1 History of Sports Drug Testing

1.1 HISTORICAL ATTEMPTS OF ARTIFICIAL PERFORMANCE ENHANCEMENT

Sports competition is initiated by many different motivations, which include, but are not limited to, mankind's desire for excellence and perfection, the enjoyment of contests, and financial as well as social benefits associated with victory and success at sporting events.[1] In particular, the latter facts are frequently mentioned as the major reasons for attempts of sportsmen to artificially increase their physical performance and that this modern and contemporary issue was not present between 776 B.C. and 393 A.D. at the ancient Olympic Games. However, the belief that athletes participating at the ancient Panhellenic Games, which included the Olympic Games as the most prestigious sporting festival, were motivated only by the glory and appreciation that they might receive, is weakened even by the interpretation of the term "athlete." The origin of this expression, the Greek noun *athlon*, means "prize" or "reward;" its verbal form *athleuein* means "to compete for a prize." Consequently, the athlete is a person who competes for a prize, which was reportedly a great value even in ancient times,[2] and sport for sport's sake was not an ancient concept.[3] In fact, even thousands of years ago, athletes were seeking competitive advantage over their rivals in many different ways, which included manipulation of equipment and corruption of judges. Moreover, the consumption of certain mushrooms as psychogenic aids was reported,[4] and Philostratus (3rd–2nd century B.C.) and Pliny the younger (1st century A.D.) wrote notes about athletes consuming bread prepared with juice of the poppy plant (opium) and the use of a decoction of the hippuris plant, respectively.[5] All was done with considerable support by the doctors and the goal to enhance the athletes' performance, which would possibly be referred

Mass Spectrometry in Sports Drug Testing: Characterization of Prohibited Substances and Doping Control Analytical Assays, By Mario Thevis

to as "doping" in a modern context. Physicians acted as coaches and doctors simultaneously, and one of the most famous medical attendants was Herodicus, the indoctrinator of Hippocrates, who was particularly interested in athletes' nutrition and rehabilitation.[6] In addition to the human competitors, horses were also the subject of treatment to increase their endurance and stamina as reported in the ancient Rome where *hydromel*, a mixture of honey and water, was administered to horses in chariot races.[7] With the growing medical and pharmaceutical knowledge in the 19th and 20th century, more and more attempts of artificial performance enhancements were assumed and reported, which were conducted with humans and animals likewise. Consequently, various initiatives were started to counteract the misuse of drugs and methods to surreptitiously increase power, strength and athletic capabilities. Such approaches were first conducted with horse saliva in the early 20th century, and, approximately 50 years later, applied also to human urine and blood specimens.

1.2 BACKGROUND AND RATIONALE OF DOPING CONTROLS

Numerous reasons for banning drugs and methods of doping and manipulation from sports were defined by anti-doping authorities such as the medical commission of the International Olympic Committee (IOC) or the World Anti-Doping Agency (WADA) and first necessitated the recognition of the issue. In 1988, the IOC medical commission drafted a charter stating that, "The use of drugs and other substances and banned methods to enhance or accentuate athletic performance is a tragic reality that must be eliminated from modern sport."[8] These fundamental words still reflect the principles that are still seminal to anti-doping programs, which have been coined to preserve the "spirit of sport." According to the World Anti-Doping Code, the spirit of sport is characterized by various values including ethics, fair play and honesty, health, dedication and commitment, and respect for rules, laws, self, and other participants, etc.[9] Doping, however, contravenes to all of these aspects and, thus, modern doping controls are focused on substances and methods of doping that meet at least two of three criteria as defined by WADA: (1) A substance or method has the potential to enhance or enhances sport performance as evidenced by medical or scientific data, pharmacological effects, or experience; (2) a substance or method represents an actual or potential health risk to the athlete as evidenced by medical or scientific data, pharmacological effects, or

experience; and (3) a substance or method violates the spirit of sport as defined in the World Anti-Doping Code.[9]

1.2.1 Cheating

Sportsmanship implements the idea of fair play and the integrity of all members of the sporting community. The ideology that only "eligible" persons should be allowed to compete was present also during the ancient Olympic Games and outlined in the facts that only athletes who were never convicted of a crime should participate. Moreover, sportsmen were requested to swear that they had trained for 10 months prior to coming to Olympia, and another 4 weeks on-site being supervised by the *Helenedonakai*—the judges.[4] Doping contravenes the most basic principles of fair play and results in beguilement of competitors and spectators. Both are hoodwinked, and in particular the deceived athlete might suffer from financial, social, and probably occupational disadvantages in addition to a personal disappointment, if he/she loses a competition against an athlete who artificially increased his/her performance. Consequently, doping must be regarded as cheating in numerous regards, and the rights of those athletes, who are devoted to clean and fair sports, must be protected.

1.2.2 Health Issues

Doping practices can compromise the short- and long-term physical and mental health of athletes; hence, health and safety concerns have been a major aspect of the fight against doping. Numerous articles were published dealing either with case reports about serious or even fatal consequences of drug abuse in professional and amateur sport as well as general undesirable effects observed and associated with doping,[10–20] which were supposedly the final trigger for international sport federations to establish anti-doping rules and test their athletes for drug abuse.[21]

1.2.3 Ethical Issues

According to the World Anti-Doping Code[9] and the common understanding of the intrinsic value of sport, doping categorically contradicts the spirit of sport. This issue has also ethically been evaluated and all values attributed to the spirit of sport have been subject of ethical considerations.[22] Fair play and honesty, character and education, and the virtue of athletes, are an integral part of sport pedagogy and pedagogic

ethics, which is complemented by numerous additional aspects of ethics in sports concerning the health and the exploitation of the human (or animal) body as well as the respect for rules, laws, self, and other participants. The violation of ethical principles is not acceptable in sport, and noncompliance is regarded as a doping offence.

1.3 EARLY DETECTION METHODS: POSSIBILITIES AND LIMITATIONS OF ASSAYS WITHOUT MASS SPECTROMETRY

Doping of animals, primarily horse and hound, has been considered a major pacemaker of doping practices in modern human sports but also as a driving force of anti-doping activities. In 1666, the first decree was enacted in England, which prohibited the administration of substances to horses aiming to improve their performance in races at Worksop,[12] and severe consequences up to the death penalty were announced and executed as reported in the late 18th century in Cambridge (Great Britain) when horses were poisoned at Newmarket.[23] In light of such regulations and their strict enforcement, it was a logical consequence that the first successful attempts to detect doping agents using bioassays and analytical chemistry were introduced in horse racing rather than in human sports.

1.3.1 First Applications Using Chemical and Biological Approaches in Horse Doping Control

In 1910, the Austrian Jockey Club hired a Polish pharmacist named Alfons Bukowski[24] (who has, at some occasions, been referred to as a Russian scientist) to establish a method that allows the detection of alkaloids such as morphine and heroin in equine saliva. He reportedly succeeded in developing such a method but never disclosed any details and returned to his home country, which prompted the Austrian Jockey Club to call in Professor Sigmund Fränkel from the University of Vienna to install a new procedure enabling saliva drug testing. Although never published by Fränkel himself, the principle procedure was later described by G. Lander, the chief chemist for the Jockey Club of England,[25] who published a method that included various consecutive extraction and concentration steps followed by chemical reactions forcing precipitation and/or color reaction of alkaloids for visual inspection. In general, the employed approach was mainly a miniaturized application of the Stas-Otto process,[26] which gained public recognition

as early as 1850 when its use helped reveal the murder of Gustave Fougnies and strongly influenced the newly born arena of forensic sciences.[27]

First, a comparably large volume of saliva was required, which was preferably obtained by washing the horse's mouth using a 0.16 M acetic acid solution over a period of up to 5 minutes. The obtained material was extracted using 90% pure ethanol and diluted acetic acid, followed by filtration and concentration of the extract *in vacuo* to an aqueous residue of approximately 5 mL. The solution was purified by ether extraction and its volume further reduced by evaporation to yield a viscous remainder, which was again extracted with small amounts of ethanol that was finally concentrated to dryness. The dry residue was extracted using 0.3 M aqueous hydrochloric acid, the aqueous layer was adjusted to alkaline pH followed by extraction using chloroform and benzene, the combined organic layers were concentrated to dryness, and the remaining residue reconstituted in 0.16 M acetic acid. The presence of an alkaloid (including cocaine, strychnine, quinine, morphine, and heroin) was visualized by formation of a precipitate or opalescence when placing approximately 5–10 µL of the saliva extract in a capillary test tube and adding different reagents as listed in Table 1.1.[25] Depending on the target analyte and the employed chemical, estimated detection limits between 0.05 and 20 µg were accomplished using diluted reference compounds. The applicability to authentic saliva specimens and, as such, the proof-of-principle, was provided using blank saliva samples of reportedly untreated animals as well as specimens derived from administration studies to outline the specificity of the method and the ability to "unambiguously" differentiate between positive and negative results.

TABLE 1.1: Estimated Detection Limits (µg) for Alkaloids Using Colorimetric Test Methods According to Lander (1930)[a]

Reagent	Alkaloid				
	Cocaine	Strychnine	Quinine	Morphine	Heroin
Iodine	0.05	0.05	0.05	10.0	0.50
Phosphomolybdic acid	0.05	0.10	0.20	1.00	0.50
Potassium mercuric iodide	0.05	0.20	0.40	10.0	2.00
Gold chloride	0.10	0.40	0.40	1.00	0.50
Tannic acid	—	0.50	0.40	20.0	20.0

[a] Ref. 25.

Chemical saliva tests underwent further developments that aimed for optimized extraction conditions of target analytes and more sensitive assays.[28] Using a defined array of tests, opiates were the first to screen for preferably by the Marquis reagent (formaldehyde and sulphuric acid), which yields a dark red-to-purple color in the presence of opiates, particularly morphine and heroin. Subsequently, analyses for strychnine (vanadic acid/sulphur-chromate test), quinine (bromine-ammonia test), cocaine, nikethamide, atropine, etc. (crystalline methods) were conducted. These and other compounds were used as mixtures, e.g. 1.5 g of heroin, 2.5 g of strychnine, 2 minims of nitroglycerine (accounting for 1/250 U.S. fluid ounces or 0.12 mL), 5 minims of *tinctura digitalis*, and 2 ounces of cola nut, and applied to race horses approximately 1 hour before a race.[23] Numerous additional concoctions made from stimulants, narcotics, herbal extracts, and organic as well as inorganic poisons were employed for horse doping purposes in the early 20th century,[29] and astonishing estimations about the prevalence of doping in equine sports were published mentioning more than 50% of doped horses in the United States in the early 1930s.[30] However, the numbers dropped in the following years possibly due to improved analytical procedures and certainly enforcement of severe punishments. Nevertheless, the ambitious efforts of pharmaceutical industries and the enormous numbers of continuously generated new drugs represented a great challenge for doping control chemists, and in particular the introduction of amphetamine and its derivatives initiated a new era of doping for human and veterinary sports. Consequently, more sensitive, comprehensive, and specific detection assays were required, which were developed and established for human and equine doping control specimens likewise (see section 1.3.2).

Besides chemical analytical options, bioassays based on mice and frogs were established by Munch[31–35] and Lucas.[28] The effect of selected drugs on the behavior of mice was observed for instance by Straub, who reported on the phenomenon that these animals carry their tail in an unmistakable S-shaped curve parallel to their backbone under the influence of minute amounts of morphine ("Straub tail response"),[36–39] which was due to a sustained contraction of the *sacrococcygeus dorsalis* muscles[40] (Fig. 1.1).[290] Moreover, Munch described the effects of barbiturates and sedatives in general as well as those resulting from stimulants such as strychnine and caffeine on mice, which were commonly recognized in a decrease in voluntary travel and an increased tendency to sleep or increased locomotion and muscular tremor, respectively.[32] In addition, the administration of strychnine and nikethamide to small frogs yielded characteristic convulsions, and movements were kymo-

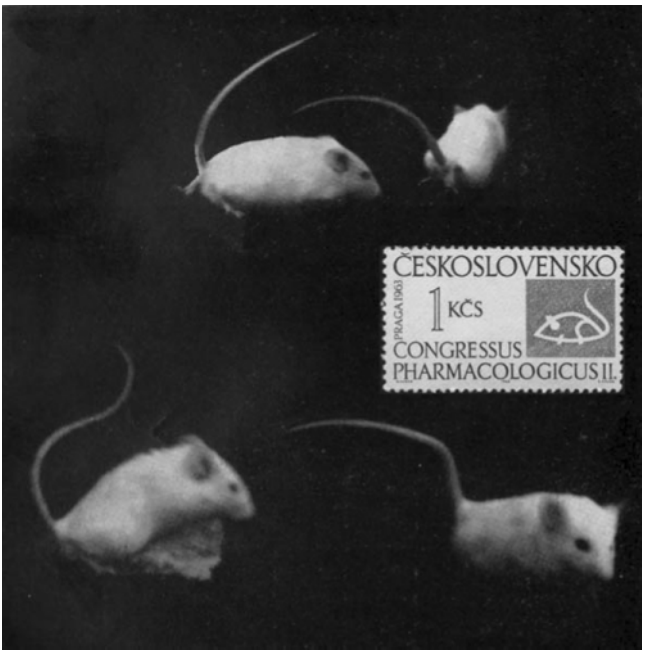

Figure 1.1: Photography of mice showing the Straub tail response to morphine applications; inset: stamp of 1963 ornamented with a Straub tail response mouse. From Klaus Starke, Die Geschichte des Pharmakologischen Instituts der Universität Freiburg, Figure 3, page 12. Reprinted by permission of Springer Science and Business Media. Copyright © Springer, Berlin 2004.

graphically recorded and evaluated.[29] Comprehensive studies were conducted on how these approaches would be useful and possibly "justiciable" for horse doping control purposes, and methods were established that required 2 mL of saliva or 1 mL of urine, aliquots of which were intraperitoneally administered to mice. Several thousand saliva and urine specimens derived from horses that did not receive any medication within a period of 24 hours were measured using the bioassay approach, and no changes in behavior, appearance, or attitude were observed within 15 minutes.[41] In contrast, samples collected from horses participating in administration studies caused positive reactions and were attributable to the applied drug(s), even when different substances were given simultaneously. Due to the fact that the injected samples were not sterilized, mice usually suffered from infections after 12–24 hours post-administration, but these reactions were differentiable from pharmacodynamic responses and not relevant for the test result. The mouse-based bioassay demonstrated reasonable sensitivity

TABLE 1.2: Estimated Detection Limits for Selected Drugs Using Mouse-based Bioassays According to Munch (1952)[a]

Drug	(μg / 20g mouse)
Amphetamine	80
Caffeine	600
Cocaine (HCl)	400
Codeine	60
Nikethamide	100
Pethidine	400
Desoxyephedrine	40
Dihydromorphinone	12
Ephedrine	200
Heroin	1
Morphine	60–80
Picrotoxin	40
Strychnine	1

[a] Ref. 32.

regarding a variety of analytes (Table 1.2), enabled fast delivery of test results, and was comparably cheap. Hence, it was considered as a rapid screening tool for horse drug testing, and the more time-consuming and laborious chemical analyses were conducted mainly when suspicious results were found. Several decades later, also the dilation of pupils of mice after subcutaneous injection of morphine and extracts derived from urine of morphine users was found to be a sensitive and specific test.[42]

1.3.2 First Applications Using Chemical Approaches in Human Doping Control

The history of drug abuse in human sports reaches back at least as far as 1865, when swimmers in the Amsterdam's canal races were competing under the influence of doping agents;[43] however, the administered drugs were never identified or disclosed. In contrast, the systematic misuse of stimulants, narcotics, and nitroglycerine by cyclists was reported particularly for participants of the infamous "Six-days" cycle races that began in 1879. Racing cyclists appeared to be the prime offenders, who used various drug cocktails consisting of coffee enriched with extra caffeine, which was further fortified with strychnine and cocaine as the race progressed. Alternatively or complementary,

sugar cubes dipped in ether were consumed, capsules of nitroglycerine (to support and ease breathing) as well as mixtures of heroin and cocaine ("speedballs") were administered, and miracle drinks were provided by trainers that supposedly contained digitalis, atropine, and camphor.[21] Most drugs were applied without any supervision and, consequently, fatalities were not surprising. The most prominent and presumably first victim of doping practices was the British cyclist Arthur Linton, who was reported to have died in 1886 during a 600 km cycle race between Paris and Bordeaux. Numerous aspects, however, disproved this anecdote, including the fact that the first Paris-Bordeaux-race was in 1891 and Arthur Linton evidently died in 1896 of typhoid fever.[44] Nevertheless, numerous athletes died young for mostly unknown reasons in the late 19th and early 20th century, which might be attributed also to the misuse of drugs. Doping agents were applied in various sports such as cycling, running, boxing, soccer, etc. and even Olympic gold medal winners such as Thomas Hicks (Marathon, 1904) were reportedly and officially using drugs such as strychnine[45] without being sanctioned. Following World War II, the use of stimulants such as amphetamine and derivatives in sports increased enormously, and in particular cyclists and soccer players were found to be regular users.[46] The great health issues associated with the uncontrolled use of these drugs in sports prompted several European countries such as France, Belgium, and Italy to install anti-doping laws in 1965[21,47] and the need for doping control analytical assays was evident. Numerous approaches were established for a variety of stimulants and narcotics employing contemporary state-of-the-art technologies.

1.3.2.1 Methods without Chromatography Drug metabolism, disposition, elimination, and analysis have been of great interest for numerous clinical applications as well as doping control purposes, and both fields of research have frequently interacted with physical, chemical, biological, and biochemical sciences. Most prominent physicochemical properties of analytes were used to differentiate and identify target compounds in urine, blood, saliva, and sweat, which yielded a variety of analytical approaches primarily based on crystallographic, chromatographic, and/or colorimetric methods. Between 1930 and 1960, only few procedures were developed and published solely for sports drug testing purposes; most inventions were generally accepted for forensic, clinical, and doping control applications.

The early characterization of drugs, in particular of stimulants, was accomplished using microscopy, the determination of melting point and the corresponding refractive index of crystals (derived from various

salts) as well as defined mixtures and their resulting melts.[48–50] In addition, microprecipitation, microchemcial tests with gold or platinic chloride and nephelometric analyses after complexation of alkaloids were reported,[51,52] but although "micro scale" methods were employed, most approaches were difficult to apply to biological matrices due to the large volumes required and the need of extensive sample preparation.

Colorimetric assays proved to be reasonably sensitive.[52] One of the first assays to determine benzedrine (the racemic mixture of d- and l-amphetamine) in human urine was reported in 1938,[53] which was based on an alkaline extraction of amines into ether, followed by a reaction with picric acid to yield a clear yellow solution, the color intensity of which was visually estimated against a calibration curve ranging from 0–20 µg/mL. The method was applied to a series of amines such as phenylethylamine, ephedrine, amphetamine, methamphetamine, mescaline, etc. in order to determine their excretion rates into urine after oral administration, and the author indicated a detection limit of 0.5 µg/mL.[54] However, the fact that various amines also occur naturally in urine specimens, which strongly influenced each individual blank value, as well as the entirely missing identification of a distinct compound made the test only moderately applicable for forensic and doping control purposes. More advanced methods with increased specificity were desired and established, commonly using extraction and re-extraction steps combined with preceding steam distillation of alkalinized urine (or other bodily specimens and extracts). Due to the volatility of amphetamines and various alkaloids as free bases, samples were usually buffered to pH 9–10 and distilled under reduced pressure into dilute acids. After re-adjusting the pH to 9–10, the distillate was extracted using organic solvents such as benzene or chloroform and the analytes were derivatized to colored compounds, for instance by means of diazotization, picric acid, or methyl orange. Following re-extraction into small volumes of dilute acid, quantitative results were obtained by colorimetry and photometry with detection limits of approximately 1 µg/mL.[51,55–60]

1.3.2.2 Methods Including Paper or Thin-layer Chromatography

Although the above-mentioned assays were comparably sensitive and optimized to ensure reduced susceptibility to interferences, the introduction of chromatography in general was considered a major breakthrough in drug testing. Various applications were established for numerous compounds, and special attention was first paid to the paper-chromatographic separation of alkaloids and stimulants[61–67] as well as diuretic agents,[68] which were, however, not prohibited in sports until

1988. Sample preparation and extraction techniques were commonly adapted from earlier methods based on colorimetry and photometry (*vide supra*), and extracts were subsequently subjected to paper chromatography followed by visualization of target analytes by means of different stains such as the Dragendorff's reagent (Bismuth nitrate and potassium iodide in dilute acetic or hydrochloric acid),[61,64] the Prussian blue reagent (also known as Berlin blue reagent, prepared of potassium ferricyanide and iron(III) chloride),[65] or an alcoholic solution of bromocresol green (3,3′,5,5′-tetrabromo-*m*-cresolsulfonphthalein).[67] Paper chromatography was rapidly complemented and/or substituted by thin-layer chromatography (TLC) in forensic and doping control analyses, in particular with regard to morphine-related narcotics and amphetamine and its derivatives,[69–85] as well as diuretics.[86] The principle sample preparation procedures remained and commonly required between 5 and 200 mL of urine (if available). After purification and extraction of target compounds, separation was accomplished by one-[75] or two-dimensional[74] TLC followed by visualization either on the plate using spray reagents or after elution of spots employing ultraviolet spectrophotometry.[73] Numerous spray reagents were tested such as ninhydrin, diphenylcarbazone/silver acetate/mercury(II) sulphate, iodoplatinate, iodine/potassium iodide, bromocresol green, and ammoniacal silver nitrate that exhibited either particular sensitivity or specificity for selected groups of compounds enabling the detection of 0.1–1.0 µg of target analytes per mL of urine.[87]

1.3.2.3 Methods Including Gas Chromatography The capability of gas chromatography (GC) to separate compounds relevant for doping controls was recognized in the late 1950s, and first proof-of-principle studies outlined the potential of GC systems to measure various classes of analytes.[88–92] Commonly, columns consisting of 1–3 m long stainless steel or glass tubing (0.3–0.6 cm outer diameter) filled with different stationary phases (e.g., 5% Carbowax 6000 and 5% KOH, 2% Carbowax 20M and 5% KOH, 2.5% SE-30, or 10% Apiezon L and 10% KOH) were employed to accomplish efficient separation of target substances of different polarities,[93] and analyzers such as flame ionization and nitrogen-phosphorus detectors (FID and NPD, respectively) as well as ionization β-ray (strontium[90]) or electron capture detectors were used to measure the eluting compounds. Initially, stimulants and alkaloids such as amphetamine, methamphetamine, caffeine, cocaine, ephedrine, strychnine, etc. and their hydroxylated metabolites isolated from biological matrices were the subject of research and routine analysis.[46,76,94–100] While sample extraction and concentration methodologies

were mostly adapted from earlier procedures and only marginally altered, major aspects of gas chromatography to improve were soon identified, which included for instance the need for supporting information that increases confidence in the identification of a target compound, better chromatographic peak shapes, and reproducible separation of isomers. All of these issues were addressed by various derivatization strategies, which first improved chromatographic properties and, second, provided additional data to characterize a substance. A strategy to identify a compound by its retention times obtained from the native as well as derivatized analyte or two different derivatives was termed the "peak-shift technique"[101] and used as a common standard in confirmatory analyses. Trimethylsilylation (using e.g., hexamethyldisilazane or *N*-methyl-*N*-trimethylsilyltrifluoroacetamide [MSTFA][102]), acylation (using e.g., acetic, propionic, or heptafluorobutyric anhydride, bis[acylamide], etc.), alkylation, formation of several Schiff-bases (e.g., acetone-, propionaldehyde-, benzyl methyl ketone-Schiff-bases),[103] or preparation of mixed derivatives were utilized to modify the physicochemical nature of substances and, thus, enhance their traceability in (sports) drug testing. The most frequently used methods to chemically modify target analytes in doping controls were finally based on trimethylsilylation or acylation according to assays established by Donike and co-workers,[104–108] which enabled detection limits of approximately 1 µg/mL and represented a central element of the first comprehensive doping control program undertaken at the Olympic Games 1972 in Munich and subsequently conducted great sporting events.[109–111] Improved GC columns, in particular using capillary tubing, further increased the sensitivity and robustness of analyses, which has made GC an invaluable tool of past and present sports drug testing approaches,[112,113] especially as a separation unit for target compounds. However, the enormous complexity of biological matrices and the continuously increasing number of therapeutics have necessitated more specific and unequivocal analyzers than for instance NPD and FID, which resulted in the highly successful combination of GCs and mass spectrometers.

1.3.2.4 Methods Including Liquid Chromatography The analysis of substances by means of GC requires vaporization, and several compounds that gained relevance for doping controls in addition to "classical" doping agents are thermolabile or possess poor gas chromatographic properties even after derivatization and, thus, insufficient detection limits. Consequently, alternative approaches were necessary, and high performance liquid chromatography (HPLC), in particular

employing reversed-phase columns, was found suitable for the detection of compounds that composed the continuously expanding list of prohibited compounds of doping such as diuretics,[114–119] (ring-hydroxylated) stimulants,[120–124] anabolic-androgenic steroids and corticosteroids,[125–138] analgesics,[139–142] etc. with and without derivatization. The commonly used detectors were ultraviolet (UV)- or fluorescence-based analyzers, depending on the nature of the analyte and/or the produced derivative.

Diuretics represent a heterogeneous class of compounds; however, most of them can be measured using conventional absorbance detectors such as UV-analyzers without further derivatization at reasonable detection limits of approximately 0.01–2.0 µg/mL.[116] Increased sensitivity and/or selectivity was reported for the loop diuretics furosemide and bumetanide as well as the potassium sparing diuretics amiloride and triamterene when using fluorescence detection due to the fact that numerous agents co-extracted with diuretics are visualized by means of UV but not fluorescence detection.[114]

Although stimulants related to amphetamine were determined using the above-mentioned GC-based approaches, options employing HPLC were evaluated and demonstrated great utility in particular regarding sensitivity and comprehensiveness. Target analytes were measured from urine either in native forms[122] or after pre- or post-column derivatization using *o*-phthalaldehyde, 4-chloro-7-nitro-benz-2,1,3-oxadiazole, sodium naphthaquinone-4-sulphonate, or bis(2,4,6-trichlorophenyl) oxalate, which allowed for detection limits ranging from 0.01–0.1 µg/mL.[120,121,124]

The analysis of steroidal agents such as androgens, corticosteroids, and anabolic androgenic steroids was of great interest in various fields including doping controls,[123] and the advantages of HPLC-based approaches over immunological methods in terms of specificity and reproducibility were major reasons for the developments of assays, enabling the detection of steroids in human and animal specimens.[135] Anabolic steroids such as metandienone were detected at concentrations of 10 ng/mL,[125] and other analytes lacking ultraviolet active functional groups were derivatized for instance by conversion into benzoates or *p*-nitrobenzoates.[130]

Few analgesics such as morphine and related opiates have been prohibited in human sports, and all members of the class of analgesic therapeutics are not allowed for the treatment of competing animals. Hence, various methods using HPLC were established to determine the presence of these compounds in urine and blood samples, and comprehensive assays enabled the detection of 10–100 ng/mL of analytes such as morphine, ketoprofen, naproxen, etc.[139,141,142]

Several of these assays are still in use in various fields of analytical chemistry but usually not in sports drug testing, where comprehensive, fast, and specific procedures are required. Although detection limits of numerous methods would fulfill so-called minimum required performance limits as established by WADA, the inferior specificity of UV-spectra compared to mass spectrometric information has led to several endeavors to combine the liquid chromatographic separation units via ionization interfaces to all kinds of mass spectrometers.

1.3.2.5 Methods Including Immunological Approaches (Radio) Immunoassays have been of great interest in endocrinology and related fields due to their capability to qualitatively and quantitatively determine trace amounts of peptide hormones.[143] Although most compounds were not prohibited in sports at the time of early detection method developments, various procedures would have been of benefit for doping controls also, e.g., assays for the detection of insulins,[144–146] corticotrophins,[147–149] human growth hormone (hGH),[150–156] human chorionic gonadotrophin (hCG),[157,158] luteinizing hormone (LH),[159,160] or erythropoietin (EPO).[161] However, their successors have been frequently used in sports drug testing and have become an important tool for the identification or purification of several drugs in doping control analyses.

Strategies for the immunological detection of substances that are prohibited in sports were introduced in 1975 by Brooks and colleagues[162] based on results first published by Sumner in 1974.[163] A major issue of the early anti-doping fight was, among others, the missing tool to uncover the assumed misuse of anabolic steroids, and the availability of radioimmunoassays represented a breakthrough in sports drug testing.[164–167] The extraction of steroids followed by acetylation and subsequent determination using radioimmunoassays targeting 17-alkylated steroids or 19-norsteroids provided a sensitive procedure enabling the detection of as low as 10 pg of metandienone per mL of urine or serum. These approaches were applied to anonymized samples collected at the 1974 Commonwealth Games (Christchurch, New Zealand) and European Games (Rome, Italy),[168] and further used officially to screen urine specimens at the Olympic Games of 1976 in Montreal (Canada).[167] Confirmatory analyses of samples that yielded adverse analytical findings were conducted by means of newly established GC-MS methods, and two samples of the 1974 Commonwealth Games as well as six specimens from the 1976 Olympic Games were reported "positive" with RIA and GC-MS. Although the quality of analytical results obtained by combinations of GC and MS considerably improved

in the following years and became more and more the gold standard for doping controls, immunological assays were employed for various analytes such as stimulants,[169–172] opioids,[173–179] β_2-agonists,[180,181] diuretics,[182,183] benzoylecgonine, etc. in human and animal drug testing for decades.[184–186] Still, the importance of immunoassays for the detection of low molecular weight drugs dropped significantly with the constantly increasing performances of mass spectrometry-based methods and requirements of unequivocal identification of target analytes; however, the fact that high molecular weight compounds were hardly or not measurable from doping control specimens using conventional MS systems has made immunological approaches an invaluable tool in the past and present fight against doping. The most prominent examples for the application of immunoassays have been the detection of misuse regarding hCG and LH,[187–191] and, more recently, hGH[192–204] and EPO.[205–213] These methods have enabled the determination of prohibited compounds (e.g., hCG in urine samples of male athletes), the quantitation of banned substances such as hGH and its natural variants, or the quantitative analysis of parameters that indirectly indicate the potential misuse of drugs and methods of doping such as the administration of hGH and EPO or blood transfusions.

1.4 INTRODUCTION OF MASS SPECTROMETRY TO DOPING CONTROL ANALYSIS

Mass spectrometry of organic compounds and natural products has greatly influenced analytical approaches and possibilities of analyte characterization in complex matrices. Numerous seminal articles were published describing the fundamentals[214–219] and, thus, the instrumentation required for applied mass spectrometry in forensics, clinical, and doping control analysis. One of the earliest successful combinations of gas chromatography and mass spectrometry consisted of packed GC column interfaced to a Type 12-100 time-of-flight (TOF) MS (Bendix Aviation Corp.), which allowed to scan from *m/z* 1 to 6000 at an enormous scan rate of 2000/s with unit resolution up to *m/z* 200.[214] Alternative approaches included conventional magnetic field mass spectrometers such as the CEC Model 21-620 cycloidal path or the CEC Model 21-103B 180-degree MS (both Consolidated Electrodynamics Corp.) that covered a scan range from *m/z* 40 to 160 within a duty cycle time of 30s at a resolving power of 600.[215] Commonly, approximately 3–10% of the GC effluent was diverted from packed columns through a capillary to the ion source of the mass spectrometer, before capillary GC columns

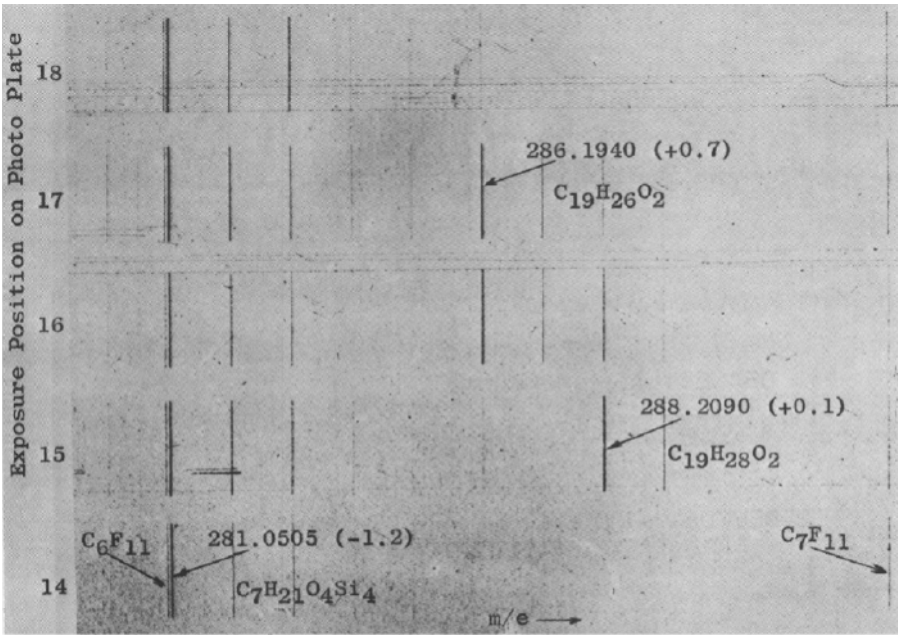

Figure 1.2: High-resolution mass spectra of androstane-3,17-dione (15) and androst-4-ene-3,17-dione (17), recorded on a GC-HRMS system (CEC Model 21-110) with a photographic plate detector. Reprinted with permission from J.T. Watson, K. Biemann, *Anal. Chem*., 1965, 37 (7), p. 850, Figure 9. Copyright © 1965 American Chemical Society.

were directly interfaced to the analyzers.[217] Methods and instruments dramatically improved within a few years and enabled, for instance, the direct recording of high resolution mass spectra of steroids such as androstane-3,17-dione, and androst-4-ene-3,17-dione, which were injected into a GC that was interfaced to a double-focusing MS (Mattauch-Herzog design, CEC Model 21-110) with a photographic plate detector (Fig. 1.2).[219]

1.4.1 First Approaches and Adverse Analytical Findings

First official anti-doping rules as established by international federations became effective in 1966 (Fédération Internationale de Football Association, FIFA) and 1967 (Union Cycliste Internationale, UCI; Union Internationale de Pentathlon Moderne et Biathlon, UIPMB). Nevertheless, pilot studies with regard to human sports drug testing started as early as 1955,[46] and systematic controls at great sporting events were initiated at the FIFA World Cup in 1966. At that time, the

FIFA list of prohibited substances included narcotics such as morphine and heroin, stimulating agents (amphetamine and its derivatives, strychnine, micorene, phenmetrazine), diethyl ether, and trinitroglycerine. In 1967, UCI and UIPMB presented lists of banned compounds and drugs accordingly, and the IOC conducted first doping controls in Grenoble (86 samples) and Mexico City (667 samples) in 1968 following their own list of prohibited substances (Table 1.3).[47]

Early approaches of sports drug testing employing mass spectrometric techniques were reported in 1967 by Beckett and associates[93] who measured amphetamine and its derivatives from spiked urine specimens on a GC-MS system. A Perkin Elmer F11 GC equipped with a packed stainless steel column (2 m, i.d. 31 mm) and interfaced to a Hitachi-Perkin Elmer RMU-6E single focusing MS was used, which scanned from *m/z* 10 to 450 within 12 seconds and allowed the detection of less than 4 µg of analyte per mL of urine. Using three different sensitivity settings of the amplifier, informative electron ionization (EI) spectra were recorded (Fig. 1.3)[93] to serve as confirmatory data. Despite these successful couplings of GC and MS, also alternative assays using offline combinations, e.g., TLC and MS, were reported for the detection of methylamphetamine in doping controls.[220] Here, a Hitachi Perkin-Elmer RMU-6D MS was used, and although detection limits of the presented assay were not evaluated, the sensitivity was considered comparable to the earlier reported GC-MS-based method. A comprehensive use of GC-MS to substantiate suspicious test results in doping controls was present at the 1972 Olympic Games held in Munich, where seven adverse analytical findings with amphetamine (1), ephedrine (3), phenmetrazine (1), and nikethamide (2) were documented.[221–223] Besides common screening procedures employing GC with nitrogen-specific alkali-flame detector (N-FID),[109] an Atlas MAT CH-5 single sector MS interfaced to a temperature-programmed Hewlett-Packard GC Model 7600, which was equipped with a packed glass column (1.06 m, i.d. 2.5 mm, 2% Igepal CO-880 and 12.5% Apiezon L), was used to unambiguously confirm the presence or absence of prohibited compounds. An EI mass spectrum of an adverse analytical finding with nikethamide is depicted in Figure 1.4a (reconstructed). Compared to an EI mass spectrum recorded in 2008 (Fig. 1.4b), no significant difference is observed.

In order to enhance chromatographic properties of many target analytes with relevance to sports drug testing, numerous derivatives were prepared in the following years for instance from stimulants[224–226] and steroids.[168,227–230] The advantages of derivatization were manifold and various modifications were tested for more efficient GC separation

TABLE 1.3: First Lists of Prohibited Substances 1966–1968[a]

Federation	FIFA	UCI	UIMPB	IOC
Year	1966	1967	1967	1968
Occasion	World Cup (UK)	General	General	Olympic Games (Grenoble / Mexico City)
	Narcotics (including morphine, heroin, etc.)	Narcotics (including morphine, heroin, etc.)	Narcotics	Narcotic analgesics (including morphine, heroin, etc.)
	Substances related to amphetamine (including amphetamine and its methylated and hydroxylated derivatives)	Stimulants such as amphetamine, ephedrine, etc.	Amphetamine and its derivatives	Sympathomimetic amines (amphetamine, ephedrine, methylephedrine, etc.)
	Strychnine	Strychnine, ibokaine	Strychnine	CNS-stimulants (strychnine, analeptics, etc.)
	Phenmetrazine		Cocaine	
	Trinitroglycerine		Nitrate and related substances	
	Diethyl ether	Diethyl ether		
		Alcohol	Alcohol	Alcohol (on request by UIMPB after shooting competition)
		Antidepressants		Antidepressants (MAO-inhibitors, imipramine, etc.)
	Micorene		Camphor and pharmacologically related substances, analeptics	
			Hormones (natural and synthetic) if not used for more than one month)	
			Lobeline and related compounds	
			Vasodilators (peripheral)	
			Tranquilizer	Tranquilizer (e.g., phenotiazine)
			Purine bases	

[a] Ref. 47.

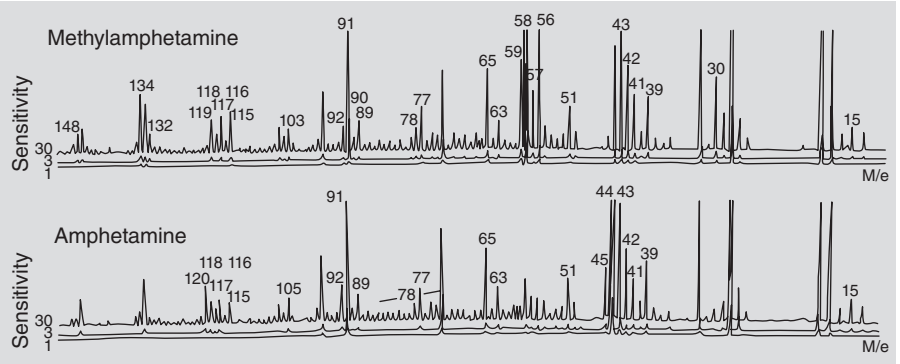

Figure 1.3: GC-EI-MS spectra obtained from urine samples containing methylamphetamine (top) and amphetamine, recorded on a Hitachi-Perkin Elmer RMU-6E single focusing MS. This figure has been reproduced with permission from Pharmaceutical Press, an imprint of RPS Publishing, the publishing organization of the Royal Pharmaceutical Society of Great Britain.

of analytes, improved peak shapes, and more characteristic mass spectra as obtained after EI. Stimulants were derivatized, e.g., to acetyl-, trifluoroacetyl-, pentafluoropropionyl-, heptafluorobutyryl-, perfluorooctanoyl-, trichloroacetyl-, or pentafluorobenzoyl-analogues[224,225] as well as mixed derivatives using trimethylsilylation and trifluoroacetylation.[102,105,226,231] Steroids were preferably acetylated, dimethylsilylated, trimethylsilylated,[168,227,230,232] or chloromethyldimethylsilylated,[228] and also mixed derivatives (methoxime/trimethylsilyl[233–235] or *N*-alkyl/trimethylsilyl[236] derivatives) were formed to yield adequate chromatographic peak shapes and informative EI mass spectra. However, also underivatized steroids were successfully measured using capillary GC and MS instruments.[237]

In 1975, the IOC list of prohibited substances was expanded by the class of anabolic androgenic steroids (AAS), and newly developed methods[168] were applied to samples collected at the 1976 Olympic Games in Montreal (Canada). From a total of 1786 specimens, which underwent conventional drug testing procedures, a selection of 275 was subjected to a special screening procedure based on GC-MS, which was dedicated to the detection of AAS.[238,239] After enzymatic hydrolysis of phase-II-metabolites, steroids such as metandienone (Dianabol), stanozolol (Stromba), 19-norethyltestosterone (Nivelar), 17α-ethyl-4-estren-17β-ol (Orabolin), 19-nortestosterone and its phenylpropionate ester (Durabolin) and respective phase-I-metabolites were fractionated on Sephadex LH-20 solid-phase extraction (SPE) columns. Aliquots were

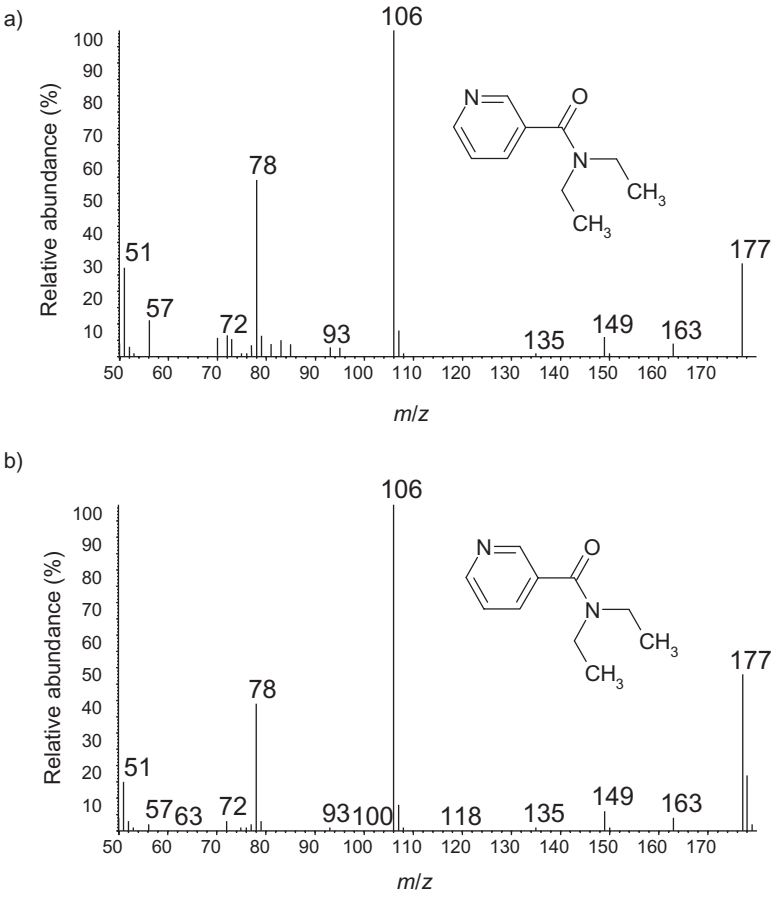

Figure 1.4: EI-mass spectra (a) of nikethamide found in a doping control specimen at the Olympic Games in Munich in 1972 (reconstructed), and (b) of a reference standard analyzed in 2008.

dried, and the dry residues were trimethylsilylated using pyridine/hexamethyldisilazane (HMDS)/trimethylchlorosilane (TMCS) followed by GC-MS analysis. The instrument used was a Pye 104 GC equipped with a 3% OV-1 column, which was connected to a Varian MAT 731 double-focusing high-resolution mass spectrometer (Mattauch-Herzog geometry), which allowed for repetitive scans every 8 seconds as well as selected ion monitoring (SIM). Using this approach, eight samples were tested positive for anabolic steroids, three of which were provided by gold- or silver medalists (all weightlifting).

Although invented as early as 1953,[240] the quadrupole mass spectrometer was not installed in doping control laboratories as detector in GC-MS systems until the late 1970s/early 1980s.[232,241–246] Providing fast

scan speeds at sufficient resolution, benchtop GC-MS systems using quadrupole analyzers soon became state-of-the-art analytical tools in sports drug testing. Implemented for the drug testing programs at the 1982 Soccer World Cup in Spain, the 1983 World Championships in Athletics in Finland,[247] and the Pan American Games in 1983 in Venezuela (Caracas), GC-MS systems helped to uncover one of the biggest doping scandals in the latter event. Using two Hewlett-Packard 5996 GC-MS instruments with a capillary GC column (OV-1, 17 m, i.d. 0.2 mm, film thickness 0.11 μm) and EI source (heated to 250°C), SIM as well as full scan data were recorded in screening and confirmation methods. Earlier sample preparation methods were improved, and urine specimens underwent SPE, enzymatic hydrolysis, liquid-liquid extraction (LLE), and finally trimethylsilylation of extracted analytes before measurement.[184,244,245] A total of 19 athletes including 11 weightlifters, one cyclist, one fencer, one wrestler, one volleyball player, and four track and field athletes,[248–250] tested positive after competition. Four were sanctioned due to the use of stimulants (two ephedrine and two fencamfamine) and 15 were convicted of the use of AAS, in particular nortestosterone (12) was well as testosterone (1), metenolone (1), and metandienone (1).[110,251] In addition to these "clear" cases, reports and rumors on 12 further adverse analytical findings (out of 13 unlabeled urine specimens) prior to the start of the 1983 Pan American Games with regard to AAS misuse among the U.S. team, which were not sanctioned, became public. Moreover, the sudden departure of 12 American athletes when being informed about the efficiency of on-site drug tests[250] caused concern and confusion followed by endless speculations and few confessions.[252]

1.4.2 Progression of Analytical Methods

The detection methods being used in sports drug testing have continuously been improved, updated, and expanded in accordance with instrumental innovations and new drugs and challenges in doping controls. New standards and milestones in terms of analytical assays and available instruments were commonly set at great sporting events such as the Olympic Games.[111] In 1984 at the Los Angeles Olympic Games, for the first time all samples (1510 urine specimens) were screened for AAS using GC-MS, which was accomplished on Hewlett-Packard 5996 analyzers operated in SIM mode. Eleven adverse analytical findings were reported, including one stimulant (ephedrine) and ten anabolic steroids (eight nandrolone cases, one testosterone, and one metenolone case).[184,253]

Four years later at the Olympic Games in Seoul in 1988, the local doping control laboratory was equipped with 12 state-of-the-art Hewlett-Packard 5890/5970B GC-MS systems plus two HP5988A instruments interfaced to GC as well as LC (via thermospray).[185] The LC-MS version of the "Engine" (HP5988A) was used to measure corticosteroids from doping control specimens.[254] Revolutionary work regarding the determination of long-term metabolites of anabolic androgenic steroids such as stanozolol and the derivatization for sensitive detection using GC-MS systems[255] allowed the confirmation of three findings of stanozolol abuse, which represented one third of all positive specimens. The methodology on how to identify particular stanozolol metabolites in urine samples was installed as early as 1986 in Seoul by Schänzer and Donike, which was subsequently and successfully employed for the Olympic Games in 1988. In addition to the stanozolol findings, stimulants such as caffeine (1) and pemoline (1) as well as four cases of furosemide misuse were reported, but no further AAS.[253]

The doping control laboratory in Barcelona (Olympic Games 1992) used 13 GC-MS systems (HP5970, HP5971) and two LC-MS systems (HP5989) with particle beam interface (HP59980B). The latter was connected to an HP1090L Series II liquid chromatograph and was used to provide the first (and possibly last) adverse analytical finding (mesocarb) that has ever been confirmed using LC-particle beam-MS in sports drug testing.[256] In total, five positive doping control samples were reported with strychnine (1), norephedrine (1), clenbuterol (2), and mesocarb (1).

In 1994, the first GC-high resolution-MS system (HP5890/Finnigan MAT 95, double-focusing sector with reversed Nier-Johnson geometry) was installed for the Olympic Winter Games in Lillehammer (Norway),[257] in particular due to its superior sensitivity and selectivity for a specific subset of analytes such as stanozolol and metandienone metabolites as well as clenbuterol.[258] The GC-HRMS was complemented by seven HP5890/5970 and 5972 systems as well as one HP5890/Finnigan SSQ7000 low resolution GC-MS instruments to cope with the constantly increasing number of compounds to be analyzed.

The use of GC/combustion/isotope-ratio mass spectrometry (GC/C/IRMS) has demonstrated distinguished capabilities to reveal misuse of endogenous steroids such as testosterone, dihydrotestosterone, dehydroepiandrosterone (DHEA), etc. and was reported for the first time in 1994.[259] The analysis of carbon isotope ratios of steroids in doping control specimens progressively improved,[260–262] and in 1998 at the

Olympic Winter Games in Nagano (Japan), the first comprehensive analyses for synthetic endogenous steroids were conducted using an HP6890 GC coupled to an Isoprime IRMS (Micromass, Manchester, UK).

Polysaccharide-based plasma volume expanders were first analyzed in urine specimens during the Nordic Ski World Championships in Lahti (Finland, 2001) using a GC-MS-based method (HP 5890/5972 GC-MSD).[263–265] Seven adverse analytical findings were reported and gold, silver, and bronze medals were stripped, all won by Finnish male and female elite athletes.[266] Consequently, the established procedure was also employed at the subsequent Winter Olympic Games in 2002 (Salt Lake City, USA), but ever since, no additional positive case was found although methods were further expanded and improved using LC-MS/MS[267,268] and MALDI-TOFMS[269] approaches.

State-of-the-art GC-HRMS systems (Autospec Ultima) double-focusing sector instruments with EBE geometry, Micromass, Manchester, UK) were also used at the Athens Olympic Games and contributed significantly to the highest number of adverse analytical findings in the history of Olympic Games. Excluding those athletes who were sanctioned due to refusal/manipulation of doping control specimens (3) or "no-show" (2), 20 prohibited substances including AAS (stanozolol, testosterone, metandienone, nandrolone, methyltestosterone, oxandrolone), clenbuterol, cathine, ethamivan, heptaminol, and furosemide were reported. First approaches to use LC-MS/MS for the detection of peptide- and protein-based doping agents in human sports drug testing was described as early as 1993,[270] but it took approximately 10 years to become part of routine procedures[271–281] as for instance in Athens 2004, where LC-iontrap systems (Agilent 1100 LC/MSD SL) were employed. Major advances in sensitivity, accuracy, and resolution of mass spectrometers, which are described in more detail in Chapter 2, provided the basis for efficient method development and more comprehensive detection tools to cover a variety of prohibited peptide hormones such as insulins,[276,282–285] corticotrophins,[286] insulin-like growth factors,[287] luteinizing hormone releasing hormone,[288] as well as methods of manipulation using proteases.[289]

The need for complementary or new methods for doping control purposes is evident and a major task of sports drug testing related research. Continuously, new methods and approaches are developed to expand the portfolio of detection procedures for drugs and methods of manipulation, and currently applied MS-based methods are described in Chapter 6.

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