Department of Environmental Analysis

Instruction for laboratory course

Practical chromatography in criminology and food safety

Exercise 1

Determination of doping substances in body fluids using
SPE-HPLC-UV method

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Determination of doping substances in body fluids using SPE-HPLC-UV methods

1. Introduction

The use of performance-enhancing substances dates back to ancient times, when herbal extracts were popular, especially among athletes. One of the most important ones was an extract from the *Ephedra sinica* plant. It took until 1924 for the stimulant found in this plant to be identified as ephedrine. Another known method to improve the performance of endurance and to combat fatigue was the use of amphetamines and the practice of blood transfusions (1). Currently, the consumption of performance-enhancing agents in humans and animals, as well as evasion of blood/urine sampling, substance trafficking, manipulation or any type of participation in anti-doping violations are prohibited and defined as doping (2). Recently, so-called gene doping, defined as the non-therapeutic use of cells, genes, genetic elements or modulation of gene expression with the ability to improve sports performance, has also become a new threat (2).

The first doping scandal to see the light of day in front of an international audience occurred during the 1960 Rome Olympics. During a summer competition, due to the consumption of vasodilators (Ronicol) or stimulants (amphetamines), a Danish cyclist collapsed and died (1). The above-mentioned event led to the creation of the International Olympic Committee's (IOC) Medical Commission in 1961, whose overarching goal was to combat doping in sports. A number of subsequent doping incidents in international arenas, including during the cycling Tour de France, contributed to the establishment of the World Anti-Doping Agency in 1999. This organization to this day promotes and coordinates the World Anti-Doping Program. As part of its activities, a list of prohibited substances and methods is updated annually (3). For example, among the substances banned in the doping sector are anabolic agents, peptide hormones, growth factors and mimetics, beta-2-agonists, hormone and metabolic modulators, diuretics, and for some substances, such as ephedrine and methylephedrine, threshold values have been established. These compounds are categorized as stimulants only when their concentration in urine exceeds 10 µg/ml (2,3).

Various body fluids are subjected to anti-doping tests. The material routinely used for anti-doping tests is urine. This is primarily due to the use of a non-invasive sampling method, the sufficiency of the material, and the fact that most metabolic products are excreted by the kidneys. Nevertheless, there are reports indicating that the excretion of some substances (including ephedrine and methylphedrine) can be clearly influenced by urine pH and urine volume (4). Blood was included in the study only in 1992. For many years the subject of blood sampling was very

controversial for religious and cultural reasons (1). It is also worth noting that other samples of a biological nature, such as hair, are also tested for prohibited substances. (5),

Sample analysis in anti-doping control is a two-step process. The first step is to perform screening tests, using rapid chromatographic methods: e.g., HPLC-UV, GC-NPD or immunoenzymatic assays, to indicate the presence or absence of a doping agent (6). Some substances such as salbutamol, morphine, cathine, ephedrine, methylephedrine, pseudoephedrine and carboxy-tetrahydrocannabinol require quantitative measurement, as they are considered prohibited substances only above a threshold concentration. After the detection of a doping agent, a confirmation step shall be carried out using methods that ensure sufficient precision, accuracy and number of identification criteria for reliable identification and determination. A commonly used technique is mass spectrometry combined with high-performance liquid chromatography or gas chromatography (7). Biological samples must undergo a special preparation procedure prior to analysis based on the physical and chemical properties of the analyte and the analytical method used. The most important step is to use a suitable extraction method to get rid of excess impurities and separate the analyte in its maximum pure and concentrated form. Among many extraction methods available, the technique of choice is usually the Solid Phase Extraction (SPE). Besides a wide range of SPE stationary phases (i.e., normal phases, reversed phases, ion exchangers, mixed mode phases) dedicated to compounds with different physicochemical properties, SPE can be easily automated, allowing simultaneous preparation of multiple samples.

Taking doping agents has tragic consequences. At the same time, it is worth noting that some of them, especially those from unknown sources, are sometimes contaminated with other harmful substances as well. This can result not only in permanent damage to health, but even death. Commonly observed side effects associated with the consumption of doping substances include (8):

- heart attack and stroke;
- kidney failure; respiratory disorders;
- aggression attacks;
- acne;
- depression, insomnia, suicidal thoughts, seizures and hallucinations;

- infertility and mammary gland hypertrophy;
- menstrual cycle disorders, lowered tone of voice and excessive body hair.

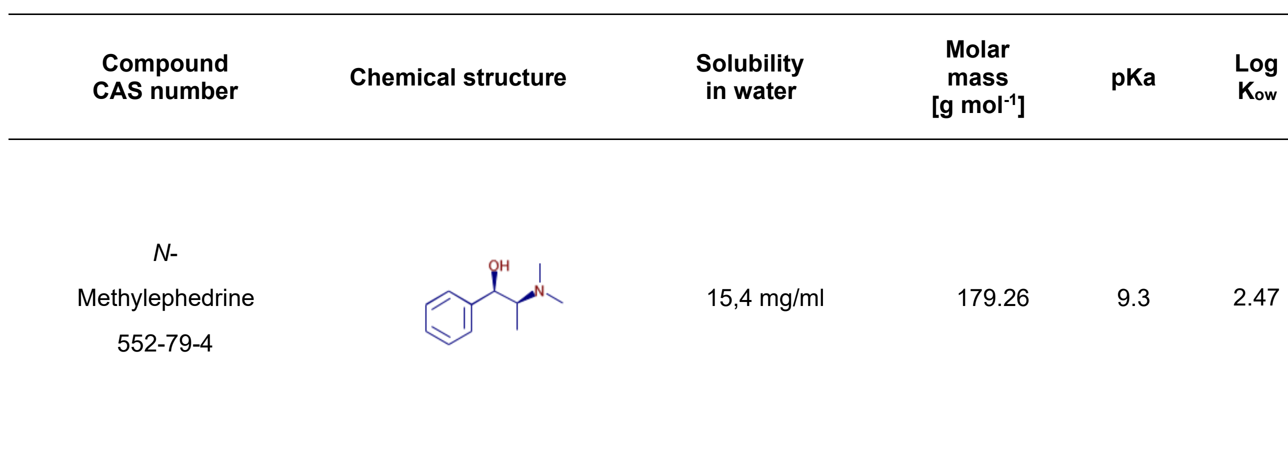
Ephedrine alkaloids, which are typical stimulants found in several plant species, are on the World Anti-Doping Agency's list of banned substances. They include ephedrine, methylephedrine, pseudoephedrine, methylpseudoephedrine, norephedrine and cathine, and are all sympathicomimetic amines used in asthma and bronchial disorders. These substances are included in over-the-counter cold medicines (4).

2. Experimental part

The aim of exercise is assessment of extraction efficiency of *N*-methylephedrine from blood samples and its determination in artificial blood using SPE-HPLC-UV technique.

The most important physicochemical properties of this substance are presented in Table 1.

Table 1. Summary of physicochemical properties of *N*-mehylephedrine (9)

Compound CAS number	Chemical structure	Solubility in water	Molar mass [g mol^{-1}]	pKa	Log K_{ow}
N-Methylephedrine 552-79-4		15,4 mg/ml	179.26	9.3	2.47

https://go.drugbank.com/drugs/DB11278

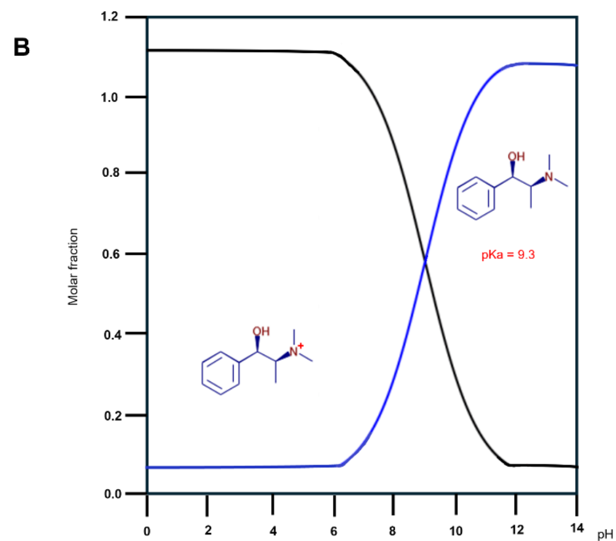
2.1. Extraction of analyte from blood samples.

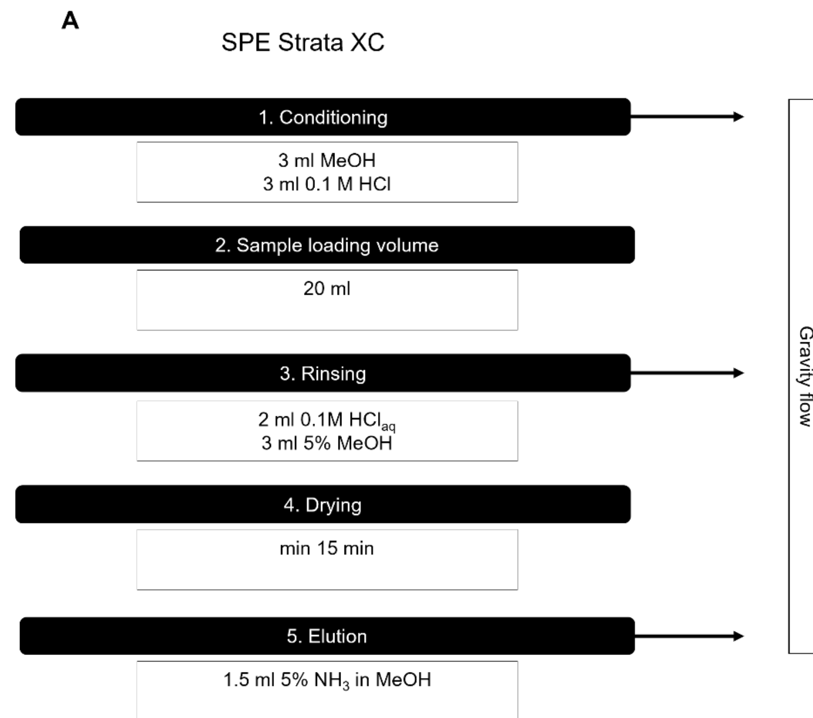
SPE technique will be used for the extraction *N*-methylephedrine from blood sample. During experiments, four different procedures will be tested. For each procedure, two types of samples should be prepared: spiked and blank sample.

Sample preparation procedures:

- Using glass syringe, add appropriate volume of *N*-methylepehdrine stock solution (C = 1000 μg mL^{-1}) to four blood samples (spiked samples for each procedure tested). The amount of analyte in blood samples should be 50 μg each.
- **First procedure:** Add 15 mL of 0.1M HCl_{aq} solution to spiked sample and to blank sample and shake them using vortex.
- **Second procedure:** Add 15 mL of H_2O (pH 11) to spiked sample and to blank sample and shake them using vortex.
- **Third procedure:** Add 15 mL of 25 mM ammonium formate to spiked sample and to blank sample and shake them using vortex.
- **Fourth procedure:** Add 15 mL of 25 mM ammonium formate to spiked sample and to blank sample and shake them using vortex.
- Load each sample to conditioned SPE sorbent using Pasteur pipette.

The first procedure of extraction:





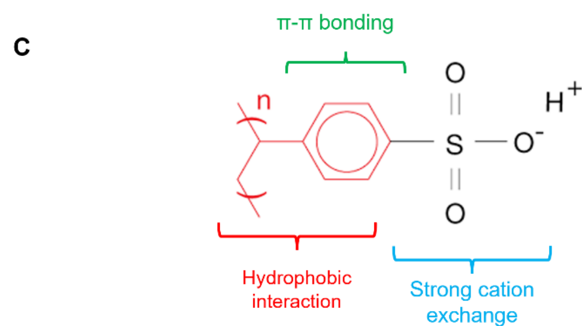
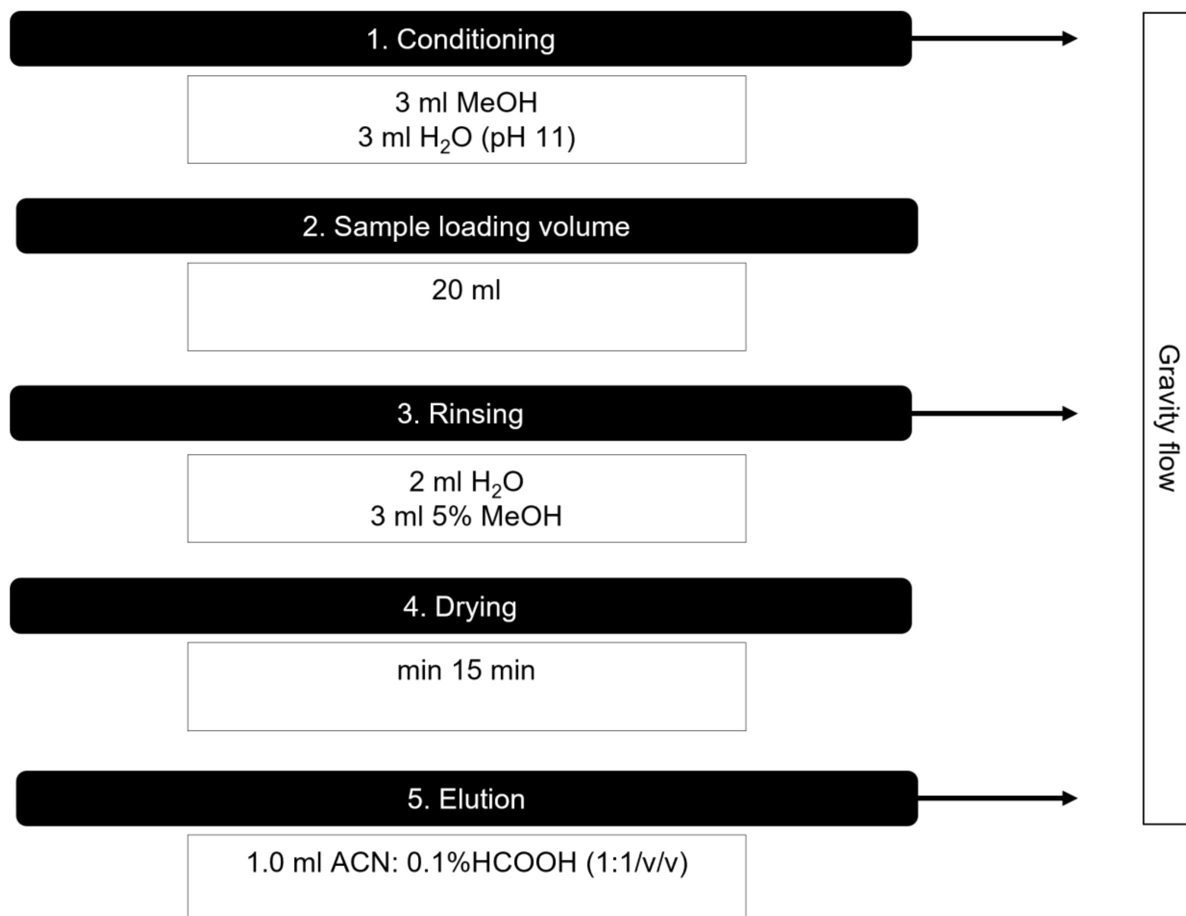
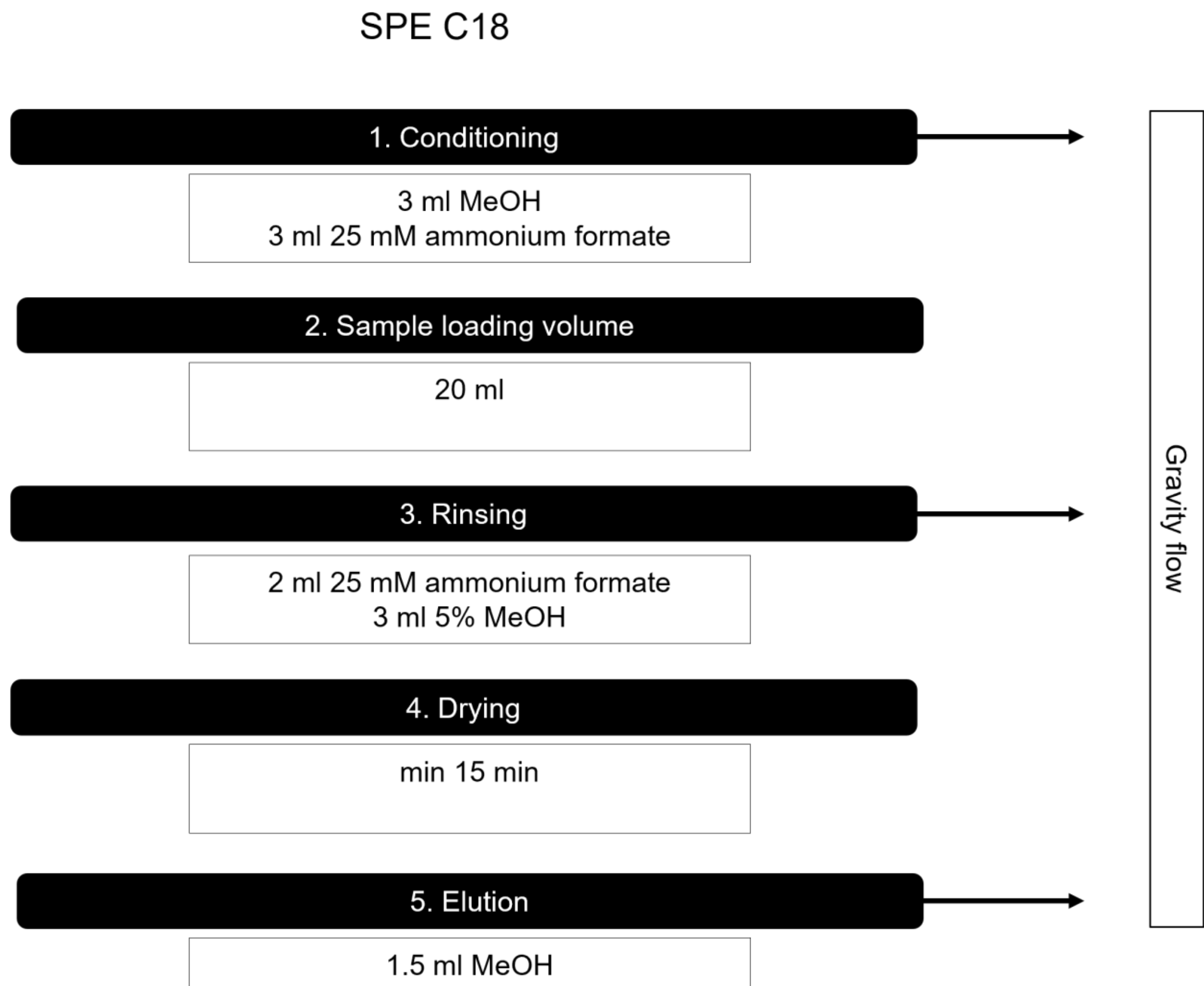


Figure 1.
A: Extraction procedure of *N*-methylephedrine from blood samples using SPE technique (modified)(10)
B: Influence of pH on the form of *N-methylephedrine*;
C: Structure of Strata XC sorbent used for extraction of *N-methylephedrine* (Phenomenex) and possible interactions between analyte and sorbent.

The second procedure of extraction:



The third procedure of extraction:



The fourth procedure of extraction:

SPE Strata X

1. Conditioning

3 ml MeOH
3 ml 25 mM ammonium formate

2. Sample loading volume

20 ml

3. Rinsing

2 ml 25 mM ammonium formate
3 ml 5% MeOH

4. Drying

min 15 min

5. Elution

1.5 ml MeOH

Gravity flow

Evaporate extracts obtained to dryness under a stream of nitrogen, dissolve them in 1 mL mixture of ACN:0.1%HCOOHaq (1:1, v/v) and filter into separate chromatographic vials using syringe filters. Next, samples prepared this way will be analyzed using HPLC-UV method (chromatographic conditions are presented in Table 2).

Use retention time to identify the *N*-methylephedrine in extracts. Concentration of compound should be determined based on calibration curve equation. Assessment of extraction efficiency, expressed as absolute recovery, should be performed based on formula presented below:

$$AR = \frac{P_{pre-ext} - P_{non-spiked}}{P_{standard}} \qquad \text{Eq. (1)}$$

where: AR – absolute recovery, $P_{pre-ext}$ is the peak area of the analyte recorded for the sample, spiked with the target compound prior to extraction; $P_{non-spiked}$ is the peak area of the analyte recorded for the non-spiked sample (blank sample); $P_{standard}$ is the peak area of the analyte recorded for the standard solution.

2.2. Calibration curve preparation

Using standard stock solution of *N*-methylephedrine (C = 1000 μg mL^{-1}) prepare six working solutions in the mixture of ACN:0.1%HCOOHaq (1:1, v/v): 0 μg mL^{-1}, 10 μg mL^{-1}; 15 μg mL^{-1}, 30 μg mL^{-1}, 50 μg mL^{-1}, 70 μg mL^{-1}. For this purpose, evaporate the methanolic portion of the stock solution under stream of nitrogen to dryness. Next, add 1 ml solvent mixture to each sample. Finally, perform the chromatographic analysis of working solutions and each of prepared extracts (twice) according to the conditions presented below (Table 2).

Table 2. Analytical conditions for the determination of *N-methylephedrine* using HPLC-UV.

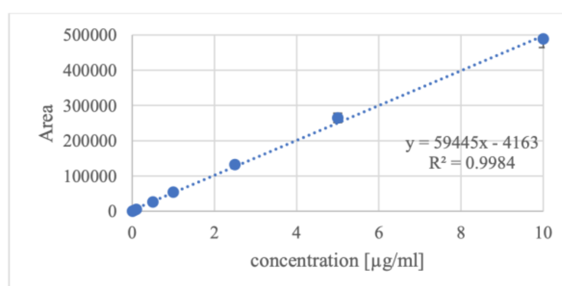
Chromatographic column	Mobile phase	Elution programme	Flow of mobile phase [mL min^{-1}]	λ [nm]
Gemini C18	(B) – ACN (A) – 0.1% $HCOOH_{aq}$	Isocratic mode: 15 % B 85% A	1.0	210

2.3 Report:

- Show experimental part in the form of scheme, taking into account the conditions of the analyses performed;
- Construct the calibration curve by plotting the calculated average values of peak areas of the analyte and its concentration in the used standard solutions; Determine the equation of the calibration curve and the value of R^2.
- Determine the concentration of *N*-methylephedrine in extracts using the equation of the calibration curve;
- Calculate absolute recoveries for extracts based on Eq. 1 and compare the SPE procedures tested;

2.4 Topics to study

- The basic information about doping substances;
- The construction of the liquid chromatograph (block diagram), HPLC working principles;
- Blank sample;
- Calibration curve method;
- Absolute Recovery;
- Solid Phase Extraction – what it is; steps; goals; type of sorbents; interactions, advantages and disadvantages;
- A sample containing an unknown amount of compound was brought to the laboratory. The HPLC measurement of the standard solutions and samples was performed at a wavelength of 272 nm. Using the calibration curve presented below, determine the concentration of the analyte in the test sample, if the mean surface area of the compound signal was 616278. Report the result in mg/l; ug/l, ng/ul, mg/ml, etc.



3. Glass and reagents:

- 0.1M HCl_{aq} (200 mL)
- 0.1 M NaOH
- 2 x ACN (1000 mL, for HPLC)
- MeOH (100 mL)
- ACN (100 mL)
- 2 x 0.1% $HCOOH_{aq}$ (1000 mL)
- 0.1% HCOOH (100 mL)
- Water for column rinsing (2 x 1000 mL)
- MeOH for syringe rinsing x 2
- Methanolic standard stock solution of *N*-methylephedrine (1000 mg L^{-1})
- 5% NH_3 in MeOH (50 mL)
- 25 mM Ammonium formate (250 ml)
- Strata XC cartridges (200 mg/3mL; 2 pieces)
- Strata X cartridges (200 mg/3mL;4 pieces)
- Strata C18 cartridges (200 mg/3mL;4 pieces)
- Vortex
- Glass flasks for SPE (4 pieces)
- Test tubes (8 pieces)
- Measuring cylinder 50 mL (3 pieces)
- Pasteur pipettes (10 pieces)
- Flasks or glass bottles (4 x 100 mL)
- Glass baguette (2 pieces)
- Chromatographic vials (1,5 ml, 20 pieces)
- Syringes for HPLC (2 items, 50 mL and 100 mL)
- Measuring pipette 2 mL (4 pieces)
- Measuring pipette 1 mL (3 pieces)
- Measuring pipette 5 mL (3 pieces)
- Marker for glass
- pH meter

5. Literature

1. Ljungqvist A. Brief History of Anti-Doping. Med Sport Sci. 2017;62:1–10.
2. Segura J, Ventura R, Marcos J, Gutiérrez Gallego R. Chapter 21 Doping substances in human and animal sport. Handb Anal Sep. 2008;6:699–744.
3. Antydopingowa ŚA. LISTA SUBSTANCJI I METOD ZABRONIONYCH. ŚWIATOWY KODEKS ANTYDOPINGOWY Stand MIĘDZYNARODOWY. 2020;1–10.
4. Kojima A, Nishitani Y, Sato M, Kageyama S, Dohi M, Okano M. Comparison of urine analysis and dried blood spot analysis for the detection of ephedrine and methylephedrine in doping control. Drug Test Anal. 2016;8(2):189–98.
5. Wong JKY, Choi TLS, Kwok KY, Lei ENY, Wan TSM. Doping control analysis of 121 prohibited substances in equine hair by liquid chromatography–tandem mass spectrometry. J Pharm Biomed Anal. 2018;158:189–203.
6. Klaus Mueller R, Grosse J, Lang R, Thieme D. Chromatographic techniques-the basis of doping control. J Chromatogr B Biomed Sci Appl. 1995;674(1):1–11.
7. Badoud F, Guillarme D, Boccard J, Grata E, Saugy M, Rudaz S, et al. Analytical aspects in doping control: Challenges and perspectives. Forensic Sci Int. 2011;213(1–3):49–61.
8. Tsakalof A, Tzatzarakis M, Tsitsimpikou C. Detection of doping substances residues in biological material: A comparative approach. Toxicol Lett. 2015;238(2):S43–4.
9. Gray N, Musenga A, Cowan DA, Plumb R, Smith NW. A simple high pH liquid chromatography-tandem mass spectrometry method for basic compounds: Application to ephedrines in doping control analysis. J Chromatogr A. 2011;1218(15):2098–105.
10. Zhang L, Wang ZH, Li H, Liu Y, Zhao M, Jiang Y, et al. Simultaneous determination of 12 illicit drugs in whole blood and urine by solid phase extraction and UPLC-MS/MS. J Chromatogr B Anal Technol Biomed Life Sci. 2014;955–956(1):10–9.