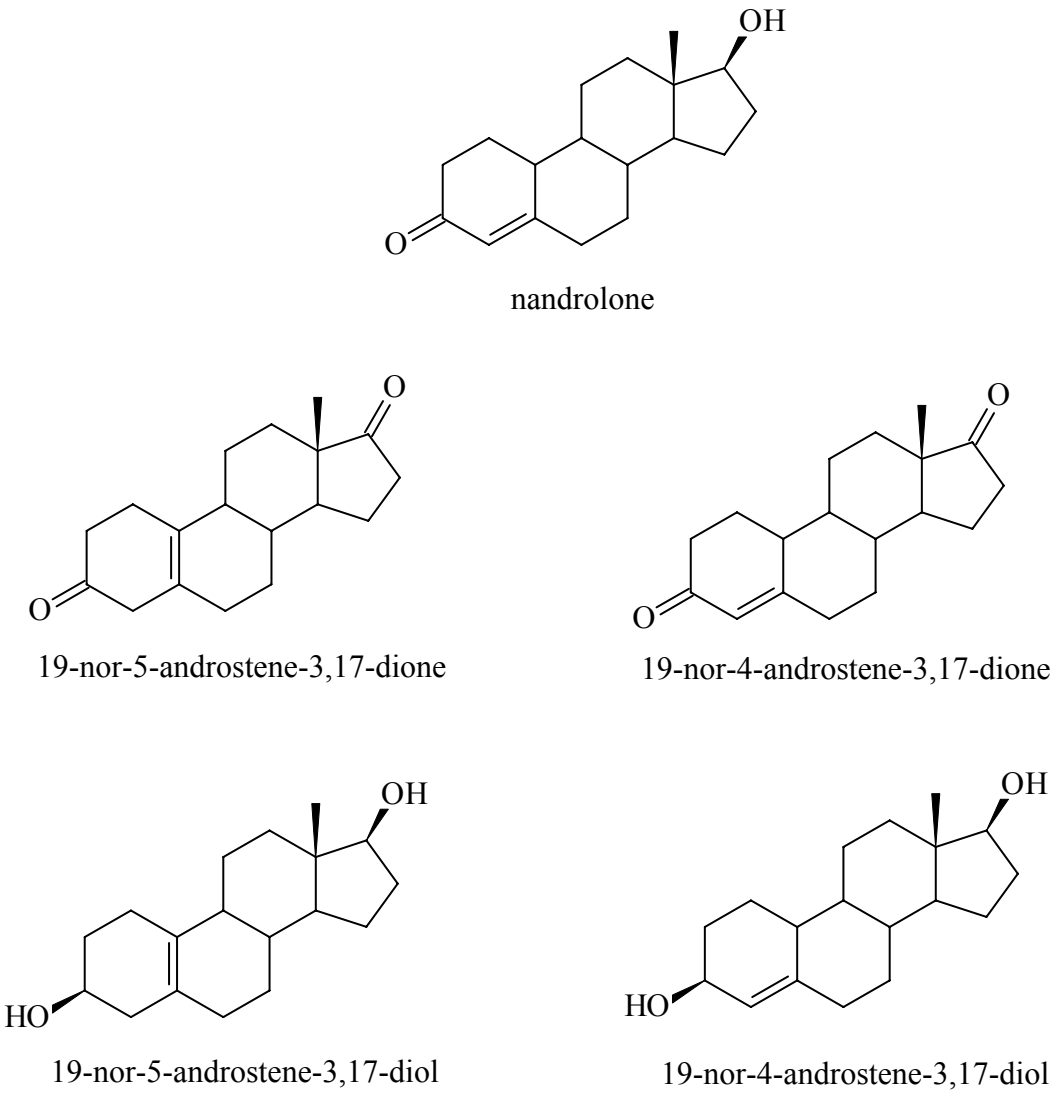



**Figure 1.3.2 Chemical structure of nandrolone and some of its precursors.**

Another issue is the labelled content of supplements. Evidence has been given that prohormones like androstenedione, sold as nutritional supplements, do not always have the content they claim. Table 1.3.1 summarises the labelled versus detected content of prohormones, promoted as nutritional supplements. The detected dose rarely approaches the labelled dose and in some cases the alleged prohormone can not even be detected. More alarming results were obtained in the same studies as it was proven that in several supplements steroids, or precursors other than the labelled prohormones were present.

**Table 1.3.1 Labelled vs. detected content of as nutritional supplements promoted prohormones.**

| Reference | Steroid and dose listed on label | Steroids detected | Detected Amount |
|---|---|---|---|
| [40] | androstenedione*, 100 mg | androstenedione* | 93.1 mg |
| | androstenedione*, 100 mg | androstenedione* | 82,8 mg |
| | androstenedione*, 100 mg | androstenedione* | 103 mg |
| | androstenedione*, 100 mg | androstenedione* | 90 mg |
| | androstenedione*, 100 mg | androstenedione* | 88 mg |
| | androstenedione*, 100 mg | androstenedione* | 85 mg |
| | 4-androstene-3,17-dione, 50 mg | 4-androstene-3,17-dione | 35 mg |
| | androstenedione*, 50 mg | none | 0 |
| | 4-androstene-3,17-dione, 250 mg | 4-androstene-3,17-dione<br>Testosterone | 168 mg<br>10 mg |
| [41] | 19-norandrostenedione, 50 mg<br>19-norandrostenediol, 50 mg | 19-norandrostenedione, 33%<br>19-norandrostenediol, 52%<br>androstenediol*, 15% | # |
| | androstenediol*, 200 mg | androstenediol*<br>androstenedione*<br>testosterone | # |
| | 5-androstenediol | 5-androstenediol, 97%<br>testosterone, 2% | # |
| | 5-androstenediol, 50 mg<br>androstenedione*, 250 mg<br>19-norandrostenedione, 50 mg<br>DHEA, 150 mg | 5-androstenediol<br>androstenedione*<br>19-norandrostenedione | # |
| | DHEA, 100 mg | DHEA, 85%<br>androstenedione*, 15% | # |
| | nor-4-androstenedione, 100 mg<br>19-norandrostenediol, 100 mg | nor-4-androstenedione, 25%<br>19-norandrostenediol, 25%<br>androstenedione*, 42%<br>testosterone, 8% | # |
| | 19-norandrostenediol, 100 mg | androstenedione* | # |
| [42] | 5-androstenediol, 100 mg | 5-androstenediol<br>androstenedione* | 77.3 mg<br>3.3 mg |
| | 4-androstene-3,17-dione, 250 mg | 4-androstene-3,17-dione<br>testosterone | 166,3 mg<br>10 mg |

| | | | |
|---|---|---|---|
| | 4-androstene-3,17-diol, 100 mg<br>androstenedione*, 100 mg | 4-androstene-3,17-diol<br>androstenedione* | 78.5 mg<br>45.5 mg |
| | androstenedione*, 100 mg | androstenedione* | 51.5 mg |
| | androstenedione*, 100 mg | androstenedione* | 84.5 mg |
| | androstenedione*, 100 mg | androstenedione* | 98.5 mg |
| | 4-androstene-3,17-dione, 50 mg | 4-androstene-3,17-dione | 35.3 mg |
| | 4-androstene-3,17-dione, 3.33 mg<br>4-androstene-3,17-diol, 83.33 mg<br>5-androstene-3,17-dione, 3.33 mg<br>5-androstene-3,17-diol, 3.33 mg<br>19-nor-4-androstene-3,17-dione, 3.33 mg<br>19-nor-5-androstene-3,17-diol, 3.33 mg | 4-androstene-3,17-dione<br>4-androstene-3,17-diol<br>19-nor-4-androstene-3,17-dione | 1 mg<br>35.8 mg<br>1 mg |
| | 5-androstene-3β,17β-diol, 50 mg<br>19-nor-4-androstene-3β,17β-diol, 50 mg<br>19-norandrostenedione, 100 mg | 5-androstene-3β,17β-diol<br>19-norandrostenedione | 44 mg<br>76.5 mg |
| | androstenedione*, 100 mg<br>4-androstene-3,17-diol, 100 mg | androstenedione*<br>4-androstene-3,17-diol | 70 mg<br>87.5 mg |
| | 5-androstene-3β,17β-diol, 50 mg<br>*Tribulus terrestris*, 250 mg | 5-androstene-3β,17β-diol<br># | 88.5 mg |
| [43] | 1-androstene-3β,17β-diol, 100 mg | 1-androstene-3β,17β-diol<br>boldenone<br>DHEA<br>19-nor-4-androstenediol<br>testosterone<br>5α-androstane-3α,17β-diol | # |

# no information available * position of double bond not specified.

Results from Kamber *et al.* [41] show that 35 % (6 out of 17) of prohormone supplements contain other substances than indicated on the label. In another study, analysis of a 1-androstene-3β,17β-diol containing nutritional supplement revealed the presence of 5 other anabolic steroids including boldenone and testosterone [43].

In several studies the presence of prohormones in non-hormonal nutritional supplements has been investigated. Results from an international study showed that 14.8% (94 out of 634 samples) of the investigated non-hormonal nutritional supplements contained one or more anabolic androgenic steroid not declared on the label [44]. The detected substances were

DHEA and other precursors of testosterone including 4-androstene-3,17-dione and 4(5)-androstene-3,17-diol and 26 supplements contained 19-nor-4-androstene-3,17-dione, a precursor of nandrolone. A quarter of the contaminated samples showed a total anabolic androgenic steroid concentration higher than 5 μg/g. The highest percentages of contaminants were found in supplements marketed in the Netherlands (25.8%), Austria (22.7%), UK (18.9%) and USA (18.8%). The vast majority of those supplements originated from the USA. Supplement producers are not obliged to disclose the information where a supplement has been produced. In this way, prohormones or contaminated supplements can enter the European market where they are prohibited. Also interesting to note is that more than 20% of the non-hormonal supplements bought from prohormone selling companies contained anabolic androgenic steroids whereas this percentage was below 10% for companies not selling prohormones, indicating that improper cleaning of machinery is responsible for an important fraction of the contamination in non-hormonal supplements.

Other studies reported on the detection of prohormones as contaminants in nutritional supplements [45-48]. One of the most commonly detected steroids was 19-nor-4-androstene-3,17-dione. Its presence may lead to a positive doping test for the nandrolone metabolite norandrosterone [40, 44-46, 49, 50]. As will be discussed in Part IV Chapter 4, approximately 4.8 mg 19-nor-4-androstene-3,17-dione per capsule was found in a supplement from U.S. origin. Its presence was not declared on the label. The recommended dosage was 7 capsules per day. Ingestion of only 1 capsule would have resulted in a positive doping test according to the WADA rules (19-norandrosterone, 2 ng/ml) up to 144 h after the ingestion. Besides these highly contaminated nutritional supplements, low concentrations of nandrolone precursors could also result in positive doping tests [44].

Following a study by Van Eenoo *et al.* [51], the intake of 4-androstene-3,17-dione, present in several nutritional supplements, could result in a positive doping test for 6 to 9 hours after administration.

After the introduction of the prohormones of testosterone such as DHEA on the market around 1996 and later prohormones of nandrolone, also boldenone and its prohormones became available on the supplement market [43]. Recently oxygenated anabolic steroids such as 7-keto DHEA [43] and 4-androstene-3,6,17-trione [52] were detected in over-the-counter

nutritional supplements. Moreover, in two recent studies [53, 54] the presence of non-labelled metandienone (17α-methyl-androsta-1,4-dion-17β-ol-3-one) in nutritional supplements was reported. The use of 17α-alkylated steroids is associated with serious adverse health effects similar to the use of other anabolic steroids but 17α-alkylation, which makes the steroid orally active, aggravates the situation because of the association with jaundice, hepatic carcinoma and peliocis hepatis, a fatal degenerative liver condition [37-39, 55, 56]. Similar remarks can be made about 17α-methyltestosterone detected in two nutritional supplements analysed in the Ghent Doping Control Laboratory (unpublished results). In addition, the intake of these supplements could lead to adverse doping findings.

Numerous dietary supplements are also marketed as containing a natural testosterone booster called *Tribulus terrestris*, a plant containing saponins and diosgenin. Diosgenin has been used as the starting material for industrial hemi-synthesis of androgens, however there is no evidence that it is metabolised to androgens in man. In one study investigating the effect of *Tribulus terrestris* combined with androstenedione supplementation no increase in serum testosterone concentrations was observed [30]. In addition no effects on the urinary steroid profile after the intake of *Tribulus terrestris* were found [34].

From January 20$^{th}$ 2005 the U.S. Congress enacted the Anabolic Steroid Control Act publishing a limited list of hormonal substances, including most previously mentioned prohormones, which are no longer allowed on the U.S. market. This list is supposed to be exhaustive but unfortunately does not contain steroids such as DHEA and the recently discovered designer steroid Madol [57]. In addition, new designer steroids such as 6-oxo steroids or 7-oxo steroids are also not included in this list.

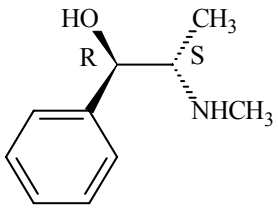
Summarising, athletes should be very careful about dietary supplements. Obviously athletes should refrain from taking supplements mentioning the presence of one or more prohormones or anabolic androgenic steroids, not only for health reasons, but also to avoid positive doping tests. In addition, athletes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

Following WADA-rules athletes remain responsible for the presence of doping substances in their biofluids. To avoid positive doping results following the use of nutritional supplements

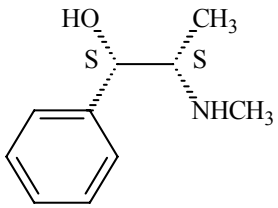
athletes should address to supplement manufacturers to get a guarantee on the purity of the products they use. Only laboratories specialised in doping analysis can provide this guarantee.

### 1.3.2. Stimulants

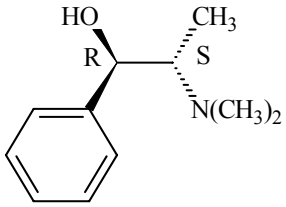
One of the oldest known medicinal herbs is Ephedra or Ma Huang. *Ephedra sinica* is the primary species that has been used in traditional Chinese medicine and is still being used in Ephedra preparations and extracts around the world. The 6 optically active alkaloids present in *Ephedra* species, ranging from 0.02 % to 3.4 % of the plant material, are summarised in Figure 1.3.3 [58].



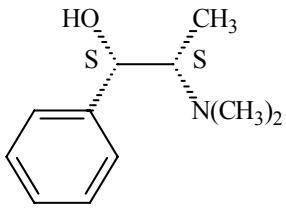

(-)-Ephedrine



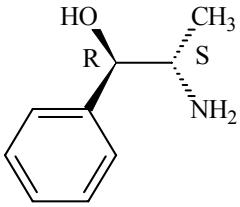

(+)-Pseudoephedrine



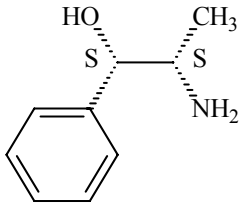

(-)-N-Methylephedrine




(+)-N-Methylpseudoephedrine




(-)-Norephedrine




(+)-Norpseudoephedrine

**Figure 1.3.3 Chemical structure of the 6 optically active alkaloids present in *Ephedra* species.**

Decades ago, ephedrine was used for its bronchodilating effect to treat asthma [59]. Today, other and more effective products, including beta-agonists, have replaced ephedrine in the treatment of asthma. Nevertheless, ephedrine is still promoted as a dietary supplement. The use of ephedrine containing supplements promoting weight loss or athletic performance enhancement has garnered a lot of success. For instance, more than U.S.$1 billion has been spent on Ephedra products in 2000 [60].

Effects of herbal ephedrine supplements on weight reduction have been well documented. Several studies indicated that the administration of ephedrine to obese people resulted in weight loss [61-63]. The influence of ephedrine on performance however is doubtful. Studies by Bell *et al.* [64-68] reported an increase in athletic performance (i.e. increase in exercise time to exhaustion, improved time in Canadian Forces warrior test, increased time to exhaustion in cycle ergometer trial), but most of those studies were performed in small populations and caffeine was frequently co-administered with ephedrine which makes it difficult to attribute the obtained effect to ephedrine. In addition, the effect of pseudoephedrine, another optically active alkaloid present in *Ephedra* species and readily available as an over-the-counter nasal decongestant, on performance is not proven [69-72]. In only one study [73] an improvement in maximum torque performed during an isometric knee extension exercise and in peak power during maximal cycle performance was found, but after doses exceeding the therapeutic dose making the results questionable.

On the other hand, numerous reports have been published on adverse effects associated with the use of Ephedra alkaloids. Haller and Benowitz [22] reviewed 140 reports of adverse effects related to the use of dietary supplements containing Ephedra alkaloids between June 1997 and May 1999. Out of them, 31 % were considered to be definitely related to the use of Ephedra alkaloids and another 31 % were deemed to be possibly related. Ten cases resulted in death and 13 others in permanent health damage. Most frequently reported adverse effects were hypertension, palpitations, tachycardia, stroke and seizures. Furthermore, ephedrine can increase heart rate as well as glucose and insulin concentrations, and in combination with caffeine it could increase systolic blood pressure [74]. Although misuse is the most probable cause of adverse effects, side effects were also observed in subjects involved in clinical trials with controlled doses [62, 65, 67]. As a result of those reports the FDA has issued warnings regarding possible side effects of Ephedra alkaloids [75] and several regulatory actions

against Ephedra and ephedrine alkaloids aiming at the regulation of these compounds have been implemented in the USA [58]. After having warned the general public of the risk associated with the use of ephedrine alkaloids and sending warning letters to manufacturers of these supplements on December 30$^{th}$, 2003 the FDA issued a regulation prohibiting the sale of dietary supplements containing Ephedrine alkaloids that became effective on April 11$^{th}$, 2004. However, traditional Asian medicine containing Ephedra alkaloids was excluded from this rule and can still be sold on the U.S. market. In addition, a U.S. federal judge struck down the FDA ban [75] on Ephedra containing supplements in favour of a supplement producing company on April 13$^{th}$ 2005. This makes the implementation of this ban doubtful and opens the way for other producers to continue their marketing strategies on ephedrine containing supplements.

Despite these regulatory efforts a study by Bents *et al.* [76] has demonstrated that the use of stimulants amongst U.S. college hockey players remains high with 46 % reporting the use of pseudoephedrine and 38 % were using ephedrine.

Similar to other supplements, variation may occur between the labelling of the dietary supplement and the content. Gurley *et al.* [77] showed that markedly different amounts of Ephedra alkaloids were found in many commercial dietary supplements and even significant batch-to-batch variation occurred. In 7 out of the 20 supplements analysed the exact amount of total Ephedra alkaloids was not given and in 80 % of the analysed samples the ephedrine content was even not mentioned on the label. Similar results were noticed by Haller *et al.* [78]. Not mentioning the active compound on the label of a nutritional supplement could result in serious complications for athletes, e.g. positive doping tests, as they are probably not aware of the fact that Ma Huang contains Ephedra alkaloids. In addition, some supplements declaring Ma Huang on the label seem not to be as “natural” as they claim, as only ephedrine was present [79], indicating an eventual deliberate addition of this substance instead of using herbal products.

Besides alkaloids originating from the Ephedra plant other substances with stimulating properties have been detected in nutritional supplements. Already in the 1980’s a Belgian female marathon runner tested positive for the stimulant phentermine. Analysis of the consumed food supplement promoting weight loss revealed the presence of the anorexic

agents phentermine and fenfluramine [80]. A study conducted by the Dutch Ministry of Health, Welfare and Sports preceding the Olympic Winter Games in Salt Lake City (2002) resulted in the detection of MDMA, or XTC, in a supplement.

In conclusion not only naturally occurring stimulants including ephedrine and analogues, but also synthetic amphetamine analogues and related compounds can be present in nutritional supplements.

Stimulants belong to the classes of prohibited substances according to WADA rules [81]. A threshold level for ephedrine and methylephedrine of 10 µg/ml and a level of 5 µg/ml for norpseudoephedrine have been introduced to avoid unintentional positive doping results. Nevertheless, because of the wide variety in ephedrine concentrations found in nutritional supplements and the numerous side effects, athletes should be very cautious using this kind of nutritional supplements and they should be aware of the possibility that supplements can be contaminated with various prohibited substances. One case of a positive doping test resulting from the administration of a botanical food supplement has been described [82].

Also, numerous nutritional supplements promoted for performance enhancement and stimulating effects contain caffeine. Some of these products comprise, besides caffeine, extracts of guarana (*Paullinia cupana*), a South American plant which has been shown to contain caffeine [83]. Caffeine has a positive influence on endurance sport. This is frequently observed in run/cycle to exhaustion tests but also in swimming and tennis [84-87]. As a result, extensive research has been done to investigate the mechanisms by which caffeine exerts its performance enhancing effects. Although not completely resolved, it appears that the caffeine antagonism of adenosine receptors is an important factor in the comprehensive mechanism of action of caffeine [88]. Before January 1$^{st}$, 2004 the use of caffeine containing nutritional supplements could have resulted in positive doping test as caffeine was listed by the IOC as a doping agent in class I A “Stimulants” with a threshold level of 12 µg/ml. This is not longer an issue because from January 1$^{st}$, 2004 on caffeine was removed from the WADA list of forbidden substances. Nevertheless, athletes should take care when using caffeine because excessive use is associated with withdrawal effects and negative effects on the cardiovascular system [88].

## *1.4. Analysis of nutritional supplements for doping contaminants*

Numerous methods have been developed to analyse herbal preparations for the presence of Ephedra alkaloids or to evaluate the labelled content of *Ephedra sinica* (Ma Huang) containing supplements. These methods include TLC [89], immunoassays [41, 82], GC [90-92], HPLC [78, 79, 93-98] and CE [99]. Identification is best achieved using mass spectrometry. Identification of the ephedrines using GC/MS is not possible without derivatisation, which could be considered as a technical drawback. On the other hand, in several HPLC methods sodium dodecyl sulphate or triethylamine as a constituent of the mobile phase are used to increase the theoretical plate number, hampering the HPLC combination with mass spectrometry due to overloading of the ion source. Only one method has been described for the identification of Ephedra alkaloids in dietary supplements using LC/MS [94].

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

Several methods for the detection of stimulants in urine using HPLC have been published [100-104]. GC-methods routinely used for the detection of stimulants for doping control purposes, have also been described [105-109]. Based on GC with NPD detection for screening purposes in urine, Parr *et al.* descibed a method for the screening of stimulants and other nitrogen containing doping relevant drugs in nutritional supplements [110]. Confirmation of positive screening results was done with GC/MS after selective derivatisation using MSTFA and MBTFA. Besides ephedrines, amphetamines and related compounds, this method also allows for the identification of methylecgonine, formed during alkaline extraction, as a marker for cocaine. The only drawback of this method is the relatively high limit of detection for some compounds including MDA, cocaine and strychnine as some authorities, such as NZVT (http://www.necedo.nl/nzvt/thenetherlandssecuritysystemnutritionalsupplements) require better analytical sensitivity. The development of an LC/MS method for the screening of stimulants in dietary supplements might be a solution.

Analysis of nutritional supplements for the presence of anabolising agents has been described in several papers. Catlin *et al.* [40] and Green *et al*. [42] used HPLC to detect contaminants in nutritional supplements marketed as prohormones but the method was restricted to 5 prohormones of testosterone and nandrolone. Using this method the detection of low concentrations of contaminants seems questionable as the dietary supplements were diluted numerous times.

A more frequently applied analytical technique for the identification of prohormones in dietary supplements is GC/MS, similar to the screening methods for anabolic steroids in urine for doping control purposes. Full scan MS methods were able to detect contaminants in the milligram range [41, 46, 53] while the use of SIM methods allowed more sensitive identification of impurities [44, 45, 47, 48].

Geyer *et al*. [44] described a method for the screening of 12 compounds including testosterone and prohormones, nandrolone and prohormones, prohormones of boldenone and 5α-dihydrotestosterone. LODs ranged between 5 ng/g and 100 ng/g. These LODs allow for the determination of trace contaminations in "non-hormonal" nutritional supplements.

Two validated methods have been published for the screening of 28 different anabolizing agents including testosterone and prohormones, nandrolone and prohormones, esters of both compounds, stanozolol and metandienone [47, 48]. Although until now only one ester of testosterone (testosterone undecanoate) is available for oral administration, these methods are capable of detecting other esters of testosterone and nandrolone as well, when they should become available on the supplement market. A distinction was made between solid [47] and aqueous [48] nutritional supplements. LODs ranged from 2 ng/g to 40 ng/g for solid supplements while for aqueous supplements LODs were between 1 ng/ml and 10 ng/ml. These LODs meet the requirements of official authorities such as the Dutch NZVT system.

Methods developed for the identification of trace contaminants rely on liquid/liquid extraction. Geyer *et al.* [44] used a method based on mixing nutritional supplements with methanol. Afterwards an extractive clean-up using pentane and methanol was used. Other methods use an alkaline extraction with NaOH and pentane/diethylether [47] or $K_2CO_3$/$NaHCO_3$ and pentane/diethylether [48].

Nutritional supplements form a heterogeneous group of products available in different forms. Consequently, analysis of nutritional supplements for anabolic steroids has proven to be very difficult due to the different matrices. Problems most frequently occur with solid nutritional supplements or oil based supplements [48]. Parr *et al.* [111] reported several possible solutions for problems occurring during extraction and analysis of supplements. The proposed modifications include extra derivatisation, additional clean-up steps, alternative sequence of injection and reduction of the matrix. In this way improvements could be obtained. However, still 10.4 % of all investigated samples could not be analysed. A possible solution for those problems could be a pre-analysis fractionation using HPLC as described by De Cock *et al.* [46]. Promising results in the clean-up step were obtained by Geisendorfer and Gmeiner [112] using GPC prior to GC/MS analysis. This method allowed for the determination of 12 different anabolic steroids and prohormones without the occurrence of matrix problems. Although the obtained LODs (20 – 50 ng/g) were not in compliance with the requirements of some authorities (NZVT) this method seems to be very promising.

## *1.5. Conclusion*

The use of nutritional supplements seems unnecessary when athletes use a well balanced diet. Nevertheless, according to supplement sales numbers the use of these supplements has exploded in the last decade. Athletes are influenced by parents, coaches, competitors and high financial stakes to improve their performance. Aggressively marketed dietary supplements promising the athlete to jump higher, run faster or throw further seem to be a helpful tool. As long as products such as vitamins or minerals are used combined with a proper medical support this use is rather harmless. The use of prohormones, available on the supplement market as a result of several loopholes in the 1996 DSHEA, is of greater risk. Besides the serious health effects associated with these products, athletes should also take into account that they have to pass a doping control test. Therefore, the use of supplements by athletes in general is dangerous as numerous nutritional supplements are contaminated with anabolizing agents or stimulants. According to the WADA doping rules athletes are held responsible for the substances detected in their biofluids, irrespective of the origin. The development of methods capable of detecting contamination of nutritional supplements with doping relevant substances by specialised doping control laboratories is of utmost importance. At present

athletes or supplement producing companies have the possibility to have their supplements tested.

Although several analytical methods are now available further improvements are needed because presently a fraction of the supplements cannot be analysed, according to the requirements of some testing authorities, due to matrix problems. In this perspective, additional research has to be done to solve these problems and to give athletes the best guarantee they can get.

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# CHAPTER 2: Detection of prohormones in nutritional supplements

Adapted from:

- W. Van Thuyne and F.T. Delbeke, *Validation of a GC-MS screening method for anabolizing agents in solid nutritional supplements*. Biomed. Chromatogr., 2004. **18**: 155-159.

- W. Van Thuyne and F.T. Delbeke, *Validation of a GC-MS screening method for anabolizing agents in aqueous nutritional supplements.* J. Chromatogr. Sci., 2005. **43**: 2-6.

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# Chapter 2: Detection of prohormones in nutritional supplements

## *2.1. Abstract*

A sensitive and selective method for the screening of 32 different compounds including testosterone, nandrolone and prohormones, stanozolol and metandienone in both solid and aqueous nutritional supplements is described. The different substances are extracted from solid nutritional supplements by liquid-liquid extraction with a mixture of pentane and freshly distilled diethylether (9/1; v/v) after dissolving the supplement in NaOH (1N). In liquid matrices the substances are extracted by liquid-liquid extraction with a mixture of pentane and freshly distilled diethylether (1/1; v/v) after alkalinisation with a $NaHCO_3/K_2CO_3$ (2/1; w/w) buffer. The anabolizing agents are derivatised with a mixture of MSTFA/$NH_4I$/ethanethiol (320/1/2; v/w/v), routinely used for the determination of anabolic steroids in urine. The TMS-derivatives are analysed by GC/MS in the SIM mode. The limits of detection were in the range 2 to 40 ng/g for solid nutritional supplements and 1 to 10 ng/ml for aqueous nutritional supplements. Examples of solid and aqueous nutritional supplements analysed by these methods were found to contain several forbidden substances according to the current WADA doping regulations. All detected compounds, except dihydrotestosterone, could be confirmed by GC/MS/MS, proving that the proposed methods are capable of detecting anabolizing agents in both solid and aqueous dietary supplements.

## *2.2. Introduction*

Nutritional supplements are food, supplying in one or more nutrients in a concentrated form such as minerals, vitamins, enzymes, that are theoretically present in a normally balanced diet [1]. Usually, they are offered in an atypical form like powder, tablets or capsules. Also commercially available and very popular are sports and energy drinks and the recently commercialised creatine serums. During the last decade the use of nutritional supplements has increased tremendously [1-3].

Several factors are at the origin of the present situation by which the use of nutritional supplements by athletes has become a matter of concern. The ever increasing aim for success by athletes is stimulated by the high financial stakes in elite sport. On the other hand, since the adoption of the DSHEA in 1994 [4], prohormones became commercially available on the U.S. supplements market. According to the rules of the WADA, and formerly the IOC, these prohormones belong to the prohibited class of anabolic steroids [5] due to potential risks associated with prohormone use similar to those observed with the use of anabolic steroids [6-8].

Recently, evidence was found that several of these prohormones were present in "non-hormonal" nutritional supplements like vitamins, minerals and amino acids, prohormones that were not even declared on the label of these supplements [9-11]. In an international IOC study 94 nutritional supplements out of 634 (14,8%) were found to contain one or more prohormones not mentioned on the label [12]. In two other studies, high doses of the anabolic steroid metandienone were found in supplements [13, 14], in both cases the presence of this anabolic steroid was not mentioned on the label. The presence of these prohormones may lead to a positive doping test especially for the nandrolone metabolite norandrosterone [9-11]. As a result of the increasing use of nutritional supplements and the detection of several prohormones in non-hormonal supplements banned by international sport federations and the lack of standard methods according to ISO17025, validated methods for the screening of anabolizing agents in both solid and aqueous nutritional supplements were needed and are described here.

## *2.3. Experimental*

### 2.3.1. Reagents

5α-androstane-3α,17β-diol, 19-nor-4-androstene-3,17-dione, 5α-androstane-3β,17β-diol, 4-androstene-3,17-dione, boldenone, androsterone and testosterone were obtained from Sigma (St. Louis, MO, USA). 19-nor-4-androstene-3β,17β-diol, 19-nor-5-androstene-3β,17β-diol, 1,4-androstadiene-3,17-dione, 1-androstene-3,17-dione, 19-nor-5-androstene-3,17-dione, 4-androstene-3β,17β-diol, metandienone, 5-androstene-3β,17β-diol, 5-androstene-3,17-dione, 4-androstene-19-ol-3,17-dione and 7-keto-DHEA were bought from Steraloids (Newport, USA), DHEA from Serva (Heidelberg, Germany) and DHT from Piette International Laboratories (Drogenbos, Belgium). Nandrolone and stanozolol were bought from NARL (Pymble, Australia). Clenbuterol was obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany). 17α-methyltestosterone, testosterone propionate, testosterone isocaproate, testosterone decanoate, testosterone phenylpropionate, testosterone undecanoate, nandrolone decanoate and nandrolone phenylpropionate were obtained from Organon (Oss, The Netherlands). Nandrolone laurate, was from Intervet International (Boxmeer, The Netherlands) and 1-testosterone was from Promochem (Molsheim, France). MSTFA was purchased from Chem. Fabrik Karl Bucher (Waldstetten, Germany). All other chemicals were of analytical grade.

Nutritional supplement X (creatine serum, aqueous dietary supplement) was from US origin. The labelled content of this supplement was: creatine serum 50 mg/ml, inositol 20 mg/ml, D-glucose 20 mg/ml, glucosamine sulphate 20 mg/ml, magnesium ascorbate 20 mg/ml, calcium pyruvate 10 mg/ml, citrusbioflavonoids 10 mg/ml, green tea extract 10 mg/ml, guarana extract 10 mg/ml, L-arginine 10 mg/ml, L-carnitine 10 mg/ml, L-glutamine 10 mg/ml, Siberic ginseng 10 mg/ml, royal jelly 6 mg/ml, vitamin B5 3.6 mg/ml, zinc gluconate 2 mg/ml, chromium gluconate 2 µg/ml, vitamin B12 0.6 µg/ml. Other ingredients were water, glycerine, sorbitol and aromas. The recommended dosage was 5 ml to be used 10 minutes before exercise, only on training days.

Nutritional supplement Y (solid dietary supplement) was also from US origin. The labelled content of the supplement was: *Mucuna pruriens* (Dopa Bean), standardized 15% L-Dopa

666.6 mg, α-glycerylphosphorylcholine 100 mg, *Bacopa monniera* extract, standardized 20% bacosides A&B 50 mg per serving. Other ingredients were fructose, sorbitol, natural flavor, steraic acid, magnesium stearate, cellulose, croscarmellose and sucralose. The recommended dosage was 4 capsules, one serving per day.

### 2.3.2. Gas chromatography-mass spectrometry conditions

The GC/MS analysis was conducted in the SIM mode on an Agilent 6890 gas chromatograph directly coupled to an Agilent 5973 mass selective detector (Agilent, Palo Alto, USA). Three ions, each with a dwell time of 25 ms, were monitored for each compound. The GC column was an HP-Ultra 1 (J&W, Folsom, USA), 100 % methylsilicone column with a length of 17 m, an internal diameter of 0.2 mm and a film thickness of 0.11 µm. Helium was used as the carrier gas (linear velocity: 41 cm/s). A total of 0.5 µl was injected splitless. The oven temperature program was as follows: 120°C (0 min), 70°C/min → 181°C (0 min), 4°C/min → 234°C (0.1 min), 30°C/min → 300 °C (10 min). The electron energy was set at 70 eV and the ion source temperature was set at 230 °C.

Confirmation of positive results was done using GC/MS/MS on a Varian 3800 gas chromatograph directly coupled to a Varian Saturn 2000 mass spectrometer. The GC-column was a Varian CP-Sil 24 CB low bleed column (50% phenyl, 50% dimethylpolisyloxane) with a length of 30 m, an internal diameter of 0.25 mm and a film thickness of 0.15 µm. Helium was used as the carrier gas (linear velocity 41.6 cm/s). The oven temperature program was as follows: 120°C (0 min), 70°C/min → 170°C (0 min), 4°C/min → 260°C (0. min), 30°C/min → 270 °C (0 min). The mass spectrometer was used in the MS/MS mode with an emission current of 80 µA. The dissociation parameters are as shown in Table 2.3.1.

**Table 2.3.1 Dissociation parameters in GC-MS/MS confirmation.**

| Component | Precursor Ion (m/z) | Excitation storage level (m/z) | Excitation Amplitude (Volts) |
|---|---|---|---|
| androsterone (IS) | 434 | 165 | 100 |
| DHEA | 432 | 150 | 65 |
| 5-androstene-3β,17β-diol | 434 | 180 | 85 |

### 2.3.3. Analysis of nutritional supplements

5 ml of the aqueous nutritional supplement were made alkaline with 1 g of a $NaHCO_3/K_2CO_3$ (2/1; w/w) buffer. After stirring, 50 µl of the internal standard androsterone (2 µg/ml, MeOH) was added, followed by 5 ml of a pentane/diethylether mixture (1/1; v/v). After extraction by rolling for 1 h and centrifugation, the organic layer was separated and evaporated under oxygen free nitrogen at 40 ± 5°C. The residue was derivatized with 100 µl MSTFA/$NH_4$I/ethanethiol (320/1/2; v/w/v) at 80°C for 30 min and transferred to an autosampler microvial.

Solid nutritional supplements (1 g) were extracted by vigorously shaking with 5 ml of 1 N NaOH in a screw capped tube. Tablets were grounded before extraction. After shaking, 50 µl of the internal standard androsterone (2 µg/ml, MeOH) was added together with 5 ml pentane/diethylether (9/1; v/v). After rolling for 1 h, 0.5 – 1.0 g of anhydrous $Na_2SO_4$ was added before centrifugation. Afterwards, the organic layer was separated and dried under oxygen free nitrogen. The residue was derivatized with 100 µl MSTFA/$NH_4$I/ethanethiol (320/1/2; v/w/v) at 80°C for 30 min and transferred to an autosampler microvial. A total of 0.5 µl was injected on the chromatographic system.

### 2.3.4. Analytical method validation

The analytical method validation for the screening of 32 compounds in both aqueous and solid supplements was performed according to Eurachem guidelines [15] on twenty different, randomly chosen aqueous and solid nutritional supplements.

To determine the LODs, ten different aqueous nutritional supplements were spiked with a reference mixture at different concentrations in the range 1 – 40 ng/ml (1, 2, 5, 10, 20 and 40 ng/ml) and 10 different solid supplements in the range 1 – 80 ng/g (1, 2, 5, 10, 20, 40 and 80 ng/g). Selectivity was tested by the analysis of a reference mixture of 10 different other anabolizing agents at a concentration of 200 ng/ml. These compounds were 19-noretiocholanolone, 17α-trenbolone, oxymesterone, 3'-OH-stanozolol, mesterolone, salbutamol, terbutaline, etiocholanolone, 5β-androst-1-ene-17β-ol-3-one and oxandrolone. Specificity was tested by the analysis of the 20 different nutritional supplements.

## *2.4. Results and Discussion*

All screened compounds are given in Table 2.4.1. They include prohormones of testosterone and nandrolone, most commonly found in nutritional supplements [9-11], esters of both compounds, stanozolol and metandienone. Under the described chromatographic conditions, the internal standard androsterone-bis-TMS gave a sharp peak with a retention time of 10.81 min. The GC relative retention times and ions monitored (3 per compound) are given in Table 2.4.1.

**Table 2.4.1 GC relative retention times and monitored m/z values for trimethylsilylated compounds.**

| Compound | RRT | m/z |
|---|---|---|
| clenbuterol | 0.46 | 335.1 , 300.1 , 86.1 |
| androsterone (IS) | 1.00 | 434.3 , 419.3 , 329.2 |
| 5α-androstane-3α,17β-diol | 1.03 | 436.4 , 331.2 , 241.2 |
| 19-nor-5-androstene-3β,17β-diol | 1.05 | 330.2 , 240.2 ; 225.1 |
| 19-nor-4-androstene-3β,17β-diol | 1.06 | 420.3 , 330.2 , 240.2 |
| 1-(5α)-androstene-3,17-dione | 1.09 | 430.3 , 415.3 , 194.1 |
| DHEA | 1.11 | 432.3 , 417.3 , 327.2 |
| 19-nor-4(5)-androstene-3,17-dione | 1.12 | 416.3 , 401.2 , 194.1 |
| 4-androstene-3β-17β-diol | 1.12 | 434.3 , 405.3 , 143.1 |
| 1-testosterone | 1.13 | 432.3 , 417.3 , 194.1 |

| | | |
|---|---|---|
| 5-androstene-3β-17β-diol | 1.14 | 434.3 , 344.3 , 239.2 |
| 5α-androstane-3β,17β-diol | 1.15 | 436.4 , 421.3 , 241.2 |
| nandrolone | 1.15 | 418.3 , 403.3 , 194.1 |
| 1,4-androstadiene-3,17-dione | 1.16 | 428.3 , 413.3 , 323.2 |
| DHT | 1.17 | 434.3 , 405.3 , 143.1 |
| 4(5)-androstene-3,17-dione | 1.19 | 430.3 , 415.3 , 234.1 |
| boldenone | 1.19 | 325.2 , 229.1 , 206.1 |
| testosterone | 1.22 | 432.3 , 417.3 , 209.0 |
| metandienone | 1.33 | 444.3 , 339.2 , 206.1 |
| 17α-methyltestosterone | 1.35 | 446.3 , 356.2 , 301.2 |
| 4-androstene-19-ol-3,17-dione | 1.36 | 518.4 , 428.3 , 415.3 |
| 7-keto-DHEA | 1.38 | 518.3 , 429.2 , 296.1 |
| testosterone propionate | 1.41 | 416.3 , 401.3 , 343.2 |
| stanozolol | 1.55 | 472.4 , 457.3 , 143.1 |
| testosterone isocaproate | 1.56 | 458.4 , 443.3 , 343.2 |
| nandrolone decanoate | 1.78 | 500.4 , 485.4 , 329.2 |
| nandrolone phenylpropionate | 1.79 | 478.3 , 463.3 , 194.1 |
| testosterone decanoate | 1.82 | 514.4 , 499.4 , 343.2 |
| testosterone phenylpropionate | 1.83 | 492.4 , 477.3 , 105.0 |
| testosterone undecanoate | 1.90 | 528.5 , 513.4 , 343.2 |
| nandrolone laurate | 1.96 | 528.5 , 513.4 , 329.2 |

4-androstene-3,17-dione and 5-androstene-3,17-dione can not be separated because of the formation of identical derivatives using the derivatisation mixture as described in §2.3.3 (Figure 2.4.1). The same applies for 19-nor-4-androstene-3,17-dione and 19-nor-5-androstene-3,17-dione.

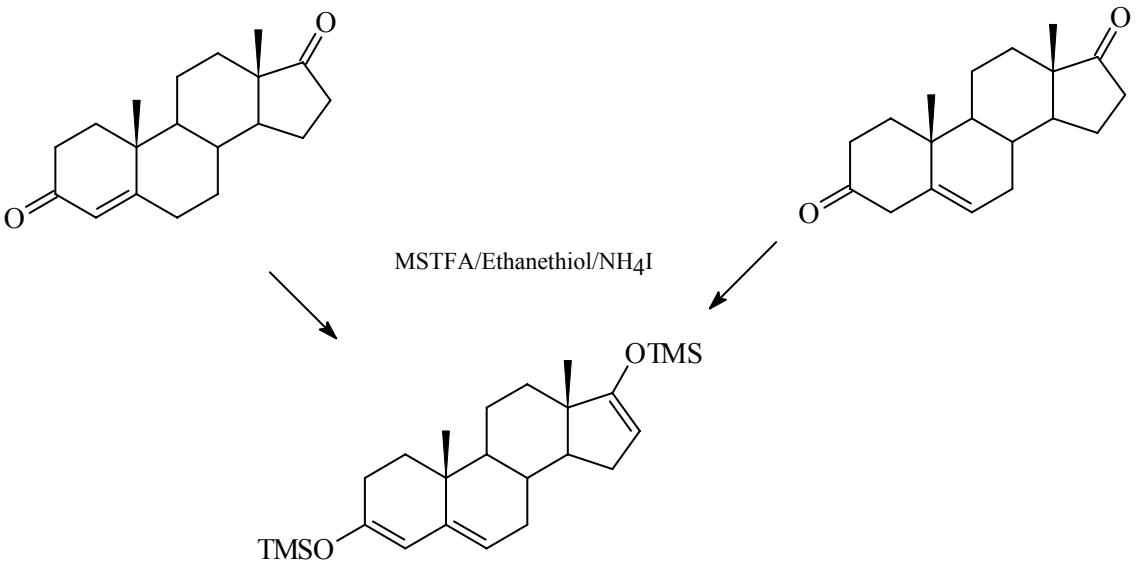


**Figure 2.4.1 Derivatisation of 4-androstene-3,17-dione and 5-androstene-3,17-dione with MSTFA/ethanethiol/$NH_4I$.**

For screening purposes at least two ion traces were monitored for every substance. The presence of a substance is suspected and the sample consequently forwarded to confirmatory analysis, if the relative abundance of the ion traces is similar to the relative abundance of the ion traces in the reference (20% relative margin). As an extra criterion, a maximal allowed deviation in relative retention time of 1% was used.

The validation was performed according to the Eurachem guidelines [15]. According to these rules, the LOD is defined as the concentration where an analyte can be detected with a certainty of 100% (using the above mentioned criteria) in ten spiked supplements. The resulting LODs are summarised in Table 2.4.2.

**Table 2.4.2 LODs for 32 different compounds in both solid and aqueous nutritional supplements.**

| compound | LOD Solid (ng/g) | LOD Liquid (ng/ml) | compound | LOD Solid (ng/g) | LOD Liquid (ng/ml) |
|---|---|---|---|---|---|
| DHEA | 2 | 1 | stanozolol | / | 10 |
| 1,4-androstadiene-3,17-dione | 10 | 1 | metandienone | 10 | 2 |
| 19-nor-4-androstene-3,17-dione | 2 | 1 | 5α-androstane-3β,17β-diol | 10 | 2 |
| 5-androstene-3β-17β-diol | 5 | 2 | testosterone phenylpropionate | 10 | 2 |
| nandrolone | 5 | 1 | testosterone isocaproate | 20 | / |
| dihydrotestosterone | 5 | 1 | testosterone propionate | 5 | 1 |
| 4-androstene-3,17-dione | 2 | 1 | testosterone undecanoate | 2 | 1 |
| testosterone | 2 | 1 | testosterone decanoate | 2 | 1 |
| 19-nor-5-androstene-3,17-dione | 2 | 1 | nandrolone decanoate | 5 | 2 |
| 5-androstene-3,17-dione | 2 | 1 | nandrolone phenylpropionate | 5 | 2 |
| 4-androstene-3β-17β-diol | 10 | 1 | nandrolone laurate | 5 | 1 |
| 5a-androstane-3α,17β-diol | 5 | 1 | methyltestosterone | 2 | 1 |
| 7-keto-DHEA | / | 2 | 1(5α)-androstene-3,17-dione | 5 | 2 |
| boldenone | 10 | 2 | 1-testosterone | 5 | 1 |
| 4-androstene-19-ol-3,17-dione | / | 2 | 19-nor-5-androstene-3β,17β-diol | 10 | 1 |
| clenbuterol | 10 | 1 | 19-nor-4-androstene-3β,17β-diol | 10 | 1 |

Several analytes can be detected at a concentration of 1 ng/ml in aqueous nutritional supplements. The highest LOD for aqueous supplements was 10 ng/ml, a concentration far below LODs obtained with a previous full scan method [9].

For solid nutritional supplements 9 analytes could be detected at a concentration of 2 ng/g. All other compounds could be detected at or below 20 ng/g.

LODs could not be defined for 7-keto-DHEA, stanozolol and 4-androsten-19-ol-3,17-dione in solid nutritional supplements and for testosterone isocaproate in aqueous nutritional supplements due to matrix interferences in one sample.

Specificity and selectivity were tested according to the procedure described by Verwaal *et al.* [16]. For a qualitative method, the analysis of different negative matrices used to determine the LOD is sufficient to test for the specificity. No matrix interferences were found at the retention times of the validated analytes nor at the retention time of the internal standard androsterone. Selectivity was tested by the analysis of several related compounds. According to Verwaal *et al.* [16] the concentration of these compounds must be at least twice the LOD of the determined analytes. In this study, a concentration of 200 ng/ml was used. No interference of the related compounds was observed at the retention times of the different compounds and the internal standard androsterone. Hence, this method seems to be specific and selective. In conclusion, it seems that this method is reliable and sensitive for the screening of anabolizing agents in both aqueous and solid nutritional supplements. The method fulfils all criteria set by NZVT (http://www.necedo.nl/nzvt/thenetherlandssecuritysystemnutritionalsupplements)

Previous analysis of nutritional supplement X with a full scan method [9] did not result in the detection of one or more prohormones. Therefore, this matrix was used as a negative matrix during the validation procedure. Surprisingly, the test for specificity resulted in the detection of DHEA in very low concentrations. Screening results for DHEA in nutritional supplement X in comparison to a reference are shown in Figure 2.4.2.

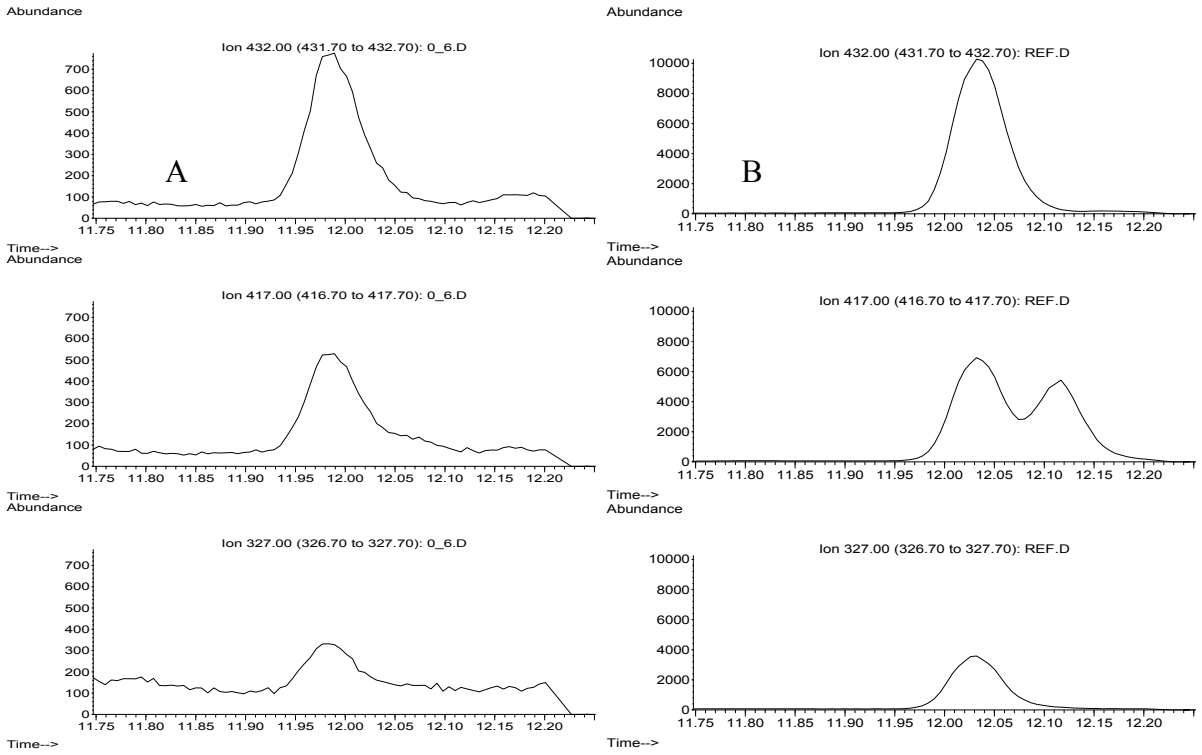


**Figure 2.4.2 Screening results for DHEA in nutritional supplement X. (A) Nutritional supplement X and (B) DHEA.**

Confirmation of this screening result was obtained using GC/MS/MS. The resulting product spectrum of DHEA in nutritional supplement X compared to a reference is given in Figure 2.4.3. Because DHEA was confirmed in nutritional supplement X, the method was further validated by replacing this matrix by another negative nutritional supplement.

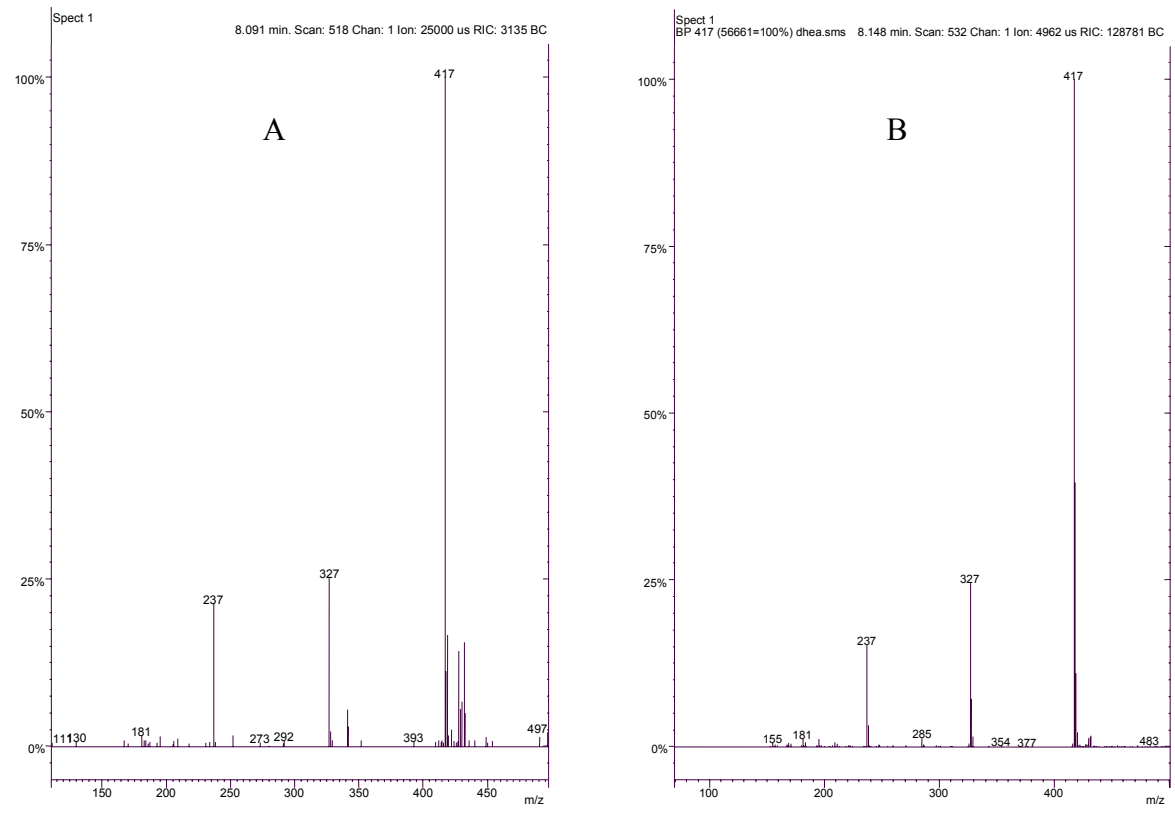


**Figure 2.4.3 Product spectrum (precursor m/z 432) of DHEA in nutritional supplement X. (A) nutritional supplement. (B) DHEA.**

The method for solid nutritional supplements was applied to nutritional supplement Y and resulted in the detection of DHEA, 19-nor-4(5)-androstene-3,17-dione, 5-androstene-3β,17β-diol, DHT, 4(5)-androstene-3,17-dione and testosterone. As an example the ion chromatogram of 5-androstene-3β,17β-diol in nutritional supplement Y in comparison to a reference is shown in Figure 2.4.4. Confirmation of the screening results was performed by GC/MS/MS. The resulting product spectrum of 5-androstene-3β,17β-diol is shown in Figure 2.4.5. All other compounds, except dihydrotestosterone, were also confirmed by GC/MS/MS.

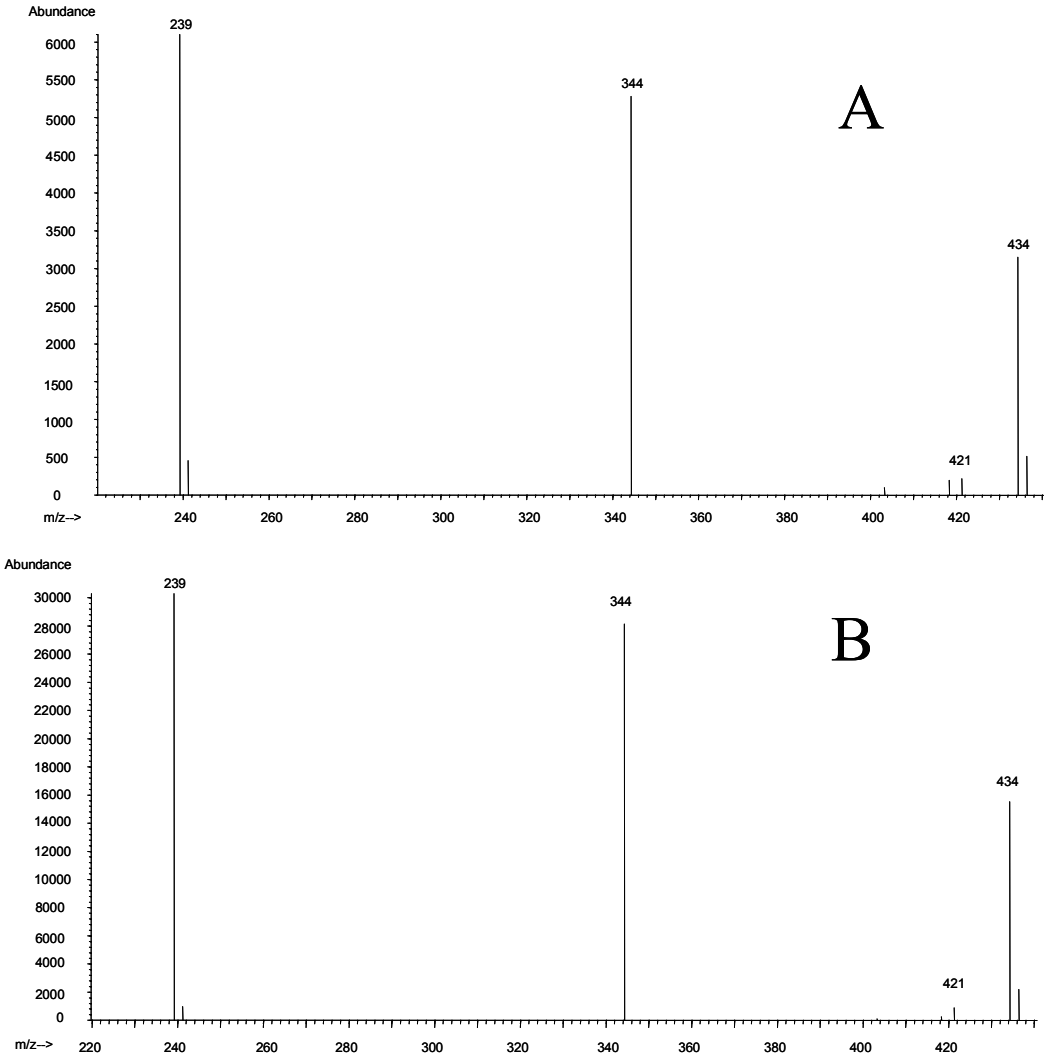


**Figure 2.4.4 Screening results for 5-androstene-3β,17β-diol in nutritional supplement Y. (A) nutritional supplement. (B) 5-androstene-3β,17β-diol.**

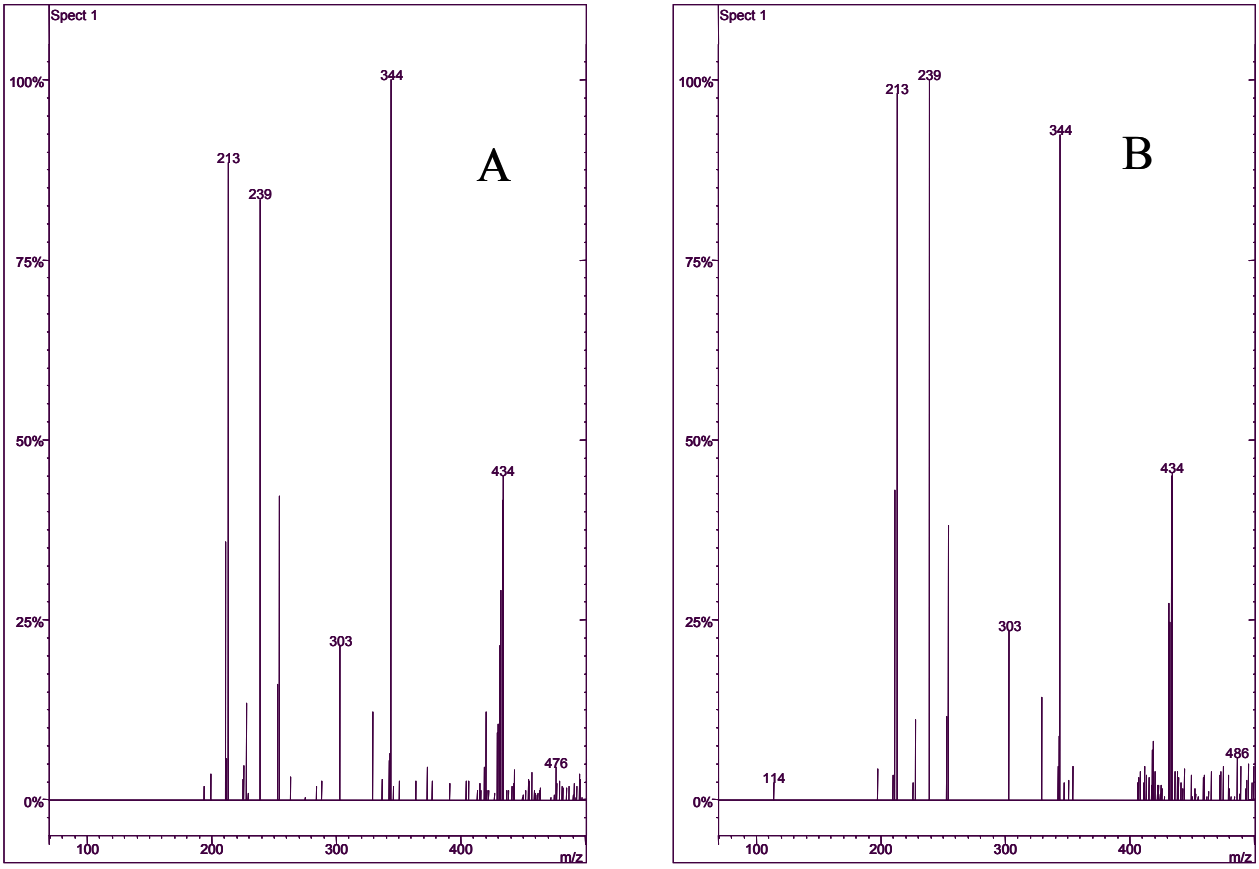


**Figure 2.4.5 Product spectrum (precursor m/z 434) of 5-androstene-3β,17β-diol. (A) nutritional supplement Y. (B) 5-androstene-3β,17β-diol.**

## *2.5. Conclusion*

Following international doping regulations, athletes remain responsible for the presence of doping substances in their biofluids. Because several studies have shown that nutritional supplements can be contaminated with prohormones, athletes should be very cautious using nutritional supplements. The developed sensitive and validated screening methods for the detection of anabolizing agents in both aqueous and solid nutritional supplements could be helpful for manufacturers and distributors to avoid unintended contamination of their products and to give that guarantee to their customers.

It can be concluded that the described screening methods are capable of detecting contamination with prohormones at very low levels as the obtained LODs of 29 analytes are

below 20 ng/g for solid nutritional supplements and below 10 ng/ml for 31 analytes in liquid supplements. The application of these methods showed the presence of low level contamination in an aqueous supplement and multiple contamination of a solid supplement.

## *2.6. Acknowledgements*

The authors wish to thank the Flemish Ministry of Health for financial support. The technical assistance of N. Desmet is gratefully acknowledged.

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# CHAPTER 3: DETECTION OF STIMULANTS IN SOLID NUTRITIONAL SUPPLEMENTS

Adapted from:

- K. Deventer, W. Van Thuyne, P. Mikulčíková, P. Van Eenoo, F.T. Delbeke, *Detection of stimulants in solid nutritional supplements by liquid chromatography-mass spectrometry.* Food Chem., submitted

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# Chapter 3: Detection of stimulants in solid nutritional supplements

## *3.1. Abstract*

Nutritional supplements are frequently used by athletes and can contain substances banned by WADA. The aim of this study was to develop a screening method for the detection of stimulants in solid nutritional supplements.

Analyses were performed using high pressure liquid chromatography coupled to an ion trap mass spectrometer with an APCI interface after acidic clean-up and basic liquid/liquid extraction with diethylether. The mass spectrometer was operated in full scan MS/MS using positive ionisation. LODs were equal to or below 50 ng/g for all compounds except strychnine (100 ng/g). The suitability of the method for routine application was illustrated by the analysis of two suspected samples.

## *3.2. Introduction*

As described in the 1994 DSHEA [1] and defined by Schröder [2], nutritional supplements are food supplying in one or more nutrients in a concentrated form, e.g. vitamins, minerals, herbs or other botanicals, amino acids, metabolites, constituents, extracts or combinations of any of such ingredients, that are theoretically present in a normal balanced diet. As a result herbal preparations, frequently promoted for their alleged preventive and/or therapeutic effects were not considered as drugs [3]. One of the oldest, and best known, medicinal herbs is Ephedra or Ma Huang (*Ephedra sinica*). This herb was frequently used as a constituent in traditional Asian medicine and is still being used in herbal preparations today. The major active components are 6 optical active compounds including ephedrine, pseudoephedrine and norephedrine [4]. Today, herbal preparations containing parts of *Ephedra sinica* are frequently promoted for their performance enhancing effects or their positive influence on weight reduction. Although the effect of ephedrine on weight reduction has been documented [5-7], the influence on performance is doubtful [8-10] while numerous reports have been published describing the health risks associated with the use of ephedrine. A study by Haller and Benowitz [11] gave an overview of 140 adverse reports associated with the use of dietary supplements containing ephedra alkaloids.

Besides alkaloids originating from the Ephedra plant, other stimulating agents have been detected in nutritional supplements. Analysis of a nutritional supplement used by a Belgian female marathon runner who tested positive during a doping control, revealed the presence of the anorexic agents phentermine and fenfluramine [12]. In addition, analysis of nutritional supplements for use by elite athletes preceeding the Olympic Games in Salt Lake City indicated the presence of MDMA, or XTC, in one nutritional supplement.

Stimulants belong to the classes of prohibited substances according to WADA doping rules [13]. Athletes using contaminated supplements are at risk of violating the current doping rules. Analytical methods for the detection of prohomones in nutritional supplements have previously been published [14, 15]. Parr et.al [16] described a GC method for the detection of various stimulating agents in nutritional supplements based upon screening methods routinely used in doping control. Detection of stimulants using GC can only be accomplished with

nitrogen-phosphorus detection (GC/NPD) or mass spectrometry (GC/MS) after selective derivatisation. Because the obtained LODs [16] are not always in compliance with requirements made by official authorities (http://www.necedo.nl/nzvt), the aim of this study was to develop a LC/MS screening method for the detection of stimulants in solid nutritional supplements as an alternative for existing GC methods.

## *3.3. Experimental*

### 3.3.1. Chemicals and reagents

The internal standard (IS) 3-bromophenethylamine and strychnine were obtained from Sigma (Bornem, Belgium). MDA, MDEA and MDMA were obtained from the Portuguese doping control laboratory (Lisbon, Portugal). Norephedrine.HCl, norpseudoephedrine.HCl, pseudoephedrine.HCl and methamphetamine.HCl were purchased from Merck (Darmstadt, Germany). Amphetamine sulphate was purchased from Smith-Kline & French Laboratory (Philadelphia, USA). Ephedrine.HCl was obtained from Hoechst AG (Frankfurt, Germany) and fenfluramine.HCl from Laboratoires Servier (Orleans, France).

Analytical grade hydrochloric acid, potassium hydroxide, acetic acid and diethylether were from Merck (Darmstadt, Germany). *n*-Pentane was obtained from Biosolve (Valkenswaard, The Netherlands). HPLC grade methanol was from Acros (Geel, Belgium) and HPLC grade water from Fischer (Loughborough, UK).

Methanolic HCl (1M) was prepared by the addition of 3.9 g acetylchloride (Sigma) using a dropping funnel, during a period of 20 minutes while stirring, to 50 ml methanol p.a. (Acros Organics, Geel, Belgium) cooled to 0°C. The solution was stored between 0 and 8 °C.

Nutritional supplement X was from Dutch origin. The labelled content was Tribulus Terrestris 750 mg. The recommended dose was 1-3 capsules per day.

Nutritional supplement Y, also from Dutch origin was promoted as a herbal mixture. The manufacturer recommended 2-4 capsules a day.

### 3.3.2. Liquid chromatography – mass spectrometry conditions

A Thermo Separation Products (TSP) Model P4000 quaternary pump equipped with a TSP Model AS 3000 autosampler with a 100 µl sample-loop and connected to a ThermoFinnigan LCQ-Deca® mass spectrometer was used (all from Thermo Separation Products, Thermo, San Jose, CA, USA).

A Nucleosil C18 column 3 mm x 100 mm, 5 µm (Varian, St.-Katelijne-Waver, Belgium), protected with a Chromsep guard column 2 x 10 mm (Varian), was used for chromatographic separations. 50 µl was injected on the chromatographic system using push loop injection.

The mobile phase consisted of 1% acetic acid (solution A) and methanol (solution B). Gradient elution at a flow rate of 0.4 ml/min was as follows: 90% A for 2 min, linear to 20% in 6 min, isocratic for 3 min followed by an increase to 90% in 0.5 min. The equilibration time before the next injection was 6.5 min and the total run time was 18 min.

Ionisation of analytes was carried out using APCI. The capillary and vaporizer temperature were set at 120 and 350 ºC, respectively. The discharge current was 5 µA. The sheath gas was maintained at 50 units and no auxillary gas was used.

Tuning of the capillary voltage, tube lens, octapole lenses and entrance lens was automatically done on the protonated molecular ion of amphetamine.

In MS/MS experiments the isolation width was set at 3.0, the activation q at 0.250 and the activation time at 30 ms. The collision energy was set in order to fully fragment the precursor ion.

### 3.3.3. Extraction procedure

Extraction was based on a previously described method [16]. Briefly, 50 µl of the internal standard 3-bromophenethylamine (10 µg/ml, MeOH) were added to 1 g of grinded supplement, followed by the addition of 5 ml of 1M HCl. Liquid-liquid extraction was performed by rolling for 10 min with 4 ml *n*-pentane. After centrifugation the organic layer was discarded. Two ml of 5 M KOH were added to the remaining aqueous layer and a second liquid-liquid extraction was performed using 5 ml diethylether by rolling for 10 min. After

centrifugation the organic layer was transferred to a new tube and 100 µl of methanolic HCl (1M) was added after which evaporation was performed under OFN at 40 ºC. The residue was dissolved in 200 µl of mobile phase.

### 3.3.4. Analytical method validation

The validation was carried out according to Eurachem validation guidelines [17] on 10 randomly chosen solid nutritional supplements. These supplements were spiked at 4 different levels with the studied stimulants. Final concentrations were 20, 50, 100 and 200 ng/g. The samples were extracted as described above. The LOD was defined as the lowest concentration where a compound could be identified in all samples. Selectivity was tested by analysing several other doping agents including other stimulants, anabolic agents, beta-blockers, narcotics, diuretics and corticosteroids. Specificity was tested by analysing 10 blank nutritional supplements used to determine the LOD.

## *3.4. Results and Discussion*

All stimulants included in this screening method contain an amine function and can be extracted with good recoveries at the alkaline pH value used in this method [18]. Nutritional supplements often consist of complex matrices and are composed of multiple ingredients, including vitamins, amino acids and herbal extracts. Because of the basic amine moiety of stimulants, a selective acid extraction was used to remove unwanted neutral and acid compounds. In doping analysis diphenylamine is commonly used as IS for the analysis of amphetamine type drugs [19, 20]. However this substance is lost during the acid clean up step. Therefore, 3-bromophenethylamine, a compound with similar functionalities as the compounds of interest was used as the internal standard.

Methanolic HCl was added to the organic phase before the evaporation step to avoid undesired loss due to the volatility of amphetamine type drugs [21].

The use of MeOH instead of acetonitrile, as organic modifier was preferred because MeOH allowed for the isomers ephedrine-pseudoephedrine and norpseudoephedrine- norephedrine to be partially separated.

For the determination of the diagnostic ions direct infusion was performed. Therefore a solution of 5 µg/ml, producing a fairly distinguished protonated molecular ion $[M+H]^+$, was directly infused into the mass spectrometer. For all compounds protonated molecular ions were observed in full scan MS. Because MS/MS often results in improved sensitivity this technique was applied for all compounds. Retention times and monitored diagnostic ions are presented in Table 3.4.1.

**Table 3.4.1 RTs, instrument parameters and LODs.**

| Substance | RT (min) | PI $[M+H]^+$ | CE | DI | LOD (ng/g) |
|---|---|---|---|---|---|
| bromophenethylamine* | 8.13 | 200 | 25 | 183 | - |
| amphetamine | 6.61 | 136 | 25 | 119 | 20 |
| ephedrine | 5.20 | 166 | 30 | 148 | 50 |
| fenfluramine | 9.44 | 232 | 30 | 159 | 50 |
| MDA | 7.10 | 180 | 25 | 163 | 50 |
| MDEA | 7.88 | 208 | 28 | 163 | 50 |
| MDMA | 7.52 | 194 | 27 | 163 | 20 |
| methamphetamine | 7.28 | 150 | 30 | 119 | 50 |
| norephedrine | 3.79 | 152 | 25 | 134 | 50 |
| norpseudoephedrine | 4.36 | 152 | 25 | 134 | 50 |
| pseudoephedrine | 5.67 | 166 | 30 | 148 | 50 |
| strychnine | 8.23 | 335 | 37 | 264 | 100 |

CE: collision energy, PI: precursor ion for MS/MS, DI: diagnostic ion
* IS

As indicated in Table 3.4.1, the obtained LODs were equal to or lower then 50 ng/g for all compounds excepting strychnine (100 ng/g). These LODs are lower than the detection limits obtained in previous studies using GC/MS [16] and are in compliance with the requirements of some testing authorities (http://www.necedo.nl/nzvt).

Ion chromatograms obtained after analysis of a quality control sample spiked with all compounds at a concentration of 100 ng/g are given in Figure 3.4.1.

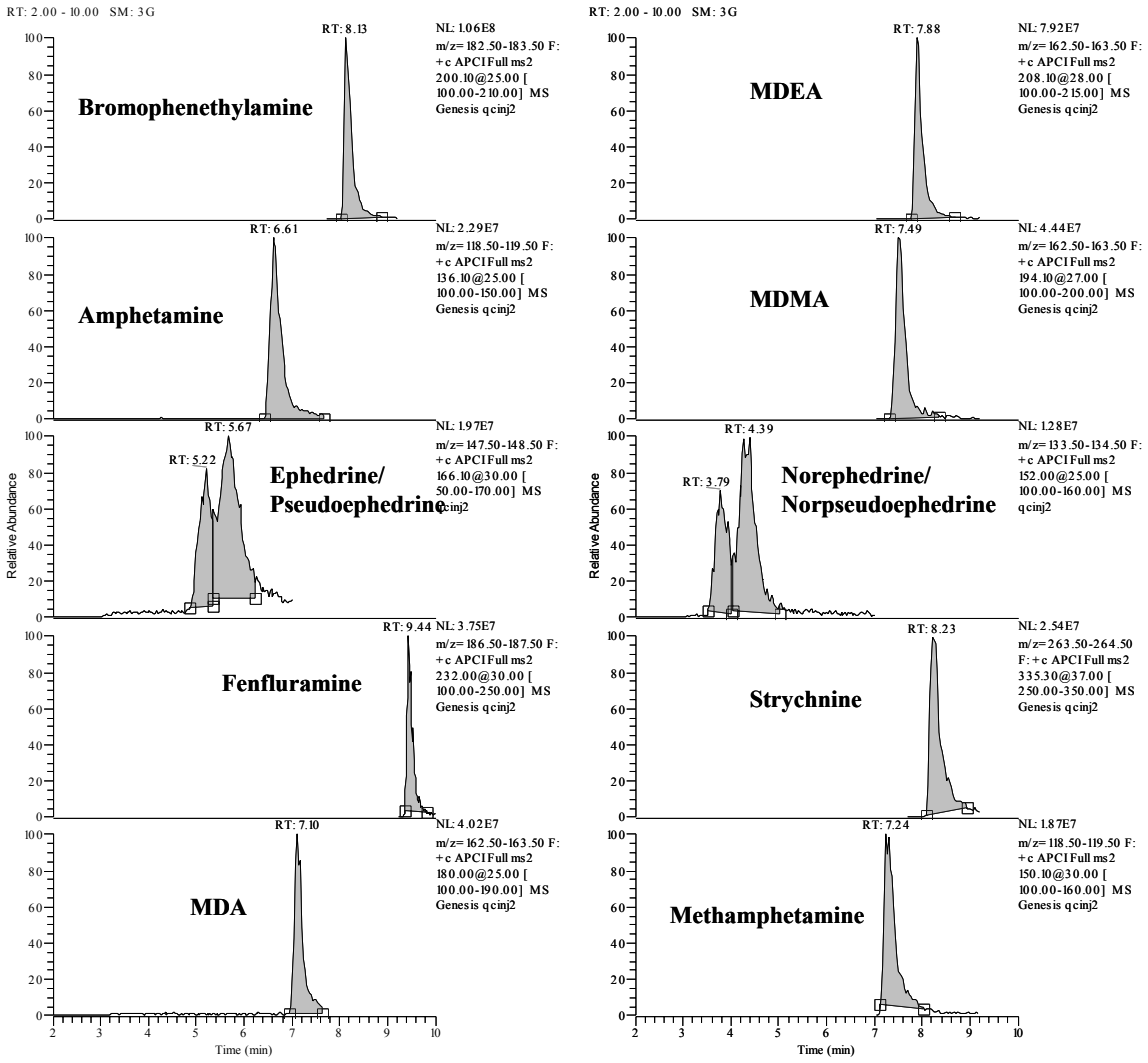


**Figure 3.4.1 Quality control sample spiked with all components at 100 ng/g.**

The method seems to be very selective as no interferences were detected when other doping products including other stimulants, anabolic agents, beta-blockers, narcotics, diuretics and corticosteroids were analysed. The specificity, tested by the analysis of 10 different blank dietary supplements, was satisfactory as no matrix interferences were noticed at the retention time of the 11 analytes tested in this method, nor at the retention time of the internal standard.

The method was applied to the analysis of two solid supplements. In the first sample from Dutch origin, containing the alleged natural steroid enhancer *Tribulus terrestris* (supplement X), ephedrine and pseudoephedrine were detected (Figure 3.4.2).

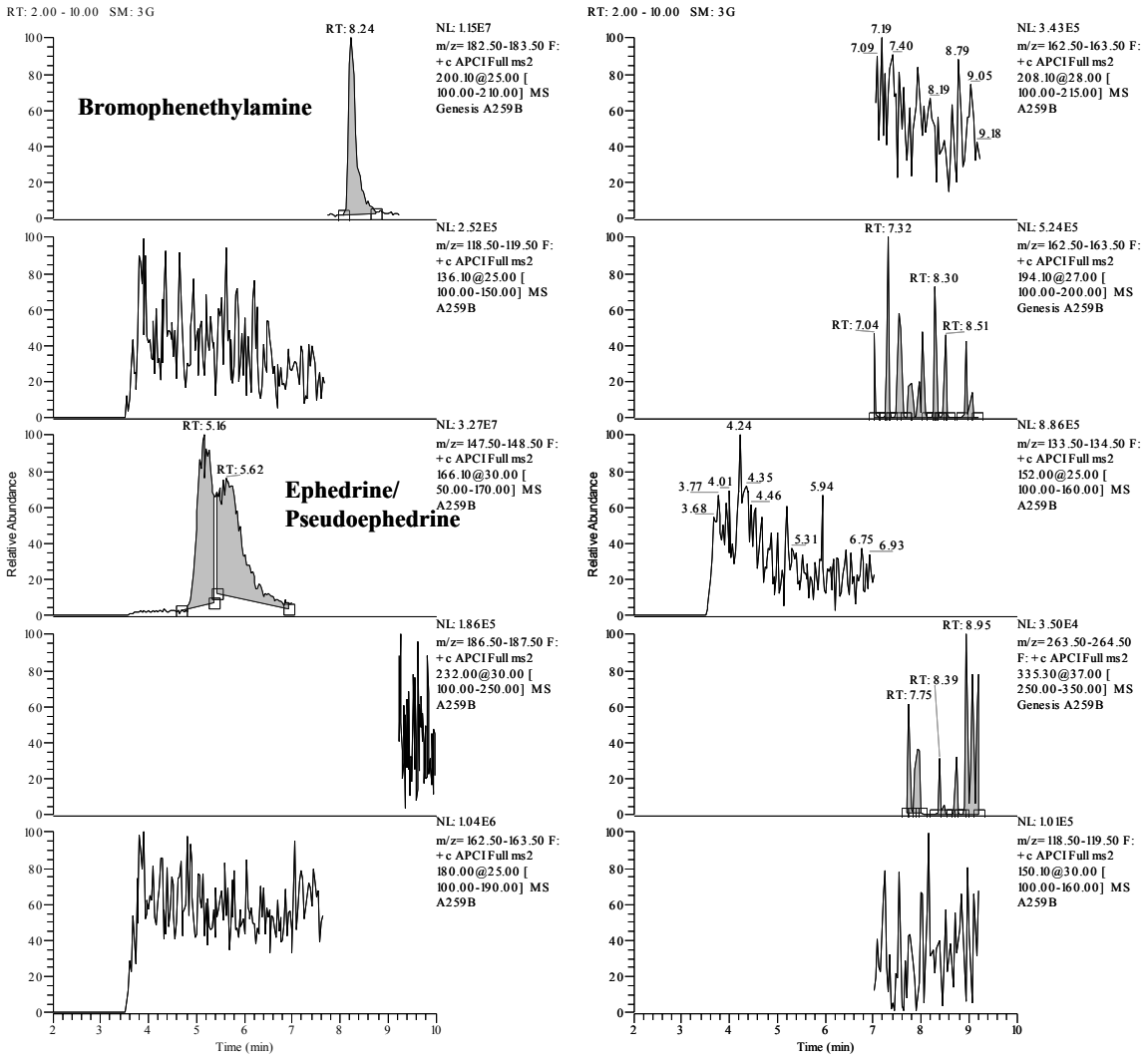


**Figure 3.4.2 Positive screening results for ephedrine and pseudoephedrine in supplement X.**

In a second sample also from Dutch origin, the presence of non-labelled strychnine was found. Although not mentioned on the label extracts from *Strychnos nux vomica*, a natural source of strychnine, were probably added to the supplement (Figure 3.4.3).

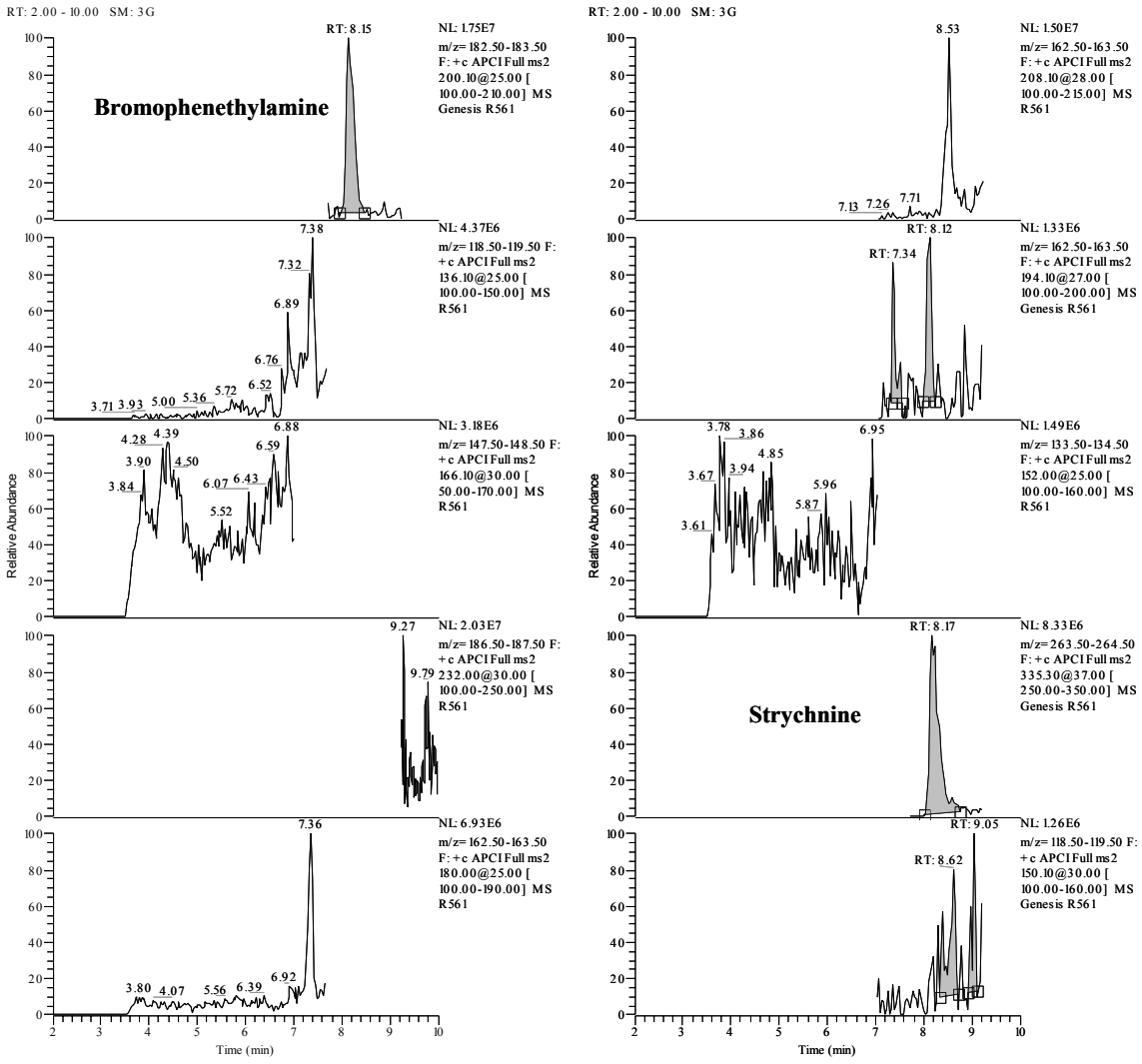


**Figure 3.4.3 Positive screening results for strychnine in supplement Y.**

## *3.5. Conclusion*

A sensitive screening method for the detection of 11 stimulants using LC/MS/MS was developed and validated. This method can be applied as a screening method for the routine analysis of stimulating agents in nutritional supplements. The obtained LODs are in compliance with the requirements of the NZVT. The method resulted in the detection of ephedrine and pseudoephedrine in supplement X and of strychnine in supplement Y.

## *3.6. Acknowledgements*

The financial support of the Flemish Ministry of Health is gratefully acknowledged. The authors are grateful to the Belgian National Lottery for the purchase of the LCQ-DECA® instrument.

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# CHAPTER 4: CASE REPORTS

Adapted from:

- W. Van Thuyne and F.T. Delbeke, *Nutritional Supplements and Doping: Non-labelled Multiple Prohormones in a Czech Nutritional Supplement*, in *Recent Advances in Doping Analysis*. Edts: W. Schänzer, H. Geyer, A. Gotzmann, and U. Mareck. 2004. **12**, Sport & Buch Strauβ: Cologne (Germany). 471-477.

- F.T. Delbeke, P. Van Eenoo, W. Van Thuyne, and N. Desmet, *Prohormones and sport.* J. Steroid Biochem. Mol. Biol., 2002. **83**: 245-251.

# Chapter 4: Case Reports

## *4.1. Introduction*

Since the introduction in the 1980s of a general screening method for anabolic steroids in doping analysis, the detection of long acting injectable preparations in urine became rather easy [1-3]. In order to circumvent a positive test athletes therefore switched to injectable testosterone or oral preparations that were rapidly cleared from the body. It was reported that in the 80s scientists in the German Democratic Republic, knowing that a test would soon be adopted to detect the use of testosterone, developed short acting testosterone preparations and nasal sprays containing the testosterone precursor androstenedione [4].

Another way to circumvent the doping tests was the use of prohormones, especially precursors of testosterone, while the orally short acting precursors of nortestosterone also seemed to be attractive to the cheating athlete.

One of the first hormonal supplements that became available in the USA was DHEA in 1996. Thereafter the list steadily expanded and now includes DHEA, 4-androstenedione, 4-androstenediol and 5-androstenediol as well as the nandrolone precursors 19-norandrostenedione and 19-norandrostenediol. Their introduction on the U.S. market and the internet trade were the direct result of the 1994 DSHEA. Originally this Act was designed to allow people the use of common vitamin supplements. However through several loop-holes in the law, any substance that is natural to the body can be sold according to this Act. In this way consumers, including professional athletes and people using substances for cosmetic reasons, are increasingly provided with untested, unproven and potentially lethal products. Moreover as these supplements contain anabolic steroids, their use can confront athletes with sanctions from international sports authorities.

Another issue with prohormones and sports is related to nutritional supplements contaminated with prohormones [5-9]. In this chapter supplements tested by DoCoLab (UGent) with in-house methods for the detection of non-labelled anabolising agents in nutritional supplements will be discussed. For some of them urinary detection times for doping substances, which were not indicated on the labels, were established.

## *4.2. Experimental*

### 4.2.1. Contaminated nutritional supplements

Two nutritional supplements from U.S. origin were bought.

The labelled content of supplement A was as follows: seven capsules contain garcinia cambogia 2000 mg, l-carnitine 1100 mg, guarana extract 1025 mg, citrus aurantium extract 300 mg, l-phenylanaline 300 mg, dandelion root 250 mg, choline bitartrate 200 mg, cayenne powder 60 mg, vanadyl sulphate 15 mg, vitamin B6 10 mg and "much more". The manufacturer recommended seven capsules on a daily basis.

Supplement B was a pyruvate supplement and the recommended dose was one tablet per day.

Two other nutritional supplements, C & D, were from Czech origin. Supplement C, was a pyruvate Ca supplement used by an athlete suspended after a positive nandrolone doping test. According to the manufacturer's recommendations the optimal dose was three capsules before exercise and three more 4 hours later.

Supplement D, was sent by the Czech Agriculture and Food Inspection Authority. This supplement was a creatine pyruvate produced by the same company as supplement C. The recommended dosage was 6 – 8 capsules per day. Supplement C and D arrived at the laboratory within an interval of 1.5 years.

### 4.2.2. Analysis of nutritional supplements

All supplements were in solid form and were extracted and analysed using GC/MS as described in Part IV Chapter 2. The GC/MS analysis of supplement A was hampered by matrix effects. To overcome this problem an additional HPLC fractionation was performed after the traditional liquid-liquid extraction. The HPLC system consisted of a SP 8800 ternary pump (Spectra-Physics, CA, USA), an SP 8880 autosampler and a Spectra Focus forward optical scanning detector (UV3000) set at a wavelength range of 220-360 nm. A 100 x 3 mm I.D. column packed with octadecyl silica (5 µm Nucleosil, Chrompack, Belgium) with an appropriate guard column was used. The column was held at room temperature. The loop

volume was 20 µl. The mobile phase consisted of acetonitrile and acetic acid (1% in $H_2O$) at a flow rate of 1 ml/min. The gradient program was as follows: initial acetonitrile 2% for 4 min, increased to 32% in 6 min, finally to 45% in 6.5 min and hold for 4 min. Afterwards the column was equilibrated for 10 min with 2% acetonitrile. The residue obtained after liquid-liquid extraction was dissolved in 200 µl of mobile phase. A total of 20 µl was injected on the chromatographic system. The fraction between 15.2 and 18.3 min was collected, evaporated and derivatised with 100 µl MSTFA/$NH_4I$/ethanethiol (380/1/2; v/w/v) for 1 h at 80°C after which the GC/MS analysis was performed in full scan mode (m/z 40 – 780).

No additional clean-up was needed for supplements B, C en D. GC/MS screening of those supplements was performed in SIM-mode as described in Part II Chapter 2.

### 4.2.3. Quantification of prohormones

The concentration of prohormones in the supplements was determined in the homogenized content of 10 capsules or tablets using an equally weighted linear calibration curve constructed in the range 0 to 500 ng/g. Therefore a negative nutritional supplement (carbo energizer orange, Performance, Nutrico, Belgium) was spiked with testosterone, DHT, DHEA, 4(5)-androstene-3,17-dione, nandrolone, 19-nor-4(5)-androstene-3,17-dione and 5α-androstane-3,17-dione and analysed at 5 different levels (0, 50, 100, 250 and 500 ng/g) according to the described method. The quantification and qualifier ions for each compound are summarised in Table 4.2.1. If necessary, samples were diluted to obtain concentrations within the range of the calibration curve. Quantification was performed using the same GC/MS method, except that the MS was operated in the full scan mode (m/z 40 – 780) instead of SIM.

**Table 4.2.1 Quantification ion and qualifier ions (m/z) used in the quantification of different trimethylsilylated compounds in nutritional supplements.**

| compound | Quant. Ion | Qual. ion 1 | Qual. ion 2 |
|---|---|---|---|
| testosterone | 432 | 417 | 209 |
| DHT | 434 | 405 | 143 |
| nandrolone | 418 | 403 | 194 |
| DHEA | 432 | 417 | 327 |
| 19-nor-4(5)-androstene-3,17-dione | 416 | 401 | 194 |
| 4(5)-androstene-3,17-dione | 430 | 415 | 234 |
| 5α-androstane-3,17-dione | 432 | 417 | 275 |

### 4.2.4. Excretion study

Excretion studies were performed with supplements A, B & C according to a strict research protocol approved by the Ethics Committee, University Hospital (Ghent, Belgium) (EC/2005-81/sdp). The purpose of the study was explained to each volunteer.

One capsule of supplement A was taken by 5 male volunteers. Urine was collected before administration and quantitatively during the first 12 h, i.e. 2, 4, 6, 9, 12 h post administration. Additional urine samples were collected after 24, 48, 72, 96, 120, 144 and 168 h.

Two tablets of supplement B were ingested by 2 volunteers. Urine sample collection was as for supplement A.

An excretion study with supplement C was performed in 3 male volunteers. Each volunteer took 3 capsules at the beginning of the study, followed by three more 4 hours later, in agreement with the recommendations on the package of the supplement. Urine was taken before administration and thereafter quantitatively up to 12 hours (0, 1, 2, 4, 6, 9 and 12 h).

### 4.2.5. Urine analysis

Quantification of norandrosterone in urine was as follows: one ml sodium acetate buffer (pH 5.2, 1M) and 50 µl of β-glucuronidase type HP-2 from *Helix pomatia* were added to 5.0 ml

urine. The mixture was hydrolysed for 2.5 h at 56°C. After the addition of 50 µl of the internal standard 17α-methyltestosterone (2 µg/ml, MeOH) and 1 g of $NaHCO_3/K_2CO_3$ (2:1; w/w) extraction was performed by rolling for 20 min with 5 ml n-pentane. After centrifugation, the organic layer was evaporated under OFN. The residue was derivatised with 100 µl MSTFA/$NH_4I$/ethanethiol (380/1/2; v/w/v) for 20 min at 80°C.

Quantification of norandrosterone and endogenous steroids was done by GC/MS in the SIM (dwell time 50 ms) mode on an Agilent MSD-5973 mass spectrometer directly coupled to an Agilent 6890 gas chromatograph (Agilent, Palo Alto, USA) equipped with a 17 m crosslinked methylsilicone Ultra-1 column (I.D. 0.20 mm, $d_f$ 0.11 µm) (J&W, Folsom, USA). The oven temperature was programmed as follows: 120°C (0 min) – 70°C/min → 181°C (0.2 min) – 4°C/min → 234°C (0.1 min) – 30°C/min → 300°C (3 min). The electron energy was set at 70 eV and the ion source temperature at 270 °C. A total of 0.5 µl was injected splitless. Quantification of norandrosterone was performed using a 5-point calibration curve in the range 1 to 20 ng/ml. Steroid profiling of the urine samples was done using the extraction and derivatisation procedure as described for the quantification of norandrosterone, except that 2 ml urine was used and extraction was performed with diethylether instead of n-pentane. Steroid profiling was done using a one point calibration. The mass spectrometer was operated in the SIM mode using dwell times of 20 ms.

## *4.3. Results and Discussion*

Calibration curves for the quantification of prohormones in nutritional supplements showed good linearity ($R^2$ between 0.977 and 0.996). A similar correlation coefficient was obtained for the quantification of norandrosterone in urine.

### 4.3.1. Supplement A

After evaporation and derivatisation of the collected HPLC fraction the presence of high concentrations of 19-nor-4(5)-androstene-3,17-dione and 4(5)-androstene-3,17-dione were found in dietary supplement A. Both substances were not mentioned on the product label and were confirmed by GC/MS in full scan mode (Figure 4.3.1).

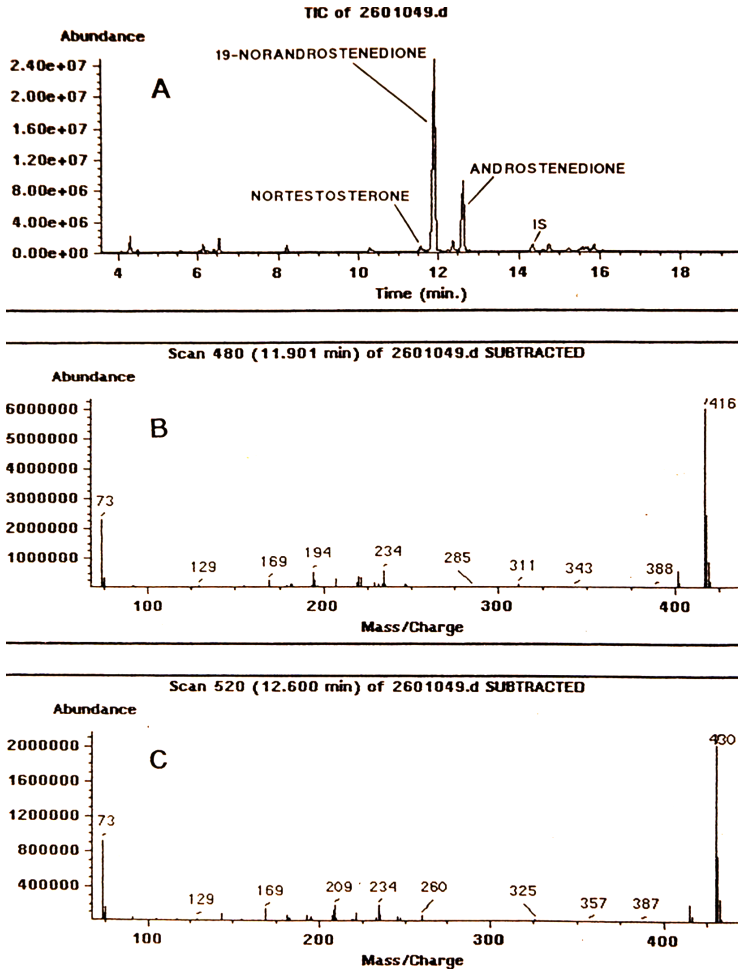


**Figure 4.3.1 Confirmation of non labelled steroids in supplement A. Total ion chromatogram of the collected HPLC fraction (A). Mass spectrum of trimethylsilylated 19-nor-4(5)-androstene-3,17-dione (B). Mass spectrum of trimethylsilylated 4(5)-androstene-3,17-dione (C).**

Quantification of the steroids present in supplement A after homogenisation of 10 capsules (7.77 g) resulted in concentrations of 0.9 and 6.3 mg/g for 4(5)-androstene-3,17-dione and 19-nor-4(5)-androstene-3,17-dione, respectively. Hence, one capsule of the dietary supplement A contained 0.7 mg 4(5)-androstene-3,17-dione and 4.8 mg 19-nor-4(5)-androstene-3,17-dione. Concentrations in the milligram range in this supplement can not be considered as contamination related to the production process. Instead they are indicative of the deliberate addition of these substances.

Because 4(5)-androstene-3,17-dione is a precursor of testosterone, an increase in urinary testosterone as well as in 4-androstene-3,17-dione concentration can be expected after the

ingestion of this supplement [10]. An excretion study conducted with one tablet of supplement A however showed no increase in the ratio of testosterone to epitestosterone. Van Eenoo et.al [10] stated that the misuse of 4-androstene-3,17-dione can be proven if the ratio of 4-androstene-3,17-dione to epitestosterone exceeds 1.2. In only one subject this threshold level was exceeded from 4 h until 6 h after the administration of one tablet of this supplement. (Figure 4.3.2). It remains unclear what the outcome of this test would be if the manufacturer's recommendation of 7 capsules was followed.

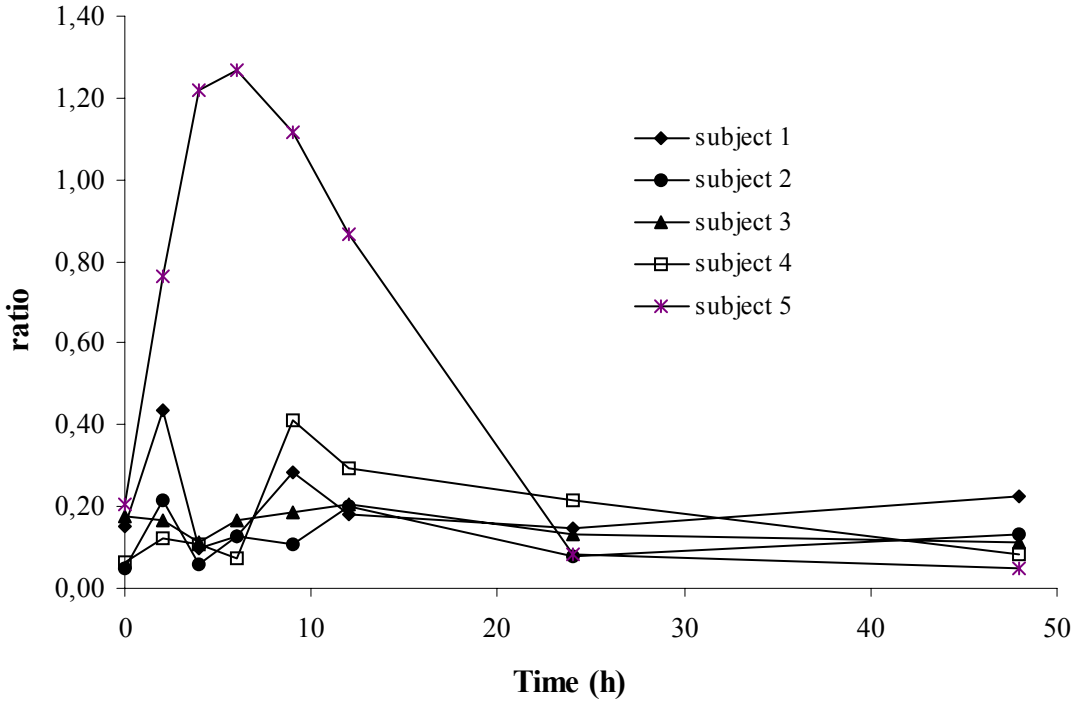


**Figure 4.3.2 Urinary 4-androstene-3,17-dione/epitestosterone ratios in 5 male volunteers after ingestion of one capsule of the contaminated dietary supplement A.**

Recently it has been shown that the misuse of exogenous 4-androstene-3,17-dione can be detected by screening for 6α-hydroxyandrostenedione [11]. Inclusion of this compound in screening methods might have indicated the use of 4-androstene-3,17-dione after the administration of one tablet of this supplement although this remains questionable as the ingested dose is far below the most frequently used dose of 50 mg [10, 11].

High amounts of 19-nor-4-androstene-3,17-dione, a precursor of nandrolone, in supplement A were detected in all subjects by the presence of 19-norandrosterone (the main urinary metabolite) (Figure 4.3.3)

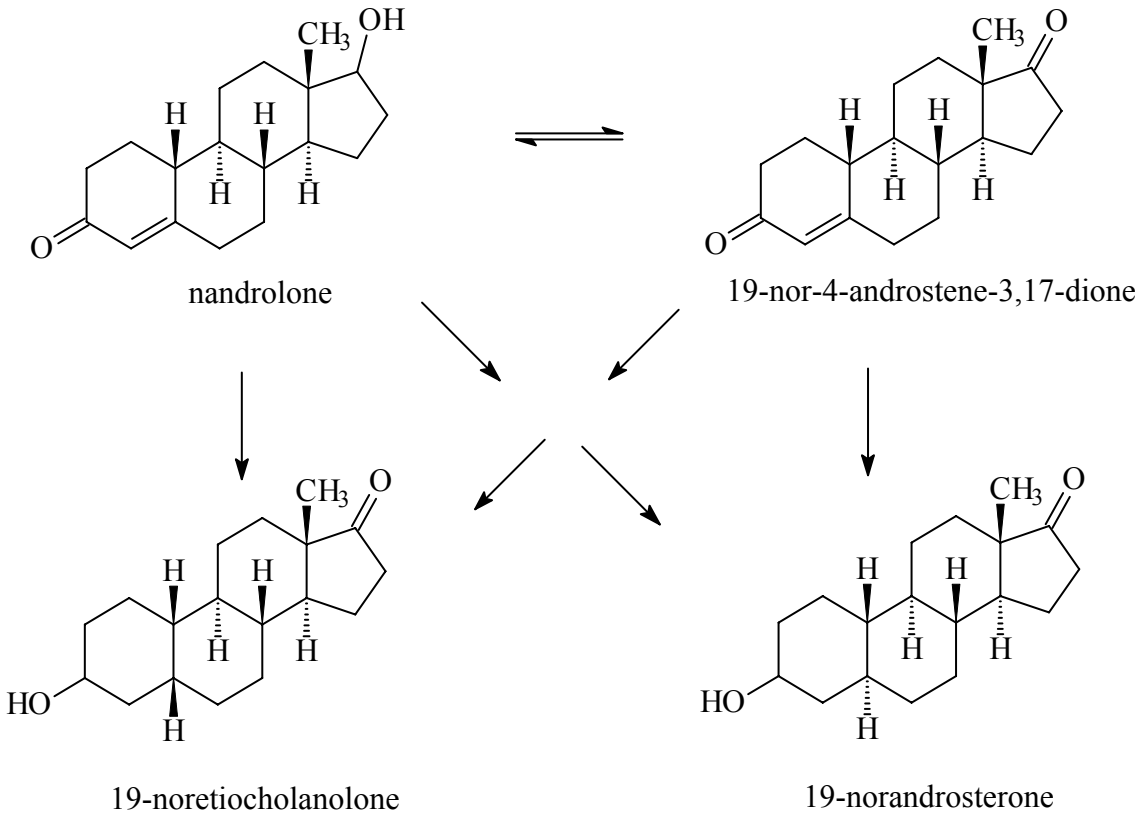


**Figure 4.3.3 Metabolic pathway of 19-nor-4-androstene-3,17-dione.**

The excretion profile of this substance after the ingestion of one capsule of supplement A is shown in figure 4.3.4.

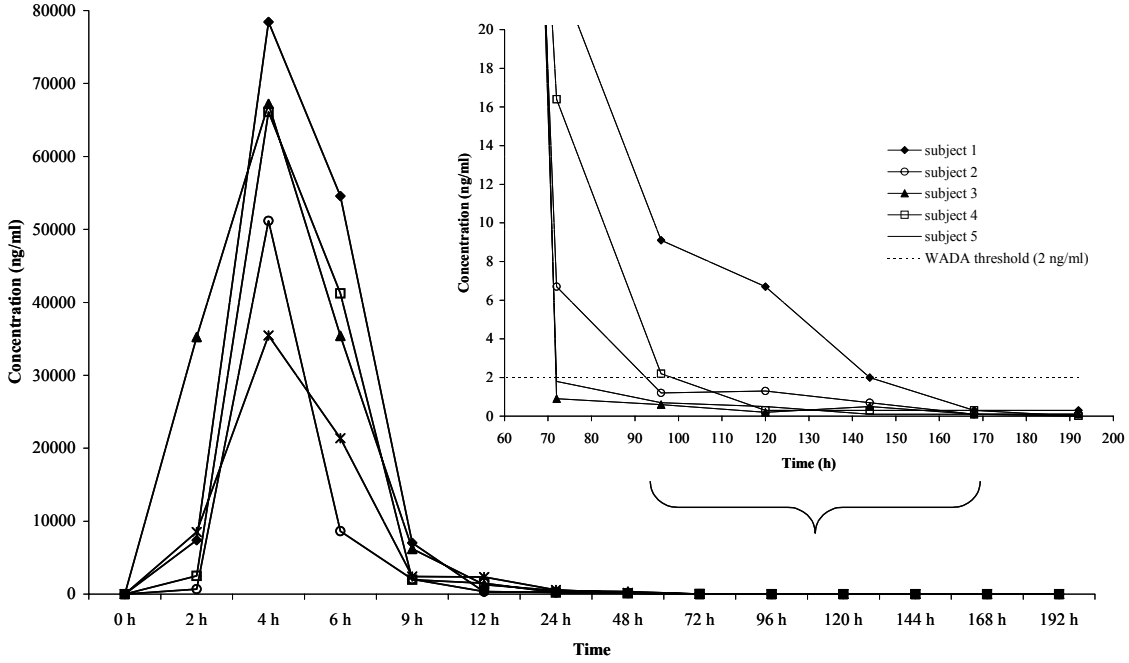


**Figure 4.3.4 Urinary excretion profiles of 19-norandrosterone in five male volunteers after the ingestion of one capsule of supplement A.**

According to the WADA urinary threshold level for norandrosterone of 2 ng/ml, doping positive results were found up to 144 h after the administration of one capsule of the supplement.

The use of 7 capsules of supplement A, as recommended by the manufacturer, could have resulted in even longer detection times and in serious health effects.

### 4.3.2. Supplement B

The analysis of nutritional supplement B revealed the presence of multiple non-labelled prohormones. These substances and their concentration, determined on the homogenised content of 10 capsules, are given in Table 4.3.1

**Table 4.3.1 Detected prohormones and concentration in nutritional supplement B.**

| Substance | Concentration (ng/g) |
|---|---|
| DHEA | 159 |
| 4(5)-androstene-3,17-dione | 78 |
| testosterone | 243 |
| 19-nor-4(5)-androstene-3,17-dione | 189 |

The concentrations are much lower than in supplement A, eventually indicative for undeliberate contamination.

Urinary steroid profiles did not change significantly after the ingestion of the recommended 2 capsules. Norandrosterone was detected in urine of both subjects in the 2 h sample. The detected concentrations were 0.11 ng/ml and 0.23 ng/ml in subject 1 & 2 respectively. Although the concentrations of prohormones in supplement B are too low to result in adverse doping findings after administration of the recommended dose, the presence of these prohormones should have been mentioned on the product label. According to the U.S. Anabolic Steroid Control Act enacted on January 20$^{th}$ 2005, the presence of these steroids is illegal and should result in the withdrawal of this nutritional supplement from the market.

### 4.3.3. Supplement C

Analysis of 10 homogenised capsules of nutritional supplement C resulted in the detection of 6 prohormones in a concentration range of 67 to 1739 ng/g(Table 4.3.2).

**Table 4.3.2 Concentration (ng/g) and average amount per capsule (ng) in nutritional supplement C.**

| Compound | Concentration (ng/g) | Amount/capsule (ng) |
|---|---|---|
| DHEA | 1047 | 761 |
| 19-nor-4(5)androstene-3,17-dione | 1739 | 1265 |
| nandrolone | 474 | 345 |
| DHT | 218 | 158 |
| 4(5)-androstene-3,17-dione | 67 | 49 |
| testosterone | 408 | 296 |

Confirmation of the screening results was obtained with full scan mass spectrometry. Figure 4.3.5 shows the confirmation results for DHT in comparison to a quality control sample spiked at 50 ng/g. Similar confirmation results could be obtained for the other detected compounds.

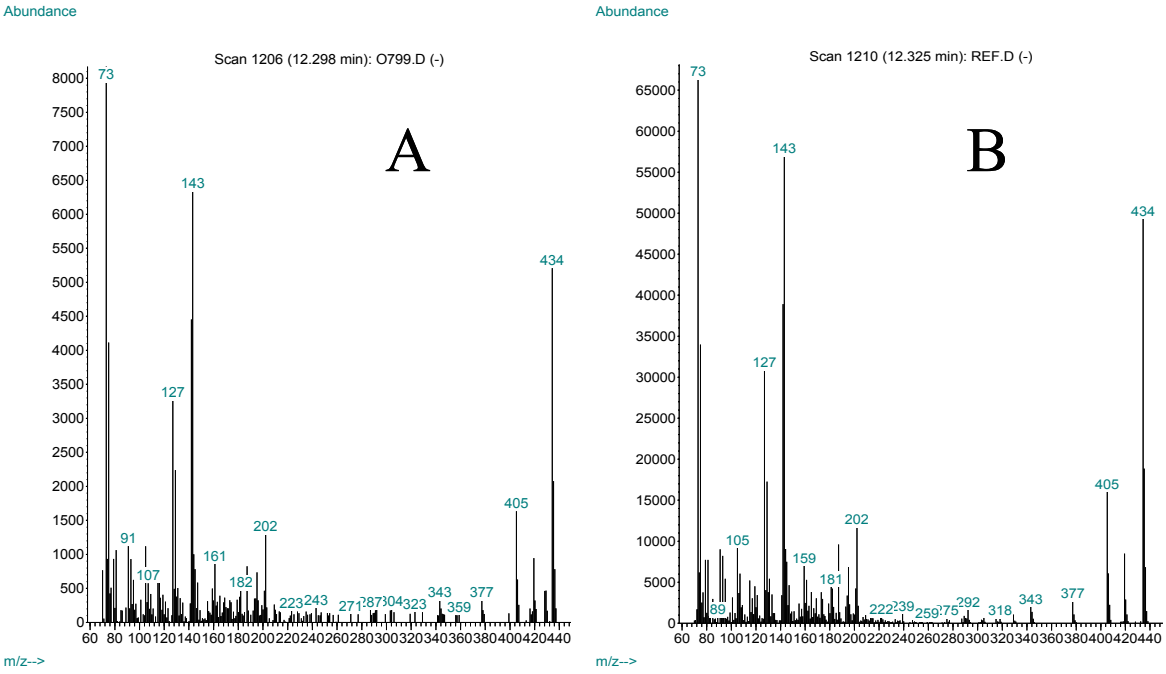


**Figure 4.3.5 Mass spectrum of TMS-derivatised DHT in the nutritional supplement (A) in comparison to a quality control sample (B).**

DHT is the active metabolite of testosterone and its androgenic activity is 2-3 times higher than the parent steroid. In humans, DHT is mainly produced by 5α-reductase from testosterone in androgen dependent target tissues. Because this process is irreversible, the administration of DHT as a substitute for testosterone may be beneficial because the undesired side effects due to the formation of estradiol (female hormone) by aromatase are avoided [12].

Parameters for the misuse of DHT are the DHT concentration, the ratio of androsterone to etiocholanolone and the ratio of 5α-androstan-3α,17β-diol to 5β-androstan-3α,17β-diol. These ratios are based upon the fact that DHT solely metabolises to 5α-metabolites and that no inter-conversion to 5β-metabolites occurs [12] (Figure 4.3.6).

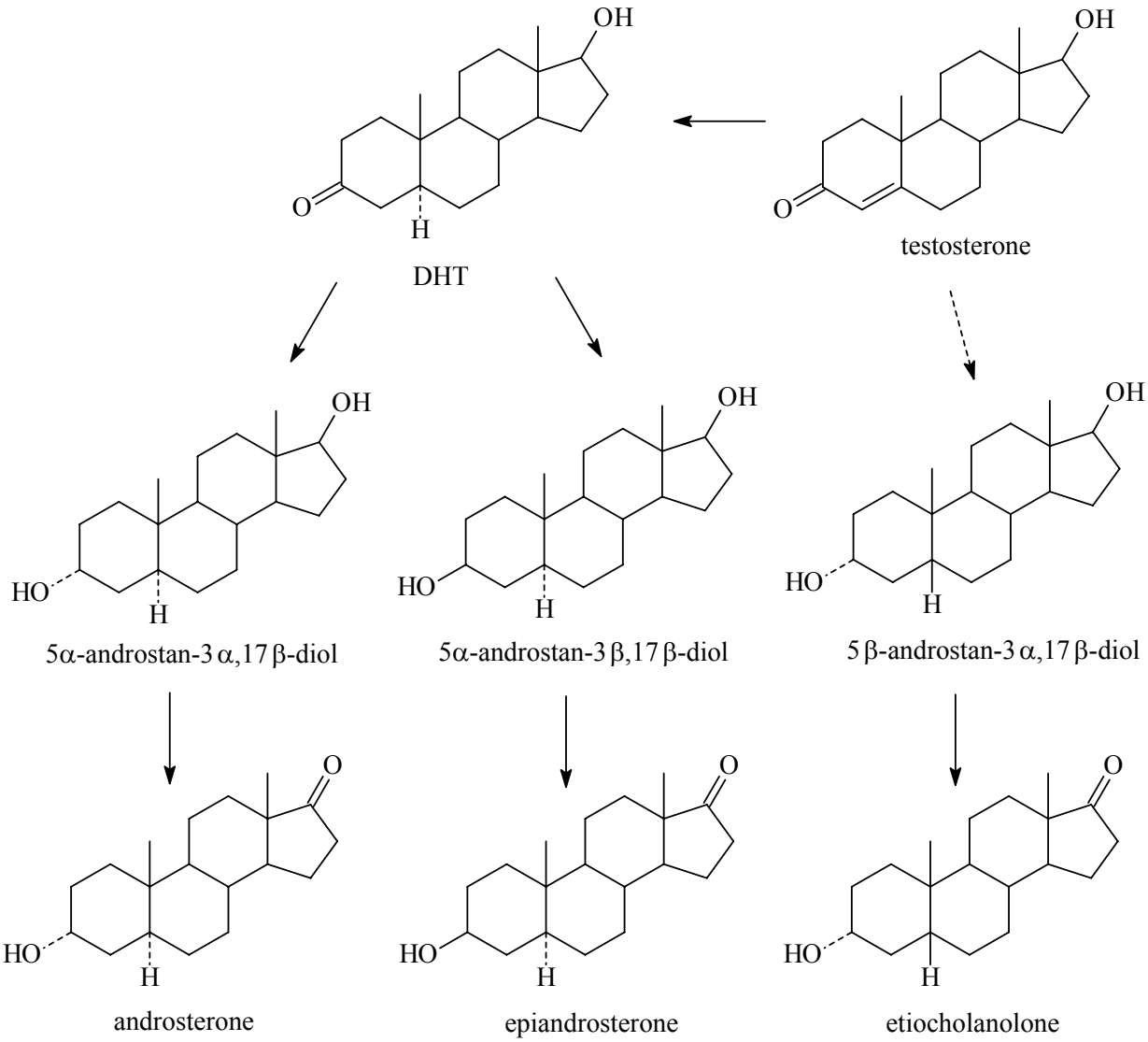


**Figure 4.3.6 Metabolic pathway of DHT.**

An excretion study performed in three male volunteers, as described in §4.2.3, did not result in an elevated T/E ratio nor in changes in the parameters indicative for DHT misuse. Also no changes were found in the urinary parameters indicative for the use of 4(5)-androstene-3,17-dione. These findings are probably due to the low amounts of these compounds in nutritional supplement C. The concentration of 19-nor-4(5)-androstene-3,17-dione however was sufficient to result in the detection of 19-norandrosterone. Urinary excretion profiles of 19-norandrosterone following the administration of nutritional supplement C according to the manufacturer's recommendations are shown in Figure 4.3.7.

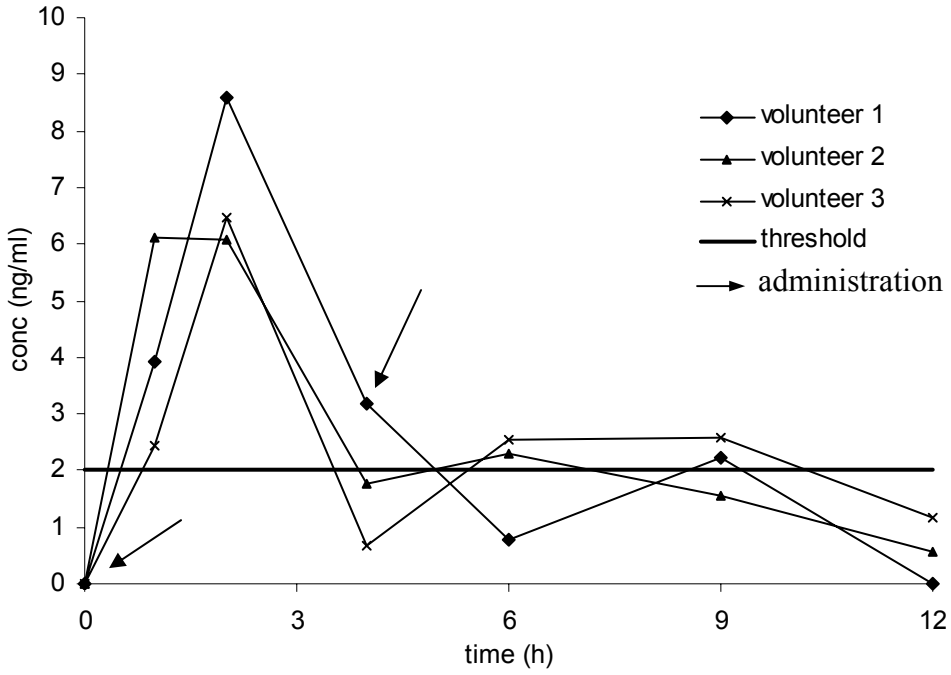


**Figure 4.3.7 Excretion profile for 19-norandrosterone following administration of supplement C.**

19-norandrosterone was detected in the urine samples of all volunteers already 1 h after the administration of the first dose. Volunteer 1 & 3 tested positive according to the WADA threshold level of 2 ng/ml until 9 h after administration. Previous findings by Geyer *et al.* [13] proving that doping positive urinary concentrations of the nandrolone metabolite norandrosterone can be obtained if the total oral intake of nandrolone precursors is higher than 1 µg are confirmed in this study.

### 4.3.4. Supplement D

Analysis of nutritional supplement D, from the same supplement producing company as supplement C, showed the presence of high amounts of 19-nor-4(5)-androstene-3,17-dione (1.7 µg/g). Based upon the results in nutritional supplement C, ingestion of this amount of 19-nor-4(5)-androstene-3,17-dione will also result in doping positive urine samples.

Besides the nandrolone precursor a large peak at a retention time of 12.21 min appeared in the TIC of the confirmation procedure. Based upon a library search on the underivatised component and the mass spectrum obtained after derivatisation, this unknown compound was

identified as 5α-androstane-3,17-dione. Confirmation was obtained by the comparison of the supplement with a reference substance as shown in Figure 4.3.8.

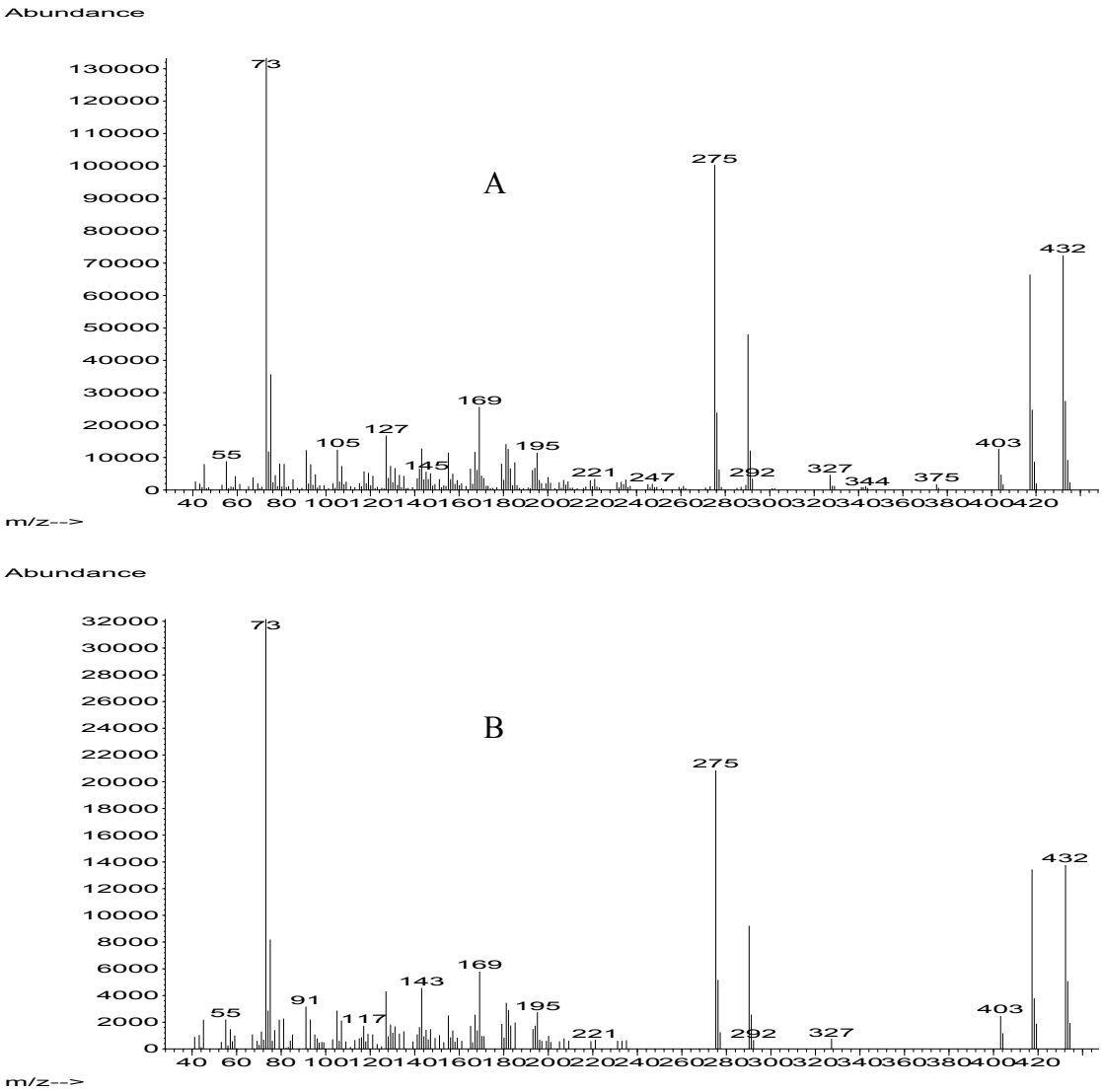


**Figure 4.3.8 Mass spectra of bis-TMS-derivatised 5α-androstane-3,17-dione (A) and of the unknown substance in supplement D (B).**

The presence of 5α-androstane-3,17-dione in nutritional supplements has never been reported. The detected amount of this substance (453 ng/g), together with the detection of 19-nor-4(5)-androstene-3,17-dione is rather indicative for the deliberate addition of these compounds instead of accidental contamination.

### 4.3.5. Overall Results

The case studies reported here are only a fraction of the results from supplement testing in our laboratory. Since December 2002 until January 2006, 367 supplements were analysed in the Doping Control Laboratory (UGent). These supplements were tested for anabolizing agents and/or stimulants according to the manufacturers demands, using the validated methods for the screening of anabolizing agents and stimulants described in Part III Chapter 2 & 3.

In most cases supplements were sent by supplements producing companies. Some were bought and some others were sent by athletes as a follow-up study after a positive doping test. The Doping Control Laboratory (UGent) also acts as one of the two laboratories involved in the NZVT-system. This system comprises the Dutch Olympic Committee, the Dutch Government, NeCeDo and the branch organisation for supplement producing companies (NPN) in a combining effort to supply athletes with supplements free of doping contaminants.

The total number of samples analysed, the number and percentage of positives analysed until January 2006 in the different categories is given in Table 4.3.3

**Table 4.3.3 Number of nutritional supplements analysed, number and percentage of positives obtained in the period December 2002 – January 2006.**

| | prohormones | stimulants | caffeine |
|---|---|---|---|
| N° samples | 347 | 108 | 16 |
| N° positives | 37 | 6 | 3 |
| % positives (%) | 10.7 | 5.6 | 18.8 |

Caffeine was detected in 3 nutritional supplements. Concentrations in these supplements never exceeded 6 mg/g. As indicated in Part II Chapter 1 these amounts would not result in positive doping findings and are of minor importance after the removal of caffeine from the WADA doping list. However, not indicating the presence of caffeine on the label, which was the case in all three supplements, can be considered as misinformation towards the consumer.

Six nutritional supplements contained one or more stimulants. Four of them contained non-labelled ephedrine. Pseudoephedrine, norephedrine, norpseudoephedrine and strychnine were

also found. Of all samples analysed within NZVT one sample contained non-labelled ephedrine and cathine.

Out of the 347 samples analysed for prohormones in our laboratory 37 dietary supplements contained one or more prohormones (Table 4.3.4).

**Table 4.3.4 Prevalence of prohormones in nutritional supplements in the period December 2002 – January 2005.**

| Compound | Frequency |
|---|---|
| DHEA | 28 |
| testosterone | 18 |
| 4(5)-androstene-3,17-dione | 16 |
| 19-nor-4(5)-androstene-3,17-dione | 10 |
| DHT | 7 |
| nandrolone | 5 |
| 5-androstene-3β,17β-diol | 5 |
| 1-androstene-3,17-dione | 3 |
| 17α-methyltestosterone | 2 |
| 4-androstene-3β,17β-diol | 1 |
| 1-testosterone | 1 |
| 5α-androstane-3,17-dione | 1 |

The results indicate that numerous dietary supplements contain more than one anabolizing agent not mentioned on the label. Prohormones of testosterone, more specifically DHEA, testosterone and 4(5)-androstene-3,17-dione, were most frequently detetected. Nandrolone and its precursors were less detected, but the results of excretion studies in this chapter demonstrate that contamination with these prohormones often results in adverse doping findings. The detection of 17α-methyltestosterone in two nutritional supplements is alarming because of the serious health effects associated with its use.

## *4.4. Conclusion*

Nutritional supplements can be contaminated as a result of improper cleaning of equipment or by the use of raw materials not tested for the presence of forbidden substances. In many cases the detected concentrations are low and inconsistent over the complete batch. Use of this kind of supplements by athletes is of lower risk compared to nutritional supplements intentionally contaminated with prohormones. Concentrations of prohormones in intentionally contaminated supplements are often very high and can result in positive doping tests if the recommendations of the manufacturers are followed.

The case reports in this chapter prove that the search for anabolizing agents in nutritional supplements is of utmost importance. Numerous dietary supplements were contaminated with multiple prohormones not mentioned on the label. In addition, a new substance, 5α-androstane-3,17-dione, previously not detected in supplements was also found.

Overall results show that a substantial fraction of the supplements tested in the Ghent Doping Control laboratory were contaminated with one or more non-labelled substances.

Besides the serious health effects associated with the use of these products, athletes should be aware that the use of contaminated supplements could result in a positive doping test. According to WADA doping rules athletes are responsible for the substances detected in their biofluids, irrespective of the origin. Hence, the development of methods capable of detecting contamination of nutritional supplements with doping relevant substances by specialised laboratories is of utmost importance. This guarantee can only be given by the specialised doping control laboratories.

## *4.5. Acknowledgements*

The authors wish to thank the Flemish Ministry of Health for financial support.

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# CHAPTER 5: ANDROST-4-ENE-3,6,17-TRIONE

Adapted from:

- W. Van Thuyne, P. Van Eenoo, P. Mikulcíková, K. Deventer, and F.T. Delbeke, *Detection of androst-4-ene-3,6,17-trione (6-OXO®) and its metabolites in urine by gas chromatography-mass spectrometry in relation to doping analysis*. Biomed Chromatogr., 2005. **19**: 689-695.

# Chapter 5: Androst-4-ene-3,6,17-trione

## *5.1. Abstract*

6-OXO®, a new nutritional supplement available on the internet, is advertised as an aromatase-inhibitor and contains androst-4-ene-3,6,17-trione as active ingredient. The metabolism and excretion of androst-4-ene-3,6,17-trione after administration of the "nutritional" supplement 6-OXO® was investigated by GC/MS. The parent drug androst-4-ene-3,6,17-trione as well as androst-4-ene-6α,17β-diol-3-one and androst-4-ene-6α-ol -3,17-dione were detected in post administration urine samples.

Androst-4-ene-3,6,17-trione is an anabolic steroid and an aromatase inhibitor and regarded as a doping agent. A selective and sensitive GC/MS method in selected ion monitoring mode for the detection of the TMS-enol-TMS –ether derivatives of this substance and the metabolites was developed and validated for doping control purposes. LODs of the investigated compounds ranged from 5 to 10 ng/ml. The detection time for androst-4-ene-3,6,17-trione and androst-4-ene-6α,17β-diol-3-one was 24 h, while androst-4-ene-6α-ol-3,17-dione could be detected up to 37 h after administration of the recommended dose.

## *5.2. Introduction*

Aromatase inhibition is a therapeutic target for the selective lowering of estrogen levels in patients with estrogen dependent tumours including breast cancer [1, 2]. Several in-vitro experiments have shown that the anabolic steroid androst-4-ene-3,6,17-trione exhibits aromatase inhibiting-properties [2-4].

Similar to many other so-called prohormones [5-7], androst-4-ene-3,6,17-trione recently became available as a nutritional supplement. This product is sold as an over-the-counter nutritional supplement under the 1994 U.S. DSHEA as androst-4-ene-3,6,17-trione was not included in the Anabolic Steroid Control Act. The global distribution of these products via the internet has resulted in a huge commercial success for products such as (nor)androstenedione, (nor)androstenediol and dehydroepiandrosterone [5].

Currently, androst-4-ene-3,6,17-trione is advertised as an aromatase inhibitor capable to cure gynaecomastia, an adverse side-effect of anabolic steroids misuse [8] and it is produced by a company that has introduced several other anabolic steroids on the supplement market [5, 9].

Because of their action and potential misuse, aromatase inhibitors are classified as prohibited substances in sports by WADA [10]. Based on its chemical and (claimed) pharmacological action, it is clear that androst-4-ene-3,6,17-trione is a prohibited substance in sports.

Because most anabolic steroids are completely metabolised and no parent drug is excreted in urine, detection methods for these metabolites need to be developed [11].

At present no data on the in-vivo metabolism of androst-4-ene-3,6,17-trione in humans is available. Based upon its structure it is highly unlikely that misuse of this steroid will be detected, using routine screening methods for anabolic steroids in WADA-accredited laboratories because these laboratories traditionally rely on GC/MS in the SIM mode [12, 13]. Although SIM displayed a higher sensitivity compared to full scan GC/MS, it only allows for the detection of known substances [12]. This study was set up to elucidate the metabolism of androst-4-ene-3,6,17-trione and to determine markers for misuse of this substance in doping control analysis.

## *5.3. Experimental*

### 5.3.1. Reagents

Reference materials of androst-4-ene-3,6,17-trione (6-oxo-androstenedione), androst-4-ene-6α-ol-3,17-dione (6α-OH-androstenedione), androst-4-ene-6β-ol-3,17-dione (6β-OH-androstenedione), androst-4-ene-6α,17β-diol-3-one (6α-OH-testosterone) and androst-4-ene-6β,17β-diol-3-one (6β-OH-testosterone) were purchased from Steraloids (Newport, USA). 17α-methyltestosterone was from Organon (Oss, The Netherlands). The enzyme preparation β-glucuronidase type HP-2 from Helix pomatia (127 300 U/ml β-glucuronidase), potassium acetate and imidazole were obtained from Sigma (St. Louis, MO). MSTFA was purchased from Chem Fabrik Karl Bucher (Waldstetten, Germany). All other chemicals were of analytical grade.

One jar of 6-OXO® containing 60 capsules was purchased from Ergopharm (Champaign, USA). The labelled content of each capsule was 100 mg of 3,6,17-androstenetrione. The other listed ingredient was micro crystalline cellulose. The manufacturer's recommended daily dose was 3 to 6 capsules in cycles of 4 to 6 weeks.

### 5.3.2. Analysis of 6-OXO® supplement

Analysis of the 6-OXO® supplement was performed according to a previously described procedure by Van Thuyne and Delbeke [14] except for the internal standard which was 17α-methyltestosterone and for the amount of supplement tested (100 mg instead of 1 g).

### 5.3.3. Urine analysis

Extraction of the urine samples was performed using liquid-liquid extraction with diethylether of 2 ml urine after enzymatic hydrolysis, as described by Van Eenoo *et al.*[9].

### 5.3.4. Derivatisation

The dried residue of the urine samples was derivatised with 100 µl MSTFA/$NH_4I$/ethanethiol (320/1/2; v/w/v) at 80°C for 60 min and transferred to an autosampler microvial for GC/MS analysis.

To elucidate the stereochemistry at C6, the samples were derivatised with 100 µl MSTFA/potassium acetate/imidazole (1000/20/20; v/w/v) at 80°C for 60 min.

### 5.3.5. GC/MS conditions

The identification of the urinary metabolites was performed in full scan mode (mass range: m/z 65-600). Determination of the detection times was done in SIM mode. Both GC/MS methods were run on an Agilent 6890 gas chromatograph directly coupled to an Agilent 5973 mass selective detector (Agilent, Palo Alto, USA). The GC column was an HP-Ultra 1 (J&W, Folsom, USA) 100 % methylsilicone column with a length of 17 m, an internal diameter of 0.2 mm and a film thickness of 0.11 µm. Helium was used as carrier gas in the constant flow mode (0.6 ml/min). A total of 1 µl was injected splitless on the chromatographic system. The oven temperature program was as follows: 120°C (0 min), 70°C/min → 230°C (0.1 min), 1.5°C/min → 232°C (0 min), 1.0°C/min → 238 °C (0 min), 30°C/min → 300 °C (2 min). The electron energy was 70 eV and the ion source temperature was 230 °C. Three ions were monitored for each compound in the SIM mode. Dwell times were set at 50 ms except for the ions 520.3, 516.3 and 518.3 which were set at 30 ms.

### 5.3.6. Method validation

To determine the LOD, ten different negative control urines were spiked with the metabolites at different concentrations in the range 2 – 50 ng/ml (2, 5, 10, 25 and 50 ng/ml).

Specificity was tested by the analysis of ten different negative urine samples. Selectivity was evaluated by the analysis of a reference mixture of several compounds. These compounds were: 9α-fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol, 17α-methyl-androst-4-ene-11α,17β-diol, 6β-hydroxy-4-chlorodehydro-methyltestosterone, 17α-ethyl-5β-estrane-3α,17β-diol, 7α,17α-dimethyl-5β-androstane-3α,17β-diol, 16β-OH-stanozolol, salbutamol,

clenbuterol, terbutaline, oxandrolone, 17α-trenbolone, pemoline, probenecid, clopamide, canrenone, amiloride, triamterene, 16β-hydroxy-furazabol, 7β,17α-dimethyl-5β-androstane-3α,17β-diol, 6β-hydroxy-metandienone, 4-chloro-androst-4-ene-3α-ol-17-one, 2α-methyl-5α-androstan-3α-ol-17-one, 1α-methyl-5α-androstan-3α-ol-17-one, 11-nor-$\Delta^9$-tetrahydrocannabinol.carboxylic acid, 19-norandrosterone, 19-noretiocholanolone, 17β-methyl-5β-androst-1-ene-3α,17α-diol, 3'OH-stanozolol, 17α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5β-androstane-3α,17β-diol, boldenone, 5β-androst-1-ene-17β-ol-3-one, oxymesterone and ethisterone. The concentrations ranged from 80 to 500 ng/ml.

### 5.3.7. Excretion study

Excretion studies were performed in two male volunteers according to a strict research protocol approved by WADA and by the Ethical Committee, University Hospital (Ghent, Belgium) (EC/2005-81/sdp).

Subject 1 ingested 1 capsule of the 6-OXO® supplement. Urine samples were collected quantitatively during the first 24 hours (0, 2, 4, 6, 8, 10, 12, 24). Additional urine samples were taken for 7 days, three times a day (morning, noon, evening).

Subject 2 took three capsules of the 6-OXO® supplement for three successive days according to the manufacturer's recommendations. Urine samples were collected quantitatively for 72 h (0, 2, 4, 6, 8, 10, 12, 24 on each of the three days). Additional urine samples were taken for 7 more days, three times a day (morning, noon, evening).

All urine samples were stored at -20°C awaiting analysis.

## *5.4. Results and Discussion*

Analysis of the nutritional supplement 6-OXO® revealed the presence of androst-4-ene-3,6,17-trione and minor amounts of 6ξ-OH-androst-4-ene-3,17-dione. No other compounds were detected as contaminants according to the in-house method for the detection of anabolising agents in nutritional supplements [14].

As described by van de Kerkhof *et al.* [15] trimethylsilylation with a mixture of MSTFA/$NH_4I$/ethanethiol, as routinely used in doping control laboratories, cannot be applied for the selective analysis of the steroids androst-4-ene-6α-ol-3,17-dione and androst-4-ene-6β-ol-3,17-dione because the stereochemical integrity at C6 is lost due to 3,5-dienol formation. Therefore derivatisation using MSTFA, potassium acetate and imidazole was used to retain the stereochemical information at C6 resulting in the formation of 2,4-dienol-TMS-derivatives [16]. The mass spectra of the 2,4-dienol-TMS-derivatives of 6β-OH-androstenedione and 6α-OH-androstenedione are shown in Figure 5.4.1 and Figure 5.4.2, respectively.

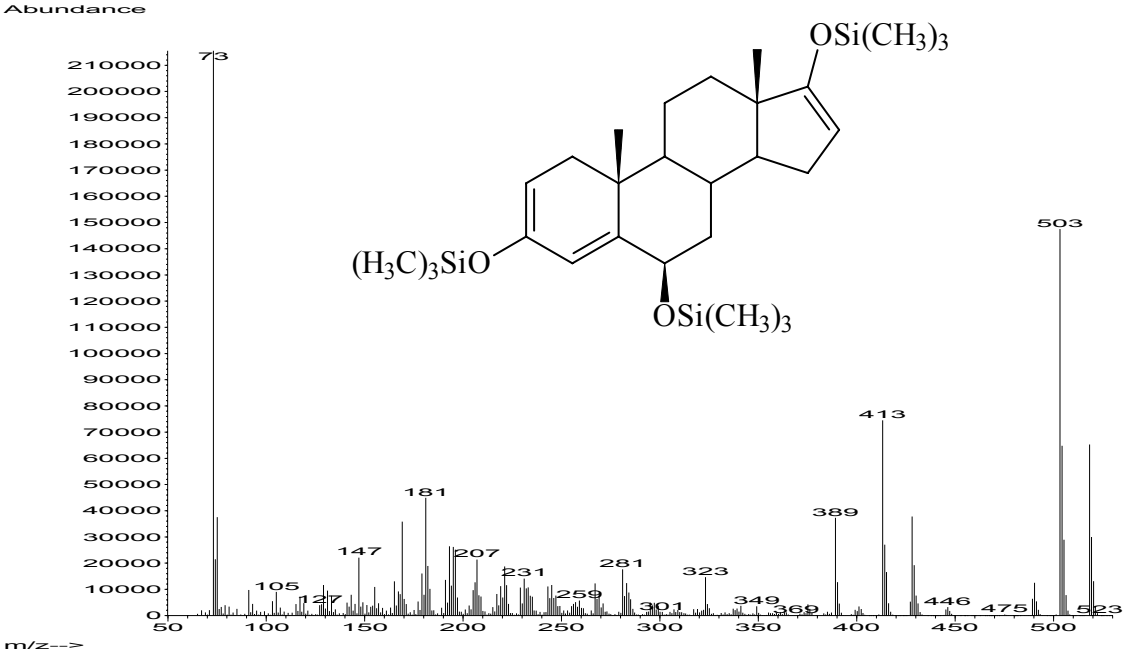


**Figure 5.4.1 Mass spectrum of MSTFA/imidazole/KOAc derivatized 6β-OH-androstenedione.**

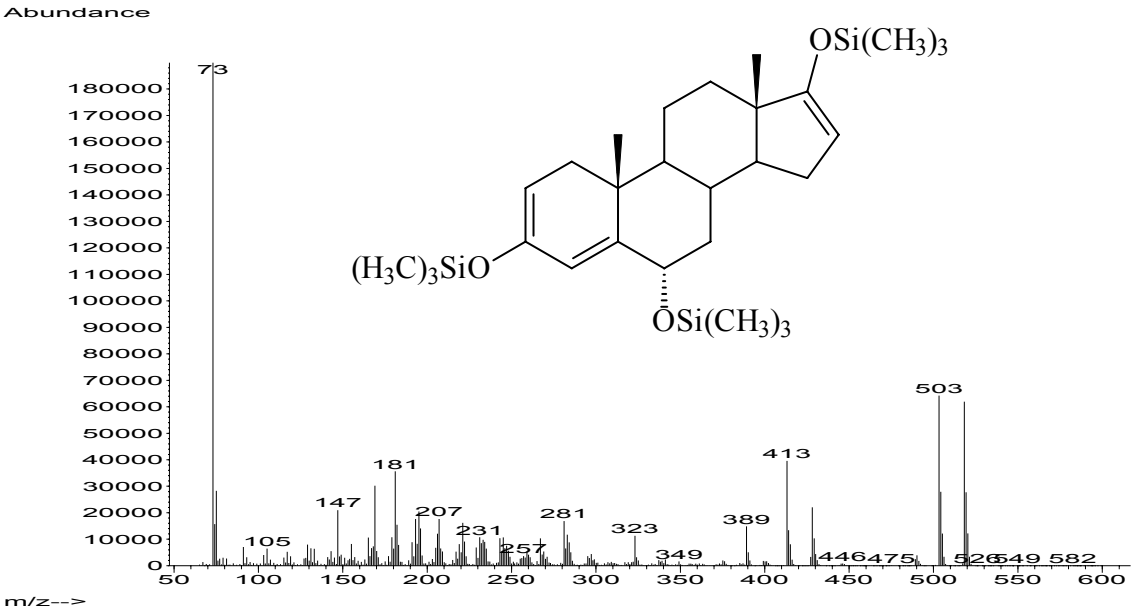


**Figure 5.4.2 Mass spectrum of MSTFA/imidazole/KOAc derivatized 6α-OH-androstenedione.**

Retention times of 6β-OH-androstenedione and 6α-OH-androstenedione were 6.0 and 6.7 min, respectively. This additional analysis revealed that the minor compound present in the capsules was androst-4-ene-6β-ol-3,17-dione (6β-OH-androstenedione).

Identification of urinary metabolites of 6-oxo-androstenedione was performed using GC/MS in the full scan mode. For this purpose, the urine sample taken 10 h after the administration of three capsules on the third day by volunteer 2 was analysed. Besides 6-oxo-androstenedione (Figure 5.4.3) the sample also contained 6ξ-OH-androstenedione (Figure 5.4.4) and 6ξ-OH-testosterone (Figure 5.4.5).

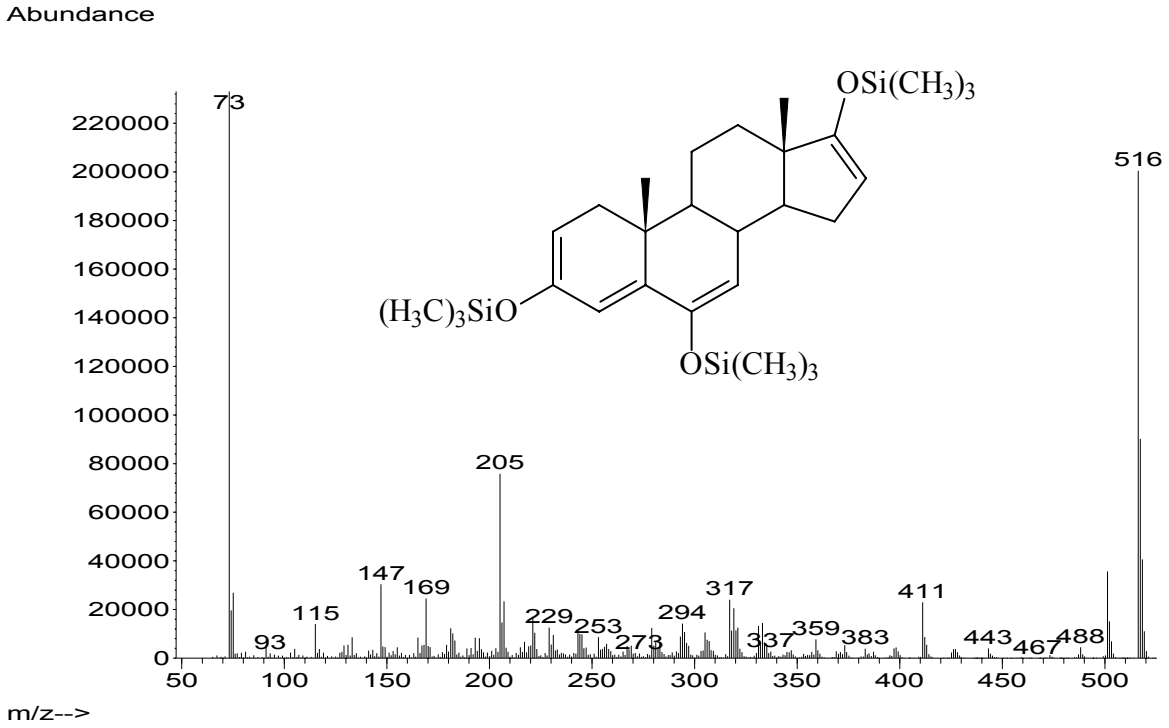


**Figure 5.4.3 Mass spectrum of MSTFA/$NH_4I$/ethanethiol derivatized 6-oxo-androstenedione.**

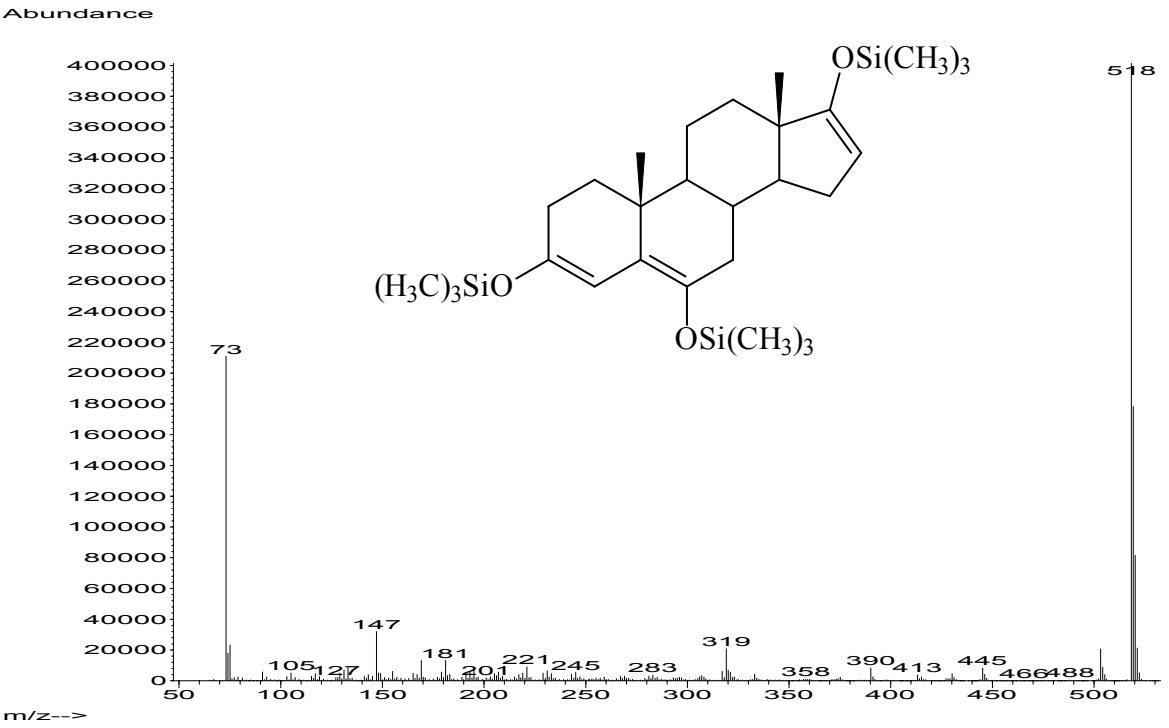


**Figure 5.4.4 Mass spectrum of MSTFA/$NH_4I$/ethanethiol derivatized 6ξ-OH-androst-4-enedione.**

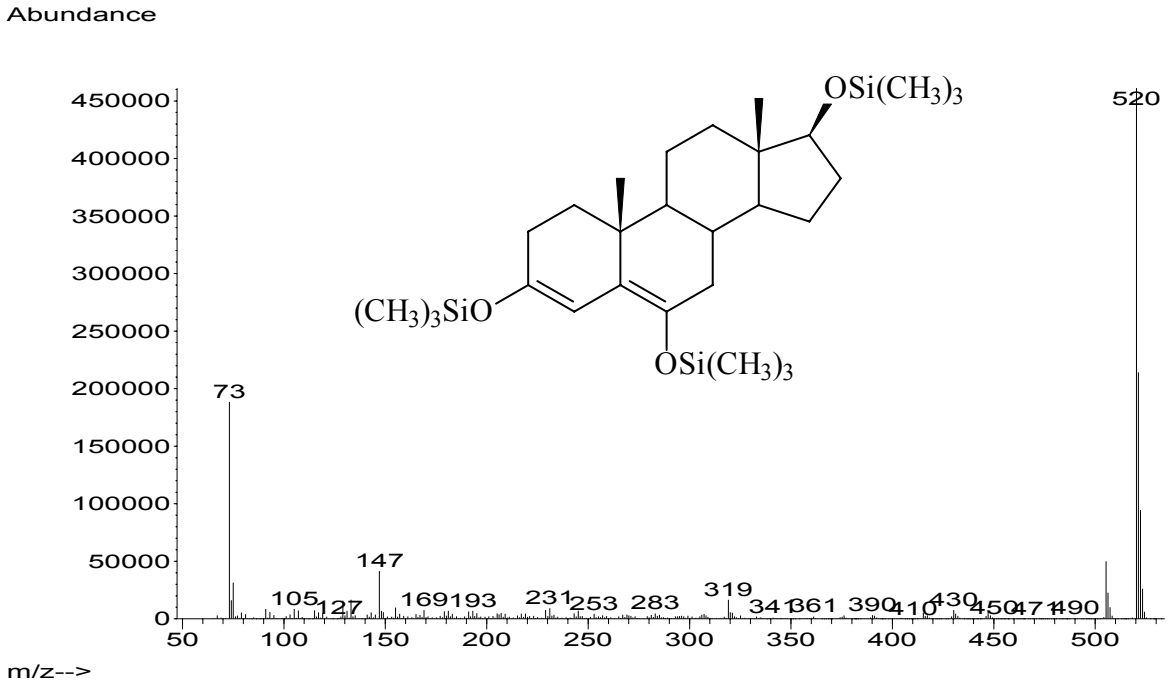


**Figure 5.4.5 Mass spectrum of MSTFA/$NH_4I$/ethanethiol derivatized androst-4-ene-6ξ,17β-diol-3-one.**

Again, similar to 6β-OH-androstenedione in the capsules, the position of the hydroxyl function at the C6 of 6ξ-OH-androstenedione and 6ξ-OH-testosterone in the urine samples could not be determined with MSTFA/$NH_4I$/ethanethiol as derivatisation reagent. An additional analysis using MSTFA/potassium acetate/imidazole however revealed that the metabolites were 6α-OH-androsterone and 6α-OH-testosterone. The corresponding 6β-isomers were not detected in the urine samples.

Lévesque *et al.* [17] and van de Kerkhof [18] showed that, in contrast to *in-vitro* experiments where 6β-hydroxylation prevails [19], orally administered androst-4-ene-3,17-dione is metabolised *in-vivo* to 6α-OH-androstenedione. From the results of this study, it can be concluded that metabolism of 6-oxo-androstenedione is similar as for androst-4-ene-3,17-dione (Figure 5.4.6).

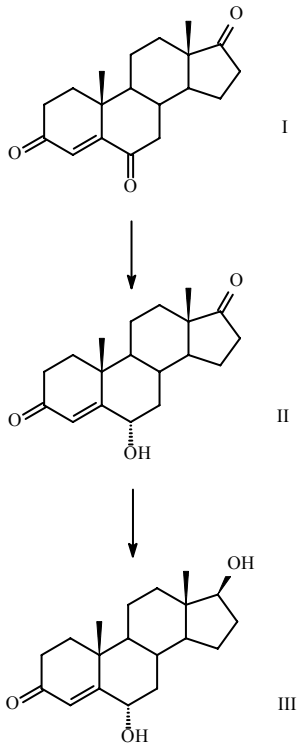


**Figure 5.4.6 In vivo metabolic pathway for androst-4-ene-3,6,17-trione (I) to androst-4-ene-6α,17β-diol-3-one (III) via androst-4-ene-6α-ol-3,17-dione (II).**

Taking into account the metabolism of androgens in man and particularly the metabolism of androst-4-ene-3,17-dione [20], metabolism of 6α-OH-androstenedione to 6α-OH-testosterone seems logical. Moreover, 6β-isomers were not detected in the excretion urine, although further research on the fate of 6β-OH-androstenedione, the minor component in the capsules, is in progress.

The appearance of 6α-OH-testosterone and especially the parent drug 6-oxo-androstenedione in a urine sample can be used as a direct indication of the intake of 6-OXO®. Although van de Kerkhof [18] speculated on the theoretical conversion of 6α-OH-androstenedione to 6-oxo-androstenedione, analogous to the formation of 7-keto-dehydroepiandrosterone from 7ξ-OH-dehydroepiandrosterone, his study showed that androst-4-ene-3,17-dione administration did not result in the detection of 6-oxo-androstenedione. Hence, the detection of 6-oxo-androstenedione can be used to discriminate between the administration of 6-oxo-androstenedione and androstenedione.

Based upon these findings, a GC/MS method in the SIM-mode was developed to determine the detection times of the metabolites after administration of 6-oxo-androstenedione. The GC (relative) retention times, monitored ions and respective relative abundances are given in Table 5.4.1. Presence of the substances was determined in agreement with criteria on chromatography and mass spectrometry as defined in a technical document by WADA [21].

**Table 5.4.1 RT, RRT, diagnostic ions (relative abundance) and LOD for the trimethylsilylated compounds analysed in the selective ion monitoring mode.**

| Compound | RT (min) | RRT | Diagnostic ions (relative abundance %) | LOD (ng/ml) |
|---|---|---|---|---|
| androst-4-ene-3,6,17-trione | 7.16 | 1.05 | 516 (100), 501 (20), 411 (12) | 5 |
| androst-4-ene-6α-ol -3,17-dione | 7.50 | 1.10 | 518 (100), 503 (6), 319 (6) | 10 |
| androst-4-ene-6α,17β-diol-3-one | 7.77 | 1.14 | 520 (100), 505 (11), 319 (5) | 5 |

The validation of the SIM-method was performed according to Eurachem guidelines [22]. As indicated in Table 5.4.1, LODs ranged from 5 to 10 ng/ml, complying with WADA rules requiring an MRPL for anabolic steroids of 10 ng/ml [23].

The procedure used to test for selectivity and specificity was the same as in Verwaal *et al.* [24]. For qualitative methods, the specificity can be tested by analysing all negative matrices used to determine the LOD. No matrix interferences were found, neither at the retention times of the analytes, nor at the retention time of the internal standard 17α-methyltestosterone. Selectivity was tested by analysing a reference mixture of several structurally related and other doping agents. According to the guidelines described by Verwaal *et.al.* [24], the concentration of these analytes must be at least twice the LOD of the determined compounds. In this study their concentration ranged from 0.8 µg/ml to 5 µg/ml, clearly complying with these requirements. As no interferences were detected at the retention times of the determined analytes and the internal standard, it can be concluded that the method is reliable and sensitive for the detection of 6-oxo-androstenedione, 6α-OH-androstenedione and 6α-OH-testosterone.

Detection times depend on the administration protocols. In the first protocol, where the subject took only one capsule of the 6-OXO® supplement, 6-oxo-androstenedione and 6α-OH-testosterone were both detectable from 2 until 12 hours post-administration and 6α-OH-androstenedione could be detected up to 29 hours. In the second protocol, where 3 capsules were taken during three successive days according to the manufacturer's recommendations, 6-oxo-androstenedione and 6α-OH-testosterone remained detectable until 24 hours after the last administration, while 6α-OH-androstenedione could be detected up to 37 h after the last intake.

## *5.5. Conclusion*

Analysis of the 6-OXO® supplement resulted in the detection of 6-oxo-androstenedione and in minor amounts of 6β-OH-androstenedione. No other contaminants were detected.

A GC/MS SIM method was developed and validated meeting WADA requirements. Using this method, the ingestion of the supplement, according to the manufacturer's recommendations resulted in the detection of 6α-OH-androstenedione up to 37 h after the administration, while 6-oxo-androstenedione and 6α-OH-testosterone could be detected up to 24 h post administration. Because of the longer detection time and its presence in urine after the administration of androstenedione, it is recommended that besides 6-oxo-androstenedione, 6α-OH-androstenedione is also included in screening methods for doping control purposes. The presence of 6-oxo-androstenedione or 6α-OH-testosterone can be used to discriminate between the administration of 6-oxo-androstenedione and androstenedione.

## *5.6. Acknowledgements*

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# Part IV: Medication use in sports

# 1. Introduction

Studies have shown that the use of several categories of medication differs from sport to sport. Regarding the percentage of inhaled β-agonist notifications during the Sydney 2000 Olympic Games for instance, 20% of the triathlon athletes and cyclists declared the use of an inhaled β-agonist. Similar results were observed in endurance sports, e.g. cross country skiing, during the Nagano 1998 Olympic Winter Games [1]. The emphasis on declared use of β-agonists in endurance sports was also noticed in Finnish athletes [2]. Also interesting was the higher percentage of inhaled corticosteroids in endurance sports compared to sports requiring motor skills [2]. Besides differences between sports, it also appears that the medication use can change over time. For instance, the percentage of analgesics declared on doping control forms by Turkish soccer players increased from 25.3 % to 34.5 % over a period of 3 years [3].

In this chapter the results of monitoring the declared medication by athletes from 2002 until 2005 will be discussed.

# 2. Monitored categories

The most frequently observed categories of medication mentioned on doping control forms are: corticosteroids, $\beta_2$-agonists and analgesic drugs which can be devided in four main categories: narcotic analgesics, local anaesthetics, NSAIDs and various central acting non-opioid drugs such as anti-depressants.

## *2.1. Corticosteroids*

Corticosteroids can be divided in two groups: the glucocorticoids and the mineralocorticoids. Mineralocorticosteroids affect the water and electrolyte balance. Use of fludrocortisone for instance results in increased $Na^+$ reabsorption and increases in $K^+$ and $H^+$ efflux [4].

Besides anti-inflammatory and immunosuppressive effects, glucocorticosteroids are so called because they stimulate the increase of glucose levels for energy [5]. Glucocorticosteroids are

very potent agents used for the short-term treatment of many inflammatory disorders. They inhibit both the early and late stage of inflammation. The use of large doses or prolonged use of corticosteroids can result in immunosupression, suppression of the patients capacity to synthesise corticosteroids, metabolic effects and effects on water and electrolyte balance.

Some therapeutically used corticosteroids are given in Figure 2.1.1.

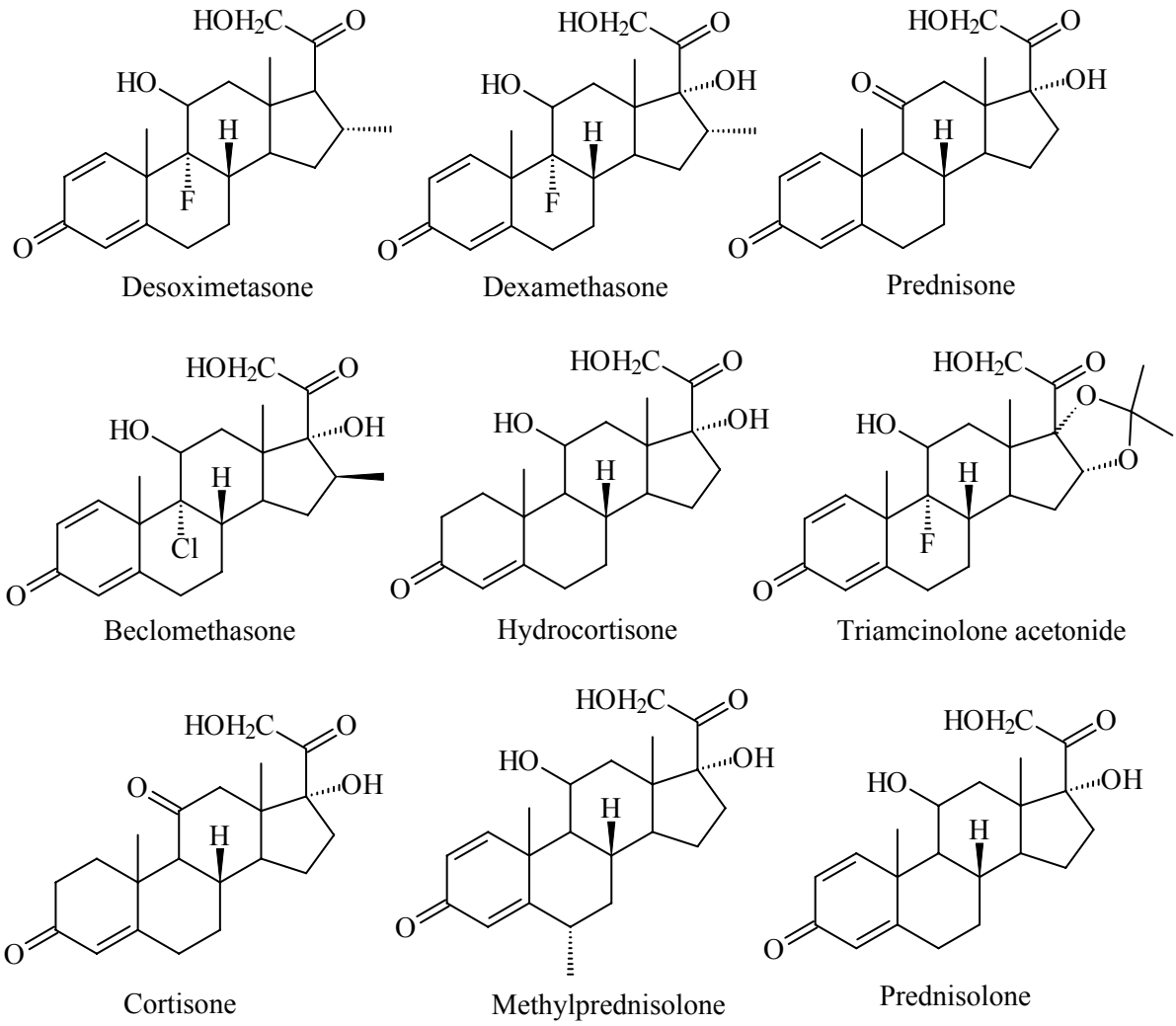


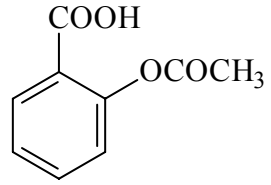

**Figure 2.1.1 Chemical structure of some corticosteroids.**

As a result of their therapeutic effects the use of corticosteroids is widespread. Due to the euphoric effects occurring with high doses, corticosteroids were included on the WADA list of prohibited substances [6]. Systemic use (intravenous or intramuscular injections, oral, rectal) is prohibited while non systemic use including topical application (buccal, nasal,

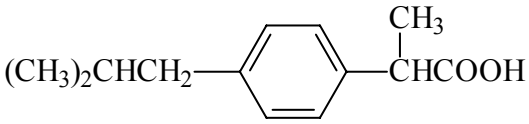
creams and eyedrops) is allowed. Other routes of non systemic applications require an abbreviated TUE. Recently, WADA introduced a scientifically unsound reporting level of 30 ng/ml. In contrast to non systemic use, this level can only be reached after the systemic use of corticosteroids. However, laboratories are supposed to detect the non systemic use as well which makes the situation even more complicated.

## *2.2. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)*

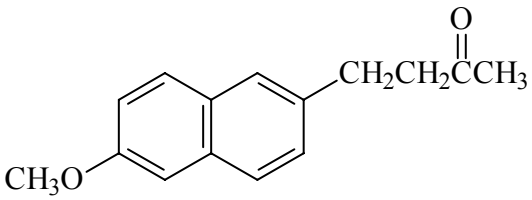
NSAIDs are among the most widely used therapeutic agents. The structure of the most important NSAIDs is shown in Figure 2.2.1.



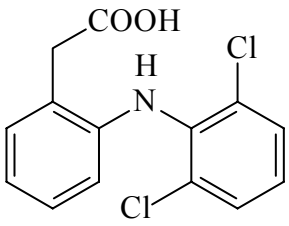

acetylsalicylic acid



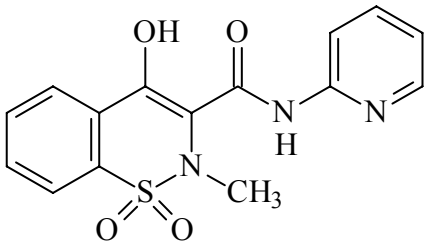

ibuprofen



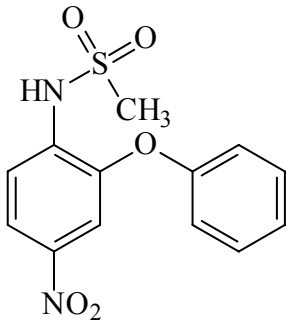

naproxen




diclofenac




piroxicam




nimesulide

**Figure 2.2.1 Chemical structure of some NSAIDs.**

Although the effectiveness of NSAIDs for the treatment of sport injuries is questionable, they are frequently used in sport medicine to treat muscle injuries [7, 8]. Minor effects have been reported on exercise [9, 10]. No ergogenic effect might be expected as confirmed in two studies [11, 12].

The frequent use of NSAIDs by athletes was shown by Corrigan and Kazlauskas who reported a percentage of incidence during the Sydney 2000 Olympics of 25.6 %. This was the highest percentage of all declared medication [13].

Paracetamol is one of the most frequently used non-narcotic analgesic and antipyretic agent. Although it only possesses non or weak anti-inflammatory activity, paracetamol shows good analgesic and antipyretic effects and is therefore often prescribed.

In this study, paracetamol will be considered in the NSAID group.

## *2.3. $\beta_2$-agonists*

Beta-agonists are bronchodilators and belong to the group of sympathicomimetics. The structure of $\beta_2$-agonists is closely related to (endogenous) catecholamines. The $\beta_2$-agonists are phenyl β-ethanolamines with different substituents on the amino-group at different positions of the phenyl ring [14]. Some of the most frequently used $\beta_2$-agonists are shown in Figure 2.3.1.

Because bronchial smooth muscle is strongly dilated by $\beta_2$-agonists the use of selective $\beta_2$-agonists is important in the treatment of asthma. These drugs are usually administered by inhalation of aerosol, powder of nebulised solution. Some are injectable preparations. A metered dose inhaler is used for aerosol preparations [15].

Two types of $\beta_2$-adrenoceptor agonists can be used in asthma therapy [16]:

- Short acting agents, such as salbutamol and terbutaline, which are given by inhalation. They are used to control symptoms when needed. Their short-acting properties are a result of their high selectivity for the $\beta_2$-receptor caused by the bulky amino substituents like tert.butyl groups [15].

- Long acting agents, such as salmeterol, which are given regularly in adjunctive therapy in patients whose asthma is inadequately controlled by glucocorticosteroids [16].

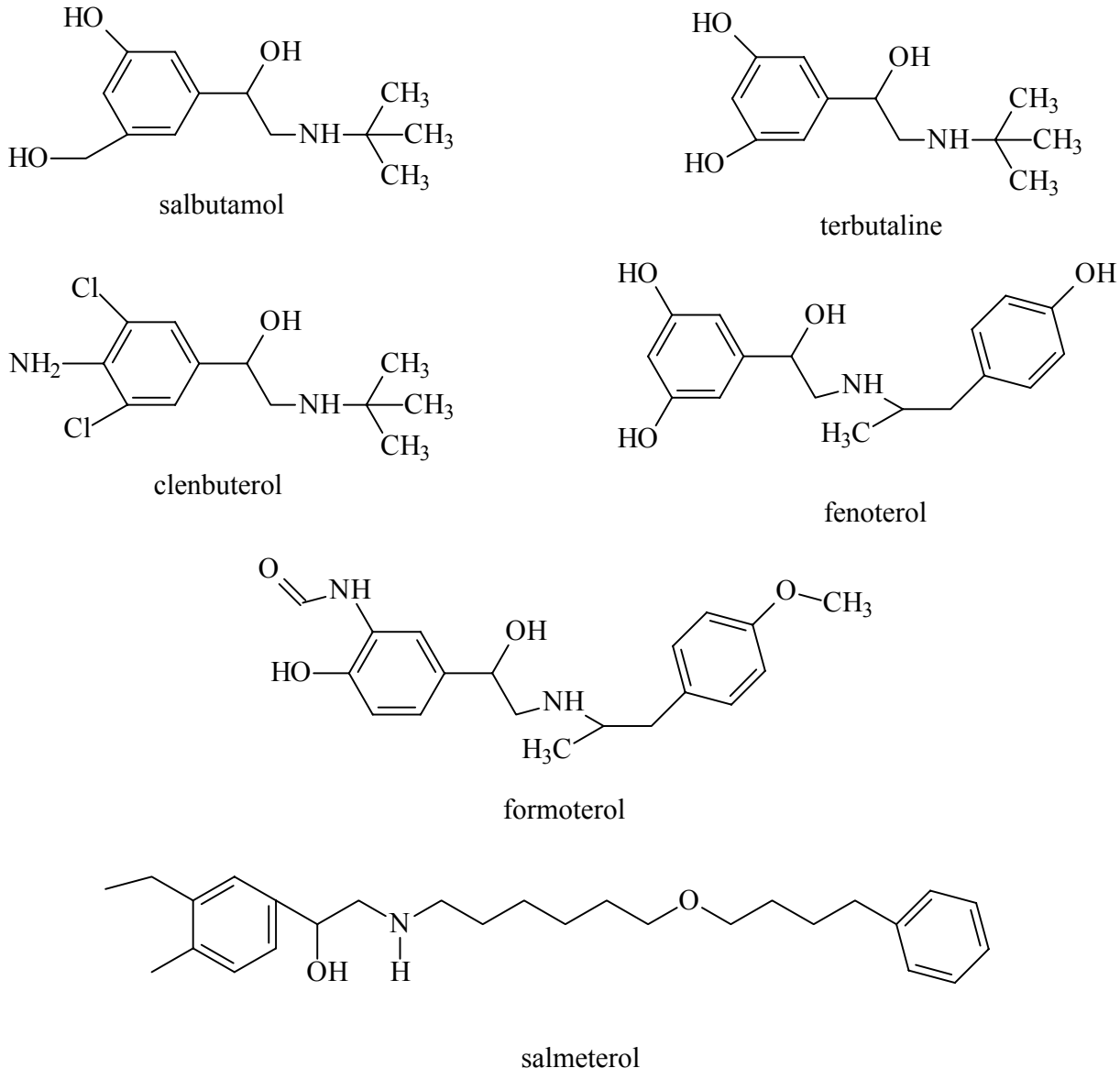



**Figure 2.3.1 Structure of several β-agonists used in human medicine.**

Besides the main pharmacological action $\beta_2$-agonists produce, at higher doses, side-effects on protein synthesis and lipolysis resulting in anabolic action [14]. Hence $\beta_2$-agonists might be misused for two reasons:

- Stimulatory effect on respiration and the central nervous system
- Growth-promoting action when administered in higher doses.

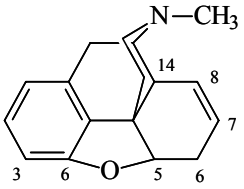
In athletes both effects are regarded as performance enhancing. Therefore, the use of $\beta_2$-agonists is prohibited in both in- and out-of competition. Exceptions on this rule are formoterol, salbutamol, salmeterol and terbutaline which require a TUE when used for the treatment of asthma or brochoconstriction [6].

## *2.4. Narcotic analgesics*

The most famous narcotic analgesic is morphine. Morphine is a pure substance obtained from opium, an extract from the plant *Papaver somniferum*. Already in ancient times opium was used in medicine to treat headaches, kidney stones and pain as well as to produce euphoria and sleep.

A lot of narcotic analgesics are morphine analogues while some others are synthetic derivatives without any structural relationship with morphine (Figure 2.4.1).

Morphine Analogues




| Drug | Substituents | | | |
|---|---|---|---|---|
| | 3 | 6 | N | 14 |
| morphine | -OH | -OH | $-CH_3$ | -H |
| heroin | $-OCOCH_3$ | $-OCOCH_3$ | $-CH_3$ | -H |
| codeine | $-OCH_3$ | -OH | $-CH_3$ | -H |
| levorphanol | -OH | -H | $-CH_3$ | -H (lacks –O- at $C_4$-$C_5$) |
| dihydrocodeine | $-OCH_3$ | -OH | $-CH_3$ | -H (lacks double bond $C_7$-$C_8$) |
| nalorphine | -OH | -OH | $-CH_2CH{=}CH_2$ | -H |
| nalbuphine | -OH | -OH | $-CH_2$-cyclobutyl | -OH (lacks double bond $C_7$-$C_8$) |
| naloxone | -OH | =O | $-CH_2CH{=}CH_2$ | -OH (lacks double bond $C_7$-$C_8$) |

Synthetic derivatives

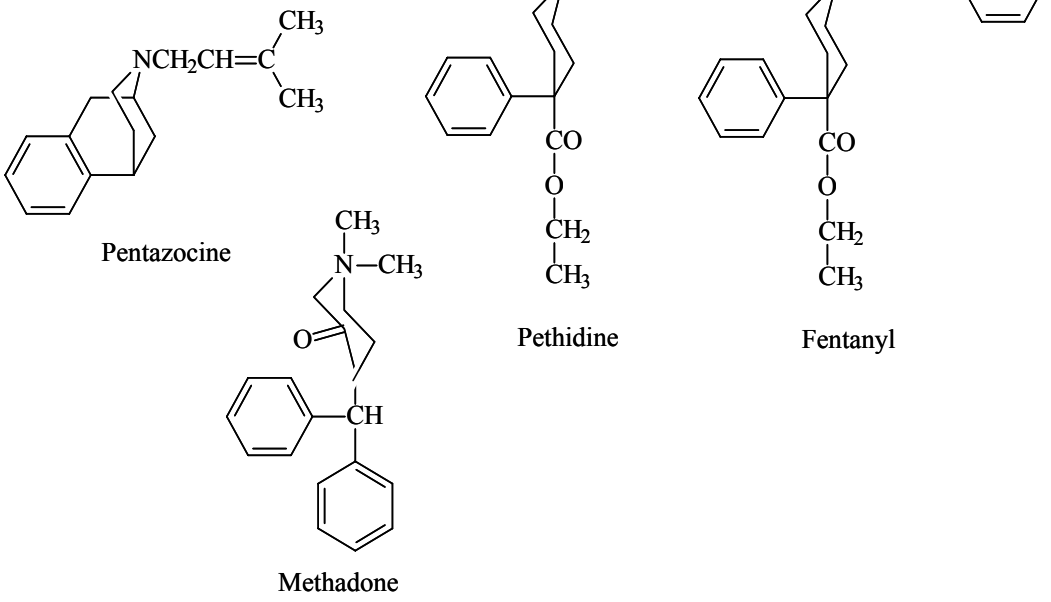



**Figure 2.4.1 Structure of several narcotic analgesics.**

Narcotic analgesics can be divided into three subcategories according to their pain killing properties. Codeine, dihydrocodeine and dextropropoxyphene can be considered as weak analgesics while pethidine, pentazocine and tilidine exhibit intermediate analgesia and buprenorphine, methadone, hydromorphone and morphine are powerful analgesics [17]. Their mechanism of action is linked to their agonist/antagonist action on the μ, δ and κ receptors [18]. The most important effect of narcotic analgesics is the influence on the central nervous system resulting in analgesia and euphoria.

As a result of their powerful analgesic effects, morphine and other narcotic analgesics are used in sport to overcome pain associated with strenuous exercise. This was the major reason why morphine was included in the IOC list of banned substances. Before 1991, the presence

of morphine in urine, irrespective of its concentration, resulted in a positive doping test. This changed in 1991 when the UCI introduced a 1 µg/ml threshold level for morphine as well as for codeine, one of the precursors of morphine.

At present the use of codeine and ethylmorphine, both morphine precursors, is allowed while morphine is forbidden in concentrations exceeding 1 µg/ml [6]. Other narcotic analgesics, such as pethidine, buprenorphine and pentazocine are prohibited.

## *2.5. Local anaesthetics*

The first applied local anaesthetic was cocaine in the eye by the ophthalmologist Carl Köller in 1884 proving the reversible corneal anaesthesia, resulting afterwards in the general use of cocaine as an anaesthetic in dentistry and surgery [19].

The mechanism of action of local anaesthetics is linked to the blocking of the $Na^+$ channels [20, 21]. These molecules consist of an aromatic ring linked by an ester or an amide bond to a basic side-chain (Figure 2.5.1). They are weak bases, with $pK_a$ values in the range 8-9 and are mainly ionised at physiological pH. The activity is strongly pH dependent [22].

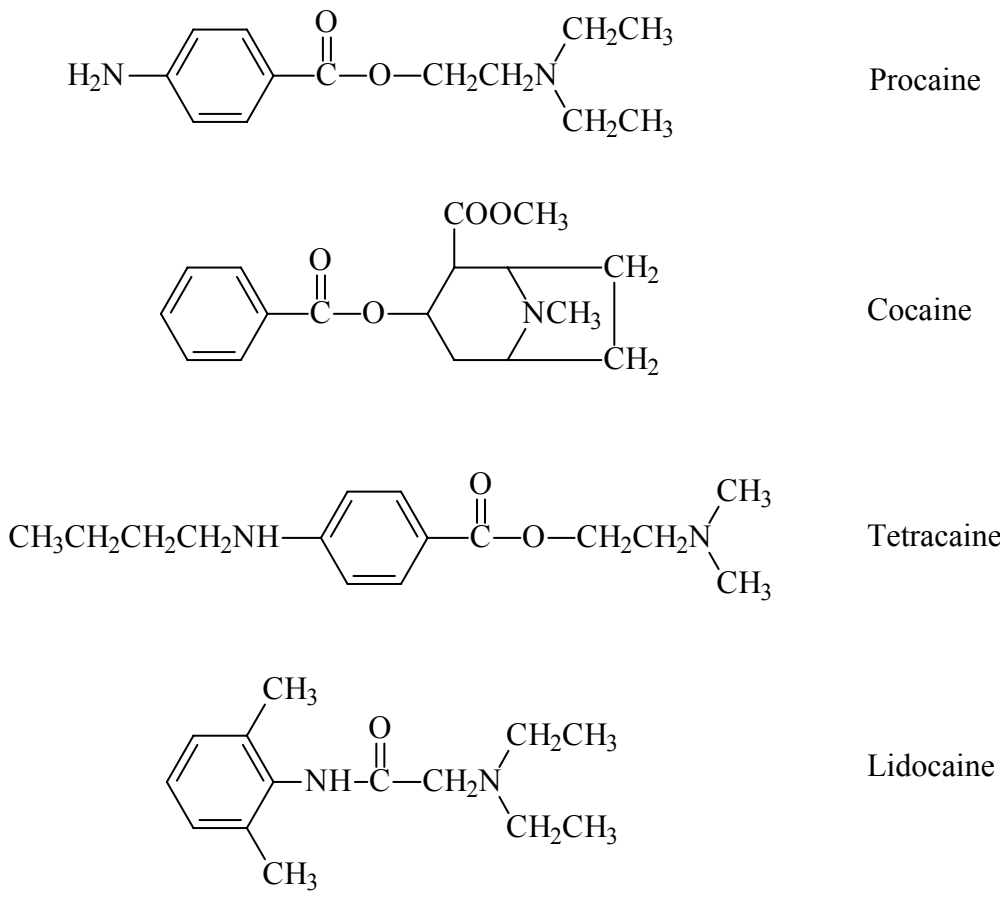



**Figure 2.5.1 Structure of commonly used local anaesthetics.**

Similar to narcotic analgesic, local anaesthetics can be used by athletes to overcome pain allowing them to better compete. Until 2004 the use of local anaesthetics was prohibited. Afterwards, their use was allowed according to WADA-rules [6]. The use of cocaine, which also shows stimulating effects, is however prohibited.

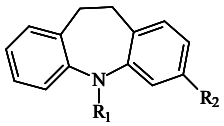
## *2.6. Antidepressant drugs*

The main biochemical theory of depression is the monoamine hypothesis, stating that depression is caused by a functional deficiency of monoamine transmitters at certain sites in the brain. In contrast, mania results from a functional excess of monoamine transmitters [23]. Antidepressant drugs can be divided into three categories: Tricyclic antidepressants (TCA) which are non-selective inhibitors of monoamine uptake, the selective seretonine reuptake inhibitors (SSRI) and monoamine oxidase inhibitors (MAOI). Their mechanism of action is

based upon the (selective) blocking of dopamine, noradrenaline and/or seretonine reuptake, increasing the concentration of these monoamines in the synaptic cleft. [24, 25]. Currently, the use of SSRI antidepressants is very popular. Fluoxetine (Figure 1.2.6) is one of the most famous anti-depressants, in general under its brand name Prozac®.

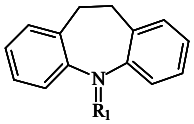
**Tricyclic antidepressants**

Diabenzazepines



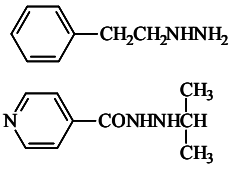
| Drug | $R_1$ | $R_2$ |
|---|---|---|
| Imipramine | $-CH_2CH_2CH_2N(CH_3)_2$ | H |
| Desipramine | $-CH_2CH_2CH_2NHCH_3$ | H |
| Clomipramine | $-CH_2CH_2CH_2N(CH_3)_2$ | Cl |

Dibenzcycloheptenes



| Drug | $R_1$ |
|---|---|
| Amitriptyline | $=CHCH_2CH_2N(CH_3)_2$ |
| Nortriptyline | $=CHCH_2CH_2NHCH_3$ |

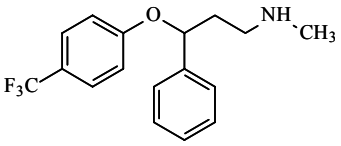
**MAOI**



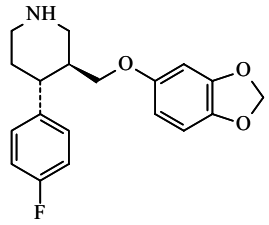
Pheneizine

Iproniazid

**SSRI**



Fluoxetine



Paroxetine

**Figure 2.6.1 Structure of frequently used anti-depressants.**

Anti-depressants are often misused for their euphoric effects. Amitriptyline is also effective in the treatment of asthma, while dopexine causes brochodilatation [26]. Despite these known beneficial effects antidepressants are not included in the 2006 WADA list of prohibited substances.

## 3. Experimental

The declared use of medication on doping control forms collected from 2002 until 2005 was examined. A database was created based on the active ingredients present in the medication.

The doping control forms originating from the Flemish Community, the UCI, KBWB, DoCoNed from 2002 until 2005 were evaluated. From 2004, samples from the Communauté Française (CF) are also included.

Other monitored parameters besides origin of the sample and active ingredient were gender and sport.

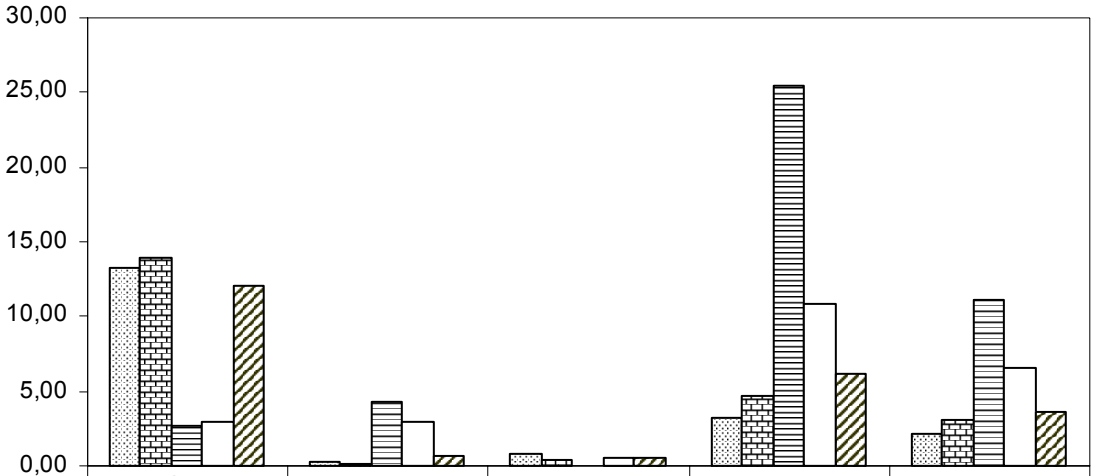
Determination of percentages of use, based upon the total number of analysed samples allowed for the comparison in time and between sports.

## 4. Results and discussion

Preliminary results obtained in 2001 showed that the incidence of use of different types of medication differs between sports [27]. 14 % of tested athletes have declared the use of one or more drugs classified in the above mentioned categories (except β-agonists and antidepressants). It was also noticed that the use of corticosteroids was more prevalent in cycling compared to other sports [27]. The use of NSAIDs on the other hand was higher in ball sports. Based on these preliminary results, a monitoring program was set up. In addition the declared use of β-agonists and antidepressants was also evaluated.

### *4.1. 2002*

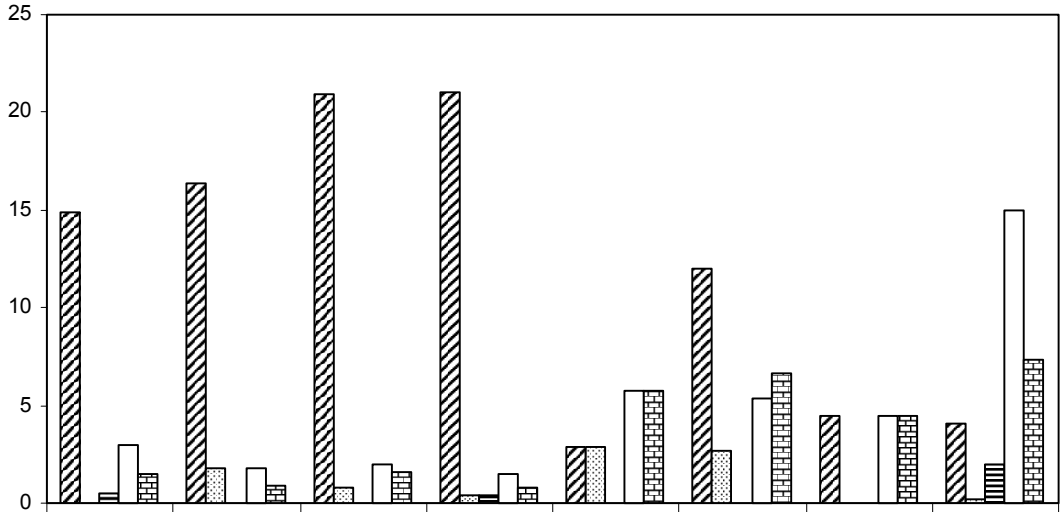
In 2002 a total of 3858 doping control forms originating from different federations and NADOs, excepting the CF, were controlled for the presence of medications belonging to one of the described categories. In total 19.8 % of the forms showed the presence of one or more drugs. In only 3 cases the use of an antidepressant agent was reported. Figure 4.1.1 illustrates the incidence per category on the doping control forms from different organisations. It appears that the declaration of NSAIDs is substantially higher on the NADO forms and that the use of corticosteroids is much higher in cycling (UCI and KBWB).




| | NSAID | anaesthetics | narc analg | cortico | beta-agonist |
|---|---|---|---|---|---|
| VI Gem | 13,23 | 0,31 | 0,78 | 3,19 | 2,20 |
| DoCoNed | 13,98 | 0,07 | 0,35 | 4,68 | 3,05 |
| UCI | 2,70 | 4,32 | 0,00 | 25,41 | 11,08 |
| KBWB | 3,01 | 3,01 | 0,60 | 10,84 | 6,63 |
| Total | 12,03 | 0,73 | 0,54 | 6,22 | 3,55 |

**Figure 4.1.1 Percentages of declared use of medication according to controlling organisation in 2002.**

Figure 4.1.2 summarises the results in the most frequently tested sports in 2002. It seems that the use of NSAIDs and paracetamol is prevalent in athletics and in ball sports with percentages exceeding 20 % in volleyball and soccer. The use of narcotic analgesics and anaesthetics follows a similar patern although the use of these drugs is low compared to NSAIDs. A higher percentage of use of corticosteroids and β-agonists was observed in tennis, handball and swimming, but the percentage of use of this type of drug is low when compared to cycling.




| | Athletics | Basketball | Volleyball | Soccer | Tennis | Handball | Swimming | Cycling |
|---|---|---|---|---|---|---|---|---|
| NSAID | 14,93 | 16,36 | 20,97 | 21,05 | 2,86 | 12,00 | 4,44 | 4,05 |
| Narcotics | 0,00 | 1,82 | 0,81 | 0,42 | 2,86 | 2,67 | 0,00 | 0,17 |
| Anaesthetics | 0,50 | 0,00 | 0,00 | 0,42 | 0,00 | 0,00 | 0,00 | 1,94 |
| Corticos | 2,99 | 1,82 | 2,02 | 1,47 | 5,71 | 5,33 | 4,44 | 14,94 |
| Beta-agon | 1,49 | 0,91 | 1,61 | 0,84 | 5,71 | 6,67 | 4,44 | 7,34 |

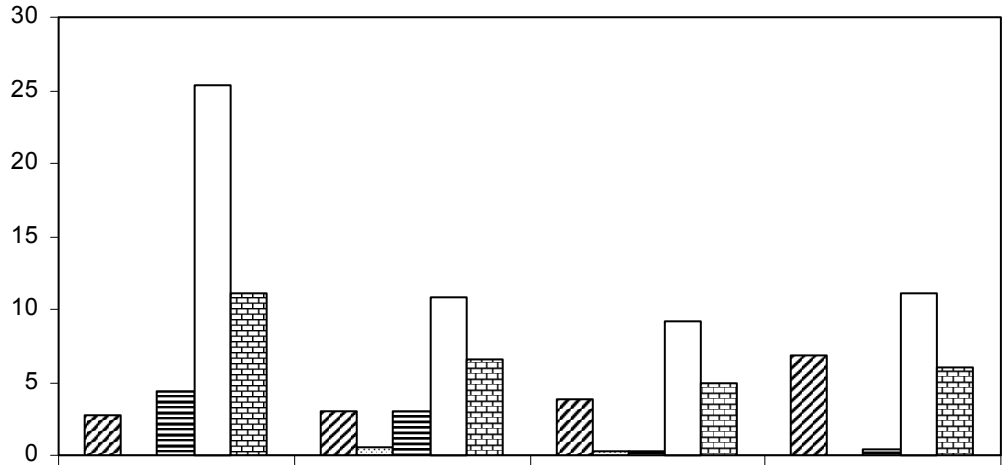
**Figure 4.1.2. Declared use of medication in different sports in 2002.**

In cycling the use of corticosteroids is more prevalent while the use of NSAIDs is substantially lower than in most other tested sports. Percentages of anaesthetics are also higher in cycling. The percentage of declared β-agonists is also substantially higher than in other sports.

Figure 4.1.3 shows the results obtained in cycling for the different testing authorities. Declared corticosteroid use on the UCI doping control forms exceeds 25 %. Also interesting to note is the difference in declared use of both corticosteroids and β-agonists in cycling amongst different controlling organisations. The higher percentages in samples originating from the UCI could be due to the fact that professional cyclists tested by the UCI have to show their medical booklet to doping control officers or because the level of the tested athletes is higher compared to cyclists selected for doping control by the NADOs.

Also interesting to note is the higher percentage of use of anaesthetics, most frequently lidocaine, in samples originating from the UCI compared to samples from NADOs. Before 2004 UCI rules stipulated that the use of local anaesthetics, including lidocaine, was only

allowed with a proper medical justification and for local use only. Other routes of administration were prohibited. This might explain the higher percentages found in samples from the UCI.

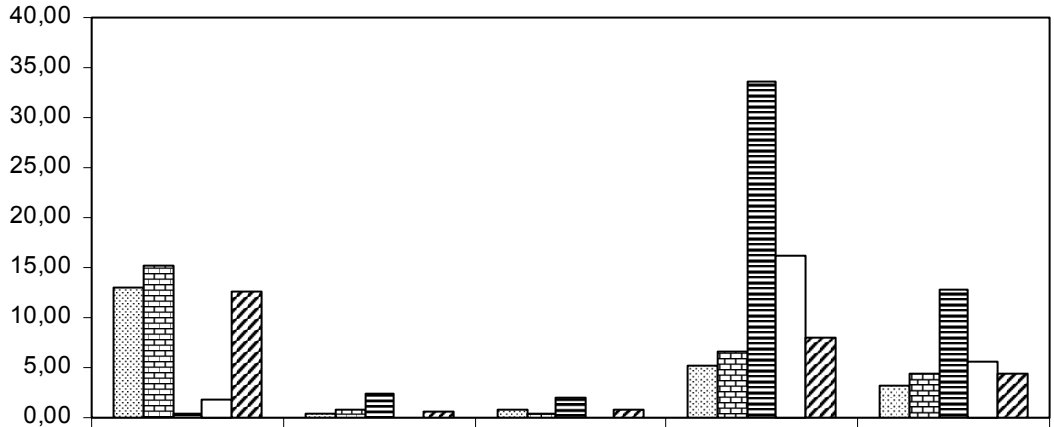



| | UCI | KBWB | VI Gem | DoCoNed |
|---|---|---|---|---|
| NSAID | 2,70 | 3,01 | 3,79 | 6,79 |
| Narcotics | 0,00 | 0,60 | 0,27 | 0,00 |
| Anaesthetics | 4,32 | 3,01 | 0,27 | 0,36 |
| Corticos | 25,41 | 10,84 | 9,21 | 11,07 |
| Beta-agon | 11,08 | 6,63 | 4,88 | 6,07 |

**Figure 4.1.3 Percentages of declared use of medication in cycling in 2002 in relation to the controlling organisations.**

## *4.2. 2003*

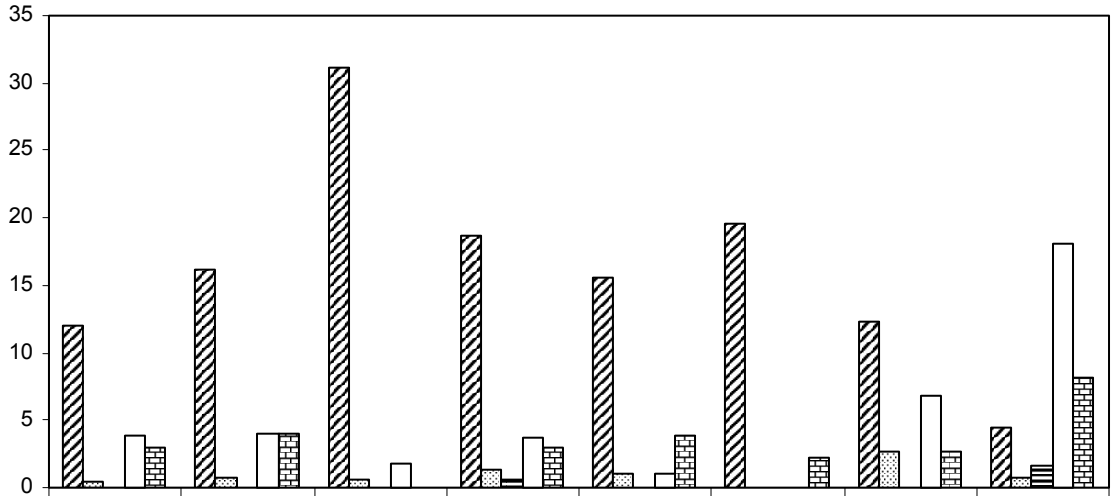
Analysis of doping control forms in 2003 from the same federations and the NADOs (N = 4417) showed that 980 athletes (22.19 %) declared the use of one or more monitored substances which is approximately 2.5 % higher than in 2002. 5 athletes declared the use of an antidepressant. Similar to 2002, the use of NSAIDs (including paracetamol) was the highest in samples originating from the Flemish Community and the Netherlands. The use of corticosteroids is significantly higher in samples originating from the UCI compared to other federations (Figure 4.2.1).




| | NSAID | anaesthetics | narc analg | cortico | beta-agonist |
|---|---|---|---|---|---|
| VI Gem | 13,00 | 0,36 | 0,81 | 5,20 | 3,18 |
| DoCoNed | 15,21 | 0,81 | 0,46 | 6,59 | 4,45 |
| UCI | 0,34 | 2,36 | 2,02 | 33,67 | 12,79 |
| KBWB | 1,88 | 0,00 | 0,00 | 16,25 | 5,63 |
| Totaal | 12,63 | 0,66 | 0,72 | 8,06 | 4,41 |

**Figure 4.2.1 Percentages of declared use of medication according to controlling organisation in 2003.**

From Figure 4.2.2 it can be seen that the use of NSAIDs in most ball sports is higher than in other sports, but the difference between sports is not as pronounced as in 2002. Percentages of NSAID use increased in 2003 and exceeded the level of 30 % in volleyball. Similar as in the previous year, the use of narcotic analgesics, anaesthetics, corticosteroids and β-agonists in the most tested sports is low.

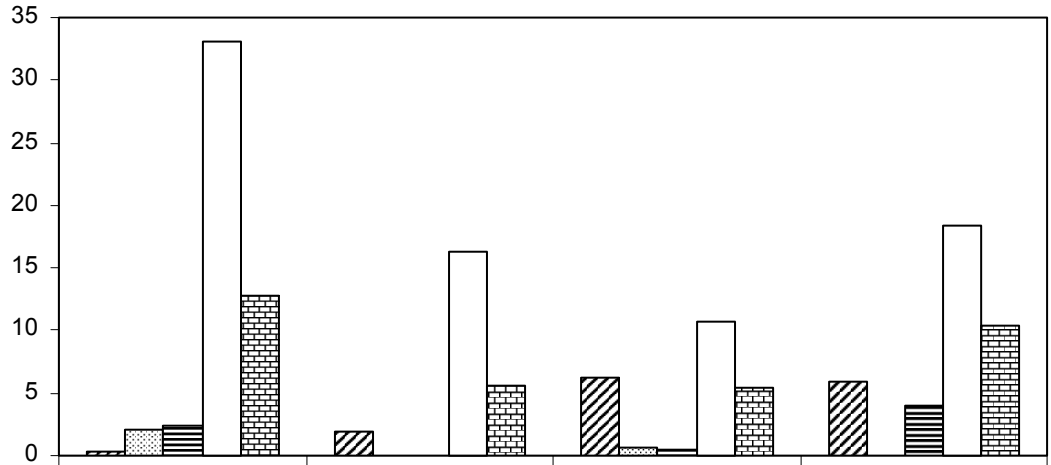



| | Athletics | Basketball | Volleyball | Soccer | Handball | Indoor soccer | Swimming | Cycling |
|---|---|---|---|---|---|---|---|---|
| NSAID | 12,07 | 16,13 | 31,10 | 18,68 | 15,53 | 19,57 | 12,33 | 4,38 |
| Narcotics | 0,43 | 0,81 | 0,61 | 1,32 | 0,97 | 0,00 | 2,74 | 0,72 |
| Anaeshtetics | 0,00 | 0,00 | 0,00 | 0,53 | 0,00 | 0,00 | 0,00 | 1,65 |
| Corticos | 3,88 | 4,03 | 1,83 | 3,68 | 0,97 | 0,00 | 6,85 | 18,03 |
| Beta-agon | 3,02 | 4,03 | 0,00 | 2,89 | 3,88 | 2,17 | 2,74 | 8,12 |

**Figure 4.2.2 Declared use of medication in different sports in 2003.**

Although the incidence of use of corticosteroids in different tested sports is the highest in swimming (6.85 %), percentages found in cycling are twice as high. Of all tested cyclists in 2003 18.03 % have declared the use of one or more corticosteroids. Declared use of corticosteroids in samples originating from the UCI is even higher than 33 %, 7.5 % higher than in 2002 (Figure 4.2.3).

Finally, the use of β-agonists is also higher in cycling compared to other sports. Percentages of use of NSAIDs, anaesthetics and narcotic analgesics are low compared to the use of corticosteroids and β-agonists.

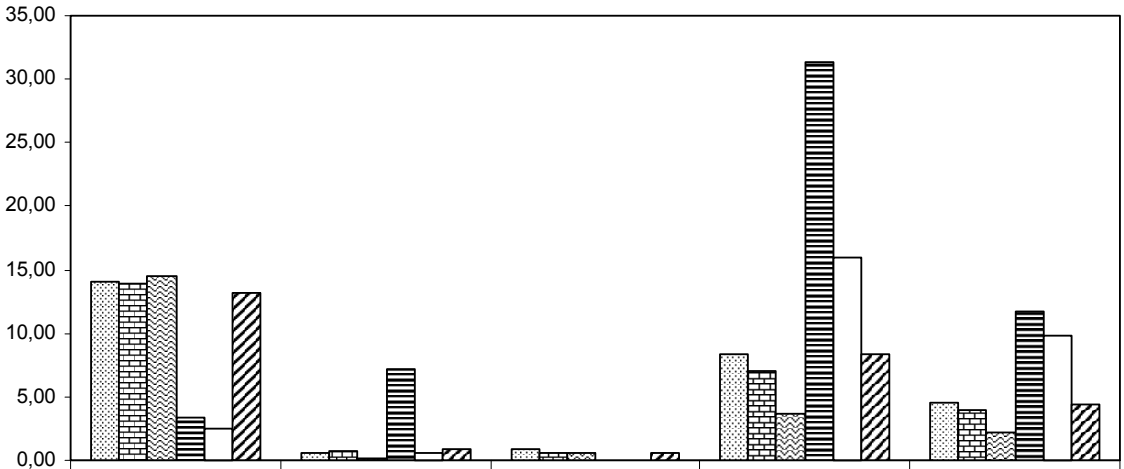



| | UCI | KBWB | Vl Gem | DoCoNed |
|---|---|---|---|---|
| NSAID | 0,34 | 1,88 | 6,18 | 5,94 |
| Narcotics | 2,02 | 0,00 | 0,65 | 0,00 |
| Anaesthetics | 2,36 | 0,00 | 0,49 | 4,06 |
| Corticos | 33,07 | 16,25 | 10,73 | 18,44 |
| Beta-agon | 12,79 | 5,63 | 5,37 | 10,31 |

**Figure 4.2.3 Declared use of medication in cycling in 2003.**

## *4.3. 2004*

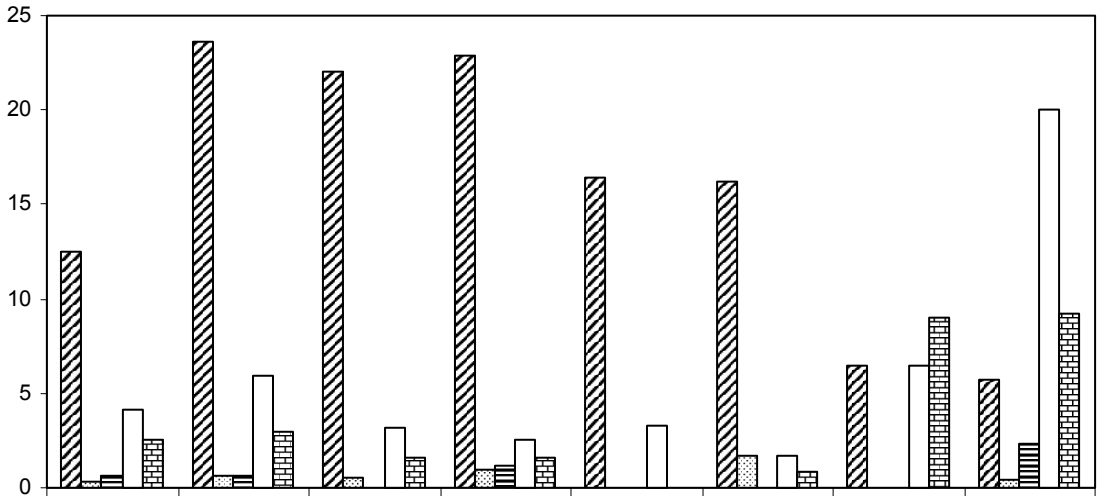
Starting from 2004 samples were also analysed for the CF, increasing the number of investigated doping control forms to 5190. Percentages of declared medication belonging to the monitored categories increased to 24.57 %, more than 2 % higher than in the previous period and even 5 % higher than in 2002. Of those athletes, 16 declared the use of an antidepressant. From Figure 4.3.1 it can be concluded that the declared use of medication on the doping control forms of the CF is similar to other NADOs (Vl Gem and DoCoNed). Similar trends as in the previous tested periods could be observed in the reporting behaviour related to the originating federation. Again, the declared use of NSAIDs is the highest on the doping control forms from the NADOs while the use of corticosteroids is highest in the samples originating from the UCI. Interesting to note is the high percentage of athletes tested by the UCI reporting the use of an anaesthetic drug, although starting from 2004, the use of local anaesthetics was not longer prohibited by WADA and the UCI.




| | NSAID | anaesthetics | narc analg | cortico | beta-agonist |
|---|---|---|---|---|---|
| VI Gem | 14,06 | 0,59 | 0,86 | 8,37 | 4,50 |
| DoCoNed | 13,93 | 0,71 | 0,57 | 7,07 | 4,00 |
| CF | 14,43 | 0,17 | 0,60 | 3,61 | 2,23 |
| UCI | 3,40 | 7,17 | 0,00 | 31,32 | 11,70 |
| KBWB | 2,45 | 0,61 | 0,00 | 15,95 | 9,82 |
| Totaal | 13,20 | 0,87 | 0,66 | 8,32 | 4,39 |

**Figure 4.3.1 Percentages of declared use of medication on forms from several controlling bodies in 2004.**

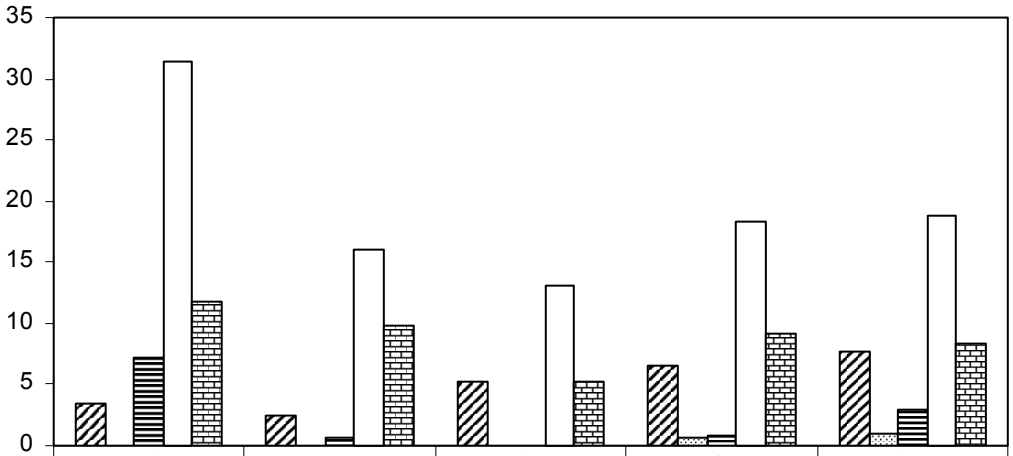
Results shown in Figure 4.3.2 confirm the results obtained in the previous periods about the use of medication is different sports. Use of NSAIDs in ball sports is again higher when compared to other sports such as swimming. It was also observed that the use of NSAIDs in volleyball decreased in 2004 to its level obtained in 2002 while the use of NSAIDs in other ball sports remained approximately the same. The use of other drugs is similar as in 2002 and 2003. Percentages of declared corticosteroid use in cycling increased to more than 20 %.




| | Athletics | Basketball | Volleyball | Soccer | Tennis | Handball | Swimming | Cycling |
|---|---|---|---|---|---|---|---|---|
| NSAID | 12,50 | 23,67 | 21,99 | 22,92 | 16,39 | 16,24 | 6,41 | 5,72 |
| Narcotics | 0,32 | 0,59 | 0,52 | 0,93 | 0,00 | 1,71 | 0,00 | 0,46 |
| Anaesthetics | 0,64 | 0,59 | 0,00 | 1,16 | 0,00 | 0,00 | 0,00 | 2,28 |
| Corticos | 4,17 | 5,92 | 3,14 | 2,55 | 3,28 | 1,71 | 6,41 | 20,02 |
| Beta-agon | 2,56 | 2,96 | 1,57 | 1,62 | 0,00 | 0,85 | 8,97 | 9,23 |

**Figure 4.3.2 Declared use of medication in different sports in 2004.**

The use of medication in cycling in 2004 was very similar to 2003 (Figure 4.3.3). Percentages found in samples originating from the CF are very similar to the percentages found in samples from the other NADOs. As for the previously tested periods, the use of corticosteroids and β-agonists in cycling is the highest in samples originating from the UCI.



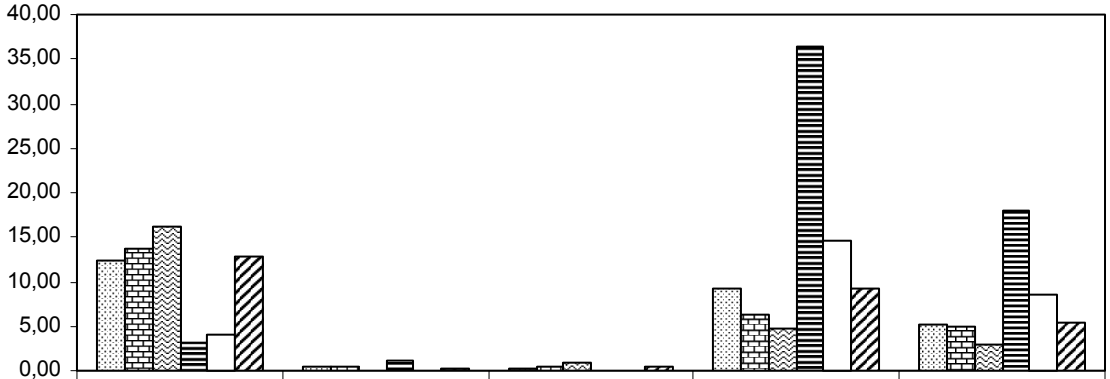

| | UCI | KBWB | CF | VI Gem | DoCoNed |
|---|---|---|---|---|---|
| NSAID | 3,40 | 2,45 | 5,26 | 6,59 | 7,67 |
| Narcotics | 0,00 | 0,00 | 0,00 | 0,59 | 0,96 |
| Anaesthetics | 7,17 | 0,61 | 0,00 | 0,88 | 2,88 |
| Corticos | 31,32 | 15,95 | 13,16 | 18,30 | 18,85 |
| Beta-agon | 11,70 | 9,82 | 5,26 | 9,22 | 8,31 |

**Figure 4.3.3 Declared use of medication in cycling in 2004.**

## *4.4. 2005*

Totally 5180 doping control forms were evaluated in 2005. 1278 athletes (24.67 %) declared the use of one or more drugs belonging to the categories in this study similar to the result obtained in 2004. Also similar as in 2004 is the total number of athletes declaring the use of an antidepressant drug.

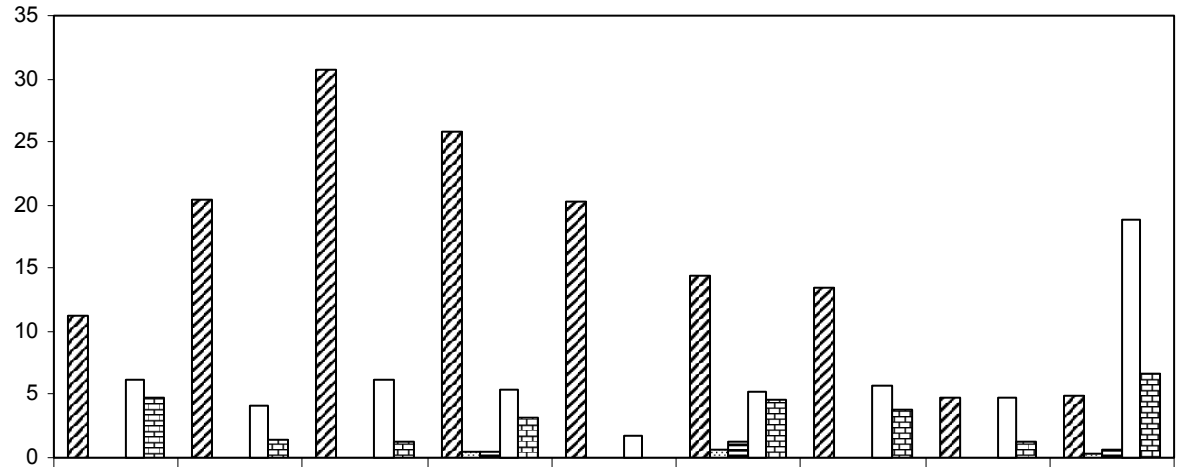
From Figure 4.4.1 it can be seen that the use of narcotic analgesics and anaesthetics is rarely reported. Use of NSAIDs is again the highest in the samples originating from the NADOs while corticosteroid use is again exuberant in samples from the UCI. In 2005 percentages even exceed 35 %.




| | NSAID | anaesthetics | narc analg | cortico | beta-agonist |
|---|---|---|---|---|---|
| Vl Gem | 12,44 | 0,35 | 0,22 | 9,26 | 5,07 |
| DoCoNed | 13,78 | 0,40 | 0,47 | 6,18 | 4,84 |
| CF | 16,26 | 0,00 | 0,82 | 4,70 | 2,86 |
| UCI | 3,04 | 1,01 | 0,00 | 36,49 | 17,91 |
| KBWB | 3,97 | 0,00 | 0,00 | 14,57 | 8,61 |
| Total | 12,76 | 0,33 | 0,39 | 9,23 | 5,42 |

**Figure 4.4.1 Percentages of declared use of medication on doping control forms of some controlling bodies in 2005.**

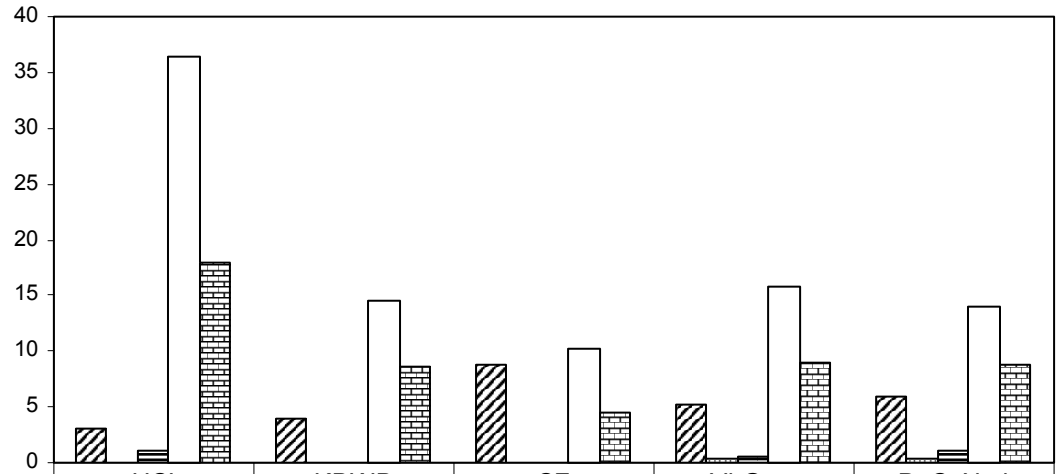
Similar observations as in the previous periods about the declared use of medication in several sports can be made (Figure 4.4.2). Levels of use of NSAIDs and corticosteroids in swimming decreased compared to 2004 and 2003 and returned to the values in 2002.




| | Athletics | Basketball | Volleyball | Soccer | Tennis | Handball | Indoor soccer | Sw imming | Cycling |
|---|---|---|---|---|---|---|---|---|---|
| NSAID | 11,28 | 20,45 | 30,67 | 25,74 | 20,34 | 14,38 | 13,46 | 4,82 | 4,95 |
| Narcotics | 0,00 | 0,00 | 0,00 | 0,54 | 0,00 | 0,65 | 0,00 | 0,00 | 0,25 |
| Anaestetics | 0,00 | 0,00 | 0,00 | 0,54 | 0,00 | 1,31 | 0,00 | 0,00 | 0,62 |
| Corticos | 6,23 | 4,09 | 6,13 | 5,36 | 1,69 | 5,23 | 5,77 | 4,82 | 18,92 |
| Beta-agon | 4,75 | 1,36 | 1,23 | 3,22 | 0,00 | 4,58 | 3,85 | 1,20 | 6,68 |

**Figure 4.4.2 Declared use of medication in different sports in 2005.**

The general use of corticosteroids in cycling in 2005 was 18.92 % or 1.10 % lower as in 2004 (20.02 %). This is caused by the decreased declaration of these drugs in samples originating from the NADOs and the KBWB as the percentage of athletes tested by the UCI declaring the use of a corticosteroid increased to 36.49 % (Figure 1.4.12). The use of β-agonists in samples from the UCI also increased to its highest level in 4 years. Combinations of both drugs are very often reported. Out of the 54 athletes declaring the use of a β-agonist, 50 simultaneously declared the use of a corticosteroid. The combination of budesonide – formoterol and fluticasone – salmeterol used for the treatment of exercise induced asthma are the most popular. Declared use of the other monitored medication in cycling is low and similar as in previous periods.




| | UCI | KBWB | CF | VI Gem | DoCoNed |
|---|---|---|---|---|---|
| NSAID | 3,04 | 3,97 | 8,82 | 5,14 | 5,96 |
| Narcotics | 0,00 | 0,00 | 0,00 | 0,37 | 0,35 |
| Anaesthetics | 1,01 | 0,00 | 0,00 | 0,49 | 1,05 |
| Corticos | 36,49 | 14,57 | 10,29 | 15,79 | 14,04 |
| Beta-agon | 17,91 | 8,61 | 4,41 | 9,06 | 8,77 |

**Figure 4.4.3 Declared use of medication in cycling in 2005.**

## 5. Conclusion

From the results obtained in this study it can be concluded that the declared use of different groups of medication depends on the origin of the samples and the tested sports. Generally, the use of local anaesthetics and narcotic analgesics is low, with the exception of lidocaine in cycling and more specifically in samples originating from the UCI. The use of other drugs is more sport depending. It was observed that the declared use of NSAIDs and paracetamol in ball sports was higher than in other sports. On the other hand, the declared use of corticosteroids in cycling is extremely high with percentages steadily increasing from 25.4 % in 2002 to 36.5 % in 2005 in samples taken by the UCI while general percentages of use in cycling decreased. The difference between elite and amateur levels may explain this trend as UCI mostly tests elite athletes while the different NADOs control athletes at both levels.

At present almost no athletes are sanctioned for the misuse of corticosteroids because of the relatively easy accessibility of a therapeutic use exemption in combination with the WADA scientifically unsound reporting level of 30 ng/ml. If trends in corticosteroid use in sports

keep on rising in the future a evaluation of the current TUE system and sanctioning should be considered.

## 6. Acknowledgements

The assistance of Mrs Trui Demey is gratefully acknowledged.

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# Part V: Summary and conclusion

## Summary and Conclusion

Since the introduction of the first doping test in the early 1960's and the development of anti-doping rules by the UCI in the same period, doping rules have been extensively revised by the IOC and currently by WADA. These rules are very extensive and include numerous classes defined as doping agents and prohibited methods.

In this thesis some substances that are situated at the borderline between allowance and prohibition are described. The complex situation with nutritional supplements and their influence on the outcome of a doping test is evaluated.

Analysis in doping control relies on strict criteria. These criteria involve both method validation and identification. These parameters are discussed in Part I.

Besides drugs allowed in sports and drugs allowed for medical treatment, the use of several drugs is restricted by a urinary concentration threshold level. Problems associated with caffeine and morphine are discussed in Part II.

Since January 1$^{st}$ 2004 the use of caffeine is allowed in sport. Before, urinary concentrations exceeding 12 μg/ml constituted a doping offence. As it is well known that caffeine exhibits performance enhancing effects, even at doses resulting in urinary concentrations far below the 12 μg/ml threshold level, the removal of caffeine from the doping list could have resulted in an increased misuse. In order to observe changes in caffeine use in different sports after the removal from the doping list, a statistical evaluation was made before and after the withdrawal of caffeine. Results obtained in this study (Part II Chapter 1) show that urinary caffeine concentrations in the period 1993-2002 were significantly higher in cycling and bodybuilding compared to other sports. A similar trend was observed in samples tested in 2004 highlighting the purported ergogenic and stimulating effects in these sports. In general, caffeine concentrations did not increase after the removal of caffeine from the WADA doping list. Calculated apparent threshold levels on the other hand show that the use of a threshold level of 12 μg/ml is capable of distinguishing use from abuse making the withdrawel of caffeine from the doping list questionable.

Morphine is prohibited in sports. A urinary threshold of 1 µg/ml is applied. The use of food products containing parts of the plant *Papaver somniferum*, the natural source of morphine is studied in Part II Chapter II. Administration of herbal teas resulted in the detection of urinary morphine concentrations up to 7.44 µg/ml. Athletes should therefore be very careful with herbal products. In addition, WADA and NADOs should warn athletes on the possible risks associated with the use of sedative teas or food products containing parts of the plant *Papaver somniferum.*

Nutritional supplements are discussed in Part III. An extensive review of the literature (Chapter 1) showed that, although nutritional supplements are allowed, their use is strongly dissuaded for two major reasons. First, it is believed that the use of nutritional supplements is unnecessary in a normal balanced diet. Secondly, some nutritional supplements have proven to contain doping substances. As a result of the 1994 DSHEA enacted in the USA, steroids and prohormones could be distributed as nutritional supplements. Previous research has shown that the labelled content of these "nutritional supplements" is not always correct. Besides steroids and prohormones, numerous nutritional supplements promoted for weight loss contain herbal formulations from *Ephedra* species. Also other stimulating agents such as MDMA (ecstasy) and fenfluramine were detected in supplements. As athletes remain responsible for the presence of doping substances in their body fluids, irrespective of their origin, it is very important to develop analytical methods capable of detecting contaminants in supplements. Two analytical methods for the detection of low range contamination of anabolising agents in both solid and liquid nutritional supplements are described in Chapter 2. These methods were the first completely validated methods in this field. Totally 32 components are screened and LODs for 29 analytes range from 2 to 20 ng/g in solid supplements and for 31 analytes from 1 to 10 ng/ml in aqueous supplements.

Besides methods for the detection of anabolising agents, a sensitive LC/MS method for the detection of stimulants in solid nutritional supplements was developed (Chapter 3). LODs of 100 ng/g are far below the requirements of supplement testing organisations.

Chapter 4 describes several case reports with contaminated nutritional supplements. Several excretion studies were carried out proving that the ingestion of contaminated supplements can result in adverse doping findings. For instance, the presence of nandrolone precursors can

result in positive findings up to 144 h. Supplement producing companies are always trying to circumvent international laws and doping rules by introducing new prohormones such as 5α-androstane-3,17-dione (Chapter 4) and 6-oxo-androstene-3,17-dione (Chapter 5) proving the need for continuing research in analysis of nutritional supplements.

The relatively high percentage of positive samples obtained during 4 years of supplement testing also proves the importance of screening for prohibited substances by specialised doping control laboratories.

Although most painkillers are allowed in sport, it seems interesting to evaluate the prevalence of these drugs in sports as declared by athletes on the doping control forms. The declared use of non-steroidal anti-inflammatory drugs and paracetamol and other frequently reported drugs such as corticosteroids, β-agonists, narcotic analgesics and local anaesthetics is described in Part IV. The reported use of anti-depressants was also included. Statistical information gathered during the period 2002 - 2005 from 18645 athletes is reported. From this data it appears that differences in reported medication were observed on doping control forms from different federations and/or NADOs and also between sports. More NSAIDs and paracetamol were reported in ball sports compared to other sports. The most pronounced difference was the high prevalence of corticosteroid use in cycling. Percentages of declared corticosteroids in samples originating from the UCI increased year after year to a maximum of 36 % in 2005. It appears that corticosteroids were often used in combination with β-agonists. Nowadays the use of corticosteroids in sport will only be allowed with a proper medical justification. This TUE will be a helpful tool to overcome the past situation of over-consumption with uncontrolled prescriptions and declarations by the athletes.

In general, this work shows that the line between allowed and prohibited medication in sport is not always clear. In most cases positive doping findings are caused by the deliberate intake of prohibited substances. Athletes using these drugs and methods should be sanctioned. Positive doping tests can also result from the accidental intake of a non-labelled prohibited substance or (deliberately) contaminated nutritional supplements. According to WADA rules, no distinction is made between those athletes and cheaters as the final responsibility relies on the athlete. As numerous athletes persist in taken supplements international organisations should warn them against the possible doping risks associated with their use. In addition,

more strict quality criteria are needed for supplement producing companies. Doping control laboratories can play an important role in this process as they have the expertise in this field. Structures similar to the NZVT-system disclosing lists of products which can be used without the risk of unwanted positive doping results on their website are of utmost importance.

## Samenvatting en Conclusies

Sinds de invoering van de eerste dopingtesten in de vroege jaren 1960 en de ontwikkeling van een antidoping regelgeving door de UCI in dezelfde periode zijn de reglementen uitgebreid gereviseerd door het IOC en momenteel door WADA. Deze regels omvatten naast verboden methodes verscheidene categorieën gedefinieerd als dopeermiddelen.

In dit proefschrift werden enkele substanties besproken die zich op de grens tussen toegelaten en verboden bevinden. Bovendien wordt de complexe situatie met voedingssupplementen en hun mogelijke invloed op het eindresultaat van een dopingtest geëvalueerd.

Analyses in dopingcontroles zijn gebonden aan strikte criteria. Deze omvatten zowel methode validatie als identificatie. Beide parameters worden besproken in Deel I.

Naast de geneesmiddelen die verboden zijn in sport en deze die toegestaan zijn voor medische redenen is het gebruik van verscheidene producten beperkt door een urinaire drempelwaarde. Mogelijke problemen geassocieerd met het gebruik van cafeïne en morfine beschreven in Deel II.

Het gebruik van cafeïne is sinds 1 januari 2004 toegelaten in de sport. Voordien werden urinaire concentraties hoger dan 12 μg/ml beschouwd als een dopingmisbruik. Aangezien de prestatiebevorderende effecten van cafeïne welbekend zijn, en dit zelfs bij hoeveelheden die aanleiding geven tot urinaire concentraties ver beneden de 12 μg/ml drempelwaarde, kan de verwijdering van cafeïne van de lijst van verboden producten aanleiding geven tot een toegenomen misbruik. Om veranderingen vast te stellen in cafeïnegebruik in verscheiden sporten na de verwijdering van de dopinglijst werd een statistische evaluatie gemaakt voor en na de verwijdering van cafeïne. De bekomen resultaten (Deel II Hoofdstuk 1) tonen aan dat de cafeïneconcentraties tussen 1993 en 2002 significant hoger waren in wielrennen en bodybuilding in vergelijking met andere sporten. Een analoge trend werd vastgesteld in de stalen geanalyseerd in 2004 wat de veronderstelde ergogene en stimulerende effecten van cafeïne in deze sporten benadrukt. Cafeïneconcentraties stegen in het algemeen niet na de verwijdering van cafeïne van de WADA dopinglijst. Aan de andere kant tonen berekende drempelwaarden aan dat de vroegere drempelwaarde van 12 μg/ml in staat is gebruik van

misbruik te onderscheiden wat de verwijdering van cafeïne van de dopinglijst twijfelachtig maakt.

Morfine is verboden in de sport boven een drempelwaarde van 1 µg/ml. Het gebruik van voedingsmiddelen die delen van de plant *Papaver somniferom* bevatten, de natuurlijke bron van morfine, is besproken in Deel II Hoofdstuk 2. Toediening van kruidenthees resulteerde in de detectie van urinaire morfineconcentraties tot 7,44 µg/ml. Omwille van dit dienen atleten voorzichtig te zijn met het gebruik van kruidenmengsels. Daarenboven dienen WADA en de NADO's de atleten te waarschuwen voor de mogelijke risico's verbonden aan het gebruik van slaapbevorderende theesoorten of het gebruik van voedingsmiddelen die delen van de plant *Papaver somniferom* bevatten.

Voedingssupplementen worden besproken in Deel III. Een uitgebreid overzicht van de literatuur (Hoofdstuk 1) toont aan de het gebruik van voedingssupplementen sterk afgeraden wordt omwille van twee redenen. Ten eerste wordt aangenomen dat het gebruik van supplementen onnodig is bij een normaal uitgebalanceerd dieet. Ten tweede werd aangetoond dat voedingssupplementen dopeermiddelen bevatten. Als gevolg van de invoering van de DSHEA in de VS in 1994 konden steroïden en prohormonen gedistribueerd worden als voedingssupplementen. Onderzoek toonde aan dat de weergegeven inhoud van deze "supplementen" niet altijd correct is. Naast steroïden en prohormonen bevatten talloze supplementen kruidenmengsels van *Ephedra* species. Daarnaast werden ook andere stimulerende middelen zoals MDMA (ecstasy) en fenfluramine teruggevonden in supplementen. Aangezien atleten verantwoordelijk zijn voor de aanwezigheid van dopeermiddelen in hun lichaam, ongeacht de oorsprong, is het van groot belang om analytische methodes te ontwikkelen die in staat zijn contaminanten in voedingssupplementen op te sporen. In hoofdstuk 2 worden 2 analytische methodes beschreven voor de detectie van lage hoeveelheden aan anaboliserende componenten in zowel vaste als vloeibare supplementen. Deze methodes waren de eerste gevalideerde methodes in dit onderzoeksdomein. In totaal worden 32 componenten gescreend en de detectiegrenzen reiken voor 29 componenten van 2 tot 20 ng/g in vaste voedingssupplementen en in vloeibare supplementen van 1 tot 10 ng/ml voor 31 componenten.

Naast methodes voor de detectie van anaboliserende stoffen werd een gevoelige LC/MS methode voor de detectie van stimulantia in vaste voedingssupplementen ontwikkeld (Hoofdstuk 3). De detectiegrenzen van 100 ng/g zijn ver beneden de vereisten van supplement testende organisaties.

Hoofdstuk 4 beschrijft verscheidene voorbeelden van gecontamineerde voedingssupplementen. Excretiestudies toonden aan dat het gebruik van gecontamineerde supplementen aanleiding kan geven tot een positief dopingresultaat. Zo kan de aanwezigheid van nandrolone precursors resulteren in positieve resultaten tot 144 h na inname. Supplement producerende bedrijven zijn ook steeds op zoek om de internationale wetgeving en dopingwetgeving te omzeilen door nieuwe prohormonen zoals 5α-androstandion (Hoofdstuk 4) en 6-oxo-androstene-3,17-dion (Hoofdstuk 5) wat de noodzaak voor onderzoek in dit domein benadrukt.

Het relatief hoog percentage aan positieve stalen bekomen gedurende 4 jaar van supplementenanalyse toont eveneens het belang van screenen voor verboden producten door gespecialiseerde dopingcontrolelaboratoria aan.

Hoewel het gebruik van de meeste pijnstillers in de sport toegelaten is, lijkt het interessant het voorkomen van deze geneesmiddelen zoals opgegeven door de atleten op de dopingcontroleformulieren te evalueren. Het opgegeven gebruik van NSAID's en paracetamol als meest gekende pijnstillers samen met andere frequent gerapporteerde geneesmiddelen zoals corticosteroïden, β-agonisten, narcotische analgetica en lokale anaesthetica is besproken in Deel IV. Het opgegeven gebruik van antidepressiva werd ook ingesloten. Statistische informatie verzameld gedurende de periode 2002 - 2005 van 18645 atleten werd gerapporteerd. Vanuit deze gegevens bleek dat verschillen in opgegeven geneesmiddelen konden worden waargenomen op de dopingcontroleformulieren van verschillende federaties en/of NADO's en ook tussen sporten onderling. Zo werden meer NSAID's en paracetamol gerapporteerd in balsporten in vergelijking met andere sporten. Het meest uitgesproken effect was het hoge gebruik van corticosteroïden in wielrennen. Percentages in stalen van de UCI namen jaar na jaar toe tot een maximum van 36 % in 2005. Deze corticosteroïden werden vaak gebruikt in combinatie met β-agonisten. Vandaag de dag is het gebruik van corticosteroïden in sport enkel toegestaan indien men beschikt over een medische

rechtvaardiging. Deze TUE zal een handig middel zijn om een einde te stellen aan de vroegere situatie van overconsumptie van ongecontroleerde voorschriften en verklaringen door atleten.

In het algemeen toont dit werk aan dat de lijn tussen toegelaten en verboden medicatie in de sport niet altijd duidelijk is. In de meeste gevallen zijn positieve dopingresultaten te wijten aan het bewust innemen van verboden substanties. Atleten die van deze praktijken gebruik maken dienen onmiddellijk gesanctioneerd te worden. Positieve resultaten kunnen ook voortspruiten uit de inname van niet gelabelde verboden substanties of van (moedwillig) gecontamineerde voedingssupplementen. Volgens de WADA regelgeving wordt geen onderscheid gemaakt tussen deze atleten en valsspelers aangezien de finale verantwoordelijkheid bij de atleet ligt. Aangezien talloze atleten doorgaan met het nemen van supplementen moeten Internationale organisaties hen waarschuwen voor de mogelijke dopingrisico's verboden aan hun gebruik. Daarenboven dienen strengere kwaliteitseisen gesteld te worden aan supplement producerende bedrijven. Door hun ervaring in dit gebied kunnen dopingcontrolelaboratoria een belangrijke rol vervullen in dit proces. Bovendien blijken structuren analoog aan het NZVT systeem, die een lijst van supplementen die zonder risico's kunnen gebruikt worden op het website plaatsen, van het grootste belang.

# Curriculum vitae

## *1. Personalia*


| | |
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| Name: | Van Thuyne |
| First Name: | Wim Robert Maurits |
| Date and Place of Birth: | Ghent, 03/09/1979 |
| Nationality: | Belgian |
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## *2. Studies*

2.1. Secondary school:

Institute: Don Bosco College, Zwijnaarde
Period: 1991-1997
Studies: Mathematics – Sciences
Degree: Secondary Education

2.2. University:

Institute: University Ghent
Period: 1997-2001
Degree: Master in Chemistry

## *3. Publications in international scientific journals with a referee system (impact factor > 0.3)*

1. Prohormones and sports.
   Delbeke, F.T., Van Eenoo, P., **Van Thuyne, W.,** Desmet, N.
   *J. Steroid Biochem. Molec. Biol*. 83 (2002), 245-251.

2. Urinary concentrations of morphine after the administration of herbal teas containing Papaveris Fructus in relation to doping analysis.
   **Van Thuyne, W.**, Van Eenoo, P., Delbeke, F.T.
   *J. Chromatograph. B*, 785 (2003) 245-251.

3. Measurement of the binding of retinoic acid to beta-lactoglobulin B by affinity capillary electrophoresis.
   Lynen, F., **Van Thuyne, W.**, Borremans, F., Vanhoenacker, G., Sandra, P.
   *J. Separation Science*, 26 (1-2) (2003), 53-60.

4. Validation of a GC-MS screening method for anabolizing agents in solid nutritional supplements.
   **Van Thuyne, W.**, Delbeke, F.T.
   *Biomed. Chromatogr.*, 18 (2004), 155-159.

5. Validation of a GC-MS method for anabolizing agents in aqueous nutritional supplements.
   **Van Thuyne, W.**, Delbeke, F.T.
   *J. Chromatogr. Sci.*, 43 (1) (2005), 2-6.

6. Distribution of Caffeine Levels in Urine in Different Sports in Relation to Doping Control.
   **Van Thuyne, W.**, Roels, K., Delbeke, F.T.
   *Int. J. Sports Med.*, 26 (2005), 714-718.

7. Distribution of caffeine levels in urine in different sports in relation to doping control before and after the removal of caffeine of the WADA doping list.
   **Van Thuyne, W.**, Delbeke, F.T.
   *Int. J. Sports. Med.*, (2006) Published online.

8. Detection of androst-4-ene-3,6,17-trione (6-OXO®) and its metabolites in urine by gas chromatography-mass spectrometry in relation to doping analysis.
   **Van Thuyne,W.**, Van Eenoo, P., Mikulčíková, P., Deventer, K., Delbeke, F.T.
   *Biomed. Chromatogr*. 19 (2005), 689-695.

9. Quantitative analysis of androst-4-ene-3,6,17-trione and metabolites in human urine after the administration of a food supplement by liquid chromatography/ion trap-mass spectrometry.
   Deventer, K., Van Eenoo, P., Mikulčíková, P., **Van Thuyne, W.**, Delbeke, F.T.
   *J. Chromatogr. B*, 828 (2005), 21-26.

10. Nutritional supplements: prevalence of use and contamination with doping agents.
    **Van Thuyne, W.**, Delbeke, F.T.
    Nutr. Res. Rev., 19 (2006), 147-158.

11. Detection of stimulants in solid nutritional supplements by liquid chromatography-mass spectrometry.
    Deventer, K., **Van Thuyne, W.**, Mikulčíková P., Van Eenoo, P., Delbeke, F.T.
    Food Chem., submitted.

## *4. Publications in proceedings of international conferences with a referee system*

1. Morphine and other painkillers in relation to doping
   **Van Thuyne, W**., Delbeke, F.T.
   Schänzer, W., Delbeke, F., Deligiannis, A., Gmeiner, G., Maughan, R. and Mester, J.
   Health and doping risks of nutritional supplements and social drugs, Cologne, 2002.

2. Nutritional supplements and doping: The Ghent Experience
   **Van Thuyne, W.**, Delbeke, F.T.
   Schänzer, W., Geyer, H., Gotzmann, A., Mareck, U.
   Recent Advances in Doping Analysis 11, Sport & Buch Strauβ, Cologne,2003, 57-66.

3. Caffeine use in sports: an overview before the removal from the doping list.
   **Van Thuyne, W.**, Delbeke, F.T.
   Schänzer, W., Geyer, H., Gotzmann, A., Mareck, U.
   Recent Advances in Doping Analysis 12, Sport & Buch Strauβ, Cologne,2004, 27-35.

4. Nutritional supplements and doping: Non-labelled multiple prohormones in a Czech nutritional supplement.
   **Van Thuyne, W.**, Delbeke, F.T.
   Schänzer, W., Geyer, H., Gotzmann, A., Mareck, U.
   Recent Advances in Doping Analysis 12, Sport & Buch Strauβ, Cologne,2004, 471-477.

5. Did caffeine use in sports change after the removal from the doping list?
   **Van Thuyne, W.**, Delbeke, F.T.
   Schänzer, W., Geyer, H., Gotzmann, A., Mareck, U.
   Recent Advances in Doping Analysis 13, Sport & Buch Strauβ, Cologne,2005, 105-114.

6. Metabolism, excretion and detection of androst-4-ene-3,6,17-trione.
   Van Eenoo, P., Mikulčíková, P., Deventer, K., **Van Thuyne, W.**, Delbeke, F.T.
   Schänzer, W., Geyer, H., Gotzmann, A., Mareck, U.
   Recent Advances in Doping Analysis 13, Sport & Buch Strauβ, Cologne,2005, 57-64.

## *5. Oral presentations at International Conferences, Symposia, Workshops and Scientific Meetings*

1. Morphine and other painkillers in relation to doping
   **Van Thuyne, W**., Delbeke, F.T.

International Symposium on health and doping risks of nutritional supplements and social drugs, Cologne, 2002, 18/07/2002.

2. Nutritional Supplements and Doping: The Ghent Experience.
   **Van Thuyne, W**., Delbeke, F.T.
   Manfred Donike Workshop, 21th Cologne Workshop on Dope Analysis. 17/03/2003

3. Caffeine Use in Sports: An overview before the removal from the doping list.
   **Van Thuyne, W**., Delbeke, F.T.
   Manfred Donike Workshop, 22th Cologne Workshop on Dope Analysis. 08/03/2004.

4. Caffeine use in sports: an overview before and after the removal from the doping list.
   **Van Thuyne, W**., Delbeke, F.T.
   Manfred Donike Workshop, 23th Cologne Workshop on Dope Analysis. 01/03/2005.

5. Nutritional supplements and doping: history, analysis and latest results.
   **Van Thuyne, W**., Delbeke, F.T.
   Bucharest, 10/07/2004 (presented by FTD)

6. Prevalence and analysis of doping agents as contaminants in nutritional supplements.
   **Van Thuyne, W**., Delbeke, F.T.
   Niterói (Brazil), 13/09/3005 (presented by FTD)

7. Comprehensive screening method for the detection of narcotics and stimulants using single step derivatisation.
   **Van Thuyne, W**., Van Eenoo,P., Delbeke, F.T.
   Manfred Donike Workshop, 24th Cologne Workshop on Dope Analysis. 06/06/2006.

8. Results of several small research projects in 2005.
   Van Eenoo, P., **Van Thuyne, W.**, Deventer, K., Delbeke, F.T.
   Manfred Donike Workshop, 24th Cologne Workshop on Dope Analysis. 05/06/2006. (presented by PVE)

9 Detection of 19-norandrosterone after ingestion of the oral contraceptives norethisterone acetate and lynestrenol and ethylestrenol
   Van Eenoo, P., Mikulčíková, P., **Van Thuyne, W.**, Deventer, K., Delbeke F.T.

Manfred Donike Workshop, 24th Cologne Workshop on Dope Analysis. 07/06/2006. (presented by PVE)

## *6. Poster Presentations at national/international Conferences*

1. Nutritional Supplements and doping.
   **Van Thuyne, W.**, Delbeke F.T.
   Manfred Donike Workshop, 22th Cologne Workshop on Dope Analysis. 07/03/2004.

2. Non-labeled multiple prohormones in a Czech nutritional supplement
   **Van Thuyne, W.**, Delbeke F.T.
   Manfred Donike Workshop, 22th Cologne Workshop on Dope Analysis. 07/03/2004.

3. Detection of contaminants in nutritional supplements (Food2Know).
   **Van Thuyne, W.**, Delbeke, F.T.
   Food2Know Intern Networking Event. 19/12/2005.

4. Czech supplements: the story continues.
   **Van Thuyne, W.**, Delbeke F.T.
   Manfred Donike Workshop, 24th Cologne Workshop on Dope Analysis. 05/06/2006.

## *7. Other*

### 7.1. Stays at other institutes

- University of Pardubice (Czech Republic): 05/02/2006 – 18/02/2006 (FWO project n°: V3/5 – KL. D 5): Exploration of SFE as a solution for extraction problems in analysis for doping agents in nutritional supplements.

### 7.2. Courses followed

- Agilent GC-MSD Chemstation and Instrument Operation "Basique" Agilent Technologies, Sint-Stevens-Woluwe, Belgium.

- Farmacokinetiek, Faculty of pharmaceutical sciences, Ghent University.
- Drugs, Faculty of pharmaceutical sciences, Ghent University.
- Kwaliteits-, milieu- en welzijnszorg in de chemische industrie, Faculty of Sciences, Ghent University.
- Agilent Technologies: Introducing the new Agilent 5975 Inert MSD, Ghent, Belgium
- Agilent Technologies: Exploring the "Terra Incognita" on your GC-MS chemstation, Kortrijk, Belgium

## 7.3. Membership of scientific associations

- Member of Food2Know