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Review

Citius, Altius, Fortius—Advanced Mass Spectrometry in Service of Forensic Analysis

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Abstract: This review presents numerous studies in which mass spectrometry has been used to assist forensic investigation. Due to its unique capabilities, mainly high-resolution mass data and structural information, high sensitivity, and cooperation with separation techniques, this method provides access to many tools streamlining and accelerating sample analysis. Low analyte consumption, advanced derivatization procedures and availability of isotopically labeled standards offer opportunities to study materials previously not considered viable evidence, opening new avenues in forensic investigations.

Keywords: forensic analysis; falsified medicine; derivatization; Isotope-Ratio Mass Spectrometry; hydrogen-deuterium exchange; liquid chromatography-mass spectrometry; quantitative LCMS analysis

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1. Introduction

Mass spectrometry (MS), particularly when coupled with liquid chromatography (LCMS), has been gaining attention as the method of choice in forensic studies [1]. Since this method could be used to analyze many compounds in parallel, it could reduce the sample processing time, thus leading to a higher throughput for forensic laboratories. Mass spectrometry is particularly useful in toxicological studies and in analysis of falsified medicines, due to the ability to extract structural data from a very limited amount of material [2].

The sensitivity and selectivity of mass spectrometry could be further enhanced by derivatization of the analytes, even allowing the detection of substances which are practically impossible to detect at low concentrations in biological matrices, such as cyanides, which are of interest in forensic studies [3].

With new sample preparation protocols, non-traditional matrices, such as ear wax [4], hair [5] or meconium [6] could also be analyzed by mass spectrometry. Such matrices often provide long-term information on drug abuse, not normally available from blood samples. The high resolution of LCMS allows analysis of polydisperse materials, making unambiguous characterization of surfactants possible, which constitute both human-driven environmental pollution and a toxicological threat [7].

Isotopic level analysis by mass spectrometry provides data unavailable by other methods, for example the ratio of certain isotopes in a sample could be used to distinguish the geographic origin of two, otherwise chemically identical, materials [8]. The hydrogen–deuterium exchange, which is also easily analyzed by mass spectrometry, can provide internal standards for quantification of biologically active compounds [9].

The innovative mass spectrometry methods, usually developed for pharmaceutical and biomedical analysis, find further application in related areas. The answers are provided faster, with higher accuracy and powerful impact, fulfilling the Olympic motto "Citius, Altius, Fortius" in the struggle to find the truth. In this review we discuss how current developments in mass spectrometry, e.g., the access to new samples and previously

overlooked substances, advanced chemical modifications for increased sensitivity and specificity, as well as unique information obtained at isotopic level change the scope of forensic studies.

2. Mass Spectrometry in Forensic Analysis

2.1. Mass Spectrometry and Analysis of Falsified Medicines

The high market value of pharmaceutical products results in various illegal actions in the production of such molecules, including low molecular weight compounds, as well as peptides, proteins and even monoclonal antibodies [10,11]. Countermeasures include development of analytical strategies for comprehensive characterization of suspicious samples [12–17]. Table 1 lists the main analytical methods used in qualitative and quantitative analysis of medicinal drugs, with their main benefits and drawbacks.

Table 1. Modern analytical methods used in medicinal drug inspection (based on [2,13,18–20]).

Analytical Method	Benefits	Drawbacks
Colorimetry	quick, low-cost	limited sensitivity
Raman spectroscopy	rapid, specific, portable, fingerprint analysis	requires chemometry for complex samples
Nuclear magnetic resonance (NMR)	direct structure elucidation	sensitivity, solvent interference
Separation methods		
High-performance thin-layer chromatography (HPTLC)	fingerprint of herbal medicines	resolution
Gas chromatography (GC)	analysis of volatiles	requires thermal stability or derivatization
High-performance liquid chromatography (HPLC)	analysis of biologicals	solvent consumption
Capillary electrophoresis (CE)	charge diversification	limited robustness
Separation methods with MS detection		
Gas chromatography-mass spectrometry (GCMS)	efficient, sensitive, databases available	thermal artefacts
Liquid chromatography-mass spectrometry (LCMS)	efficient, sensitive, suitable for labile molecules	ion suppression, size

Mass spectrometry has achieved a leading role in characterization of falsified medicines, due to the specificity and sensitivity provided by current MS instruments, but its applications in forensic science include not only analysis of drugs, but also warfare agents [21], gun-shot residues [22,23], ink differentiation [24], and other forensically relevant samples [1]. Mass spectrometry offers the identification of unknown samples, while allowing both qualitative and quantitative analysis. Accuracy is enhanced by the use of different ionization sources and hyphenation with separation techniques [25–27]. The samples of potentially falsified drugs may or may not contain the actual active pharmaceutical ingredient (API), and if contained it is often adulterated or mixed with other substances. The effect of a matrix often creates a hurdle for rapid yet accurate analysis, making the combination of mass spectrometry with a separation method (GC, LC) a necessity [28].

2.1.1. Falsified Lifestyle Drugs as a Global Problem

World Health Organization (WHO) defines a counterfeit drug as one "which is deliberately and fraudulently mislabeled with respect to identity and/or source" [29]. In the developing world illegal actions concentrate on lifesaving medicines, such as anti-malaria drugs, whereas in high income countries, performance and image enhancing drugs (PIEDs), including lifestyle drugs, such as phosphodiesterase type–5 (PDE–5is) inhibitors for erectile dysfunction (ED), are targeted [30].

Counterfeit PDE-5 inhibitors, sold as dietary supplements or functional foods, are becoming a global problem because of the high demand, low cost, and simple manufacturing. Many methods for identifying PDE-5 inhibitors in complicated matrices have been developed (IMS; vibrational, X-ray, and NMR spectroscopy; immunological tests; HPLC-UV or LCMS) [31].

In one of the recent studies, 181 counterfeit and illicit sexual enhancement drugs were subjected to HPLC and LCMS/MS. It was found that 86.2% of all samples (156 out of 181) contained PDE–5is and/or their analogs, with 49.4% containing two or more components. Sildenafil, tadalafil, and other such drugs were detected at concentrations exceeding 2–7 times the recommended maximum doses, with multiple compounds frequently detected in single samples [32].

In another study, targeted, suspected-target, and non-targeted strategies based on liquid chromatography-high-resolution mass spectrometry (LCHRMS) were utilized to analyze erectile dysfunction (ED) drugs and their analogs in powdered drink mix, honey, jelly, hard candy, and sugar-coated chewing gum samples selected based on their brand names, label claims, images, herbal ingredients, or advertising materials with connotations to male sexual performance. The method was optimized and validated using 23 target analytes, representing structurally similar ED drugs. The modified QuEChERS extraction provided complete coverage of target analytes. Validation of the method covered 25 food samples that claimed to enhance male sexual performance. The LCHRMS analysis identified and detected 10 ED drugs from 24 adulterated food samples, with 11 of them quantified at supratherapeutic levels [33].

Recently, we have studied 21 dietary supplements available through online stores and marketed as sexual enhancement supplements containing only natural compounds (herbs and animal extracts). Even a simple visual inspection of the samples, in some cases, revealed that the chemical content of the pills was inconsistent with the declaration on the seller's website and the description on the package. Instead of the expected greenish-brown extract, they contained a white solid or powder. Pills and capsules were extracted with 50:50 methanol:water mixture with the aid of an ultrasound bath, filtered and subjected to LCMS/MS analysis on the Shimadzu qTOF mass spectrometer working in a positive ion, DDA mode. Sildenafil, which was undeclared by neither manufacturer nor the seller, was found as the main ingredient in five of the analyzed products (Figures 1 and 2). Two other samples contained undeclared icariin flavonoid glucoside derived from the Epimedium plant genus, used in traditional Chinese medicine to enhance erectile function. Figure 3 presents the results of LCMS/MS analysis of one of these supplements, performed in our research group. The results were in agreement with the theoretical fragmentation scheme and spectra published by Qian et al. [34].

The analysis of multiple isobaric peaks by LCMS QTOF proved to be highly efficient in prediction of the API origin and was suggested as a promising tool in tracing drug sources [35].

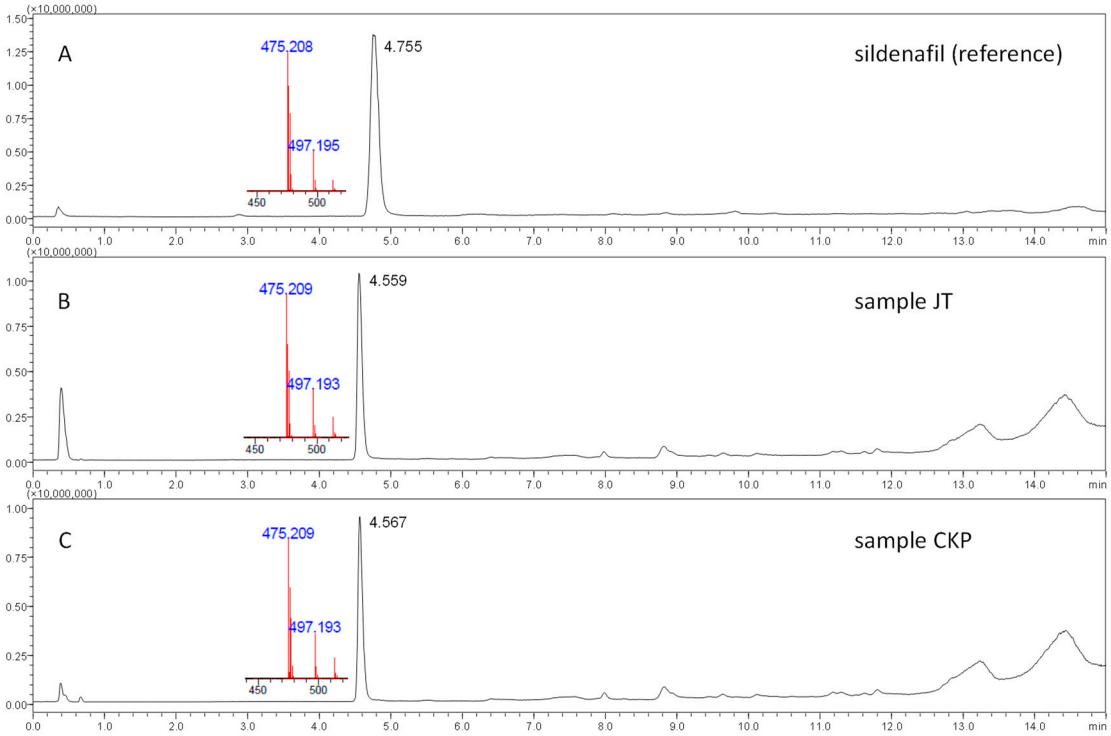


Figure 1. LCMS chromatograms of (**A**) sildenafil standard and two dietary supplement samples: JT (**B**) and CKP (**C**). Inserts included in all three panels show MS spectra collected at 4.75/4.56/4.57 min. Peak m/z = 475.208 corresponds to the $[M + H]^+$ ion for sildenafil, m/z = 497.193 and 513.167 to $[M + Na]^+$ and $[M + K]^+$, respectively.

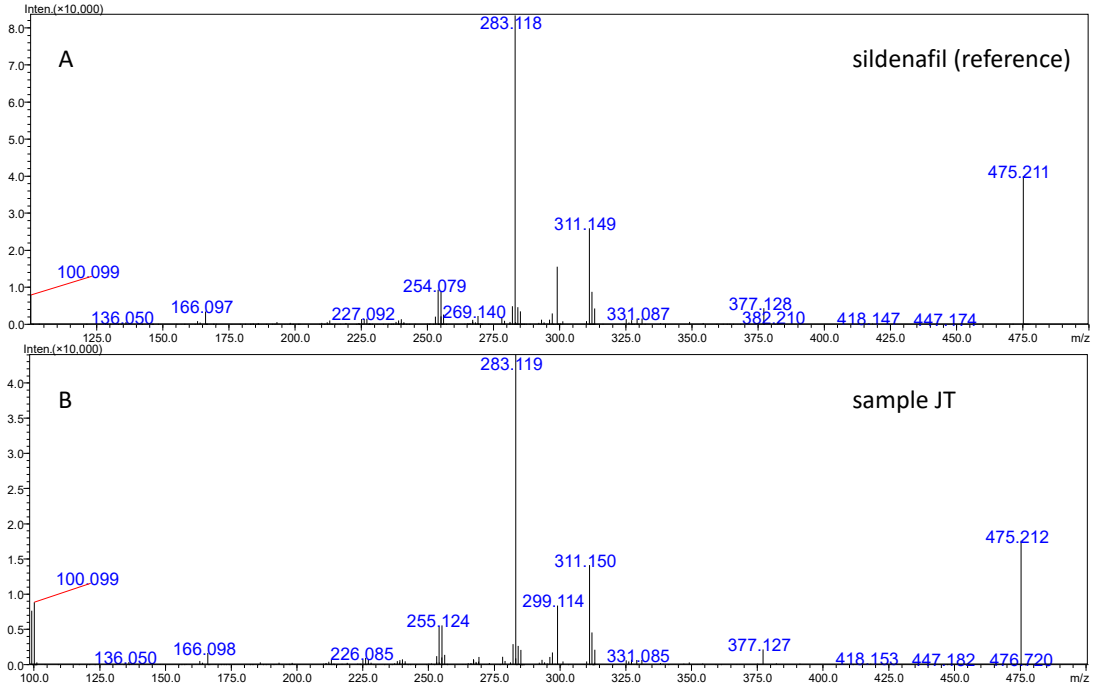


Figure 2. Comparison of the MS/MS spectra of (**A**) sildenafil standard and (**B**) dietary supplement sample JT. Precursor ion m/z = 475.210, CE = 40 ± 10 eV.

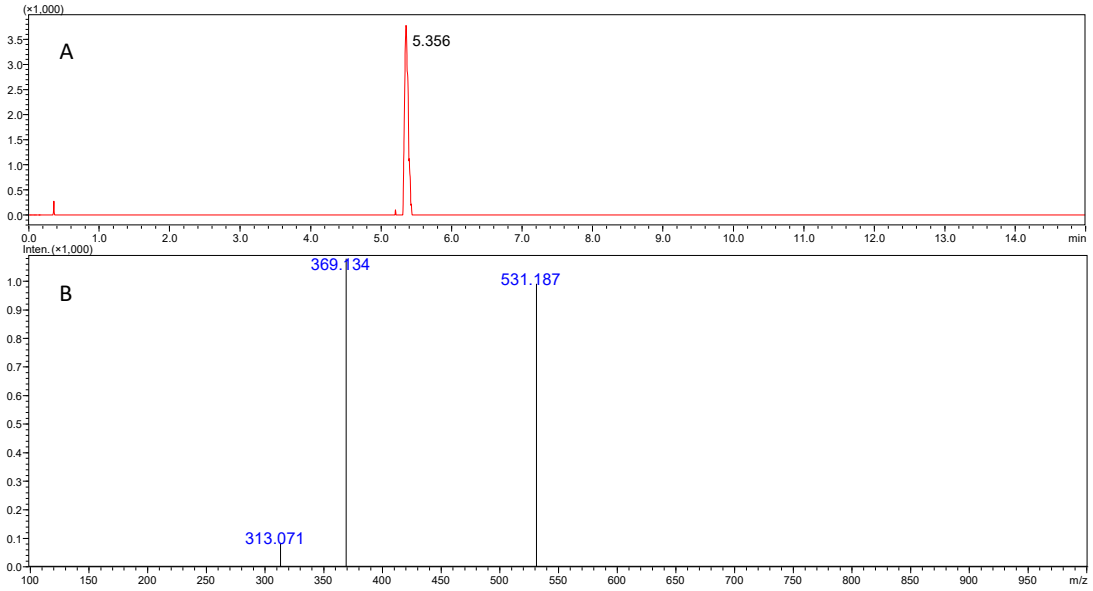


Figure 3. (**A**) Extracted ion chromatogram of sample JD for m/z = 677.2406–677.2474 corresponding to icariin. (**B**) MS/MS spectrum for the precursor ion m/z 677.249, at retention time 5.356 min, CE = 30 ± 15 eV. Fragments m/z 313.071, 531.191and 369.136 confirm presence of icariin in the sample.

2.1.2. Technical Advances in Falsified Drug Analysis

Laser Desorption/Ionization-Mass Spectrometry Imaging (LDI-MSI) was used to study authentic and falsified sildenafil citrate containing tablets. This method does not require chromatographic separation and tangible results were obtained in less than three minutes per sample. The acquired mass data and multivariate analysis led to successful differentiation of pharmaceutical products with the same API, but in different formulations. Established quality markers were used to distinguish the reference drug from the generic and falsified versions. Given the sensitivity and specificity provided by the LDI-MSI and MS/MS, this approach is capable of identifying adulterants used in the falsification process [36].

Direct analysis in real-time mass spectrometry (DART-MS) is a relatively new technique for rapid ambient MS, including thermal-desorption-DART-MS, infrared-thermal-desorption-DART-MS, Joule-heating thermal-desorption-DART-MS, etc. The main benefit of this technique results from a transfer of the ionization process into the open air, with no sample preparation, no sample carryover and the ability to probe the surface of samples of any shape and size. Practically all types of forensic traces were successfully investigated, including drugs, explosives, gunshot residues, flammable liquid residues, inks, paints, polymers, lubricants, bank dyes, beverages, and insects. Samples can be rapidly and effortlessly screened and analyzed from various delicate surfaces like skin, glass and clothes. Recent reports suggest that DART-MS may be capable of providing quantitative or confirmatory results [25,37,38].

2.1.3. Falsification of Supplements in Doping Cases

Due to its stimulating properties, 3-dimethylamylamine (DMAA) has been used as an additive in dietary supplements. The World Anti-Doping Agency (WADA) prohibited its use in 2010, with several countries following with its complete removal from the market due to toxicity cases. Dietary supplements seized by the Brazilian Police were subjected to DART-MS/MS analysis, with the panel of DMAA, ephedrine, synephrine, caffeine, sibutramine, and methylphenidate. DMAA was detected in 20% of products, frequently in combination with sibutramine and caffeine. Sibutramine was found in 50% of the 108 seized samples, whereas methylphenidate was found in 10% of the material [39].

Several products (75 pharmaceuticals and 35 dietary supplements) were seized from on-line sales addressed to bodybuilders in France and submitted for identification and quantification of active compounds using GCMS and LCHRMS. Among the pharmaceuticals, 33% were substandard (wrong dosage), 32% were counterfeit (different formulation components) and only 19% were original, with remaining products acceptable only at qualitative stage. Fifty-four pharmaceuticals contained anabolic–androgenic steroids (AAS) with 80% AAS being non-original, whereas only 3% of dietary supplements were adulterated with a doping substance 1,3-dimethylbutylamine (DMBA) [40]. Anabolic androgenic steroid products were analyzed by UPLC-QTOF-MS/MS in a report presented by Smit et al. [41]. The expected components were detected in 50% of samples. However, other undeclared steroids were frequently detected.

The vast number of samples subjected to analysis in the case of falsified drugs inflicts a strain on analytical laboratories, with demand for methods offering fast and reliable results. In the case of mass spectrometry, coupled to chromatography, UHPLC methods have gained importance, although ambient methods (DART etc.) may be the procedures of choice in the near future [1].

2.2. Forensic Applications of IRMS

Isotope-Ratio Mass Spectrometry (IRMS) is the name of the branch of MS which is focused on determining the abundance of specific isotopes of the elements present in a given sample. This method is further subdivided into analysis of stable isotopes and radioisotopes. The latter is mainly used in radiometric dating. The former has found use in many areas, ranging from earth sciences, to archaeology, and to forensics. In particular, it has been utilized in food quality control and, by extension, in tracking food adulteration [42]. IRMS has been applied to a wide variety of food types, ranging from wine, vegetable oil, animal and cereal products, to organic food [43]. The two main kinds of tampering with food are introducing artificial additives or falsifying the origin of certain foods. For example, the EU has defined designations of Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Speciality Guaranteed (TSG). The first two concern the place of production, while TSG designation guarantees the way of production, including the ingredients used.

2.2.1. The Origin of Isotopic Differences

The difference in isotopic contents of the same molecules stemming from various sources is caused by the difference of processes leading to their formation. For example, the ^{13}C content in plants is lower than that of the atmospheric CO_2, which they base their metabolism on. The difference is caused by a discrimination against ^{13}C and preference for ^{12}C of the enzymatic and physical processes occurring in the plants, such as membrane permeation. The variability between various species can be accounted for by the differences in their metabolic pathways. On the other hand, the changes between geographic places of origin within the same species can be explained by regional differences in availability of certain isotopes. For example, there is an isotopic effect observed when water is evaporated, so places with higher evaporation, and those with higher precipitation, will exhibit differences in the availability of hydrogen and oxygen isotopes. The level of discrimination, and, therefore, the difference in the isotopic ratios, is very small, but sufficient to be detected by a well-tuned mass spectrometer [44].

The instrument of choice for performing IRMS studies is the magnetic sector-type MS, also known as Nier-type MS. In this mass spectrometer, ions travel a circular path in a magnetic field, and the radius of their path depends on the intensity of the field and the m/z ratio of the ions. Such spectrometers can be equipped with several detectors, and, thus, detect ions of various m/z ratios simultaneously, since they are separated in space, not in time. This, in turn, makes measuring the intensity ratios more reliable and reproducible. The sample, before entering the magnetic sector, must be first ionized, and, before that, it needs to be converted into gas. For analyzing organic molecules, gases such as N_2, CO_2

and SO_2 are normally used, and the samples are converted into them by burning, oxidation and/or reduction [45]. Due to low sample consumption and high accuracy, IRMS has been utilized extensively in forensic studies on foods, drugs and other products (Table 2) [8].

Table 2. Selected examples of IRMS applications in forensic analysis.

Area of Application	Example	Reference
Food origin	Wine	[46]
	Olive oil	[47]
	Wheat	[48]
Alcohol tampering	Wine	[49]
	Vodka	[50]
Drug analysis	Testosterone doping	[51]
	Drugs of abuse	[52]
Explosives	Triacetone triperoxide	[53]

There are many examples showing the usefulness of the method for food analysis. In the case of wine, ^{13}C content in ethanol is accepted as an EU-standard for validating that no extra alcohol has been added. In order to simplify the sample preparation step, the ^{13}C content in volatile compounds, such as limonene or ethyl octanoate, can be measured as well [49]. Other isotopes, such as ^{85}Rb, ^{24}Mg and ^{18}O, can be used to distinguish the geographical origin of the wine [46].

2.2.2. Food Authentication by IRMS

Olive oil belongs to the most falsified agricultural products. Therefore, EU regulations place very specific requirements on labeling Extra Virgin Olive Oil and Virgin Olive Oil, including precisely marking the area in which the oil is produced. The fats in olive oil are composed of four main types of fatty acids, and the exact content of each one depends on many factors, including weather conditions and the maturity of the olives [54]. Therefore, while the exact proportions of these fatty acids can be repeated year-to-year in a certain area, there is no guarantee they will [55]. However, the ratio of particular isotopes in the oil can be used as a fingerprint indicating the area of origin of the product [47]. In particular, since the four main kinds of fatty acids are generated by different metabolic pathways, the isotope ratios differ between them. Therefore, in addition to the IRMS of the bulk oil, IRMS of specific extracted fatty acids can be used to provide an additional degree of confirmation of the place of origin, and, thus, increase confidence in the results [54].

Similarly, in the case of cereals and products obtained from them, isotope ratios can be used to verify the identity as well as the place of origin. For example, in the case of buckwheat the contents of ^{13}C, ^{15}N and ^{34}S can be used to determine not only what species it belongs to, but also whether it has been cultivated organically [56]. Similarly, isotope composition has been used to detect the geographic origin of wheat, while investigating both intact kernels and the products at various stages, up to cooked noodles. It was shown that the year-to-year variability is low enough for the region-to-region variability to be clearly detectable [48]. When it comes to the analysis of products obtained from cereals, IRMS has been used to detect adulteration of Polish vodka by the addition of maize-derived ethanol. The study concluded that the method is comparable to NMR in its precision [50]. In fact, the method can generally be used to distinguish between various plants, stemming from different geographical areas, which have been used for the production and distillation of spirits. In particular, it is relatively easy to differentiate between plants which conduct photosynthesis according to the C3 or C4 mechanisms. Moreover, alcohol synthesized chemically can be distinguished from that obtained from natural sources [57].

2.2.3. Endogenous and Exogenous Compounds Distinguished by IRMS

IRMS can also be used in other forensic applications, where it is necessary to detect the source of a certain molecule, not just its presence or absence. Anti-doping studies can

serve as an example, since some of the doping strategies are based not on supplying the patient with drugs, but on providing higher amounts of molecules, which their bodies produce endogenously. Among others, testosterone and its derivatives can be supplied to patients in order to improve their performance. However, the sole presence of testosterone cannot be a doping marker, since it is naturally present in a human body. Still, due to variations in both carbon sources and the isotopic selectivity of the processes involved, the ^{13}C to ^{12}C ratio of endogenous and synthetic anabolic hormones is different [51]. A method for determining the origin (endo/exogenous) of prednisone and prednisolone has been developed and validated to WADA standards [58]. Similar methods have been developed for other doping agents [59] and could be also used to verify the origin of drugs of abuse, such as γ-hydroxybutyric acid [52].

Other areas in which IRMS can be used includes the tracing of illicit explosives. While regulated factories add tracing markers to the explosives they produce, it is much more difficult to find the source of illicitly-made explosives, such as triacetone triperoxide (TATP). Analysis of isotopic ratios can be used here, since acetone produced in different parts of the world differs slightly, and it is the sole source of carbon in TATP [53,60]. The method can also be utilized in post-conflict human identification, where it can help to find out which part of the world a certain person comes from, or whether a victim travelled before dying, or even how many peoples' remains are collected in one place [61].

2.3. Alternative Matrices in Toxicological Analysis

The selection of samples in toxicology studies is a critical step in a forensic analysis. The most commonly tested biological matrices for the detection of drug use or exposure are blood and urine. These matrices are well understood and standard operating procedures exist. However, the Scientific Committees of Judicial Organizations (OSAC) recommends the search for alternative biological matrices to improve the results of forensic toxicology analyses [62].

Alternative biological matrices are fluids or biological tissues, which can provide additional information compared to blood and urine analysis. The choice of a matrix is often determined by easier and less invasive sampling, larger detection windows and the complexity of preparation of the analysis [63]. Occasionally, blood or urine samples are unavailable or degraded. In addition, some alternative matrices, such as hair and nail clippings, provide information that blood and urine cannot provide. This is because the hair and nails grow out of the body over time, which makes it possible, through segmental analysis, to detect substances introduced at different times [64].

When selecting a matrix, consideration should be given to the fact that the concentration of the analyzed compounds in such matrices is often significantly lower compared to blood or urine. Therefore, it is extremely important to select appropriate analytical methods. Sensitive analytical instruments and methods using derivatization of investigated compounds allow the detection of drug concentrations at the pg/mL level [62].

Some of these matrices, such as saliva and hair, are already well established and have been implemented in drug testing laboratories. Others, such as meconium, breast milk, and bone marrow, attract attention only in certain situations [65].

2.3.1. Forensic Hair Analysis by Mass Spectrometry

Hair analysis is now routinely used as a tool for detection of xenobiotics (drugs of abuse, pharmaceuticals, environmental contaminants, doping agents, etc.) in forensic science, traffic medicine, occupational medicine, and clinical toxicology. Hair analysis is a powerful tool for toxicological analysis due to the advantages of this type of sample, such as high stability (weeks to years), non-invasive sampling and easy storage [5]. Many drugs are metabolized and excreted in the urine within 2–3 days, whereas their presence in the blood is detectable for approximately 24 h (Figure 4). In chronic (daily) drug users, the expulsion of the metabolite may take up to several weeks. Therefore, even after quantification in urine, it is not possible to determine the amount of drug used and the duration of its

use. In addition, the indicated amounts can be confusing due to the diluting effect of the urine. In such situations, hair is often the only way to detect retrospective drug and/or alcohol consumption.

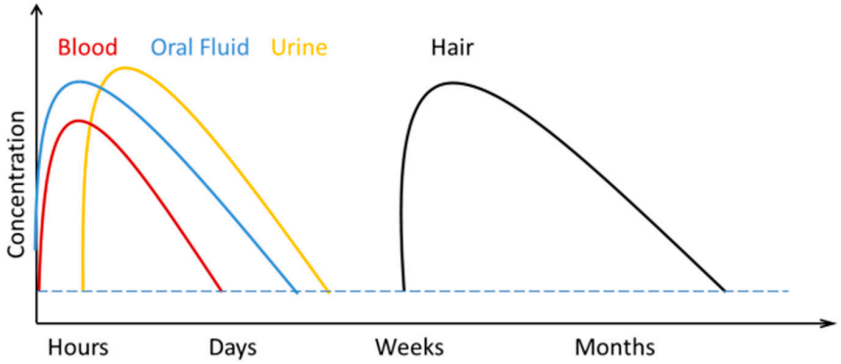


Figure 4. General drug detection window (based on [66]).

Substances from blood are incorporated into the hair by melanocytes or keratinocytes and spread along the hair as it grows. Drug concentrations in hair have been shown to be related to the melanin content of the hair (dark hair tends to bind more drugs), ethnicity, and the lipophilicity, polarity, and alkalinity of the drug or its metabolites [67]. Melanin is acidic and hydrophobic and therefore has an affinity for alkaline drugs, such as cocaine, codeine, and ketamine.

The average rate of hair growth is about 1–1.5 cm per month, therefore hair analysis makes sense only after a few weeks of taking the drug, but at the same time, the hair makes it possible to identify drugs many months after administration (hair has a large detection window). Moreover, chronic drug use can be assessed by segmental analysis.

Cosmetic treatments, such as regular shampooing, dyeing or bleaching, can have a detrimental effect on the concentration of the drug in the hair. Intense bleaching changes the physical properties of the hair (e.g., higher porosity) and causes partial or complete degradation of melanin, resulting in a reduced drug concentration. Therefore, when analyzing the hair, cosmetic treatments should be considered that can lower the concentration of the drug below the detection limit and thus cause false negative results [68]. Dyed or bleached hair may still be used for drug detection and ethyl glucuronide (EtG) analyses, but it often requires comparison with another sample, e.g., hair from other anatomical areas like pubis, arms, armpits or face [69,70]. Body hair is also used if regular head hair is not available, due to baldness, haircut, and religious, cosmetic or aesthetic reasons [71,72].

The standard use for analyzing hair xenobiotics is gas chromatography and mass spectrometry (GCMS) or tandem liquid chromatography mass spectrometry (LCMS/MS). Such analyses are laborious and time-consuming as they require advanced sample preparation. Hair testing requires the use of sensitive analytical methods that make the cost of the analysis higher than that of other biological samples. All information on the collection, testing, cut-off and presentation of validated results can be found on the websites of the Society of Hair Analysis and The European Workplace Drug Testing Society [73,74].

Sensitive and specific analytical equipment enables the testing of samples in which a low concentration of analytes is expected. Hair analysis can reveal very small amounts of a drug of abuse (Table 3). For carboxy-THC these are pg values and the obtained result is the definitive evidence of marijuana consumption. On the other hand, the segmental hair analysis shows an increase in the dose of the drug or the frequency of use. The study described by Wang et al. [75] showed that the analysis of hair samples of chronic methamphetamine abusers (MA) identified which isomer of the drug was used and confirmed that all respondents used S-isomer of MA (S-MA) continuously. Another example is the detection of gamma-hydroxybutyrate (GHB). It is an endogenous compound that is present in small amounts in the body, but it is also a substance used in drug-facilitated sexual assault (DFSA). Since GHB is endogenous, it can be detected in the hair in low concentrations,

which is not crime-related. Therefore, to distinguish endogenous and exogenous GHB, an increase in GHB concentration in the hair segment is required in accordance with the approximate time interval of the event [76]. The analysis of GHB concentration in other matrices is less reliable.

Despite their numerous advantages, the described techniques require a large number of samples and complex preparation processes, including rinsing, derivatization, pulverization, milling, filtration, liquid-liquid extraction, and solid-phase extraction [73]. The whole process is time-consuming and costly, so innovative techniques are sought that can analyze and detect drugs in a single hair.

2.3.2. Special MS Techniques in Hair Analysis

The new analytical approach is a method that uses thermal desorption and ionization mass spectrometry (TDI-MS) to analyze drugs directly in a single bristle. In this method, single hairs are placed on a heated metal ceramic heater, and then a high-voltage direct current and solvent are applied. The xenobiotics in the hair are thermally desorbed and then ionized, transferred to the MS inlet and detected. Typical hair analysis can be done in minutes [77].

Other studies have shown that the DI-SPME/LC-TOFMS method (Direct immersion-solid phase microextraction/LC-TOFMS) is just as fast and effective. It allows simplifying of the labor-intensive and usually complicated process of isolating analytes from a complex biological matrix. This method is suitable for quantitative analyses and SPME fibers can be used repeatedly as no damage is observed after cleaning and conditioning [78].

Another example of a single hair analysis is the laser desorption-assisted ionization mass spectrometry imaging technique (MALDI-MSI). This method proved to be a suitable tool for the analysis of drugs and their metabolites in biological samples [79]. Direct real-time analysis (DART) has also been shown to be a useful technique for rapid screening of drugs of abuse (DOA) in hair samples with minimal sample preparation [80].

Due to the emergence of innovative analytical methods, the amount of hair in the tested samples, as well as the analysis time, is significantly reduced.

Table 3. Recommended substances and maximum cut-off concentrations for confirmation tests in urine, oral fluid and hair. Guidelines for drug testing published by the Society for Toxicological and Forensic Chemistry [81].

Drug Name	Abbreviation	Cut off Level ng/mL	Approximate Urine Detection Times	Approximate Saliva Detection Times	Cut off Level in Hair ng/mg
Amphetamine	AMP	1000	2–4 Days	1–3 Days	0.2
Barbiturates	BAR	300	3–10 Days	1–2 Days	
Benzodiazepine	BZO	300	1–14 Days	1–2 Days	0.05
Buprenorphine	BUP	10	3–5+ Days	1–2 Days	0.01
Cocaine	COC	150	1–7 Days	1–2 Days	0.5
Ecstasy	MDMA	500	1–3 Days	1–2 Days	0.2
Ethyl Glucuronide	EtG	500	1–3 Days	6–24 h	0.3
Fentanyl	FEN	50 or 100	1–4 Days	1–3 Days	
Heroin metabolite	6-MAM 6-AM	10	1–2 Days	8 h	0.2
Marijuana Tetrahydrocannabinol	THC	50	3–30 Days	6–12 h	0.0002
Methadone Metabolite	EDDP	300	1–7 Days	1–2 Days	0.05
Methadone	MTD	300	1–7 Days	1–2 Days	0.2
Methamphetamine	mAMP, MET	500	1–4 Days	1–3 Days	0.2
Morphine	MOP, MOR, MOP-300	300	1–3 Days	1–2 Days	0.2
Nicotine Metabolite	COT	200	2–4 Days	2–4 Days	
Opiates	OPI MOP NOR	2000	1–3 Days	1–2 Days	0.2
Oxycodone	OXY	100	1–3 Days	1–2 Days	
Phencyclidine	PCP	25	2–30 Days	1–2 Days	
Propoxyphene	PPX	300	1–7 Days	1–2 Days	
Tramadol	TRA	100	13 Days	1–2 Days	
Tricyclic Antidepressants	TCA	1000 ng/mL	1–14 Days *	1–2 Days	

* detection times depend on specific substance, dose, frequency of use and body parameters.

2.3.3. Unusual Matrices for Special Toxicological Cases

Meconium is the first stool excreted by a newborn. Meconium begins to form around weeks 1–12 of pregnancy and is a very complex matrix whose contents mainly come from the amniotic fluid collected by the fetus. The main advantage of meconium as a biological matrix is a wide measuring window. In addition, the technique is non-invasive and easy, and provides a large amount of sample compared to the amount required for the analysis [6]. Nevertheless, like all complex matrices, meconium requires extensive preanalytical processing to minimize matrix interference and improve detection potential for interesting analytes [82]. The subject of toxicological studies is the assessment of fetal exposure to alcohol and drugs [83]. In meconium, alcohol metabolites EtG or FAEE (fatty acid ethyl esters) can be detected. FAEE do not cross the placenta and are, therefore, found in meconium only if produced by the fetus from ethanol that has passed through the placenta, which reflects the actual fetal alcohol exposure [84]. Other biomarkers are used to identify fetal exposure to harmful chemicals: tobacco (nicotine and cotinine), cocaine, cannabis (THC-COOH), amphetamine, *p*-hydroxyamphetamine, methamphetamine, heroin, morphine, and codeine [85]. Meconium is a very interesting matrix, but more research is still needed to be able to unambiguously relate the concentration of the drug from meconium to the degree of exposure.

In most cases, blood remains the reference matrix that provides the best toxicological profile of the victim at the time of death. However, blood samples may be limited, unavailable, or heavily denatured. In such cases, matrices alternative to blood, such as vitreous humor (VH), bile, liver tissue or bone marrow (BM), become particularly useful and important materials for toxicological analyses. Bone marrow analysis is especially important because the qualitative interpretation of BM analysis is well established for many xenobiotics [86]. Another alternative specimen recently studied is earwax. Literature reports show that cannabinoids, benzodiazepines, antiepileptic and antipsychotics drugs can be detected in earwax [4]. Its collection is minimally invasive and the detection window is longer than that of urine. Another example of an alternative matrix is synovial fluid obtained mainly from the knee joints. It has been proposed as an alternative matrix for drug/metabolite testing, such as morphine, codeine, cocaine, 6-MAM, benzoylecgonine and ecgonine methyl ester [87].

2.4. Surfactants in Forensic Toxicology—A New Analytical Challenge

The presence of permanent charge in a molecule makes it a preferred object for mass spectrometry analysis. If the charge is accompanied by more hydrophobic structural elements, certain physicochemical properties are expected for compounds consisting of polar and nonpolar fragments. Nowadays, surfactants are found in numerous products, from personal hygiene to industrial materials [88]. The broad distribution and structural verisimilitude of these compounds has resulted in the demand for efficient analytical methods for their identification and quantification. Surfactants have been studied as environmental pollutants of human origin [89]. However, toxicological cases of detergent poisoning have drawn attention to their biological activity.

Anionic surfactants, mostly alkyl- and alkylbenzene-sulfonates affect living organisms directly, for example, causing skin irritation (lauryl sulfates), but also increase the solubility of hydrophobic compounds, affecting their bioavailability and potential toxicity. The antimicrobial activity of quaternary ammonium compounds explains their frequent application as efficient disinfectants [90]. The increase in demand for strong cleaning products for both medical and domestic use has resulted in higher environmental impact of quaternary salts, growing bacterial resistance and increased risk of accidental contamination and consumption [91]. At the same time, the development of drug and gene nanocarriers with more biocompatible cationic surfactants requires adequate analytical methods [92].

2.4.1. Mass Spectrometry in Surfactant Analysis

The procedures used in surfactant analysis include electrochemical methods, involving ion-selective electrodes, infrared and UV-vis spectroscopy, and color tests and histochemical examination [93–95]. The complex matrices usually require sample preparation and analyte separation before detection. Capillary electrophoresis, as well as gas and liquid chromatography, are used in surfactant analysis [96,97], with a wide range of detectors reported for various applications, including mass spectrometry.

Mass spectrometry is frequently used to study surfactants and products of their degradation [7], due to characterization of molecules by high resolution m/z value (HRMS) and structural information from tandem methods, low detection limits and the benefit of analysis of polydisperse materials even with limited separation. Therefore, mass spectrometry is a method of choice in environmental studies and, with growing impact, in biological systems, especially clinical and forensic toxicology [98,99]. Anionic and cationic surfactants are ideally suited for soft ionization methods in mass spectrometry, with the analysis of $[M]^+$ and $[M]^-$ ions in positive or negative ion modes, respectively, whereas the non-ionic surfactants could be identified as $[M + H]^+$, $[M + NH_4]^+$ or $[M + Na]^+$ ions [100].

There are reports of technical problems associated with LCMS surfactant analysis. Clusters resembling micelles or reversed micelles may affect chromatographic separation, and moreover, studies on gas phase aggregation in ESI-MS and MALDI-MS indicate the formation of aggregates in vapor phase before the high vacuum stage of MS [101,102]. The application of supercritical fluid chromatography (SFC) may ameliorate some of the separation problems and environmental concerns related to HPLC [103,104].

In Figure 5 we present the example of LCMS analysis of ionic surfactants to visualize the discussed problems. The sample of a shampoo was separated using reversed phase HPLC column in water-acetonitrile gradient. The mobile phase was acidified with formic acid (0.1%), which facilitated the zwitterionic betaine detection (panel A) in positive ion mode, and simplified the charge of sulfosuccinate. Although mass spectra confirm the presence of the expected compounds, the chromatogram indicates unwelcome interactions with stationary phase.

Polydisperse materials create additional problems during analysis. The declared composition usually represents the average values of monomer numbers, affecting characterization procedures. Mass spectrometry, especially combined with adequate separation (liquid chromatography in normal or reversed phase mode, size exclusion chromatography) could resolve the series composition. A constant difference between MS signals usually suggests the polymeric nature of the substance, and in high resolution spectra the charge of ions could be calculated, pointing at the mer composition. However, the analysis of the spectra requires a combinatorial approach, as different charges and ionization schemes could be detected. Figure 6 shows an example of polyethylene and polypropylene glycol copolymer (PEG/PPG) analysis, performed in our research group. The mass spectrum was obtained by averaging scans from 0.1 min chromatogram time range. Two series of $[M + 2H]^{2+}$ ions could be identified, as a combination of 5 PPG units with 8–20 PEG units (green) and 4 PPG units combined with 17–29 PEG units (red). It was interesting that the declared composition was PEG/PPG-17/6. To complicate the issue, in other scans different combinations of monomers as well as ions, formed by protonation and sodium ion capture, were observed.

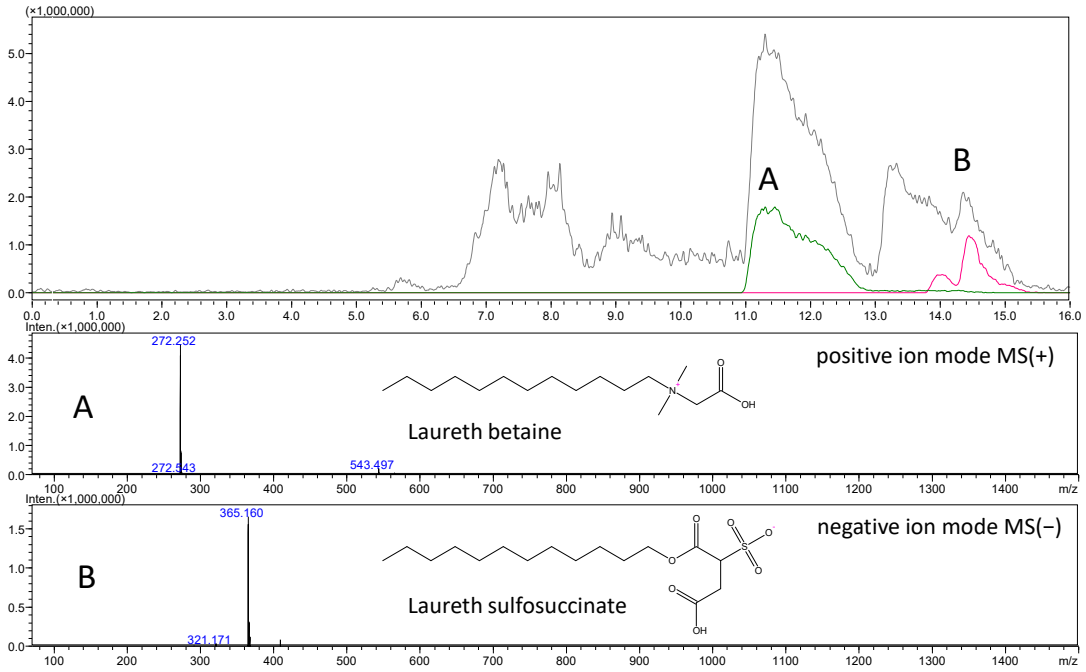


Figure 5. LCMS chromatogram of cosmetic hair product. Top panel: gray line indicates total ion current (TIC), whereas green line (A) represents extracted ion chromatogram (XIC) for laureth betaine $[M]^+$ m/z = 272.252, red line (B) represents XIC for laureth sulfosuccinate $[M - H]^-$ m/z = 365.163. Mass spectra corresponding to the indicated peaks are shown in the panels. LC-ESI-MS system (Shimadzu LC-IT-TOF) was used.

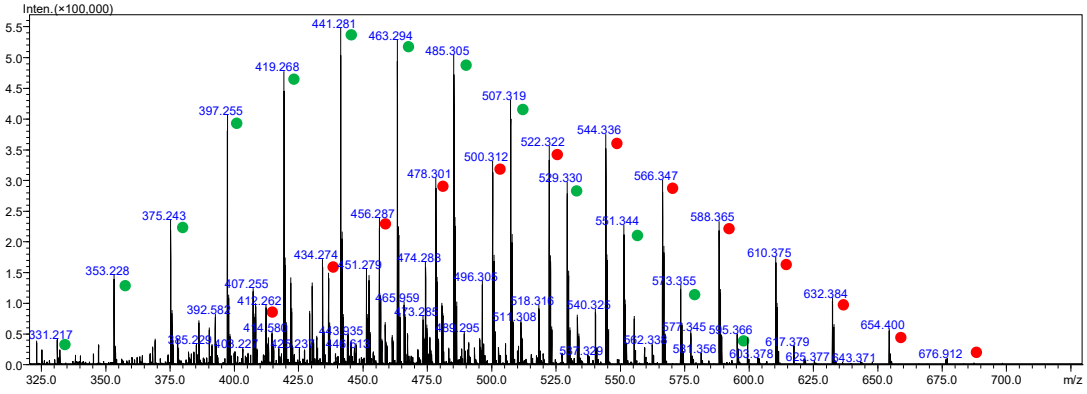


Figure 6. Mass spectrum averaged for 0.1 min time period of LCMS chromatogram of facial moisturizing toner, containing PEG/PPG-17/6 copolymer. Green dots indicate a series of protonated ions $[M + 2H]^{2+}$ consisting of 5 PPG units and 8–20 PEG units (PEG/PPG (8–20)/5), whereas red dots indicate PEG/PPG (17–29)/4. LC-ESI-MS system (Shimadzu LC-IT-TOF) was used.

2.4.2. Surfactants in Forensic Cases

Although the ingestion of anionic or non-ionic surfactants is not deemed dangerous, in certain cases the result could be fatal. According to a report by Hitosugi et al. [105], polyoxyethylene 9-lauryl ether from air freshener was detected in serum and stomach contents of an elderly person with a history of cardiac problems after acute death related to the product's ingestion. The analysis was performed using LCMS and spectra typical for polydisperse materials were recorded, with the characteristic 44 difference between signals, typical for polyoxoethylene materials, detected.

Another reported fatality resulted from ingestion of an agrochemical spreading agent containing nonionic nonylphenol ethoxylates and anionic sulfonated naphthalene-formaldehyde condensates [106]. Attention was also drawn to adulteration of infant milk

formula by household detergents [107]. The studies were directed at reducing the complexity of matrix by extraction and selection of representative surfactant dodecylbenzenesulfonate (C12-LAS) for screening of products. Selected structures of studied surfactants are presented in Figure 7.

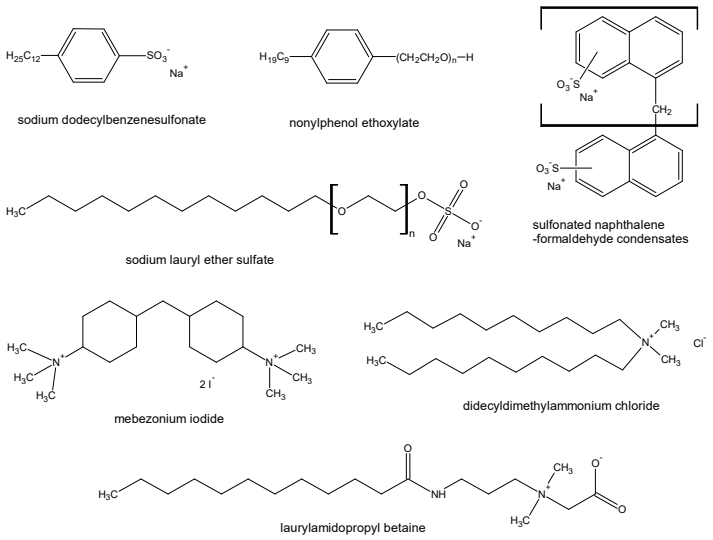


Figure 7. Structures of selected surfactants.

Cationic surfactants cause more concern due to biological activity of several quaternary ammonium compounds. Mebezonium iodide is a competitive acetylcholine inhibitor, used in veterinary euthanasia drug T61. Analytical procedures developed for succinylcholine [108] were applied in the case of T61-related suicide [109]. The main problems including hydrophilicity and lack of UV absorbance were countered with SPE on C18 cartridges with heptafluorobutyric acid counterion (HFBA) and LCMS/MS detection. Didecyldimethylammonium chloride (DDAC), used in disinfectants for medical instruments and surfaces, was one of biocides studied in hospital sewage system in Poitiers (France) in 2016 and 2017, using UHPLC-ESI-MS [90].

The deposition of two common components of household washing products, sodium lauryl ether sulfate (SLES) and laurylamidopropyl betaine (LAPB) on human skin was studied using LC-ESI-MS/MS. These surfactants require different approaches for efficient detection in MS, i.e., highly acidic conditions for full protonation of amphotheric LAPB and higher pH for stable ammonium adduct of SLES [110].

The current methods for surfactant analysis and the problems resulting from polydispersion of commercial products were discussed in a study on fingerprinting of coceth sulfates in three commercial biodegradable detergents [111]. The direct MS analysis of these sulfate esters of the polyethylene glycol ethers of coconut alcohols confirmed differences in ionization preferences in homolog series.

It is worth adding that mass spectrometry imaging could be used in analysis of surfactants in tissues. The surfactant replacement therapy in lambs with Respiratory Distress Syndrome (RDS) was studied by MALDI imaging after administration of phospholipids and analogs of hydrophobic surfactant proteins (SPs). Both qualitative and quantitative results were obtained, correlating with pulmonary physiological outcomes [112].

2.5. Derivatization as a Tool in Mass Spectrometry Analysis in Forensic Chemistry

Liquid chromatography coupled with mass spectrometry (LCMS) is recognized as a "golden standard" for both qualitative and quantitative analysis [27]. It is capable of separating analytes (LC) and measuring mass-to-charge ratio with high selectivity and sensitivity (MS). The choice of a widely applicable ionization technique, such as electrospray ionization (ESI), also affects the versatility of this method [113]. However, poor ionizability,

low ESI-MS response, and decomposition in the ion source or during the separation, can negatively impact LCMS analysis. Sometimes problems occur due to lack of an ionizable functional group, rigid structure or high polarity of compounds, as well as matrix effects that influence ionization efficiency [114].

It is possible to improve LCMS analysis performance by conducting chemical derivatization. The chemical labeling reaction relies on adding a derivatizing reagent to the analyte, which contains in its structure a marker part (chromophore, fluorophore or a charge tag) and an active fragment through which the connection with the analyte occurs. As a result of the performed reaction, a derivative is formed containing a group in its structure that gives an analytical signal compatible with the detector [115].

A good derivatizing reagent should be characterized by high selectivity, as it must produce derivatives only with one compound or compounds with a selected functional group. The reaction itself should be efficient and conducted in mild conditions, and the obtained product should produce a linear detector response with increase in concentration [115].

Although different analytical methods (HPLC, GC, MS) have different requirements towards derivatization, the reagents can be divided according to their properties [115]:

- compounds absorbing light in the UV-Vis range, introducing chromophores into the molecule;
- fluorogenic reagents, compounds which do not exhibit fluorescence on their own, but they acquire this ability under the influence of reactions with appropriate compounds;
- fluorescent reagents introducing fluorophores;
- reagents capable of redox reactions;
- reagents that introduce a permanent charge to the molecule

The derivatization reaction may be performed before or during the analysis of the test sample. When discussing this process in relation to liquid chromatography, we can consider pre-column, column and post-column derivatization [115]. In forensic analysis and toxicology, low sample concentration and poor analyte ionizability are among the biggest obstacles, therefore many new derivatizing reagents are developed.

2.5.1. Derivatization in Cyanide Analysis

Cyanide is a well-known and highly toxic compound that is used as a poison and a chemical warfare agent. Nowadays, it is present in many industrial areas, for example smelting, mining or plastic production, which may result in environmental pollution. Whereas high dosage poisonings are relatively easy to detect, chronic poisoning resulting from trace level contamination can be more challenging. Additionally, poor retention in chromatography and low mass mean it is difficult to detect the cyanide ions directly [116]. Moreover, in the human body cyanide ions undergo various transformations and can be stored or dissolved in blood, at different concentrations in specific parts of the circulatory system. Derivatization can solve problems of cyanide detection [3].

Giebułtowicz et al. [3] took advantage of cyanide ion conversion into 2-aminothiazoline-4-carboxylic acid (ATCA) and used it as a marker of cyanide exposure. Unfortunately, the analysis of unmodified samples showed many interferences in proximity to ATCA signal. By derivatizing the matrix components with *N*-(4-nitrophenoxycarbonyl)-L-phenylalanine-2-methoxyethyl ester (S-NIFE) and 5-(dimethylamino)naphthalene-1-sulfonyl chloride (DNS) (Figure 8) they were able to purify the sample, and develop and validate a quantitative method of ATCA analysis in postmortem blood.

Another example of cyanide ion derivatization is presented by Madmon et al. [116]. They used *N*-(2-(bromomethyl)benzyl)-*N*,*N*-diethylethanaminium bromide (CAX-B) as a derivatizing reagent to develop a method for analysis of a low concentration of free cyanide ions in water. The reaction (Figure 9) was conducted without previous purification. The limit of detection (LOD) was reported at 0.02 ng/mL.

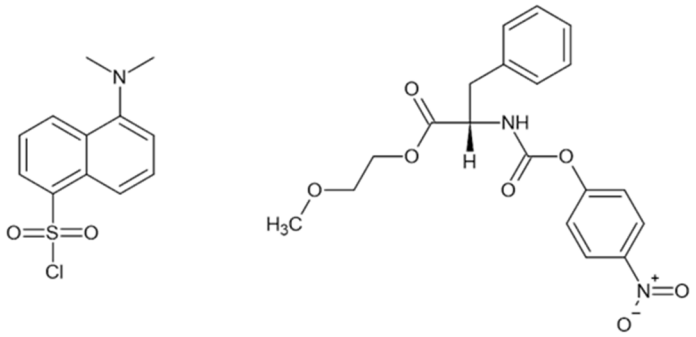


Figure 8. Chemical structure of 5-(dimethylamino)naphthalene-1-sulfonyl chloride (DNS) and *N*-(4-nitrophenoxycarbonyl)-L-phenylalanine-2-methoxyethylester (S-NIFE).

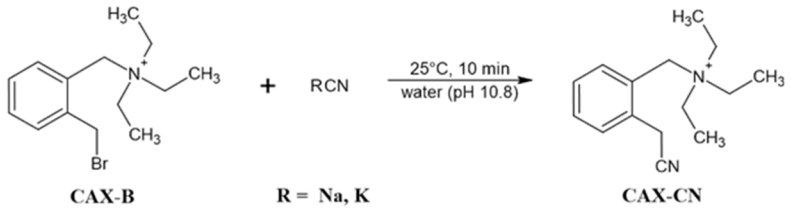


Figure 9. Reaction scheme of cyanide derivatization by *N*-(2-(bromomethyl)benzyl)-*N*,*N*-diethylethanaminium bromide (CAX-B).

2.5.2. Derivatization of Propofol

Among hypnotic agents used in anesthesia, propofol is favorable, due to its mild side effects, causing lower induction of nausea, vomiting and postoperative drowsiness. However, usage of propofol can lead to developing dependence and nonmedical use, which can then lead to overdosing. Propofol influences the central nervous and respiratory systems and overdose can result in respiratory failure, cardiac arrythmia or death. Due to the ecstatic and euphoric feeling it provides, propofol can be consumed occasionally as a recreational drug, adding importance to development of a fast and selective analysis method [117].

Due to its chemical properties (low polarity and volatility) propofol is difficult to ionize in an electrospray ion source (ESI) in both positive and negative ion modes. Vaiano et al. [117] tried to improve ionization and detection of propofol by using a new type of derivatization agent–diazonium salts. They used azo coupling reaction to attach aniline based diazonium salt to propofol (Figure 10), which resulted in retention time shift and improvement in ionization yields, both for positive and negative MS ion mode. A quantitative method of propofol analysis was developed using this ionization yield improvement, with pg/mL concentrations in urine and ng/mL in blood. In 2017 Vaiano applied this derivatization method to detecting propofol in hair [118].

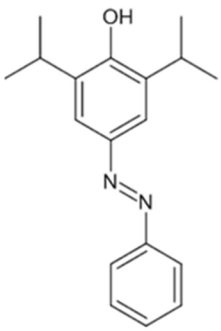


Figure 10. Chemical structure of the product of propofol derivatization using azo-coupling reaction.

2.5.3. Derivatization of Carbonyl Compounds

Compounds containing a carbonyl group are widely present in organisms as metabolites of multiple pathways, such as the citric acid cycle, glycolysis and fatty acid oxidation, resulting in production of aldehydes, ketones, keto acids and other carbonyl compounds [119]. Ingestion of exogenous substances, including some performance-enhancing drugs [120], psychoactive substances, cathinone and cathinone derivatives, also delivers chemicals with carbonyl moiety into organisms [121]. Carbonyl compounds constitute a large group, highly diverse in their physical and chemical properties. The differences in their solubility, stability, volatility and concentrations pose a great challenge in quantitative analysis. Therefore, many new derivatizing agents have been developed [119]. Additionally, Sun et al. investigated differences between pre-column and post-column derivatization in LCMS analysis [119]. In Table 4, some examples of reagents used to derivatize carbonyl compounds are presented.

Table 4. Derivatizing reagents, their structure and targets.

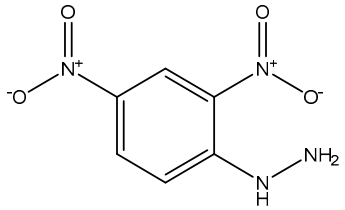
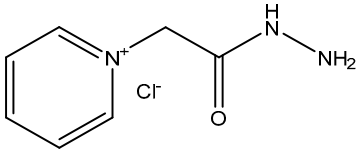
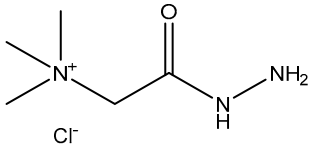
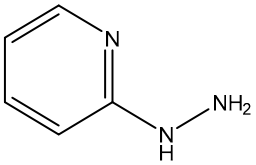
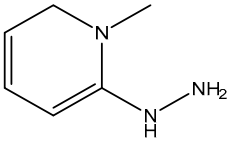
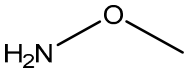
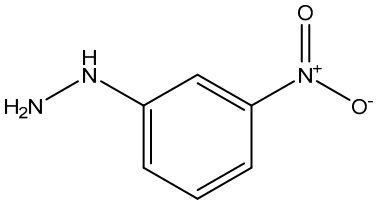
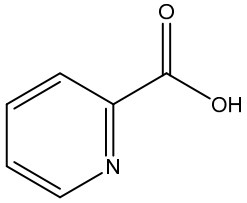
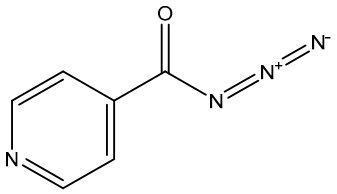
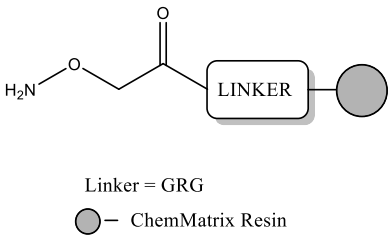
No.	Name	Chemical Structure	Application	Reference
1	2,4-Dinitrophenylhydrazine		Carbonyl compounds	[119]
2	Girard P reagent		Steroids	[119]
3	Girard T reagent		Ketosteroids	[120]
4	2-Hydrazino pyridin		Ketosteroids	[120]
5	2-Hydrazino-1-methyl pyridin		Neurosteroids Ketosteroids	[119,120]
6	Methoxyamine		Ketosteroids	[119,120]
7	3-nitrophenylhydrazine		Malondi-aldehyde	[119]

Table 4. *Cont.*

No.	Name	Chemical Structure	Application	Reference
8	Picolinic acid		Steroids hormones	[120]
9	Isonicotinoyl azide		Dihydroxy-steroids	[120]
10	AOA-GRG-CMRR		Steroids	[121]

2.5.4. Derivatization in Analysis of Neurotoxins

Sarin, Soman, Cyclosarin and Tabun are G-type nerve chemical warfare agents. They are potent neurotoxins that bind to acetylcholinesterase (AChE) blocking its activity, manifesting in devastating SLUDGE syndrome, which can result in death. Sarin is the most frequently used, as well as the most reactive of all G-type nerve agents. In general, G-type nerve agents are highly unstable and bind to AChE and other proteins leaving only a small amount of non-bound molecules in blood. Aviram et al. [122] tried to develop a new method for the analysis of trace amounts of free G-type nerve agents from dried blood spots (DBS). The derivatization reaction was conducted directly on DBS and then product was extracted and analyzed by LCMS. The 2-((dimethylamine)methyl)phenol (2-DMAMP) was used as a derivatizing reagent (Figure 11). It was discovered that the best method of derivatization was to prime DBS paper with 2-DMAMP first and then apply the blood sample. That approach not only resulted in good yield of derivatized free G-type nerve agent, but also allowed storage of the sample for up to 19 days at room temperature.

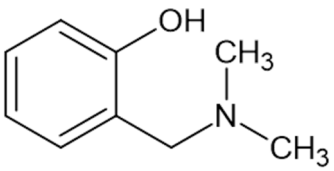


Figure 11. Chemical structure of 2-((dimethylamine)methyl)phenol (2-DMAMP).

2-DMAMP was also used for detection and identification of G-type nerve agent traces in environmental matrices. During their research, Weissberg et al. tested various solvents, obtaining the best results for water. This method was used to analyze environmental samples exposed to G-type nerve agents, such as asphalt, linoleum, cloth and concrete, which resulted in LOD between 0.8 and 20 pg/cm^2. The other sample was earth, in which they detected nerve agents on a 4 pg/g level [123].

Shellfish and seafood are popular foods that can be contaminated by ingesting diatoms. The contamination may result in the presence of domoic acid–a natural neurotoxin found in

Pseudo-nitzschia (of diatom genus) (Figure 12). Consumption of the contaminated food can cause acute intoxication or even death, therefore the upper limit of domoic acid in shellfish has been established at 20 mg/kg of edible tissue. Beach et al. [124] developed a method of qualitative analysis of domoic acid by derivatization with dansyl chloride, which resulted in a 5-fold increase in molar response in MS, as well as improved chromatographic behavior.

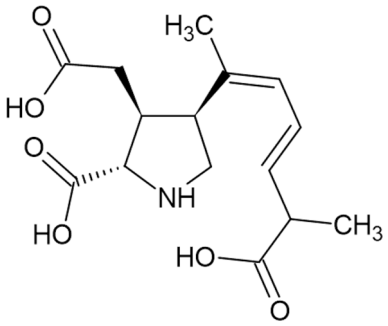


Figure 12. Chemical structure of domoic acid.

2.5.5. Derivatization of Alkaloids: Atropine

Atropine is a tropane alkaloid which occurs naturally in certain plants from the nightshade family (*Solanaceae*), such as belladonna (*Atropa belladona*), jimsonweed (*Datura stramonium*) and henbane (*Hyoscyamus niger*) [125]. It is used in medicine and is on the WHO Model List of Essential Medicines, the most important medications needed in a basic health-care system [126]. While atropine saves life, it is still a health and life-threatening alkaloid [127–130]. Moreover, the Report of the European Centre for Drugs and Drug Addiction (EMCDDA) for 2004–2005 showed that cocaine distributed on the European market contained 28–72% atropine and caused numerous deaths and acute intoxications [131]. For these reasons, atropine identification and quantitation are important in clinical and forensic toxicology.

Several analytical techniques were developed for quantification of atropine in a variety of matrices [132–137]. GCMS is a frequently used method for determination of atropine [137,138]. However, atropine is thermally unstable and could be overlooked due to its degradation, therefore other analytical methods which provide simple, sensitive and selective determination of atropine are needed [138].

Since it is not easy to analyze extremely small quantities of atropine, new tags for selective detection of this compound are required. Recently, we developed new ionization reagents based on quaternary ammonium salt (QAS) that can successfully be attached to the hydroxyl group of atropine [139].

Our previous study evidenced that the introduction of a tertiary amine, such as 1,4-diazabicyclo[2.2.2]octane (DABCO) or 1-azabicyclo[2.2.2]octane (ABCO), to peptides increases ionization efficiency in ESI-MS and lowers the detection limit to low attomole [140,141]. Moreover, our results indicated that QAS-peptides containing ABCO or DABCO groups were more stable during MS/MS experiments [140]. Therefore, we designed and synthesized new ionization tags based on 1,4-diazabicyclo[2.2.2]octane as an ionization enhancer group. Each of our new reagents consisted of 1,4-diazabicyclo[2.2.2]octane moiety combined with a dipeptide or tripeptide linker. As shown in Figure 13, the hydroxyl group of atropine could react with the carboxyl groups of the derivatization reagent to form a covalent compound with EDC and DMAP, used as the coupling agents. The obtained mass spectra revealed the characteristic signal corresponding to the molecular ion of the desired compound.

Our preliminary results clearly indicated the possibility of using the proposed ionization tags for the chemical derivatization of atropine.

The derivatization methods for the LCMS analysis of compounds important in forensic studies have been intensively studied, resulting in procedures for detection of GHB [142], cannabinoids [143,144], amphetamine, methamphetamine [145,146] and other drugs of abuse [147].

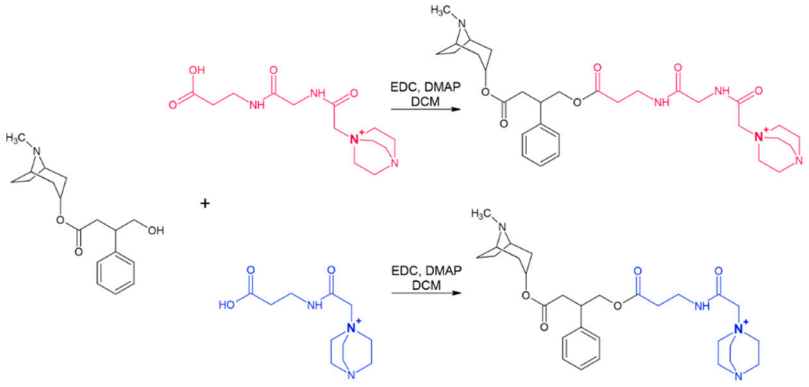


Figure 13. Scheme of derivatization reactions of atropine (DCM, dichloromethane; DMAP, 4-dimethylaminopyridin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide).

2.6. Internal Standards in Quantitative LCMS Analysis in Forensic Toxicology

2.6.1. Isotope Labeling: Hydrogen-Deuterium Exchange

Hydrogen atoms located in the backbone and side-chain functional groups of chemical compounds are easily exchanged with protons from the solvent [148]. In the presence of a deuterating agent, the source of dissociating deuterons, i.e., deuterium oxide (D_2O), these hydrogens can be replaced by a deuterium within a few minutes in a process called hydrogen–deuterium exchange (HDX) [149]. The basic mechanism of this process includes acid–base catalysis, where the reaction rate depends on the pH of the reaction mixture, with the characteristic increase observed with increasing pH. The acidity of the hydrogens subjected to the exchange, as a consequence of electronegativity differences between the hydrogen bounded to the heteroatom, is another factor affecting the HDX. The simplicity of isotopic exchange of heteroatom-bounded hydrogens serves as a tool in the conformation analysis of biologically active compounds. Additionally, the isotope exchange reactions enable the analysis of the mechanisms of chemical processes [150].

Carbon-bounded hydrogens, due to the low electronegativity difference, in most cases do not undergo acid- or base-catalyzed HDX. However, the specific molecular structure and appropriate reaction conditions may promote HDX [151–154]. Additionally, the deuterons connected with carbon centers usually do not undergo back exchange to hydrogens under neutral and slightly acidic conditions. Therefore, such isotopically labeled derivatives may serve as internal standards in quantitative liquid chromatography-mass spectrometry (LCMS) analysis. While the classical methods of deuterium introduction at the carbon atoms are expensive and time-consuming, new, rapid and cost-effective methods of HDX at the carbon atoms of specific groups of chemical compounds seem to be an important issue. Liquid chromatography-mass spectrometry (LCMS) in quantitative mode usually requires isotopically labeled standards which should fulfill certain criteria, including mass shift, chromatographic behavior practically identical to the analyzed compound (co-elution) and stability of the introduced isotopes during the experiment (lack of back-exchange) [155–157]. Quantitative analysis is performed by comparing the peak area of the labeled and non-labeled compound. Commonly used isotopically labeled standards contain ^{2}H, ^{13}C, ^{15}N and/or ^{18}O isotopes, which are introduced using de novo synthesis. The synthetic strategies used in the preparation of isotopically labeled standards are usually complicated and expensive, therefore the development of new strategies is required. Moreover, the commercially available popular deuterated standards may affect co-elution during LC-MS, due to the isotope effect depending on the number of introduced deuterons and part of the molecule where they are located [158–161]. The methods of deuterium incorporation into the target molecules, besides the de novo synthesis, may involve pH dependent and metal-catalyzed HDX reactions at the carbon centers. Although the incorporation of deuterium into the target molecules may present some drawbacks, nevertheless, the advantage of HDX procedure is its low cost and simple preparation procedure in comparison to the previously

developed techniques. Therefore, deuterated standards have found numerous applications in quantitative LCMS analysis of different drugs in forensic toxicology (Table 5).

Table 5. Selected examples of deuterated internal standards used in forensic toxicology for quantitative LC-MS analysis of various drugs.

No.	Internal Standard	Analyzed Compounds	Sample	Reference
1	morphine-d_3	morphine, morphine-3-glucuronide, morphine-6-glucuronide, and 6-monoacetylmorphine	urine	[162,163]
2	morphine-d_3, codeine-d_3	morphine, codeine	urine	[164]
3	morphine-d_3, morphine-6-glucuronide-d_3	morphine, morphine-3-glucuronide, morphine-6-glucuronide, and	plasma	[165]
4	cocaine-d_3 benzoylecgonine-d_3 ecgonine methyl ester-d_3	cocaine and its metabolites	-	[166]
5	benzoylecgonine-d_3 norcocaine-d_5	cocaine and its metabolites	blood spots	[167]
6	cocaine-d_5 benzoylecgonine-d_3, ecgonine methyl ester-d_3	cocaine and its metabolites	plasma	[168]
7	THC-COOH-d_3	THC-COOH	urine	[169]
8	methamphetamine-d_{10}, amphetamine-d_{11}, 3,4-methylenedioxyethyl-amphetamine-d_5, 3,4-methylenedioxymethyl-amphetamine-d5	Amphetamine, methamphetamine, illicit designer phenethylamines	serum	[170,171]
9	morphine-d_3, morphine-3-glucuronide-d_3, morphine-6-glucuronide-d_6, codeine-d_6, codeine-6-glucuronide-d_3, cocaine-d_8, benzoylecgonine-d_8	morphine, morphine-3-glucuronide, morphine-6-glucuronide, 6-monoacetylmorphine, codeine, codeine-6-glucuronide, dihydrocodeine, dihydromorphine, buprenorphine, methadone, tramadol, ibogaine, cocaine, benzoylecgonine ecgonine methyl ester lysergic acid diethylamide	serum, blood, urine	[172]

2.6.2. Isotopically Labeled Standards: Glycine Deerivatives

In our previous work, we described the possibility of hydrogen–deuterium exchange at the alpha carbon atom (α-C) in *N*-methylglycine (sarcosine) and *N*-benzylglycine residues in peptides under basic conditions at room temperature [173]. The obtained data confirmed that the rate of the observed exchange reaction was much slower compared to the HDX of heteroatom-bounded hydrogens. This work was a milestone in our research on the possibility of isotopically labeled standards preparation. In our further research we developed methods of deuterated standard preparation of cyclosporine A (cyclosporine A-d_3) [174], creatinine (creatinine-d_2) [175] and other *N*-substituted glycine derivatives [176].

2.6.3. Denatonium Benzoate

In 2015 we developed a method of denatonium benzoate (Bitrex) deuterium labeling [177]. Bitrex, (*N*,*N*-diethyl-*N*-[(2,6-dimethylphenylcarbamoyl)methyl]benzylammonium benzoate), is the most bitter compound, which found application in the denaturation of industrial alcohols and in making potentially harmful household products extremely unpalatable [178]. It is an important aversive agent used in prevention of accidental consumption of liquids, such as cleaning agents, windshield washer fluids, disinfectants and horticultural products. The European Union Regulations specify the level of Bitrex in several formulations of denatured alcohol to be 1 g per hectoliter of absolute ethanol. However, in some Member States, the amount of added Bitrex is even up to 2 g [179]. The procedure of deuterated Bitrex analog preparation, developed by us, is based on the H/D exchange process of hydrogen atoms in the CH_2 group situated between the carbonyl

group and the quaternary ammonium group. The complete deuteration occurs at room temperature under basic conditions (1% solution of *N*,*N*,*N*-triethylamine in D_2O, Figure 14). We found that the introduced deuterons do not undergo back exchange under acidic and neutral conditions and that the isotopologues co-elute. The developed deuterated Bitrex standard was successfully applied in the quantitative LCMS analysis of Bitrex in several Bitrex-containing household products, including alcoholic thinner and winter/summer windscreen washer fluids.

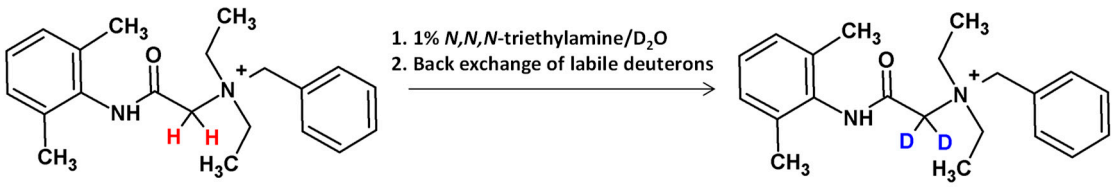


Figure 14. Preparation of deuterated Bitrex standard for quantitative analysis by mass spectrometry.

The level of detected denatonium cations in the prepared samples was determined by the isotopic distribution observed in the ESI-MS spectra according to the algorithm described by Mirgorodskaya and co-workers [180]. In this algorithm, the relative concentrations of the non-deuterated sample and isotopically labeled standard can be determined by comparing the isotopic distributions of "natural" and labeled compounds with the isotopic pattern measured for an analyzed sample containing a known amount of deuterated standard. It should also be mentioned that the cost of preparation of 10 mg of the proposed deuterium labeled standard is about 10 EUR, taking into account the costs of the reagents needed for its preparation, which, compared to the price of commercially available denatonium d_5 benzoate (480 € for 10 mg), allows a significant reduction in the costs of analysis.

2.6.4. Tadalafil and Racetams

Nowadays, our investigations concentrate on the development of the preparation methods of deuterated analogs of compounds, including tadalafil, racetams, modafinil, omeprazole, and their metabolites and analogs. Tadalafil (Figure 15) presents several side effects, including cardiovascular disorders, headaches, dyspepsia, and retinal disturbance. Additionally, the toxicity or pharmacological safety profile of its analogs is less specified and poses a significant risk to the public health. They were found in some herbal dietary supplements for improved sexual performance [181,182]. As was mentioned in chapter 2.1, illicit products containing phosphodiesterase type 5 inhibitors and their analogs create social problems, such as increasing sexual crimes and black markets (both on the internet and offline), due to their high demand as an alternative to erectile dysfunction drugs that require prescriptions. Therefore, breakthrough analytical techniques that can detect potential tadalafil analogs with updated simultaneous analysis and identification are needed. Our preliminary results clearly indicated the possibility of exchange of three hydrogens to deuterons in the presence of the source of deuterium and a catalyst (Figure 15).

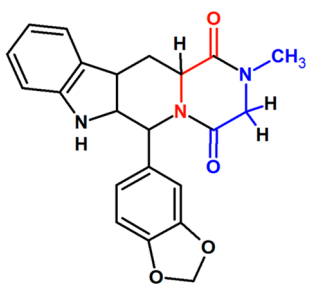


Figure 15. Schematic presentation of tadalafil. *N*-methylglycine moiety with α-C hydrogen atom marked in blue, *N*-substituted glycine moiety marked in red.

Racetams are a class of compounds belonging to the nootropic drugs, also known as cognitive enhancers, neuroenhancers or, only recently, 'smart drugs'/They were initially designed to be used in the treatment of gerontopsychiatric patients, as their main function is memory improvement [183,184]. Over the years, several racetam derivatives have been developed, including piracetam [185], phenylpiracetam [186], methylphenylpiracetam, coluracetam [187], pramiracetam [188] and oxiracetam. Nowadays, racetams are misused by students as 'brain doping' substances. Hence, it is reported that their prevalence among European students was in the range of 1–16%, while in the United States it reached even up to 35% [183]. The media attention on racetams has increased within the last few years. The drugs have developed an underground following and are commonly sold online and in illicit supply chains. Most have not been approved or scheduled in the US and are therefore of concern to regulators, such as the Food and Drug Administration (FDA) and Drug Enforcement Administration (DEA). Some of them are also prohibited by the World Anti-Doping Agency (WADA), due to the presented side effects (i.e., nephrotoxicity, increase of creatinine level). Therefore, there is a strong need for the development of a method for their qualitative and quantitative analysis. Additionally, racetams are excreted in the urine mostly as I-phase metabolites containing sarcosine moiety. Our preliminary results clearly indicated the possibility of exchange of two hydrogens to deuterons in the presence of the source of deuterium and a catalyst (Figure 16).

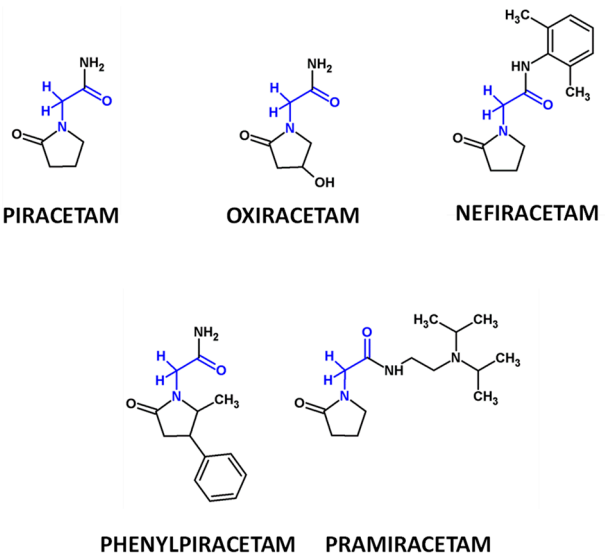


Figure 16. Schematic presentation of different racetams. *N*-substituted glycine moiety with α-C hydrogen atoms able to undergo hydrogen-deuterium exchange under basic conditions is marked in blue.

2.6.5. Armodafinil

Armodafinil is the R-enantiomer of modafinil (diphenylmethylsulfinylacetamide), a wake promoting agent that is pharmacologically distinct from CNS stimulants, such as amphetamine, dexamphetamine and methylphenidate. It is approved for use in the US and certain European countries in patients with excessive sleepiness associated with obstructive sleep apnea/hypopnea syndrome (OSA) (despite treatment of the underlying condition), narcolepsy or shift work sleep disorder (SWSD) [189,190]. Armodafinil and/or its major metabolite, modafinil acid, may be quantified in plasma, serum or urine to monitor dosage in those receiving the drug therapeutically, to confirm a diagnosis of poisoning in hospitalized patients or to assist in the forensic investigation of a vehicular traffic violation. Instrumental techniques involving gas or liquid chromatography are usually employed for these purposes. As of 2011, it is not specifically tested for by common drug screens (except for anti-doping screens) and is unlikely to cause false positives for other chemically unrelated drugs, such as substituted amphetamines [191]. The regulation of modafinil

as a doping agent has been controversial in the sporting world, with high profile cases attracting press coverage, since several prominent American athletes have tested positive for the substance. Some athletes who were found to have used modafinil protested that the drug was not on the prohibited list at the time of their offenses. However, WADA maintains that it was related to already banned substances. The Agency added modafinil to its list of prohibited substances on 3 August 2004 [192]. Armodafinil has been used non-medically as a "smart drug" by students, office workers, soldiers and transhumanists [193,194]. As a 'smart drug' it allegedly increases mental focus and helps evade sleep, properties which attract students, professionals in the corporate and technology fields, air force personnel, surgeons, truck drivers and call-center workers. In contrast, when more complex assessments are used, modafinil appears to consistently stimulate enhancement of attention, executive functions, and learning. Importantly, no preponderances for side effects or mood changes were observed [195]. A 2019 review of studies of a single dose of modafinil on mental function in healthy, non-sleep deprived people found a statistically significant but small effect and concluded that the drug has limited usefulness as a cognitive enhancer in non-sleep deprived persons [196]. A 2020 review concluded that users' perceptions that modafinil is an effective cognitive enhancer is not supported by the evidence in healthy non-sleep deprived adults [197].

In our previous study [198] we demonstrated the possibility of deuterated armodafinil derivative preparation, based on the base-catalyzed hydrogen–deuterium exchange at the carbon atom (Figure 17). Applied reaction conditions allowed the introduction of three deuterons which did not undergo back-exchange under neutral and acidic conditions. Additionally, we found that deuterated and non-deuterated armodafinil isotopologues co-eluted during LCMS analysis. The prepared armodafinil-d_3 was used in the LCMS quantification of armodafinil in human urine samples. The obtained data were in agreement with the known pharmacokinetic profile of armodafinil. The proposed method of armodafinil deuteration is simple, rapid and cost-efficient, which makes it a novel tool for researchers, clinicians and forensic scientists working on the improvement of diagnostic accuracy and quantitative forensic LCMS investigation of armodafinil.

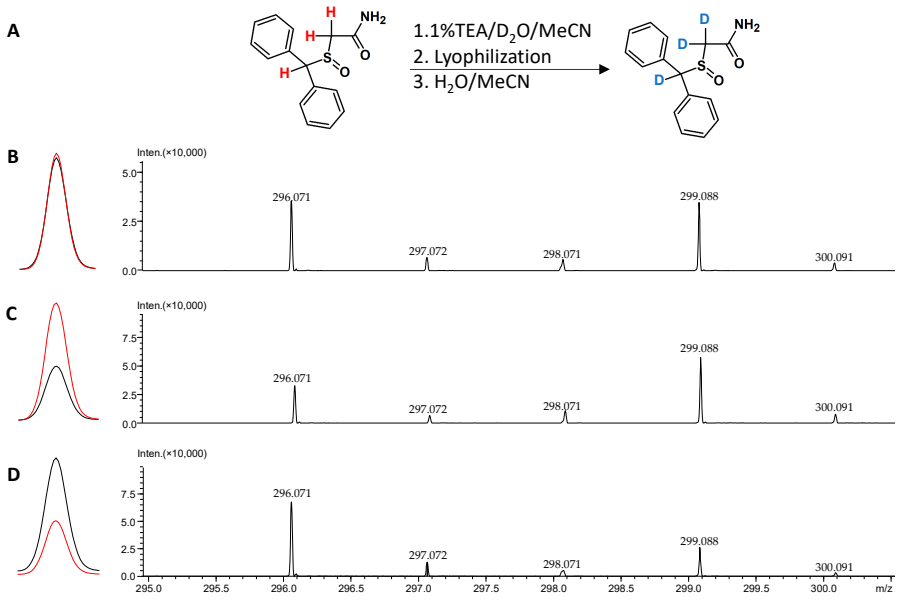


Figure 17. Schematic presentation of deuterated armodafinil preparation (**A**) and the extracted ion chromatograms with corresponding fragments of mass spectra of the analyzed samples containing armodafinil (black line) and armodafinil-d_3 (red line) mixed in (**B**) 1:1 ratio (**C**) 1:2 ratio, (**D**) 2:1 ratio [198].

It may be expected that HDX as a source of affordable references may provide new insights into the qualitative and quantitative analysis of these compounds, creating a novel tool for researchers, clinicians and forensic scientists working on improvement of diagnostic accuracy, evaluation of treatment efficacy, early diagnosis of disease and forensic investigation.

3. Conclusions

Our review clearly indicates that mass spectrometry is very useful in forensic studies. The full advantage of MS is revealed in its combination with separation methods. Both gas and liquid chromatography could be employed, providing fractionation of complex samples and benefits origination from retention time analysis. The requirements of separation methods include volatility and thermal stability for GC, and solubility and solution stability for LC. There are several reports discussing the preferences for a particular separation method in forensic cases, with LCMS with soft ionization methods gaining influence, due to the growing demand for analysis of biomolecules [19,20,27].

In general, the hyphenated methods (GCMS, LCMS) offer direct unambiguous information on analyte composition and structure, low sample consumption, reduced sample purity requirements, and the possibility of studying isotopic contents of various molecules for an additional level of characterization.

In this review, we concentrated on LCMS and LCMS/MS, presenting the specific features of these methods that make them versatile and innovative. The discussed methods indicate new possibilities in obtaining data on the presence of certain substances, their origin and concentration levels, and in providing answers to questions resulting from forensic investigations. It is the deep understanding of interaction between analytical instrument and the studied molecule that leads to the design of new solutions to forensic problems. The challenges resulting from low concentrations, complicated structures and metabolic transformations could be met by chemical modification of analyzed compounds, opening new venues for innovative derivatization, including isotopic labeling for structural studies and quantitative analysis. The progress in mass spectrometry, with ambient ionization methods and intelligent data analysis systems, is balanced by the cooperation of organic and bioorganic chemistry in molecular modification. The future forensic applications of mass spectrometry must provide faster, accurate and ingenious answers, following the Olympic motto "Citius, Altius, Fortius".

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