Dr. Eng. Dorota Kwiatkowska
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APPENDIX No. III

SUMMARY OF PROFESSIONAL ACCOMPLISHMENTS

University of Warsaw
Faculty of Chemistry
Warsaw 2016

Contents

1. Personal data

Name: **Dorota Kwiatkowska** (maiden name **Wojkowska**)

2. Diplomas and scientifics degrees

2007	**National Chamber of Laboratory Diagnosticians** Warsaw, Konopacka 4 Laboratory diagnostician
2004	**Military Institute of Hygiene and Epidemiology** Warsaw, Kozielska 4 Doctoral degree in Biology Dissertation thesis: „Steroid profil in Polish athlets" Thesis supervisor: Prof. R. Grucza, PhD. doctorate awarded by the Scientific Council of Military Institute of Hygiene and Epidemiology
1997 - 1998	**University of Warsaw – Faculty of Chemistry** postgraduate studies - chromatography and mass spectrometry
1989 - 1995	**Warsaw University of Technology – Faculty of Chemistry** Department of Technology and Characterization of Solids Warsaw, Noakowskiego 3 Master's degree in chemistry, thesis title: „Research on methods for the determination of gallium" Thesis supervisor: Prof. K. Kasiura

3. History of employment:

from 2012 (IV)	**Institute of Sport (from November 2015 Institute of Sport – National Research Institute) – Department of Anti-Doping Research** - position (apart from head of Department of Anti-Doping Research): member of technical management of Department of Anti-Doping Research
from 2006 (I)	**Institute of Sport – Department of Anti-Doping Research** - assistant professor (adjunct)
from 2003 (VI)	**Institute of Sport – Department of Anti-Doping Research** - position: head of Department of Anti-Doping Research
from 2002 (XII)	**Institute of Sport – Department of Anti-Doping Research** - position: acting head of Department of Anti-Doping Research
1998 – 2002 (IX – XII)	**Institute of Sport – Department of Anti-Doping Research** - position: deputy of head of Department of Anti-Doping Research
1996 – 2003 (IV – X)	**Institute of Sport – Department of Anti-Doping Research** - position: quality manager of Department of Anti-Doping Research
1996 (IV)	**Institute of Sport – Department of Anti-Doping Research** - assistant
1995 (IX)	**Institute of Sport – Department of Anti-Doping Research** Warsaw, Trylogii 2/16 - engineering and technical specialist

4. List of publications comprising the scientific achievement referred to in Art. 16(2) of the Act of 14 March 2003 on scientific degrees and titles and the degrees and titles in arts (Journal of Laws no. 65, item 595, as amended) selected as the basis for the habilitation proceedings

4.1 Title of scientific achievement

Analytical methods in anti-doping testing, selection, interpretation of results, search for new solutions

4.2 Publications comprising the scientific achievement

No.	Author(s), title, date of issue, journal or publishing house, volume, pages.	IF	Number of citations (03.06.2016) Web of Science/Scopus / Google Scholar
H1	Gronowska A., **Kwiatkowska D.**, Pokrywka A., Koteras M., Turek-Lepa E., Szutowski M.: *The alteration of the urinary steroid profile under the stress*. Biology of Sport, 2010, 27: 3-9.	IF (2010) = 0.15	4/4/4

My contribution was to formulate the research problem associated with the topic, arrange testing of volunteers, perform GC-MS analyses, formulate conclusions, co-write the manuscript. I estimate my relative contribution at 60%.

H2	Thevis M., Sigmund G., Gougoulidis V., Beuck S., Schlörer N., Thomas A., **Kwiatkowska D.**, Pokrywka A., Schänzer W.: *Screening for benfluorex and its major urinary metabolites in routine doping controls.* Analytical & Bioanalytical Chemistry, 2011, 401: 543-551.	IF (2011) = 3.778	5/7/9

My contribution was to cooperate with the foreign research centre (Institute of Biochemistry - Center for Preventive Doping Research, German Sport University Cologne, Germany) and co-write the manuscript. I estimate my relative contribution at 10%.

The article was written as part of the Manfred Donike Institute (MDI) project titled:

Development of a method for the detection of Benfluorex and its major urinary metabolites for implementation into routine doping control procedures:

- Germany - Institute of Biochemistry, Laboratory for Doping Analysis, German Sports University Cologne,
- Poland – Department of Anti-Doping Research, Institute of Sport, Warsaw,

where I was the coordinator and executive investigator for Poland.

H3	Thomas A., Höppner S., Geyer H., Schänzer W., Petrou M., **Kwiatkowska D.**, Pokrywka A., Thevis M.: *Determination of growth hormone releasing peptides (GHRP) and their major metabolites in human urine for doping controls by means of liquid chromatography mass spectrometry.* Analytical & Bioanalytical Chemistry, 2011, 401: 507-516.	IF (2011) = 3.778	36/36/50

My contribution was to cooperate with the foreign research centre (Institute of Biochemistry - Center for Preventive Doping Research, German Sport University Cologne, Germany) and co-write the manuscript. I estimate my relative contribution at 10%.

The article was written as part of the project: Manfred Donike Institute (MDI): Determination of growth hormone releasing peptides (GHRP) and their major metabolites in human urine for doping controls by means of liquid chromatography mass spectrometry:

- Germany - Institute of Biochemistry, Laboratory for Doping Analysis, German Sports University Cologne,
- Poland – Department of Anti-Doping Research, Institute of Sport, Warsaw,

where I was the coordinator and executive investigator for Poland.

H4	Parr M.K., Opfermann G., Geyer H., Westphal F., Sönnichsen F.D., Zapp J., **Kwiatkowska D.**, Schänzer W.: *Seized designer supplement named "1-Androsterone": Identification as 3β-hydroxy-5α-androst-1-en-17-one and its urinary elimination.* Steroids, 2011 May, 76(6): 540-547.	IF (2011) = 2.829	17/17/21

My contribution was to obtain the approval of the Committee for Scientific Research Ethics, co-operate with the foreign research centre (Institute of Biochemistry -Center for Preventive Doping Research, German Sport University Cologne, Germany), interpret the results, co-write the manuscript. I estimate my relative contribution at 20%.

H5	Beuck S., Sigmund G., Koch A., Schänzer W., Pokrywka A., **Kwiatkowska D.**, Thevis M.: *Identification and characterization of urinary prenylamine metabolites by means of liquid chromatography-tandem mass spectrometry*. Drug Testing and Analysis, 2012, 4: 701-716.	IF (2012) = 3.167	2/2/2

My contribution was to cooperate with the foreign research centre (Institute of Biochemistry - Center for Preventive Doping Research, German Sport University Cologne, Germany) and co-write the manuscript. I estimate my relative contribution at 10%.

The article was written as part of the Manfred Donike Institute (MDI) project titled: Analysis of Prenylamine in Human Control Samples:

- Germany - Institute of Biochemistry, Laboratory for Doping Analysis, German Sports University Cologne,
- Poland – Department of Anti-Doping Research, Institute of Sport, Warsaw,

where I was the coordinator and executive investigator for Poland.

H6	Chołbiński P., Wicka M., Kowalczyk K., Jarek A. , Kaliszewski P., Pokrywka A., Bulska E., **Kwiatkowska D.**: *Detection of β-methylphenethylamine, a novel doping substance, by means of UPLC/MS/MS.* Analytical & Bioanalytical Chemistry, 2014, 406: 3681-3688.	IF (2014) = 3.436	6/6/7

My contribution involved formulation of the research problem associated with the topic with particular emphasis on legislation issues, joint identification of the substance, interpretation of initial results using LC-MS, drafting conclusions and co-writing the manuscript. I estimate my relative contribution at 30%.

H7	Thevis M., Sigmund G., Thomas A., Vogel M., Walpurgis K., **Kwiatkowska D.**, Geyer H., Schänzer W.: *Isotope-dilution mass spectrometric quantification of the prodrug lisdexamfetamine in human urine in doping control analysis.* Rapid Communications in Mass Spectrometry, 2014, 28: 781-786.	IF (2014) = 2.253	2/2/3

My contribution was to cooperate with the foreign research centre (Institute of Biochemistry - Center for Preventive Doping Research, German Sport University Cologne, Germany), synthesize the substance in cooperation with that institution, and co-write the manuscript.

I estimate my relative contribution at 15%.

H8	Bulska E., Gorczyca D., Zalewska I., Pokrywka A., **Kwiatkowska D.**: *Analytical approach for the determination of steroid profile of humans by gas chromatography isotope ratio mass spectrometry aimed at distinguishing between endogenous and exogenous steroids.* Journal of Pharmaceutical and Biomedical Analysis, 2015 Mar 15, 106: 159-166.	IF (2014/2015) = 2.979	3/1/3

My contribution involved leading the science project involving research described in this paper (No. N N404 061939), formulate the research problem associated with the topic, interpret the results using GC-C/IRMS, draft conclusions, co-write the manuscript. I estimate my relative contribution at 40%.

The paper was supported with the Ministry of Science and Higher Education grant N N404 061939: The effects of ethanol as a factor modifying the steroidal profile in humans; I acted as project leader. It was also the result of cooperation as part of the project of Manfred Donike Institute (MDI): Harmonization of steroid-profiling:

- Germany - Institute of Biochemistry, Laboratory for Doping Analysis, German Sports University Cologne,
- Poland – Department of Anti-Doping Research, Institute of Sport, Warsaw,

where I was the coordinator and executive investigator for Poland.

H9	Jarek A., Kowalczyk K., Chołbiński P., Chajewska K., Turek-Lepa E., Pokrywka A., Bulska E., **Kwiatkowska D.**: *Analytical procedure for steroid profiling valid for Athlete Biological Passport.* Chemical Papers, 2015, 69: 254-261.	IF (2014/2015) = 1.468	0/0/0

My contribution involved leading the science project involving research described in this paper (No. N N404 061939), formulate the research problem associated with the topic, interpret the results using GC-MS, draft conclusions, co-write the manuscript. I estimate my relative contribution at 40%.

The paper was supported with the Ministry of Science and Higher Education grant N N404 061939: The effects of ethanol as a factor modifying the steroidal profile in humans; I acted as project leader.

H10	**Kwiatkowska D.**, Wójtowicz M., Jarek A., Goebel C., Chajewska K., Turek-Lepa E., Pokrywka A., Kazlauskas R.: *N,N-dimethyl-2-phenylpropan-1-amine – new*	IF (2014/2015) = 2.506	4/4/7

	designer agent found in athlete urine and nutritional supplement. Drug Testing and Analysis, 2015 Apr, 7(4):331-335.		

My contribution involved formulating the research problem associated with the topic, work together with foreign research centres (National Measurement Institute, Australian Sports Drug Testing Laboratory, Australia; BDG Synthesis Ltd, New Zealand), jointly identify the substance, interpret research results using GC-MS, draft conclusions, co-write the manuscript. I estimate my relative contribution at 60%.

H1-H10	**Sumaryczny dla wybranych publikacji**	**IF = 26.344**	**79/79/106**

In the above papers I was the author of the concept or directed the research proper. I was leading the conduct of experiments from obtaining the approvals by the Scientific Research Ethics Committee, through selecting volunteers and supervising the entire experimental portion performed using the equipment available at the Department of Anti-Doping Research (performing some experiments myself), to interpreting the results and drafting conclusions. Copies of scientific manuscripts constituting a monographic series of publications are included in Appendix No. VI, and the co-author statements specifying individual contribution of each one into the creation of individual papers are included in Appendix No. VII.

General information on the publications [H1 - H10] is presented below:

Total impact factor (IF) by year of publication	26.344
Total 5-year impact factor (IF5)	25.62
Total Ministry of Science and Higher Education points	274
Number of citations (Web of Science/Scopus/Google Scholar)	79/79/106
Mean impact factor (IF)	2.63
Mean 5-year impact factor (IF5)	2.56
Mean number of Ministry of Science and Higher Education points	2.74
Mean number of citations (Web of Science/Scopus/Google Scholar)	7.9/7.9/10.6

4.3 Discussion of the scientific goal of publications submitted and the key results

Since the adoption of the *Physical Culture Act of 3 June 1984*, the use of pharmacological and other performance enhancing substances has been officially banned in Poland. This approach has been reasserted by further regulations: *The Physical Culture Act of 1996, the Qualified Sport Act of 2003,* and finally *the Sport Act of 25 June 2010, with later amendments*. In addition, Poland signed and ratified the UNESCO International Convention against Doping in Sport (Journal of Laws of 8 August 2007, no. 142, sec. 999) and the Anti-Doping Convention of the European Council (Journal of Laws 2001, no. 15, sec. 149). Both conventions require their signatories to implement and follow the *World Anti-Doping Code* published by the World Anti-Doping Organization (WADA) [1].
Testing laboratories are a key part of that system. Consequently, in September 1987 the Doping Control Laboratory was established at the Institute of Sport in Warsaw, renamed the Department of Anti-Doping Research in 1995.

Doping is defined as violation of one or more rules specified in articles 2.1 – 2.10 of the *World Anti-Doping Code* [2].
The *fair play* principle, which is one of the cornerstones of sport, means, among other things, a ban on the use of prohibited performance enhancing substances. The fight against this ill practice has been going on for centuries. The first historical accounts on the subject refer to the attempts to root out unfair conduct as early as 6th century BC in Thebes: participants in the games were not allowed to drink alcohol and had to undergo a breath test by a priest. Thus, we can say that the first doping detector was the human nose.

Among the pioneers of modern anti-doping research was a Pole, the Warsaw-based pharmacist Alfons Bukowski, who performed the first anti-doping tests in 1910. He tested the saliva of horses that took part in races and found evidence of alkaloid use. Unfortunately, the exact method he used is not known [3].

The beginning of the 20th century saw a rapid development of analytical methods that has continued until the present day. A number of researchers in that area have been awarded the Nobel Prize.
In 1903 Mikhail S. Tsvet invented chromatography, in 1911 Joseph J. Thompson constructed the first mass spectrometer, and in 1919 Francis W. Aston built the first spectrometer using speed-based focusing. In the 1950s, Anthony T. James and Archer J. P. Martin used gas and liquid chromatography for separation. In 1953 W. Paul and H. Steinwedel constructed a quadrupole analyser and ion trap. Five years later, in 1958, R. Gohlke and F. McLafferty combined a gas chromatograph with a mass spectrometer, and in the 1960s the first

combination of liquid chromatograph with mass spectrometer was built. In 1975 capillary columns were introduced to standard testing, and are still in use today.
Other analytic methods were advancing simultaneously. A. Tiselius developed electrophoresis, paving the way for the first protein separation in 1937. Other substance separation and identification techniques were also being developed. This progress has been and is still closely observed by the anti-doping community, which is not only using new solutions, but driving them through extensive research.

The development of anti-doping methods is closely associated with the demands posed for anti-doping laboratories. Only those accredited by the World Anti-Doping Agency (WADA) are authorised to test samples collected from athletes. Today, there are 34 such centres in the world, among them the laboratory in Warsaw, which I am heading [4]. Along with WADA accreditation, our laboratory is also accredited by the Association of Official Racing Chemists (AORC), making it qualified to test samples from competition animals. Both WADA and the International Federation for Equestrian Sports (Fédération Équestre Internationale –FEI) maintain lists of banned substances and methods which are updated on a yearly basis [5,6].

The need to detect constantly emerging new doping substances and prohibited methods and the increasingly strict requirements of WADA have become the driving force in the development of analytic methods introduced in anti-doping testing. Since 1972, when professor Manfred Donike for the first time used a gas chromatograph coupled with a mass spectrometer in anti-doping tests during the Olympic Games in Munich, the GC-MS system has been the basic analytical tool used in anti-doping laboratories. The next step was the tandem mass spectrometer (MS/MS) and high-resolution mass spectrometer (HRMS), followed by the introduction of liquid chromatographs coupled with spectrometers, initially with the tandem mass spectrometer (LC-MS/MS), and then an ultra-high performance mass spectrometer combined with a tandem mass spectrometer (UHPLC-MS/MS).

The anti-doping laboratories of today, however, use multiple device-based methods, such as gas chromatography combined with isotope-ratio mass spectrometry (GC-C/IRMS), liquid chromatography with a time-of-flight detector (LC-Q-TOF), electrophoresis, chemiluminometry, fluorometry, colorimetry, immunochemistry, luminometry, flow cytometry, impedance, cumulative electrical impulse counting. The use of such a wide array of methods is necessary due to the variety of prohibited substances and methods used by athletes. As mentioned before, the List of Prohibited Substances and Methods (Prohibited List) is updated annually by WADA and published along with WADA Technical Documents

and WADA Guidelines that contain information on recommended testing methods and minimum required performance levels (MRPL) [5,7-17].

As I have already mentioned, the introduction of new techniques at a testing laboratory is in strict connection with amendments to the *Prohibited List* and WADA requirements. In 1967 the International Olympic Committee (IOC) established the Medical Commission to organize and supervise the fight against doping. The Committee published the first list of substances prohibited by IOC, containing only stimulants and illicit drugs. Through the years, the list has been greatly expanded. At that time, analyses were performed predominantly using gas chromatographs, and, since 1972, gas chromatographs combined with mass spectrometers. Since 2004, when WADA took over from the IOC Medical Commission as the main coordinating body of the international anti-doping system, it has been responsible for the preparation and publication of the Prohibited List. By lowering the limits of detection [15], WADA forced the laboratories to bring in MS/MS and HRMS detectors coupled with gas chromatographs, and by including in the 2004 *List* glucocorticosteroids and two steroid substances, gestrinone and tetrahydrogestrinone (THG), induced them to perform testing using liquid chromatography combined with tandem mass spectrometry [18]. Subsequent additions of substance classes accelerated the introduction of the following methods:

- electrophoresis to test for the presence of erythropoietin,
- time-separation method of immunofluorometry and immunoluminometric method to assess the luteinizing hormone concentration,
- colorimetric method to test for the presence of hydroxyethyl starch,
- the immunoluminometric method and the time-separation method of immunofluorometric analysis to test for the presence of human chorionic gonadotropin,
- immunoluminometry and radioimmunology to test for the presence of somatotropin,
- flow cytometry to detect the presence of antigenically different red blood cells,
- fluorescence flow cytometry, impedance, cumulative electrical impulse counting to analyse hematologic parameters.

The Prohibited List is updated annually with groups of new substances and new methods. In addition, it is open-ended [19]. It contains, along with examples of specific substances in the anabolic agents class: anabolic androgenic steroids (S.1.1), peptide hormones, growth factors, related substances and mimetics (S.2), diuretics and masking agents (S.5); stimulants: specified stimulants (S.6.b), a note: "(...) and other substances with a similar chemical structure or similar biological effect(s)." The *Prohibited List* also includes non-approved substances (S.0): "Any pharmacological substance which is not addressed by

any of the subsequent sections of the List and with no current approval by any governmental regulatory health authority for human therapeutic use (e.g. drugs under pre-clinical or clinical development or discontinued, designer drugs, substances approved only for veterinary use)." In addition, some groups are appended with a note that reads "(...) including, but not limited to (...)." Such wording poses a huge challenge to laboratories, forcing them to look for new solutions.

Besides techniques and methods for detecting and testing for multiple compounds, there is a need to differentiate between endogenous and exogenous substances, such as steroids, e.g. testosterone - using the GC-C/IRMS method [9], erythropoietin using electrophoresis [12], growth hormone – immunoluminometry, radioimmunology [13,17]. It is also necessary to develop new models for interpreting results, find new metabolites of known substances, e.g. in order to expand detection windows (detecting xenobiotics for a longer time following ingestion) and to identify new compounds, some of which are developed on the basis of the already existing ones. This is why laboratories are working together as teams in order to constantly improve their methods. This is also the essence of my role as coordinator in Poland. On the other hand, WADA, in an attempt to establish best practices and impose uniform testing methods, forces laboratories to cooperate and exchange ideas. Hence the importance of direct interaction between the laboratories and of the role of the head of an anti-doping laboratory [20].

The duties described above are only part of my activities at the Warsaw laboratory aimed at combatting the pathology that is doping as effectively as possible. I use testing results both for standard work associated with anti-doping human and animal sample analysis, and in toxicological research commissioned by governmental agencies and courts.

As mentioned above, work at the laboratory, besides implementing novel testing methods and the latest technological solutions, involves modernisation of screening methods for detecting prohibited substances, developing new methods for detecting performance enhancing drugs, identifying new substances that have doping potential, prolonging detection windows for already banned substances by monitoring more of their metabolites, testing compound metabolism, looking for factors that can effect changes in the human body, and, consequently, searching for new and more reliable models for interpreting analytical findings. All projects of which I have been part had those goals in mind, and I have presented their results at multiple conferences and personally notified other laboratories of emerging risks, enabling them to modify their routine procedures.

The World Anti-Doping Agency, and, by extension, anti-doping laboratories are always on a lookout for indirect evidence for the use of prohibited substances and methods. One example is the introduction by WADA of the *Athlete Biological Passport* [21]. ABP is a system for long-term assessment of biological markers in athletes. It is based on a statistical model, the so-called adaptive model, which makes it possible to calculate quantitative ranges of analysed markers and exceeded limits that suggest the use of doping substances or methods. Currently there are two biological passport modules in place – the haematological and the steroidal module. A third, peptide module, is also considered.

Unfortunately, evaluation of ABP is a complex matter. Many different factors that could affect each marker must be taken into account. One such factor is stress, as demonstrated by my team in the article [H1]. Urine samples were collected from a group of healthy volunteers experiencing intense stress 30 minutes before an important exam and one month after the event, when they were relaxed. We measured concentrations of the following endogenous hormones: 11beta-hydroxy-androsterone, 11ß-hydroxy-etiocholanolone, androsterone, etiocholanolone, 5ß-androstan-3ß,17ß-diol, 5α-androstan-3alfa,17ß-diol, 5ß-androstan-3alfa,17ß-diol, 5α-androstan-3ß, 17ß-diol, testosterone, epitestosterone, dihydrotestosterone, dehydroepiandrosterone, and on that basis calculated testosterone to epitestosterone ratios and androsterone to etiocholanolone ratios. The level of stress was determined by a questionnaire – a self-reported subjective feeling. The study showed that in females the concentration of testosterone and epitestosterone changed. These changes had no effect on the testosterone to epitestosterone ratio. In the case of males, the ratio decreased. Our findings attracted the interest of WADA and other laboratories to the extent that the influence of competition stress on parameter variation is now considered in the assessment of profile changes in the steroidal module. The article was recently cited at the meeting of WADA experts that assessed the steroidal module, the *Steroid Passport Management Meeting* (Lausanne 2016), which I attended as well.

Creation of an athlete's passport requires reliable, reproducible and validated methods to measure all parameters taken into account in the abovementioned adaptive model. This means that the method used must ensure the consistency of results obtained in various laboratories. One example of such procedure was demonstrated by my team in [H9]. We showed the importance of reference material selection for quantitative measurements in this type of procedure. We also indicated the critical importance of inter-laboratory testing when the results from one laboratory are entered in the biological passport, followed by the findings from other laboratories.

Each individual result is evaluated on an individual basis, as well as together with all parameters of interest. Any apparent changes warrant further investigative action [9,10]. One possible step is the so-called IRMS. The test uses a gas chromatograph coupled with a combustion chamber and isotope mass spectrometer to observe the values of the 13C/12C isotope ratio. However, in order to perform the test, the sample needs to be properly prepared. In the paper [H8] I analysed the utility of various preparatory procedures by using different fillings for liquid-solid extraction, enzymes and a variety of solvents for the liquid-liquid extraction. With the new protocol it was possible to achieve reproducible analytical results for anabolic-androgenic steroids, in the case of which it is vital to determine their endogenous or exogenous origin (Fig. 1).

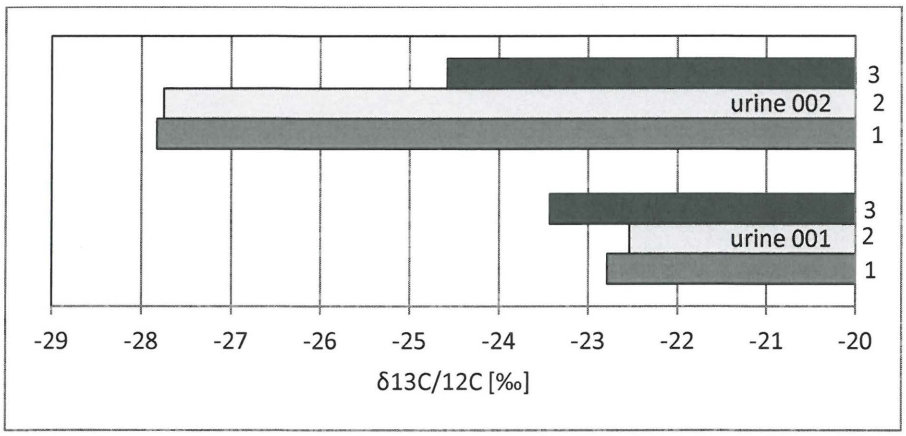

Fig. 1. The values of δ13C/12C [‰] for selected compounds (steroids) in 2 example urine samples; Urine 001 (negative finding) Urine 002 (positive finding). 1—androsterone, 2—etiocholanolone, 3—11-OHAndrosterone -ERC.

Another considerable analytical challenge in anti-doping research is testing the effects of unknown substances and interpreting the results. I have discussed the examples of such compounds in papers [H6] and [H10]. Both articles discuss modifications of phenethylamine, which is a substance so amenable to alteration that it has attracted the attention of the black market. In the first article I analysed a substance which is an isomer of amphetamine, namely β-methylphenethylamine (BMPEA). Our experience lead us to the conclusion that many toxicological laboratories do not differentiate between these two isomers even though it is vital for interpreting the results and determining whether the detected substance is a narcotic, a psychotropic drug or precursor for the manufacture of such substances in the light of existing regulations laid down in the *Act on counteracting drug addiction of 29 July 2005*. I the aforementioned paper, my team presented various procedures for preparing a sample: direct injection method, extraction method, and a method using hydrolysis. Our research was conducted using LC-TOF (Fig. 2) and LC-MS/MS (Fig. 3).

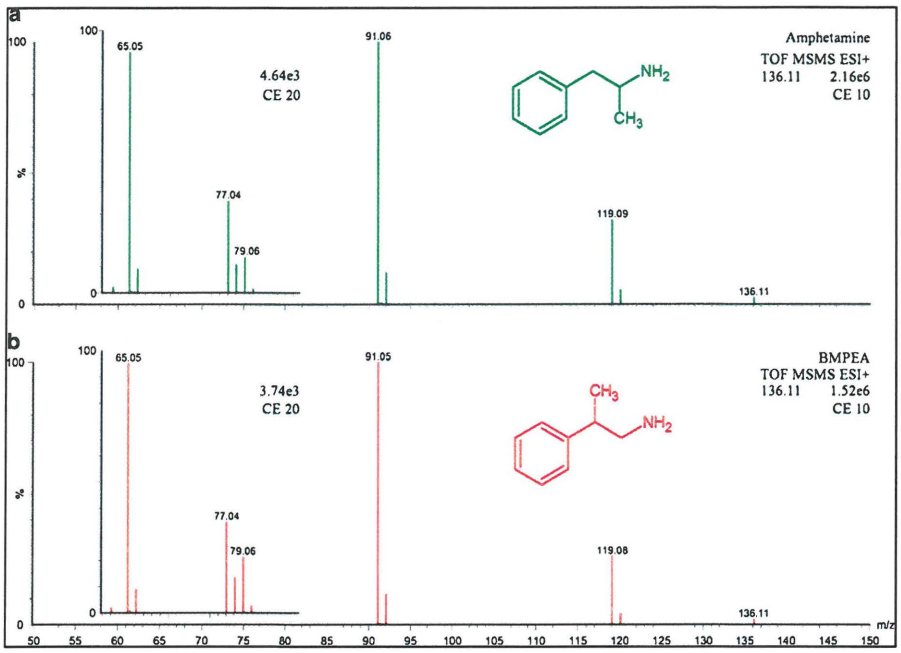

Fig. 2. ToF MS/MS spectra for the m/z 136.11 precursor ions of amphetamine (a) and BMPEA (b)

We showed that there is a simple and quick way to differentiate between these two compounds (Fig. 3).

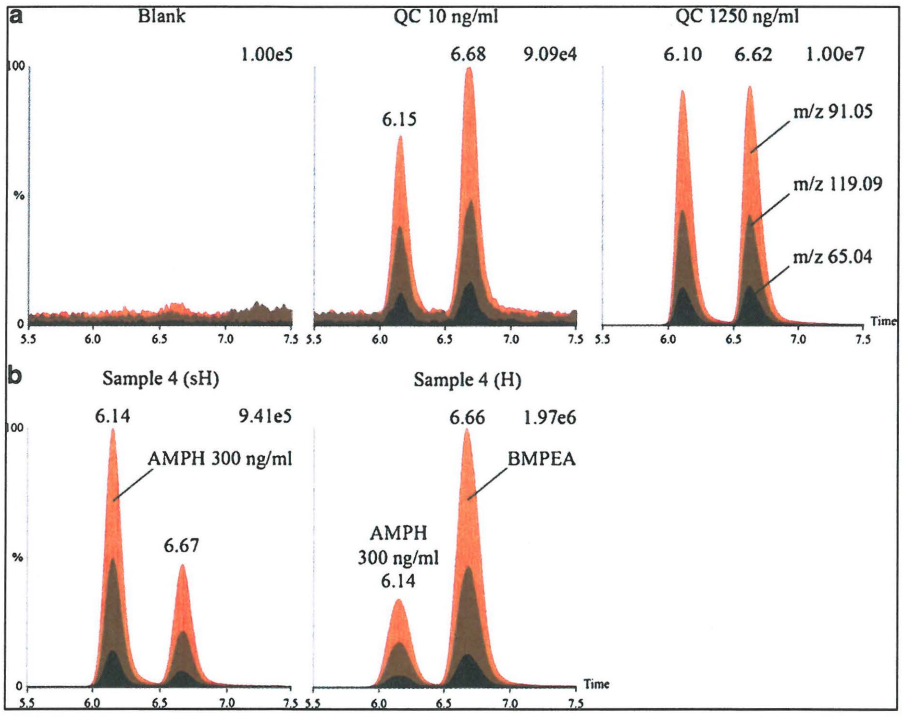

Fig. 3. Discrimination capabilities of the method at different concentration levels (a) and chromatograms of a real case sample prepared with hydrolysis (H) or shortened hydrolysis (sH) of metabolic conjugates (b).

The first step was vitally important for the whole process of data interpretation. As I have already mentioned, many laboratories fail to differentiate between these two isomers, a fact that is significant for legal as well as health-related reasons. That step was done under my supervision and in constant consultation with WADA and the Polish agency qualified to collect samples, i.e. the Polish Commission against Doping in Sport. During preliminary investigation we tested the supplements used by the athletes, which gave a focus to our further research. Today many countries are introducing legislation to remove supplements containing that substance from the market because many toxicologists consider it to be more harmful than amphetamine [22-24].

In paper [H10] I analyse a new compound, structurally similar to phenpromethamine (Fig. 4).

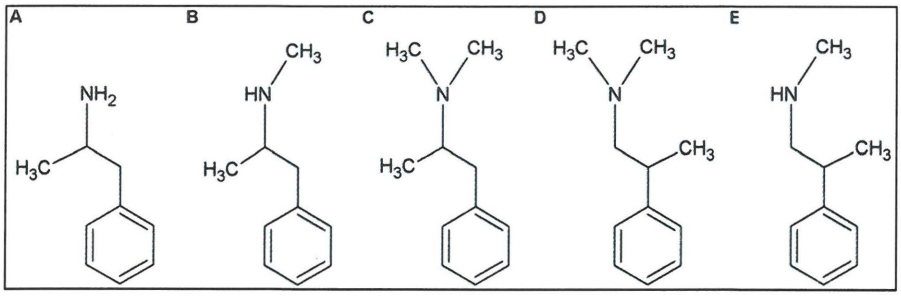

Fig. 4. Chemical structures of amphetamine (A) and its analogs: methamphetamine (B), dimethylamphetamine (C), N,N-dimethyl-2-phenylpropan-1-amine (D) and phenpromethamine (E).

Analyses were done by GC-MS, which discovered for the first time a previously unknown peak, later identified as N,N-dimethyl-2-phenylpropan-1-amine (NN-DMPPA). The findings from that study are shown in Fig. 5.

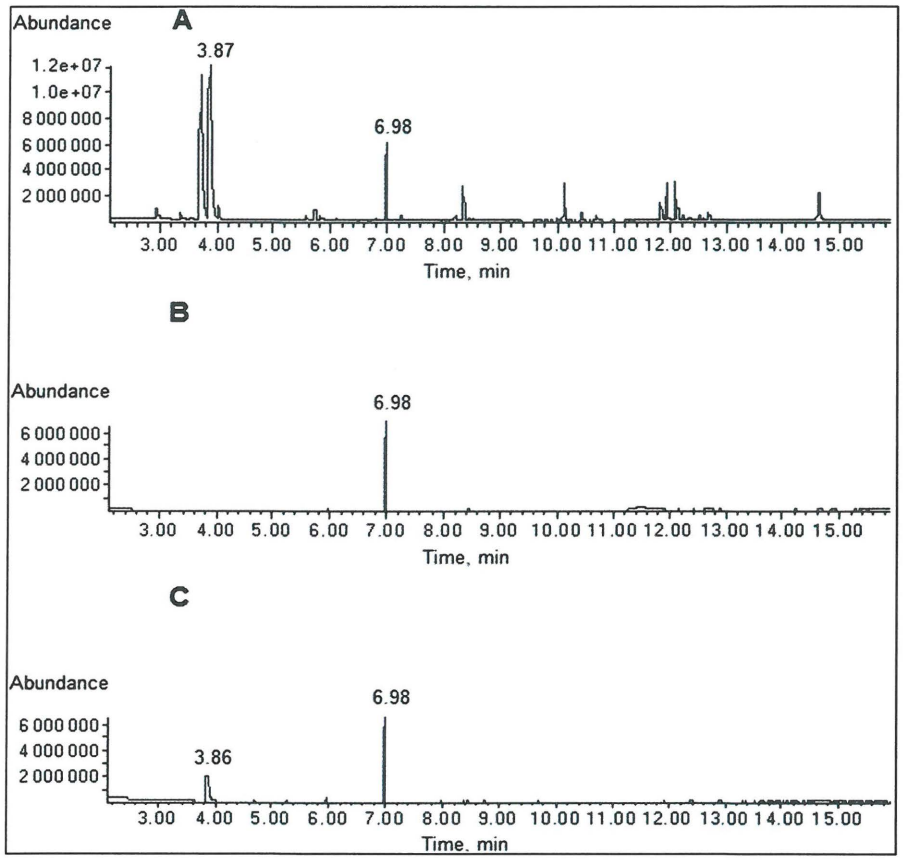

Fig. 5. Total ion chromatograms for urine sample (A), urine blank (B) and blank urine sample spiked with NN-DMPPA standard solution (C). Substances and their retention times: NN-DMPPA at 3.87 and 3.86 min, diphenylamine (ISTD) at 6.98.

To identify the substance we began by predicting its structure and then synthesising the compound. The whole process was conducted by my team. The results for an unknown peak in the analysed sample and for the synthesized reference substance are shown in Fig. 6.

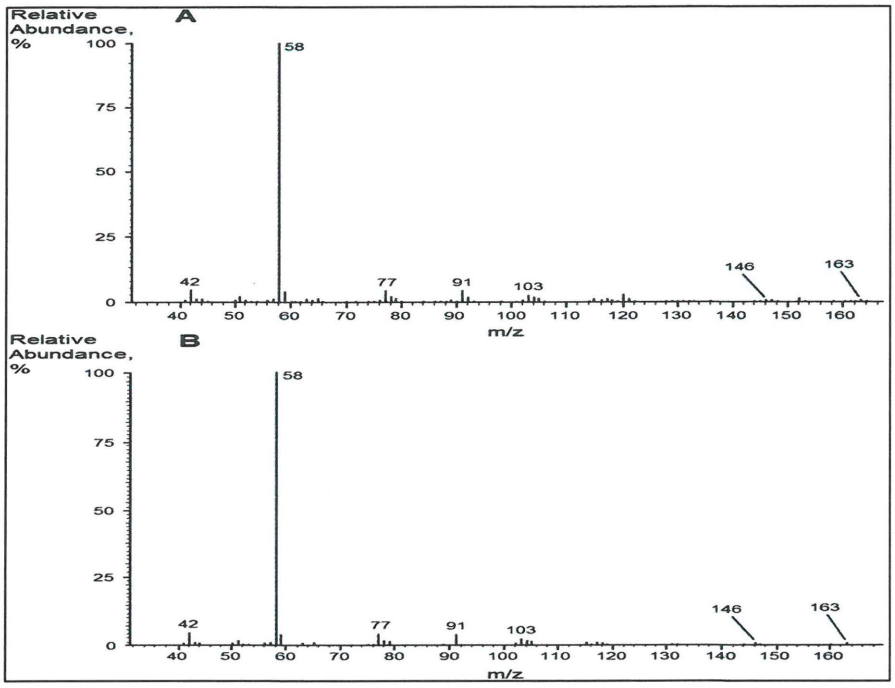

Fig. 6. Mass spectra of compound identified as NN-DMPPA in urine sample (A) and NN-DMPPA in quality control sample (B).

The process of identification involved me working together with the anti-doping laboratory in Australia (National Measurement Institute, Australian Sports Drug Testing Laboratory), which in turn kept in touch with a laboratory in New Zealand (BDG Synthesis Ltd). I have also determined the exact supplements used by a given athlete and, in cooperation with my Australian colleagues, pinpointed the suspect product.

The procedure for analysing an unknown substance presented in papers [H6] and [H10] ensures easy and reproducible identification compounds. It would not have been possible, however, without close cooperation between multiple research centres worldwide. Thanks to our research both substances have been included in routine anti-doping testing. This is particularly important considering easy access to those compounds, found, among other things, in food supplements [25].

Besides substances classified by WADA as stimulants, such as amphetamine, food supplements sometimes contain steroidal compounds that can affect the steroidal profile [25]. One such substance is discussed in paper [H4]. We identified it as 3ß-hydroxy-5α-androst-1-en-17-one (1-Androsterone). During research, approved by the Institute of Sport Committee for Scientific Research Ethics, we tested changes in the main metabolites of testosterone. Identification was done both by NMR and GC-MS. This was of particular importance due to very limited information on how this substance was metabolised. Urine

samples from a volunteer were prepared in accordance with a standard procedure for anabolic substances for GC-MS.

The identified substance present in food supplements can be referred to as a prohormone because it triggers changes in the steroid profile at the initial phase of testosterone's metabolic cycle. The most profound changes were seen in the value of the androsterone to etiocholanolone ratio (A/Etio), 5α/5ß-androstan-3α,17ß-diol (5aA-diol/5bA-diol) and dihydrotestosterone (DHT) concentration. In addition, the urine samples showed peaks identified as metabolites of 3ß-hydroxy-5α-androst-1-en-17-one (Fig. 7).

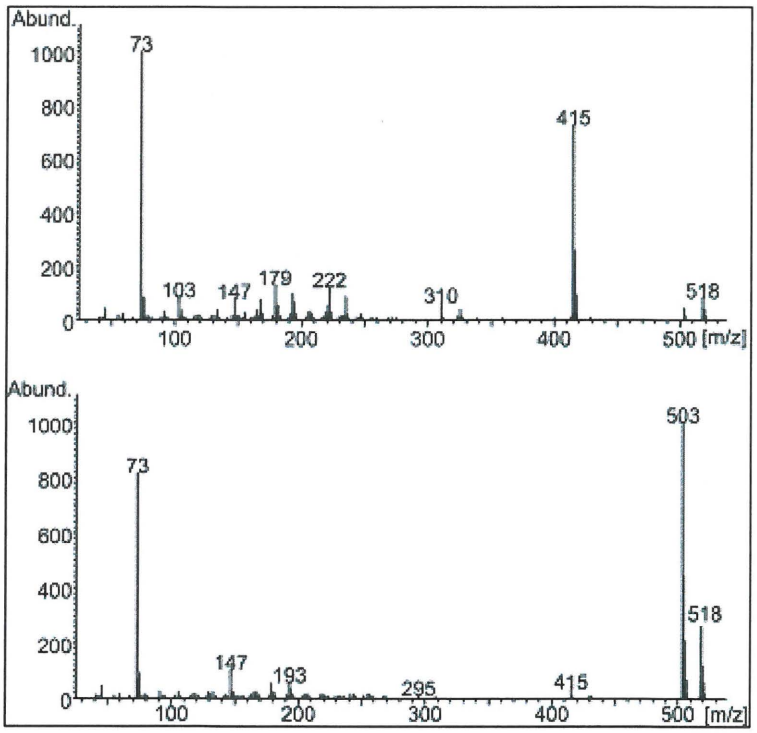

Fig. 7. Mass spectra of hydroxylated metabolites as tris-TMS derivatives upper: HO-Dione1 (M^+ = 518, RRT = 1.14), lower HO-Dione2 (M^+ = 518, RRT = 1.26).

Based on our findings a metabolism route for 3ß-hydroxy-5α-androst-1-en-17-one was proposed (Fig. 8).

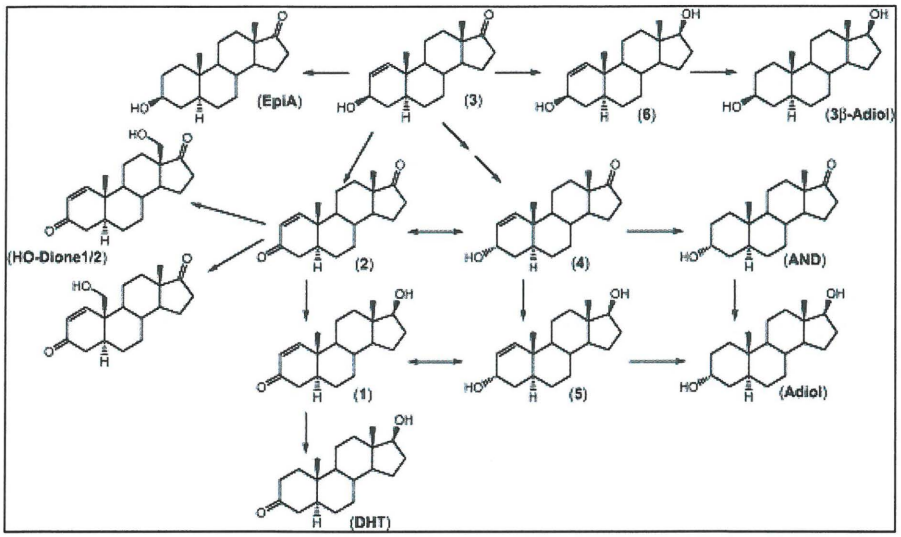

Fig. 8. Proposed metabolism of 3-beta-hydroxy-5-alfa-androst-1-en-17-one (3).

Another analytical challenge is posed by substances used in the treatment of various diseases using medicinal products included on the *Prohibited List* [27-29]. An example of such disease is the Attention-Deficit Hyperactivity Disorder (ADHD), for which some treatment protocols include amphetamine and methylphenidate, and recently also lisdexamfetamine, a novel psychoactive substance, so-called prodrug, i.e. has extended activity. Our research presented in [H7] was undertaken to find a way to differentiate between lisdexamfetamine and amphetamine use. We assumed that the metabolism of the substance should be similar to that of amphetamine, meaning that besides amphetamine, hydroxyamphetamine sulphate would be produced. The analyses were conducted using GC-MS and LC-MS/MS. For better identification, we used commercial reference materials and reference material synthesized especially for that study, namely 2H4-lisdexamfetamine. Cooperation with other laboratories was needed, among other things due to legal authorizations and permissions granted to the German laboratory (Institute of Biochemistry – Center for Preventive Doping Research, German Sport University Cologne) and the Warsaw laboratory, related to legal regulations concerning narcotic drugs and psychotropic substances or precursors for their manufacture. Identifying data is shown in Fig. 9.

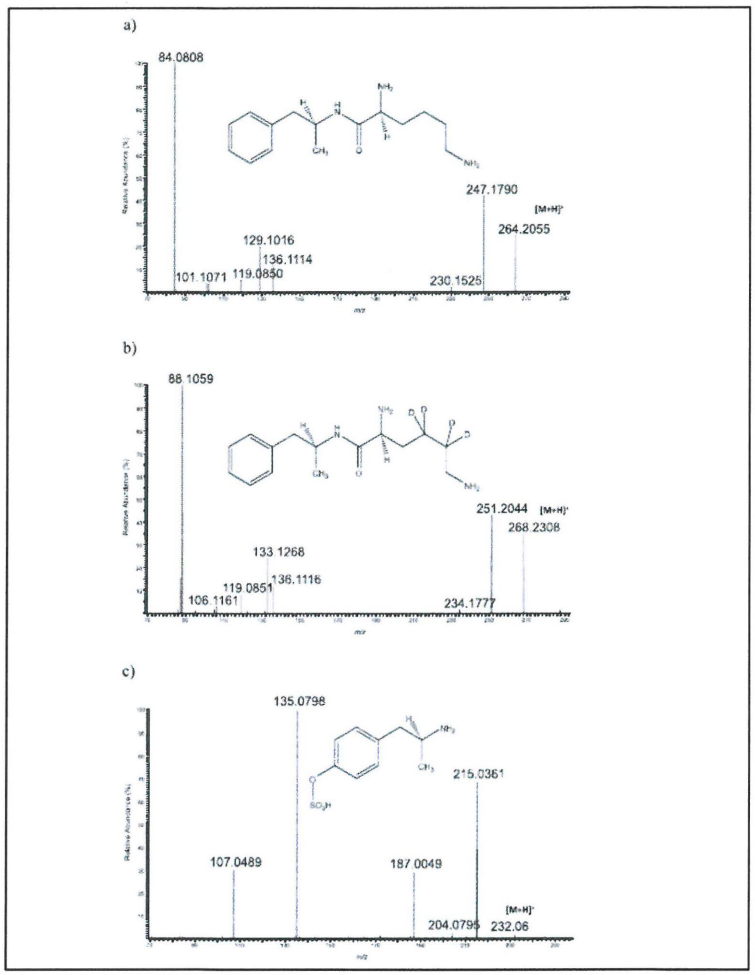

Fig. 9. Product ion mass spectra of protonated molecules $[M+H]^+$ of (a) m/z 264 of lisdexamfetamine, (b) m/z 268 of 2H4-lisdexamfetamine, and (c) m/z 232 of 4-hydroxyamfetamine sulfate, recorded at collision energies of 30 eV.

It was clear from our findings that it is difficult to determine which substance was taken by a given individual because the original compound is excreted rapidly and at low concentrations. Thus, further research, and possible new technological solutions, are necessary.

As I have argued above, information on substance metabolism are very important for selecting the correct procedure for sample preparation, choosing the right analyser and for interpreting the results. [H2] describes the pursuit of the optimum method for detecting the use of benfluorex, a stimulant according to WADA, used in medicine as an anorectic agent. The study compared the possibilities afforded by GC-MS and LC-MS/MS in screening and confirmatory tests for that compound. We have demonstrated that GC-MS is sufficient for screening purposes, as it makes it possible to detect two benfluorex metabolites: hydroxyethyl norfenfluramine (M1) and norfenfluramine (M3). We demonstrated that

stronger evidence of benfluorex use was afforded by LC-MS/MS, which detects four metabolites:

- hydroxyethyl norflenfuramine (M1),
- carboxymethol norflenfuramine (M2),
- norflenfuramine (M3), and
- the M1 metabolite combined with glucuronic acid (M4).

Moreover, with LC-MS/MS we can identify benfluorex, as the M1, M2 and M4 metabolites are specific for this compound, while the M3 metabolite may arise from other substances, e.g. fenfluramine.

In paper [H5] I presented the metabolism of prenylamine, a substance used in coronary artery disease, classified as stimulant by WADA. Prenylamine metabolism has been studies for many years [30-32], but only with the latest technical advances currently at the disposal of anti-doping laboratories can we truly analyse it in depth. Accurate interpretation of results must take into account potentially significant variations in metabolite elimination due to individual differences. This is why the search for new metabolites is so important. Research presented by our teams in [H5] was done using LC-MS/MS. Both metabolic phases were analysed. A protocol was developed that can be used equally well in anti-doping and toxicological research. The most specific metabolites were selected, namely: p-hydroxy-prenylamine (M1) and p-hydroxy-prenylamine-glucuronide (M1-gluc.); they are shown in Fig. 10.

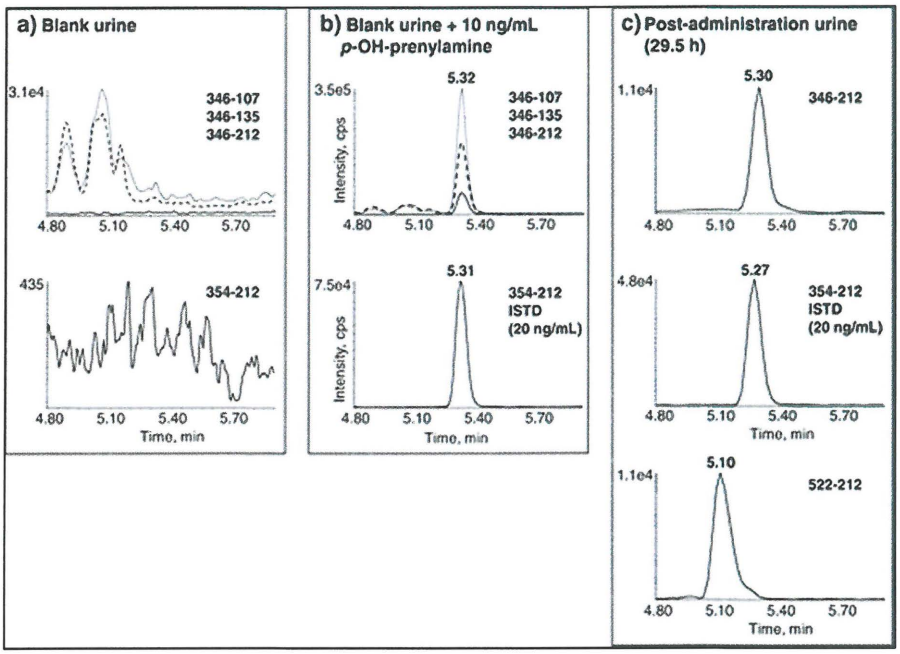

Fig. 10. Illustration of the specificity of the adapted routine screening method showing: (a) the extracted MRM ion chromatograms of phydroxyprenylamine (M1) and the internal standard (ISTD) d8-M1 in a blank urine sample. No influence of the ISTD on the signal of M1 was observed (data not shown). (b) A urine specimen fortified with 10 ng/mL of M1 and 20 ng/mL of the ISTD and (c) the selected screening ion transitions of a post-administration urine sample (29.5 h after drug administration) including the transition 522-212 for the M1-glucuronide.

In [H3] were present a testing method for the so-called small peptides, growth hormone releasing peptides (GHRP). These are substances that stimulate the production of endogenous growth hormone (GH) following ingestion. We studies such substances as: GHRP-1, GHRP-2, GHRP-4, GHRP-5, GHRP-6, alexamorelin, ipamorelin, and hexarelin.

Table 1. Characteristics of growth hormone releasing peptides: amino acid sequence, monoisotopic masses, elemental composition and dominant charge state

Name	Amino acid sequence	Monoisotopic mass [Da]	Elemental composition	Dominant charge state ESI
1	2	3	4	5
GHRP-2	(d-Ala)-(*d*-β-Nal)-Ala-Trp-(*d*-Phe)- Lys-NH_2	817.427	$C_{45}H_{55}N_9O_6$	2+

1	2	3	4	5
GHRP-1	Ala-His-(*d*-β-Nal)-Ala-Trp-(*d*-Phe)- Lys-NH_2	954.486	$C_{51}H_{62}N_{12}O_7$	2+
GHRP-6	His-(*d*-Trp)-Ala-Trp-(*d*-Phe)- Lys-NH_2	872.444	$C_{46}H_{56}N_{12}O_6$	2+
GHRP-5	Tyr-(d-Trp)-Ala-Trp-(d-Phe)-NH_2	770.354	$C_{43}H_{46}N_8O_6$	1+
GHRP-4	(d-Trp)-Ala-Trp-(d-Phe)-NH_2	607.29.2	$C_{34}H_{37}N_7O_4$	1+
Alexamorelin	Ala-His-(*d*-Mrp)-Ala-Trp-(*d*-Phe)- Lys-NH_2	957.497	$C_{50}H_{63}O_7N_{13}$	2+
Hexarelin	His-(*d*-Mrp)-Ala-Trp-(*d*-Phe)-Lys-NH_2	886.460	$C_{47}H_{58}N_{12}O_6$	2+
Ipamorelin	Aib-His-(*d*-2-Nal)-(*d*-Phe)- Lys-NH_2	711.385	$C_{38}H_{49}N_9O_5$	1+/2+
GHRP-2 metabolite	(d-Ala)-(*d*-β-Nal)-Ala-NH_2	357.168	$C_{19}H_{23}N_3O_4$	1+
ISTD1	(d-d3Ala)-(*d*-β-Nal)-Ala-NH_2	360.187	$C_{19}H_{20}{}^{[2]}H_3N_3O_4$	1+
ISTD2	(d-Trp)-d4Ala-Trp-(d-Phe)-NH_2	611.315	$C_{34}H_{33}{}^{[2]}H_4N_7O_4$	1+

Nal - naphthylalanine, Mrp - 2-methyltryptophane, Aib - aminoisobutyric acid

Apart from developing an identification method, the goal was to investigate these peptides' metabolism.

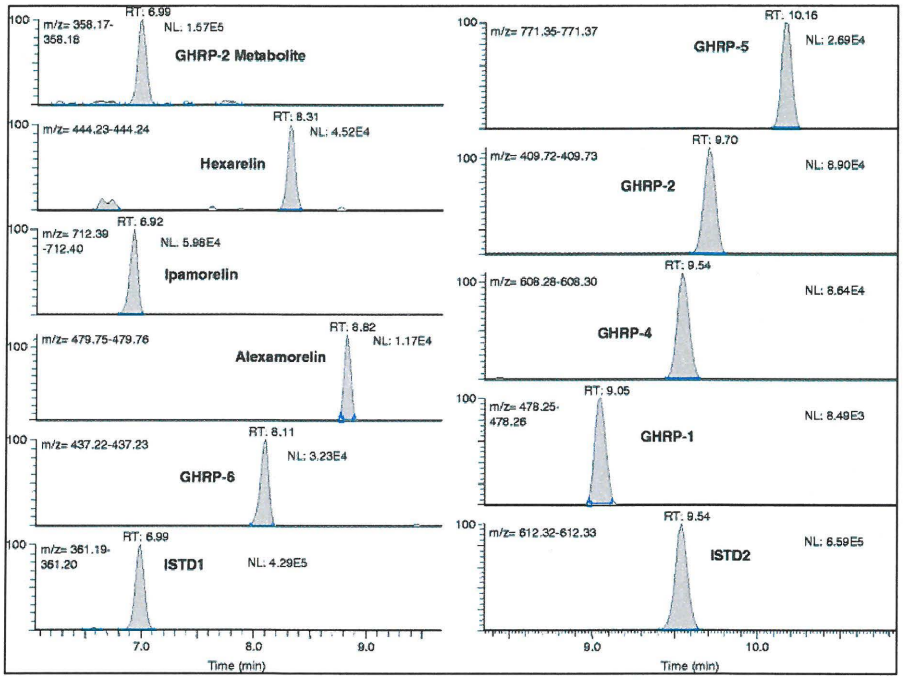

Fig. 11. Extracted ion chromatograms (width 0.01 Da) of a fortified urine sample (0.5 ng/mL) with abundant signals for GHRP-1, GHRP-2, GHRP-2 metabolite, GHRP-4, GHRP-5, GHRP-6, alexamorelin, ipamorelin, hexarelin and the ISTDs 1 and 2.

As a result, we developed a method to detect 8 peptides, including a GHRP-2 metabolite (Fig. 11). Our method paved the way for the introduction of GHRP detection protocol for standard urine sample testing. It is currently under development by anti-doping laboratories worldwide [33, 34].

4.4 Conclusion

As I have already mentioned, my work at an anti-doping laboratory began directly after I graduated the Warsaw University of Technology. In my initial projects I used GC-MS, GC-NPD, and LC-DAD systems. My further research was closely related with the development of analytical techniques and methods introduced to our laboratory, and the interpretation of test results, whether it was identifying substances, finding new metabolites or analysing factors potentially affecting biological markers.

Currently the laboratory I am heading is conducting scientific research to help combat doping more effectively and contribute to toxicological studies. The development of methods and the scope of testing is part of my ongoing duties at the laboratory.

Since 2014 I have cooperated with research centres in the Netherlands (RIKILT, Wageningen) and Germany (Freie Universität, Berlin) on the project aimed at finding a way to determine the particular route of administration of clenbuterol in humans. This is particularly important due to problems associated with meat contamination with this substance and the consequences for athletes who eat such meat.

The research was supported with a WADA grant. It was conducted to find a way to determine whether clenbuterol was administered as a pharmaceutical product or ingested with contaminated meat. In addition to looking at the clenbuterol molecule itself (its isomers), attempts were made to identify potential markers in urine that could conclusively prove whether clenbuterol was taken as a product or with meat. The second part of the project was to try and develop a decision model that could be applied to determine the source of clenbuterol if detected in an anti-doping test.

On the basis of the results obtained by our laboratories we can conclude that it is possible to determine the route of administration for a substance such as clenbuterol. We proposed models for finding interpretation that will be presented to WADA. If approved, they will be implemented across all anti-doping laboratories. The proposed model will become part of standard procedures at the laboratory I am heading.

The next step in research on contaminated meat will be to look at other animal products. In cooperation with partners from Berlin and the centre in Dresden (Dresden University) I have submitted a grant application for that research with WADA. It is currently undergoing review.

Other ongoing research projects at the laboratory concern contamination of nutrients and food supplements. As I have demonstrated above and in papers listed in Exhibit III, the issue is present and must be closely monitored. This is the motivation for the research project conducted together with the centre in France (Groupe de RMN Biomédicale Laboratoire SPCMIB (UMR CNRS 5068) Université Paul Sabatier, Toulouse).

An interesting subject pursued at our centre is the research related to clomifene. To our surprise we are dealing with this substance here in Poland very frequently compared to the rest of the world. This is why, in order to obtain reference materials and urine samples following secretion studies, we initiated cooperation with a centre in Germany (Margarete Fischer-Bosch Institute of Clinical Pharmacology and University Tuebingen). The project includes research on clomifene metabolism and development of a testing procedure to detect its use for a much longer time than the methods currently in use.

Another issue attracting our attention is research on the effects of oral administration of a growth hormone releasing peptide Hexarelin on the levels of hGH tropic hormones - IGF1 and PIIINP in serum and the GH-2000 score and to describe the elimination profile for Hexarelin in urine following oral administration. This is a continuation of research

presented, among others, in papar [H3], aimed at evaluating the efficacy of Hexarelin in stimulating GH secretion and determining how effective the anti-doping test used to detect somatotropin use based on the measurement of IGF-1 and PIIINP biomarkers can be in detecting Hexarelin use. Furthermore, we are planning to define the urine elimination profile for orally administered Hexarelin.

Other research currently conducted at the laboratory focuses on identifying cathinones and synthetic cannabinoids. Both classes of substances are closely monitored both by the anti-doping community and governments combatting drug analogues, the so-called "legal highs." This research has been going on for several years, and our results are becoming increasingly used by, for example, the prosecutor's office in order to identify specific substances.

My scientific accomplishments include:

- 25 articles published in journals listed in the Journal Citation Reports (JRC) database;
- 28 articles published in other peer-reviewed foreign journals and journals of countrywide distribution;
- 37 chapters in monographs and academic reference manuals;
- 50 abstracts and scientific meeting communiques
- 1 set of information for a website and educational materials as part of an EU project;
- participation in 12 research projects;
- participation in 7 research topics pursued at the Institute of Sport;
- 97 presentations given at international and national scientific meetings.

Information about my current scientific accomplishments was presented in the following list (prepared on the basis of databases: Web of Science Core Collection, Journal Citation Reports and the list of journals of the Ministry of Science and Higher Education), updated 3 June 2016.

Total number of publications	53
Publications in journals from JCR list	25
Other publications	28
Chapters in books and monographs	37
Total Impact Factor (IF)	44.732
Total 5-year impact factor (IF5)	49.99
Mean number of Ministry of Science and Higher Education points	600
Mean impact factor (IF) (publications in JCR)	1.789
Mean 5-year impact factor (IF5) (publications in JCR)	2.00
Mean number of points of the Ministry of Science and Higher Education (all publications)	11.11

List of bibliometric data (number of publications, citations, *h*-index) in various science databases, i.e. Web of Science Core Collection, Scopus, Google Scholar. (updated 3 June 2016).

Detailed list	Web of Science	Scopus	Google Scholar
Indeks Hirscha (h-index)	5	6	7
Number of publications	23	24	52
Number of citations	109	116	163
Mean number of citations	4.74	4.83	3.13

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