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REVIEWS

Scopes of Bioanalytical Chromatography–Mass Spectrometry

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Abstract—The areas of application of modern bioanalytical chromatography–mass spectrometry are so extensive that any attempt to systematize them becomes subjective. It would be more correct to say that there is no such area of biology and medicine where chromatography–mass spectrometry would not find application. This article focuses on the areas of application of this technique that are either relatively new or insufficiently covered in recent reviews. State-of-the-art bioanalytical techniques have become multitargeted in terms of analytes and standardized in terms of matrices. The ability to detect trace concentrations of analytes in the presence of a huge number of biomatrix macrocomponents using chromatography–mass spectrometry is especially important for bioanalytical chemistry. In the target-oriented determination of persistent organic pollutants by chromatography–mass spectrometry, the main problem is the expansion of the list of analytes, including isomers. In the detection of exposures to unstable toxicants, the fragmented adducts of xenobiotics with biomolecules become target biomarkers along with hydrolytic metabolites. The exposome reflects the general exposure of a human being to total xenobiotics and the metabolic status reflects the physiological state of the body. Chromatography–mass spectrometry is a key technique in metabolomics. Metabolomics is currently used to solve the problems of clinical diagnostics and anti-doping control. Biological sample preparation procedures for instrumental analysis are being simplified and developed toward increasing versatility. Proteomic technologies with the use of various versions of mass spectrometry have found application in the development of new methods for diagnosing coronavirus infections.

Keywords: bioanalytics, chromatography–mass spectrometry, biomarker, exposome, metabolic status, coronavirus infection

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According to Milman and Zhurkovich [1], the rapid development of bioanalytics is a consequence of the dominance of biomedicine in science as a whole. Analysis procedures are bioanalytical ones if the test materials are of biogenic origin. Analytes can be biogenic or abiogenic; in either case, the variety of their molecular forms depends on the variety of biotransformation processes. The applications of chromatography–mass spectrometry (CMS) analysis discussed below relate to in vivo sampling. Biomatrices are multicomponent mixtures of organic compounds. For this reason, highly efficient CMS methods are widely used to detect, identify, and quantify analytes in biological samples. The postmortem analysis of biosamples has specific features the discussion of which is beyond the scope of this review.

The appearance of columns with fine-grained sorbents (grain sizes smaller than 3 μm), which made it possible to shorten the duration of analysis with a simultaneous increase in sensitivity due to a decrease in peak widths, was an important stage in increasing the efficiency of liquid CMS. Mass measurement accuracy, resolution, scan rate, and sensitivity are the main characteristics of a mass-selective detector. High values of the first two characteristics ensure the selectivity of analysis and the possibility of establishing empirical formulas for identification, and the other two characteristics are especially important for multipurpose analysis or screening.

The methodological support is provided to the greatest extent for the determination of substances the use of which is restricted or prohibited by conventions and laws in biological samples. These substances include persistent organic pollutants (POPs), chemical weapon components, doping drugs, and narcotic and potent substances. A rapidly developing area is the determination of biomarkers that characterize endogenous processes or external influences, including the effect of a chemical factor on an individual or a group of people.

The intake of toxic compounds into the human body from the environment not always can be detected. The chemical body burden can be more reliably estimated by means of biomonitoring [2]. The main task of biomonitoring is to evaluate the chemical body burden at both individual and population levels.

PERSISTENT ORGANIC POLLUTANTS

The main analytes in biomonitoring are toxic metals or POPs; that is, persistent toxicants capable of being deposited in biological tissues are priority pollutants [3]. The biomonitoring of POPs is carried out using regulated analytical procedures based on the use of both gas and liquid CMS analysis [4]. The regular expansion of the list of target analytes for the biomonitoring of POPs is a problem; these are often isomeric groups of compounds, of which only a few have so far been characterized by toxicity parameters. The identification and quantitative determination of compounds from a group of new POPs were considered in a monograph [5]. Lipophilic POPs are usually determined in blood (plasma, serum, and, less often, erythrocytes) and breast milk [6] and, much less often, in bioptic material [7]. The coordinated analysis of environmental and biological samples for the presence of POPs [8] is most in demand because it allows one to assess the contribution of different sources of exposure to the total chemical body burden and to characterize this burden. Comprehensive approaches to the assessment of chemical hazard require the development of unified procedures applicable to the analysis of samples of various origins and matrix compositions (water, soil, food products, and biological samples). Among POPs, polychlorinated biphenyls, organochlorine pesticides, and brominated flame retardants, mainly represented by polybrominated diphenyl ethers, are most often identified. Recently, this group has been supplemented with perfluoroalkylated compounds, in particular, perfluoroalkyl sulfonates and perfluorooctanoic acid. Svarcova et al. [9] performed the multianalyte analysis of biological samples for POPs based on an innovative approach to the simultaneous determination of 78 halogen-containing organic POPs in human blood serum, namely, 40 flame retardants (including 7 new brominated and chlorinated flame retardants), 19 perfluoroalkanes, 11 organochlorine pesticides, and 8 polychlorinated biphenyls. The following two fractions of analytes were extracted from blood serum in the course of sample preparation for analysis: (I) a nonpolar hydrophobic fraction and (II) a more polar and hydrophilic one. Fraction I was extracted from blood serum by three-stage extraction with a mixture of hexane and diethyl ether (9 : 1, by volume) followed by purification of the extract on Florisil. Fraction II was recovered from the serum residue after the extraction of nonpolar fraction I using a modified QuEChERS procedure. Biologically relevant detection limits and satisfactory metrological characteristics required for quantitative analysis were obtained for of all the analytes. Russkikh et al. [10] reported the results of the individual determination of compounds included in the HELCOM list of the Helsinki Commission for the Protection of the Marine Environment by liquid CMS with tandem mass-spectrometric detection (HPLC–MS/MS) and proposed a procedure for the simultaneous determination of a group of perfluoroorganic acids, estradiol derivatives, hexabromocyclododecane, and triclosan. Breast milk, blood, and umbilical cord blood are commonly used to determine polychlorinated biphenyls, dioxins, organochlorine pesticides, bromine-containing flame retardants, perfluorinated compounds, and organotin compounds [11, 12]. Urine samples are analyzed for bisphenol A, organophosphates, hydroxylated metabolites of polyaromatic hydrocarbons, and phthalates [13, 14]. Error in the results of analysis depends on the biological material: it is maximal, intermediate, or minimal in the analysis of urine, blood, or breast milk, respectively. In addition, the variability of the results is lower in the determination of lipophilic compounds or higher in the determination of polar and hydrolytic metabolites. Gas CMS (GC–MS) predominates in the determination of POPs in blood and breast milk, and HPLC–MS^n, in the analysis of urine. This distribution [15] is due to the fact that hydrophilic compounds were excreted in urine, while hydrophobic ones were gradually released into blood from the organs and tissues in which they were deposited.

UNSTABLE HIGHLY TOXIC ORGANIC COMPOUNDS

Unlike POPs, unstable organic compounds undergo rapid metabolic transformations in the body and hydrolytic metabolites are excreted with urine, or they form adducts with biomolecules. To determine these compounds, two independent approaches are used: the determination of free metabolites and biomolecular adducts [16]. Chemical warfare agents (CWAs) are the most prominent representatives of unstable organic supertoxicants; after the completion of the program for the destruction of chemical weapons, the CMS analysis of these toxic substances remains of considerable current importance within the framework of verification activities. Until recently, it was believed that the determination of intact CWAs in biological media is generally impossible due to their rapid biotransformation, including postmortem changes. At the same time, the determination of intact CWAs is a problem of current interest not only for verification purposes but also for toxicokinetic studies. For the successful determination of intact CWAs in biological media, it is necessary not only to ensure the high sensitivity and selectivity of analysis but also to stop the bioconversion of CWAs in an already taken sample. In 2020, a method for the determination of intact G-type organophosphorus nerve agents (OPNAs) in whole blood was reported [17]. The derivatization of OPNAs with 2-[(dimethylamino)methyl]phenol was carried out in dried blood spots. After drying, the resulting derivatives were extracted and determined by HPLC–MS/MS. The calibration function was linear in a concentration range of 3–300 ng/mL. The reported average analyte recovery was 34% over the entire linear range. The

limit of detection was 0.7 ng/mL. As a derivative, sarin was stable in dried blood spots at room temperature for 19 days. Thus, a drop of whole blood can be taken in a field hospital, transferred to a paper carrier, dried, and treated with 2-[(dimethylamino)methyl)]phenol. The biosamples preserved in this way can be sent to stationary laboratories without special requirements for transportation conditions, for example, in postal envelopes. Earlier, a technology was proposed for the determination of OPNA adducts with albumin in the dried spots of blood plasma [18]. The approach based on the use of dried blood spots is interesting primarily due to the easy stabilization of the test sample, which makes it possible to avoid degradation in the course of storage and transportation. In this case, the completeness of analyte desorption from the paper carrier is a key criterion.

Procedures for the detection and identification of hydrolytic metabolites and biomolecular adducts of highly toxic compounds are being developed and examined in international professional tests in large analytical centers. In this case, the covalent adducts of xenobiotics with proteins and DNA are considered as retrospective markers of exposure. Their lifetime is comparable to the lifetime of biomolecules forming adducts in the body, but it is limited by aging processes in the course of which the attached xenobiotic residue or its metabolite is transformed. The consequence of aging is the loss of structural features of the starting substances, which complicates their unambiguous identification, and the loss of their ability to reactivation. The main blood proteins albumin and hemoglobin, the concentrations of which in the human body fluctuate at levels of 40 and 150 mg/mL, respectively, are affected by a portion of unstable toxicants that did not undergo hydrolysis immediately after entering the body. Because the average lifetimes of hemoglobin and albumin molecules in the human body are 120 and 20 days, respectively, the use of adducts with them as the markers of organophosphorus poisoning is very promising. At the same time, albumin is the main protein of blood plasma, which, unlike whole blood, tolerates freezing/thawing well and is convenient for transportation and preparation for analysis. On the other hand, the DNA content of blood is relatively small (0.05 mg/mL), and DNA is mainly contained in leukocytes and its isolation is a labor-intensive process. Therefore, the main trend in the development of methods for the detection and identification of unstable toxicant biomarkers is the improvement of technologies for studying blood albumin and hemoglobin addductomes [19] and, as justified below, urinary DNA adductome.

The term *adductomics* added to other omics technologies is applicable to not only establishing the effect of a toxicant on the body but also assessing the consequences of this effect. The term *adductome* has been used in bioanalytics since the early 2000s by analogy with metabolome, transciptome, etc. As an example, Kanaly et al. [20] carried out the biomonitoring of the adducts of alkylating agents with DNA in lung tissues using HPLC–MS/MS analysis. Recently, the term *adductomics* was used by Golime et al. [21], who determined the biomolecular adducts of CWAs. The list of exposure biomarkers is expanding due to the identification of new covalent adducts of xenobiotics with proteins and DNA [22].

Adductomic technologies are used not only for establishing the fact of exposure but also for performing the molecular biomonitoring of its consequences [23]. Both the residents of megalopolises and the employees of chemical enterprises are exposed to the combined action of various toxicants. Toxicological interferences and a possible cumulative effect of this action should be taken into account [24]; therefore, the determination of a large set of analytes within the framework of a single procedure seems an effective approach to biomonitoring. At the same time, even these procedures do not allow one to evaluate the effect of toxicants that were not taken into account in advance and remained outside the controlled list on the body.

It is impossible to fully take into account the human body burden of chemicals from different sources (water, food, air, industrial emissions, passive unintentional consumption of drugs, road transport, etc.) within the framework of targeted analysis. As a result, it is impossible to reasonably predict the health risks resulting from unknown chemical body burden. In response to this challenge, the scope of the CMS analysis of biological samples has expanded from the determination of an individual organic compound to the study of the exposome and metabolic status of the body.

EXPOSOME

The exposome is the sum of xenobiotics and their biomarkers in the body. Lexically, *exposome* is a derivative of exposure. The exposome characterizes the total chemical body burden, which can be partially assessed by the direct detection of known xenobiotics or their metabolites in the body or by the detection of a metabolic response to the burden; in this case, it is characterized by indirect signs. In the latter case, it is not always possible to separate the effect of a chemical factor itself from biological and even psychological stress [25].

Jones [26] attempted to describe the exposome in quantitative terms. The total number of compounds that make up the exposome is estimated at 400000, while the human metabolome contains more than 1 million compounds. The exposome can be characterized as a part of the metabolome that includes compounds coming from the external environment and their metabolites. In recent years, the term *chemical exposome* was used to avoid confusion [27].

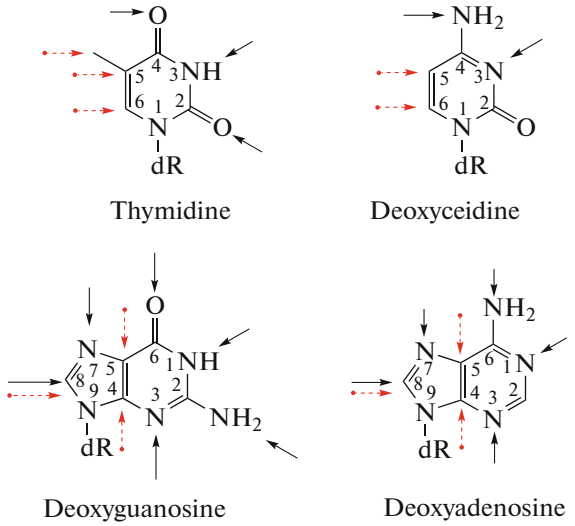

Fig. 1. The sites of nucleic bases most vulnerable to ([INSERT FIGURE AnChem2108013Saveleva-F1]) oxidation and/or (⋯→) alkylation with the formation of adducts (dR is deoxyribose).

In a study of the composition of the exposome formed by natural and anthropogenic organic environmental pollutants, the required sensitivity and selectivity are achieved by the use of high-resolution CMS analysis [28]. Modern bioanalytics provides an opportunity not only to establish the composition of xenobiotics and their transformation products in the human body but also to characterize the body's response to the combined effects of xenobiotics and to evaluate the state of vital systems [29]. Based on the use of a complex diagnostic apparatus, it is possible to give reasonable recommendations for detoxification of the body and early prevention of the development of pathologies that can be triggered by a combined effect of external factors. Concepts such as genome, microbiome, metabolome, transcriptome, proteome, immunome, exposome, adductome, and other omes, which are rooted in modern medicine and biology, are developed mainly with the use of different versions of CMS analysis.

Of the many compounds that enter the human body from the environment, special attention has been paid in recent years to those that exert a damaging effect on DNA [30]. Many cancer, neurodegenerative, and cardiovascular diseases are based on DNA damage [31]. Only an insignificant part of toxicants directly affects DNA; more often, metabolic activation with the formation of an electrophile or the induction of an active radical with a high oxidative potential occurs first. Then, these active species act to damage the structure of nucleic bases and cause their chemical modification and the breakdown of DNA chains and the disruption of their conjugations [32]. According to current concepts, if the natural repair system cannot control damage and the accumulation of mutations in genes responsible for cell growth, proliferation, programmed differentiation, and death, there is a risk of cancer [33]. It is well known that nucleotides are formed by the combination of a nucleoside and a phosphate and, in turn, nucleosides consist of a monosaccharide and a nitrogen base, which are the main targets for the action of alkylating agents. Usually, the damage to nitrogen bases consists in oxidation, deamination, alkylation, and cross-conjugations arising at their most vulnerable sites, as illustrated in Fig. 1 based on data published by Yin et al. [34]. As can be seen, the most vulnerable sites are O2 and O4 in thymine, N7, O6, C8, and N2 in guanine, N1, N3, and N7 in adenine, and O2 and N4 in cytosine.

It is believed that negative consequences occur when even a very small amount of nucleotides is broken. These changes can be detected only by supersensitive methods. An important task is to increase the sensitivity of HPLC–MS/MS analysis in relation to nucleosides, whose ionizability is relatively low.

Unlike other biomolecules, DNA is found in almost all biological media of the body. The renal excretion of the adducts of xenobiotics with DNA occurs in a depurinated form; for this reason, the DNA exposome can be more easily evaluated in urine analysis [35]. Cooke et al. [36] presented a method for studying the urinary DNA adductome as the most sensitive part of the exposome using the HPLC–MS/MS analysis of urine. In recent years, the use of high-resolution mass spectrometers (time-of-flight and orbital traps) has made it possible to obtain new data on DNA damage caused by various chemical compounds [37]. For example, covalent DNA adducts with deoxyguanosine at the N2 position are biomarkers for assessing the damaging effects of acetaldehyde [38], acrolein [39], and crotonaldehyde [40] on DNA; for sulfur mustard gas, these are adducts with guanine at the N7 position [41]. These adducts can be determined in biological samples by multipurpose quantitative CMS analysis; it is likely that this approach will be complemented in the future by a set of methods for the biomonitoring of genotoxic compounds.

In the context of the assessment of general DNA damage resulting from the sum of factors, including unknown ones, the problem of non-targeted DNA adductomics arose. In 2006, Kanaly et al. [42] were the first to attempt to solve this problem using three-quadrupole HPLC–MS/MS analysis in the electrospray ionization mode with selected reaction monitoring (SRM) and the detection of $[M + H]^+ \rightarrow [M + H - 116]^+$ transitions, where m/z 116 corresponds to the elimination of 2-deoxyribose. More recently, Chang et al. [43] combined the targeted and non-targeted determination of DNA adducts in one analysis in the neutral loss scan mode using similar instrumentation. The excretion of fragmented adducts with urine

reflects the repair process, assuming that the repair occurs due to the excision of individual sections of DNA. As a result of this process, the adducts of deoxyribonucleosides and nitrogen bases modified by alkylation or oxidation (Fig. 1), which form the so-called urinary DNA adductome, are excreted with urine. Chang et al. [43] presented this adductome in the form of five three-dimensional maps for each urine sample. The abscissa was the retention times, the ordinate was the mass numbers m/z, and the z axis was the normalized peak areas. It is shown below that they used the standard mode of HPLC–MS/MS analysis, and the novelty of the approach was in the volume and structuring of its results. In the course of sample preparation for analysis, urine samples were applied onto C18 cartridges, washed with water, and eluted with methanol. The eluate was evaporated and repeatedly dissolved in 100 μL of deionized water; thereafter, the solution was analyzed by HPLC–MS/MS in a gradient reversed-phase mode using a triple quadrupole–linear ion trap hybrid mass spectrometer. Electrospray ionization was carried out in positive polarity with the following characteristics over the entire range of scanned mass numbers: declustering potential, 40 V; entrance potential, 10 V; and collision energy, 30 eV.

The procedure was adjusted with 6 model 2'-deoxyribonucleosides and 10 nitrogen bases. The limits of detection for analytes ranged from 0.2 to 7 ng per injection. To 5000 useful peaks were identified after treatment and filtration. Without dwelling on data processing methods, we note that, after interlaboratory intercalibration and further adjustment, this method can be suitable for describing (mapping) the human urinary adductome, and it is the first, albeit under development, attempt to describe the human DNA exposome.

Studies oriented to the identification of large groups of analytes and even omes—exposome, metabolome, lipidome, etc.—were considered as nonstandard but, nevertheless, chemical analytical procedures. It is still difficult to understand whether mass-spectrometric methods for the direct monitoring of a biological process, status, or problem (deviation from the norm) can be considered in this series. Thus, Sans et al. [44] proposed a method for the direct mass-spectrometric analysis of biological tissues. With the use of a piezoelectric dispenser, the surface of a biological sample is treated with solvent nanodroplets, which dislodge and simultaneously ionize nanoparticles from the sample, and these nanoparticles are transferred to the mass-selective detector. A generalized profile of the mouse brain tissue was obtained using this approach. According to Sans et al. [44], in this case, it is possible to reliably distinguish between the profiles of healthy and tumor tissues. Such approaches are presented in the literature under the general name *high-throughput screening* [45], the main purpose of which is the rapid analysis of complex biological samples. Attempts are being made to implement such technologies in the study of the biotransformation of xenobiotics, in the detection of gross metabolic shifts in the framework of targeted metabolomics, and in the assessment of the distribution of xenobiotics in the body. Isotopic labels are applicable here, and this should be fast and, ideally, multiwell (plate) analysis.

METABOLIC STATUS

The terminology prescribing to refer to the conversion products of substances in the environment and in biological materials as markers and biomarkers, respectively [46], implies that analytically detected forms of a certain substance in a biological sample serve as biomarkers. The term *biomarker* as an indicator of physiological and pathological biological processes or pharmacological responses to therapeutic intervention [47] was proposed in 2001 by the US National Institutes of Health. Any characteristic that can be objectively measured and which can serve as an indicator of a particular process can serve as a biomarker in this interpretation. Thus, we can consider the biomarkers of metabolic disorders, oxidative stress, chronic fatigue, and other processes. If so, the biomarker may be a chemical compound, but this concept can be considered broader in some cases. In a review devoted to the clinical applications of mass spectrometry, Milman and Zhurkovich [48] proposed to consider the groups of compounds and the metabolome (for low-molecular-weight compounds) or proteome (for proteins) as a whole as biomarkers.

The metabolic status of an organism is understood as a multidimensional picture of the qualitative and quantitative composition of biogenic components. In most cases, gross deviations of the metabolic status from the norm are determined in order to diagnose diseases. The diagnosis of congenital metabolic disorders by tandem mass spectrometry is the best known and developed embodiment of this line. This vital application of CMS analysis is extremely useful, and it is continually being improved. Ma et al. [49] proposed a technology for the rapid determination of an extended list of metabolic diseases in dried blood spot analysis and tested it in the analysis of more than 1000 samples. A huge number of works were devoted to the early diagnosis of cancer diseases by CMS analysis based on the metabolomics. The currently available results of these studies are too controversial to recommend a particular CMS technology as a reliable diagnostic method.

In recent years, chemical analytical criteria corresponding to a functional state characterized as fatigue, overtraining, and chronic fatigue have been substantiated in a number of publications. Literature sources for the last five years (2017–2021) found in the ScienceDirect database upon a contextual search for the keywords *mass spectrometry, exercise, fatigue*, and

Table 1. Lines of research on exercise metabolomics carried out using mass spectrometry in 2017–2021

Area of research	Fraction of the total number of publications, %
Exercise-induced changes in urine or blood metabolic profiles	42
Metabolic status of the body corresponding to the peak of physical fitness	18
Effect of nutritional support on exercise tolerance	12
Metabolomics of fatigue and overtraining in the absence of pathology/illness	12
Changes in the metabolic profiles of urine and blood under the influence of physical activity against the background of diseases (cardiovascular diseases, metabolic syndrome, diabetes, neurological diseases, and geriatric diseases)	9

metabolomics can be divided into four main groups (Table 1). A total of 93 publications were found.

From Table 1, it follows that biomarkers that are clearly associated with exercise have not yet been established because general studies oriented to a search for these biomarkers prevailed (42 of 93). It is likely that these biomarkers will include concentration ratios or more complex multidimensional indicators rather than the absolute concentrations of biogenic substances in urine or blood. CMS methods provide a huge amount of information. Currently, a key problem is the development of algorithms for comparing this information with a wide range of physiological indicators taking into account their different significance. The main strategy of metabolomics in sports is a study of the metabolic signatures of blood plasma [50, 51]. It is believed that metabolic profiling provides indirect information on the metabolic phenotype and direct information on the concentrations of low-molecular-weight metabolites involved in the development of a physiological effect [52].

Armstrong with coauthors [53, 54] used NMR spectroscopy to collect primary information on low-molecular-weight metabolites responsible for the state of fatigue. The advantage of capillary electrophoresis in metabolic profiling is the ability to determine high-molecular-weight markers [55]; however, it is used relatively rarely and mainly in targeted metabolomics due to the high cost and insufficient availability of instrumental complexes that combine capillary electrophoresis with spectroscopic methods. Thus, various versions of CMS analysis obviously prevail in omics technologies. Kamrath et al. [56] determined the concentrations of target metabolites by GC–MS analysis and used metabolite concentration ratios as diagnostic criteria. A joint group from seven US universities [57] conducted a large-scale study using HPLC–MS/MS analysis in order to establish a relationship between the functional state of highly trained athletes and the metabolic profiles of their blood. This study was performed using the non-targeted metabolomics technique. Statistical data processing was carried out using the method of invariant sets of families of linear and nonlinear discrete systems. A total of 743 metabolites were identified. The concentrations of substances from the group of gamma-glutamic acid were significantly higher in the groups of both high-power and high-endurance athletes. This was explained as a consequence of the active work of the glutathione cycle. High endurance was associated with an increased production of the sex hormones testosterone and progesterone, and the levels of diacylglycerides and eicosanoids in the blood of high-endurance athletes were significantly reduced. High power was also associated with the high blood levels of phospholipids and xanthine. In 2019, Bongiovanni et al. [58] published a review that summarized the results of metabolomic studies on biofluids in athletes who experienced extreme stress. As a general conclusion, they noted that high power and high endurance were associated with biochemical processes such as steroid biosynthesis, fatty acid metabolism, oxidative stress, and energy metabolism. In the metabolomics applied to sports, as in the metabolomics in general, the sensitivity of analysis is not a priority because biogenic analytes (metabolites) occur in the samples in high concentrations. The highest requirements are imposed on the reliability of quantitative determinations in targeted metabolomics and on the reliability of metabolite identification and the productivity of analysis in non-targeted metabolomics. Table 2 summarizes the results of some CMS studies in the metabolomics of the consequences of extreme loads. As can be seen, the regularities noted by Bongiovanni et al. [58] were generally confirmed.

Continuing the theme of sport, it should be noted that anti-doping control is based almost exclusively on CMS analysis, and this is a well-tuned routine analysis system that combines screening and confirmation analysis procedures. Both of these lines are constantly being improved [71, 72]. New data on the long-lived metabolites of prohibited substances make it possible to expand the time window for their detection. Both standard and innovative procedures in anti-doping CMS analysis remain outside the scope of this review because they are extremely widely presented and summarized in the literature [73]. Metabolomic approaches in doping control are classified as promising development works, and they are not used in a rou-

Table 2. Results of metabolomic chromatography–mass spectrometry analysis of the biological fluids of persons with various training loads

Biological matrix	Technique	Biomarkers	Reference
Blood plasma	GC–MS	Alanine, β-dimethylglucopyranoside lactate, pyroglutamic acid, cysteine, glutamic acid, glutamine, free fatty acids, and valine are sensitive to age and fitness level	[59]
Blood plasma	HPLC–MS	At moderate loads, the concentrations of octanoyl, decanoyl, and dodecanoyl carnitines in blood plasma increase	[60]
Blood plasma	HPLC–MS	Marathon runners have higher levels of glycerol, niacinamide, gluco-6-phosphates, pantothenate, and succinate, as compared to those of ordinary people	[61]
Blood plasma	HPLC–MS	After exercise associated with hypoxia, the level of lysophosphatidylcholines, lysophosphatidyldiethanolamine, lysoplasmalogens, and metabolites associated with hemolysis is increased	[62]
Saliva	HPLC–MS	Creatinine, glucose, and antioxidant metabolite contents increased after extreme load	[63]
Blood plasma	GC–MS, HPLC–MS	13- and 9-hydroxyoctadecanoic acids were associated with loading	[64]
Blood plasma	GC–MS	After extreme exercise, the plasma levels of tricarboxylic acids and monounsaturated fatty acids increased	[65]
Urine	HPLC–MS	1-Methyladenosine, 5-methylthioadenosine, 3-indoleacetic acid, and 1-glutamic acid were associated with hypoxia	[66]
Urine	NMR, HPLC–MS	Trimethylamine oxide, phenylalanine, lactate, alanine, trimethylamine, malonate, taurine, and glycine were associated with load	[67]
Urine	HPLC–MS	Purines, tryptophan, carnitine, cortisol, amino acid oxidation products, and intestinal microflora metabolites were associated with exercise	[68]
Blood plasma	HPLC–MS	After a four-day marathon, the plasma levels of free fatty acids, tricarboxylic acids, and branched-chain amino acid metabolites increased and the monoacylglycerol and lipid levels decreased	[69]
Blood plasma	HPLC–MS/MS	Exercise increased the plasma levels of carnitine, 3-methylmyristic acid, and sebacic acid	[70]

tine analysis mode. An exception is the urinary steroid profile [74].

It is well known that a negative result of targeted doping tests does not always guarantee the absence of prohibited substances in the body, and this led to the appearance of the athlete biological passport paradigm. The steroid module of the biological passport is especially important because the designer anabolic steroids, which are not covered by the WADA list, are among the most common doping substances [75].

The steroid module of the athlete biological passport is based on the determination of urine biomarkers involved in the metabolism of endogenous steroids. The determination of concentrations of these urinary biomarkers is a difficult task because steroids are excreted mainly in a conjugated form (glucuronides or sulfates) in urine [76]. Thus, specific sample preparation [77], which includes the enzymatic hydrolysis of urine and the extraction and derivatization of particular endogenous steroids, is required before the GC–MS/MS analysis, which is recommended by WADA as a standard method for the determination of endogenous anabolic steroids. Kuuranne et al. [78] noted that, with the accumulation of experience in the control of the urinary steroid profiles of athletes, the most important problem is the interpretation of the results of the determination of concentrations and marker

ratios of endogenous steroids in urine because of their significant dependence on genetic polymorphism.

Isotope ratio mass spectrometry, which is most often oriented to the detection of pseudoendogenous steroid hormones [79], is used upon the detection of an atypical result in a urine sample (the concentrations or concentration ratios that are beyond the reference values).

The further development of the steroid module consists in the development of a personalized approach to the interpretation of criterion indicators of the steroid profile and isotope GC–MS arbitration analysis [80, 81]. A method was proposed for the monitoring of steroids in saliva with sampling performed immediately before and after (and sometimes in the course of) a competition or training [82]. Despite the fact that huge funds are invested in anti-doping control, the rate of detection of prohibited drugs remains low; because of this, the development of metabolomics in sports is of considerable current importance. In all likelihood, the role of the athlete biological passport in the overall anti-doping control scheme will increase in the future.

Anti-doping control intersects with chemical toxicological analysis in terms of not only common CMS methods and analytes (as is well known, stimulants and drugs are prohibited in sports) but also a high degree of responsibility for the results of analysis, which are often contested in court. At the same time, the methodology of chemical toxicological analysis, which is one of the most ancient areas of analytical chemistry, differs significantly from the methodology of anti-doping control. Criteria and accreditation system in anti-doping control are the same for all laboratories. Chemical toxicological analysis is also highly regulated, but regulatory systems have significant national characteristics. Without going into detail on this topic, which is far from analytical chemistry, note only some current trends in chemical toxicological analysis.

CHEMICAL TOXICOLOGICAL ANALYSIS

Classical chemical toxicological analysis is primarily aimed at the determination of relatively small molecules. GC–MS analysis with mono-quadrupole mass-selective detection has held the position of the gold standard in screening studies for almost half a century because it is based on extensive and constantly updated databases of mass spectra and retention indices. It is difficult to overestimate the importance of the quickly updated non-commercial library of mass spectra available on the website http://sudmed-ms.info. This is the most efficient and affordable tool for the detection of new psychoactive compounds in biological samples. The GC–MS screening is extremely effective for the detection of small molecules of moderately toxic compounds, for example, in the identification of overdose cases or the nonmedical use of pharmaceuticals and designer drugs [83].

The sensitivity of the mono-quadrupole mass-selective detector is insufficient for the detection and identification of biomarkers of more toxic compounds, especially when the examination is carried out long after poisoning and the main dose of a toxicant is removed from the body. Due to its high selectivity, tandem mass-spectrometric detection makes it possible to detect toxic compounds and their metabolites in biological matrices with high sensitivity [84]. As a rule, low-molecular-weight metabolites are more polar than the substances from which they were formed. The solubility of metabolites in aqueous media and, accordingly, the rate of excretion from the body through kidneys with urine increase with the polarity of metabolites [85]. Because of this, the chances of detecting urinary metabolites increase in the early stages after poisoning and decrease in the long term. Unlike blood plasma, urine does not require special processing immediately after sampling; the composition of organic compounds in urine is less susceptible to distortions in the course of sampling, storage, and transportation. As a rule, the concentrations of biogenic analytes in urine did not change significantly after several freeze–thaw cycles. In comparison with blood, urine is less saturated with organic compounds, which can not only undergo oxidation but also act as oxidation promoters [86]. Thus, urine as a biomatrix has many advantages in the determination of small molecules: noninvasive sampling, large volume, minimal interfering effects of proteins and lipids, and high concentrations of most xenobiotics due to their concentration in the kidneys [87]. The disadvantage of urine as a biomatrix is a high concentration of urea, which interferes with both direct GC–MS analysis and the complete derivatization. The elimination of the interfering effect of urea by mineral or enzymatic hydrolysis can be recommended for targeted analysis. In the survey analysis, mineral hydrolysis can distort the component composition of urine and lead to the partial or complete loss of some analytes due to their decomposition. As a rule, the use of enzymatic hydrolysis (treatment with urease) leads to an increase in the matrix effect in the total ion current mode and, as a consequence, to the loss of minor analytes because of their increased limits of detection. Algorithms for optimizing pH and selecting an extractant were proposed to perform the targeted determination of small molecules in urine [88, 89]. Salting out or freezing in acetonitrile is often used in sample preparation for survey GC–MS and HPLC–MS^n analysis. In this case, the losses of even polar analytes such as alkylmethylphosphonic acids were avoided [90].

In most cases, the instrumental part of a CMS analysis procedure is formalized and carried out in accordance with manufacturer's recommendations, whereas biological sample preparation methods for CMS analysis are under development and optimiza-

tion. The technique of solid-phase extraction with the use of multilayer columns and fractional elution is being improved. A generalized line in the development of microextraction methods in bioanalytical chemistry cannot yet be traced. Various versions of liquid [91] and solid-phase [92] microextraction were considered and systematized in the surveys of reviews. The procedures of microextraction with the use of polymer tablets, granules, powders, and magnetic particles; dispersive liquid–liquid and single-drop microextraction, including solidification of floating organic droplet microextraction; various versions of membrane and electromembrane microextraction; and hollow fiber microextraction were almost equivalently described in the literature. On the one hand, these are not new procedures, and they have not yet found wide application in routine stream analysis on the other hand. A relatively new and promising approach is the use of switchable hydrophobicity solvents [93]. By the addition of carbon dioxide, a solvent is protonated and converted into bicarbonate, and it becomes miscible with water; the solvent is separated from water after the removal of carbon dioxide by heating or passing an inert gas. The process is based on an acid–base reaction. Amidines, secondary and tertiary amines [94], and diamines [95] are used as switchable polarity solvents.

If a biomonitoring procedure requires quantitative analysis and analytes are known in advance, the main tasks in the determination of the biomarkers of toxic substances, doping agents, and psychoactive drugs are the detection and evidential identification of biomarkers that unambiguously indicate the fact that a controlled substance has entered the body. The final step in an analytical process is confirmatory analysis with the use of a reference sample. In this case, the initial stage, which consists in the detection of desired biomarker traces in a complex mixture of biomatrix macrocomponents, is the most difficult. Despite a large number of regulatory documents, the assessment of the uncertainty of this analysis cannot be performed within the framework of a general approach [96]. Because of the rapid expansion of illicit traffic in narcotic drugs, the procedures used for detecting biomarkers and establishing their belonging to a certain group of prohibited substances require the development of new approaches to improve the efficiency of laboratories at the stage of detecting analytes that will or will not be classified as target biomarkers [97]. Grigor'ev et al. [98] noted the prevalence of the survey direction in chemical toxicological analysis because the list of controlled compounds is continuously expanded and formulated 17 limitations of the HPLC–MS/MS technique in the survey analysis. It is likely that these limitations will not be completely overcome in the near future. In particular, low scanning speed, contaminants from a mobile phase observed in mass spectra, insufficient efficiency of chromatographic separation, and other technical difficulties will be overcome due to the competition between the manufacturers of HPLC–MS/MS systems. At the same time, the availability of different design solutions for a mass-spectrometric process at different companies prevents its unification, and this seems an important limitation of the capabilities of survey HPLC–MS/MS analysis. The libraries of reference mass spectra are developed as supplements to particular instruments. With a vide variety of GC–MS analysis techniques, a classical approach to the implementation of this analysis is based on the total ion current scan mode (standard 70-eV electron ionization) and chromatographic separation on a weakly polar (5%-phenyl)-methylpolysiloxane stationary phase with the determination of linear retention indices under temperature-programming conditions. The databases of identification characteristics for GC–MS analysis are rapidly updated and successfully applied regardless of the instrumentation used. If a compound was not detected in the screening procedure, it either cannot be detected at all under these experimental conditions, or it was lost in the course of preparation for analysis, or it is absent from the library, or the library mass spectrum differs significantly from the experimental one. The latter circumstance is essential for HPLC–MS/MS analysis due to the high variability of the mass spectra obtained on different instruments and the absence of an interlaboratory agreement on standard analysis conditions. For these reasons, laboratories still prefer to orient to home-made libraries.

MASS SPECTROMETRY AGAINST CORONAVIRUS INFECTION

Bioanalytical mass spectrometry has proven its ability to respond quickly to the challenges of global threats, as confirmed by a number of original ideas in opposition to the coronavirus pandemic. The technologies developed are based on well-proven methods for the separation and structural identification of proteins. Rana et al. [99] considered various proteomic technologies differing in methods used for the implementation and combination of high-throughput separation methods for proteins, their mass-spectrometric description, and the processing of experimental data arrays. Most of the efforts were directed to the development of new methods for diagnosing coronavirus infections. In particular, Nachtigall et al. [100] proposed a test with the use of MALDI mass spectrometry as an alternative to classical tests based on the polymerase chain reaction (PCR). In the development of this method, it was important not only to standardize conditions for the measurement of mass spectra but also to propose an acceptable method for data processing. The support vector machine with a radial kernel was optimal for the processing of mass spectra. As a result, it was possible to achieve 90% reliability in the

analysis of both positive and negative SARS-CoV-2 samples.

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Ihling et al. [101] applied classical proteomic analysis to the detection and identification of SARS-CoV-2 in a liquid obtained after rinsing the throat. The procedure proposed included the following stages: precipitation of proteins with acetone, deglycosylation of proteins, pepsinolysis, treatment with dithiothreitol and iodoacetamide, and HPLC–MS/MS analysis. The priority task for further research is to shorten the analysis time. Currently, the analysis of a sample takes about 3 h.

Mahmud and Garrett [102] noted that metabolomic and lipidomic studies with the use of high-resolution ultra-HPLC–MS are a powerful tool for the differential diagnosis of infections and the detection of yet unknown biomarkers of pathogens. Great hopes are related to hyphenated techniques combining PCR and mass spectrometry. The possibilities of mass-spectrometric identification of viral DNA and RNA are limited by their low concentrations in biomaterials. This problem is successfully overcome by their preconcentration using PCR. Diagnostic procedures should evolve both toward the rapid and reliable identification of known viruses and toward the detection of genetic markers of previously unknown pathogens. For the development of mobile diagnostic complexes, it is necessary to miniaturize mass spectrometers and ensure their stable operation under conditions close to field conditions.

Analytical mass spectrometry is promising in research aimed at finding new targets for the action of vaccines against SARS-CoV-2. Poran et al. [103] used a mass spectrometry–based bioinformatics predictor to demonstrate the efficiency of their algorithm proposed for predicting the reaction mechanism of T cells to SARS-CoV-2 and the formation of T-cell immunity.

The ability to detect and identify the proteins of the virus, to characterize their uniqueness, and to predict the mechanisms of cell immunity formation opens up prospects for diagnosing viral infections and blocking and preventing their development.

* * *

The combination of survey and targeted GC–MS analysis at the stage of detecting previously unknown analytes still occupies the position of the golden ratio. Standardized conditions of analysis performed using mono-quadrupole mass-selective detectors provide access to extensive and constantly updated databases of mass spectra and retention indices. HPLC–MS^n analysis with electrospray ionization can already be considered as a classical approach to the determination of both small molecules and supramolecular complexes in various complex biological materials. The capabilities of survey HPLC–MS and HPLC–MS/MS analysis are still limited to multipurpose screening, in which the formation of a list of candidate analytes should precede the analysis. GC–MS analysis remains the most rational approach to the determination of organic compounds with low electrospray ionization efficiency (for example, steroids). Medicine and biology pose the multiparametric tasks of establishing biological effects and biological states (statuses) and even collecting key information for making diagnoses to modern CMS analysis. In this case, these solutions are still based on the measurement of analytical signal intensities caused by the distribution of the mass numbers of ions. The mass-spectrometric process is steadily improving; however, in order to obtain results of diagnostic value, it is necessary to develop approaches to the standardization of the results of CMS analysis (including survey analysis) and their integration into omics technologies and the efficient processing and interpretation of large bodies of experimental data.

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