MASS SPECTROMETRY BASED ANALYTICAL APPROACHES AND PITFALLS FOR TOXICOMETABOLOMICS OF ARSENIC IN MAMMALS: A TUTORIAL REVIEW

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Abstract

The present review focus on the analytical platforms and the workflow for toxicometabolomics with a special emphasis on their strengths and pitfalls presenting as a case study the toxicometabolomics of arsenic in mammals.

Although powerful analytical methods and techniques are currently available for metabolomics, the main "bottleneck" is still the absence of unified protocols for sample preparation (e.g. quenching, solvents used) as well as several important factors in toxicometabolomics, which drastically affect the metabolism (e.g. selection of model organisms, xenobiotic doses, chemical form of the xenobiotic, exposure route, biological sample). In addition, the selection of model organisms, the xenobiotic dose and administration route are of pivotal importance in toxicometabolomic studies. In this context, the applicability to complex samples, higher sensitivity, specificity and the possibility to perform quantitative analysis offered by MS is crucial to probe xenobiotic induced metabolic changes to evaluate the stress responses.

Nowadays, the use of different metabolomic platforms allowed determining important changes in the metabolism induced by arsenic in mammals such as alterations in the energy (e.g. Glycolysis, Kreb´s cycle), amino acid, lipid, nucleotide and androgen metabolisms.

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1. Introduction

Arsenic is a global toxic element with an important impact in human health [1], being responsible of some types of cancers such us skin, lung, urinary bladder and liver cancers [2]. Humans can be exposed to arsenic by contaminated water, soil, atmosphere and food, especially seafood [3], as well as products or supplements based on algae (especially Hijiki seaweed) and cereals or cereals derived foods [4].

In the postgenomic era, the use of methods of massive information, the -omics, are very useful to study the biological response of organisms against toxic effects and to fully understand the action mechanisms. Likewise, during the last decade, the -omics technologies provide massive information generating methods that allow comprehensive description of nearly all components within the cell. To this end, genomics reveals the characteristics of the information contained in the cellular core that determines the cell function and behavior, transcriptomics examines the gene expression, and proteomics involves the analysis of protein synthesis and cell signaling. In addition, in 1999, J. Nicholson defines metabonomics as “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification” [5], while metabolomics may be defined as the measurement of all the metabolites in a specified biological sample [5]. However, both terms are commonly

used imprecisely. On the other hand, whereas metabolomics allows understanding the variation in low molecular mass metabolites in complex multicellular organisms and their response to change, the additional mentioned "-omic" sciences are related with cellular macromolecules. Thus, to understand a cell, tissue or living organism behavior it is also necessary to consider low molecular mass molecules since they represent the last action mechanism of the organisms. Figure 1 shows the omics cascade and how metabolomics is the omic science considered to be closer to the phenotype.

In the case of proteomics, it allows identifying important roles of proteins in cell homeostasis, quantitative analysis and it has been shown to generate protein biomarkers for *in vivo* toxicity, but there are a large number of proteins and post-translational modifications, and not all proteins in a sample can be identified. In relation to transcriptomics and genomics, they allow efficient sequencing of complete genomes, the study of polymorphisms can give insight into the role of genetics in toxicology and it can explain differences in susceptibility, but alterations in gene expression do not always lead to adverse health effects and it is often difficult to translate genomic results to *in vivo*, for example human toxicity or disease. Moreover, post-translational modifications, as phosphorylation and glycosylation of proteins determine their function and it is well-known that a lot of environmental factors or multigenic processes cannot be explained only with a genomics basis (e.g. disease and aging). Finally, metabolomics allows the simultaneous measurement of hundreds of metabolites *in vitro* cell cultures, *in vivo* tissue and even in non-invasive blood and urine applications, and it has been shown to predict *in vivo* liver and kidney toxicity. Conversely, metabolomics is not exempt of shortcomings, being the most remarkable the limited detection of metabolites caused by quenching and metabolite extraction procedures as well as the complexity of data analysis

and interpretation (e.g. metabolic pathways) and that *in vivo* approaches are influenced by variability factors (e.g. age, gender, diet, stress, housing conditions, health status) as well as *in vitro* ones (e.g. cell culture conditions, metabolic competence, media formulations, serum additions, treatment vehicle). In this scenario, the combination of omics seems to be the most suitable approach. But it is important to consider that the combination of proteomics with metabolomics has some drawbacks since the temporal space is different (i.e. metabolomics gives information about what happens right now, but it should be related with numerous post-translational modifications happened previously). In this sense, it seems that the combination of genomics with metabolomics is easier. Nevertheless, when metabolomics data are interpreted in combination with genomic, transcriptomic and proteomic results, in the so-called systems biology approach, a holistic knowledge of the organism/process under investigation can be achieved.

In addition, generally, there are two major approaches used in metabolomics studies: targeted and untargeted (global). A targeted approach is a qualitative or quantitative determination of specific metabolites and requires the comparison of analytes with commercial or synthetized compounds, thus it depends on the availability of standards, while untargeted metabolomics (global metabolomics) is the comprehensive study of all metabolites in a biological sample with the main aim to find differing metabolites based on their relative quantitation by using as many chromatographic/spectroscopic peaks (‘features‘) as possible. The disadvantage of untargeted metabolomics is that this approach is a relative quantification, not an absolute one. Furthermore, some of the significant features/peaks are not identifiable [6].

Although metabolomics and metabonomics, are the most common strategies for metabolomic analysis, there are other important approaches, some of them can be classified as targeted and/or untargeted [7–9] (i) Metabolite profiling: the identification and quantification of a selected number of pre-defined metabolites, generally related to a specific metabolic pathway, (ii) Metabolic fingerprinting: high throughput, rapid, global analysis of samples to provide their classification, usually without quantification and metabolic identification, (iii) Metabolite footprinting, the study of metabolites in extracellular fluids, and other that depends on the study goals: (iv) Metal-metabolomics, the study of metal or metalloid containing metabolites in a biological system (i.e. selenometabolomics) [10], (v) Toxicometabolomics, metabolomic analysis applied to toxicology (i.e. metabolomic analysis of living organisms exposed to xenobiotics) [6], etc. Figure 2 shows a scheme about the different types of metabolomic approaches.

On the other hand, metallomics is an important omic technique which can be combined with toxicometabolomics when the organism is exposed to a metal or metalloid and allows identifying and/or quantifying metal-metabolites (i.e. arsenic species, iAs^{V}, MA^{III} (monomethylarsonous acid), MA^{V} (monomethylarsonic acid), etc) or metalloproteins (i.e. selenoprotein P, metallothioneins, etc). Several papers in which both techniques have been applied together in arsenic exposed organisms are discussed in the text. In this way, it is important to consider that approximately one third of proteins need the presence of metals as cofactors to develop their function (metalloproteins) and that metals influence on more than 50% of the proteins [11]. These metals are responsible of catalytic properties or structure of proteins and the presence in molecules is determined in many cases by the genome [12]. The metallome was defined by Williams as the distribution of elements, concentration at equilibrium of free metallic ions or free elements in a cellular

compartment, cell or organism [13] and refers to the identity and/or quantity of metals/metalloids and their species [11,14–16]. Then, metallomics considers that the identification of a metal cofactor into a protein is of great importance to assign its function and to place it in the context of known cellular pathways [17,18]. Metallomics uses metal or metalloids as heteroatomic markers or tags to track these molecules on complex matrices [19–21] and provides a good alternative to deep insight into the fate of elements in exposed organisms giving information about metal trafficking, interactions and homeostasis. Then, since chemical species are "the specific forms of an element defined to isotopic composition, electronic or oxidation state and/or complex or molecular structure" [22], the line between metallomics, metal-metabolomics and chemical speciation is absolutely thin. Then, it is difficult to answer which is the real difference between them: is the analytical technique used? (i.e. atomic techniques like inductively coupled plasma-mass spectrometry or atomic fluorescence spectroscopy in speciation/metallomics and organic MS or NMR in metabolimics combined with discriminant analysis and data base searching) or is the presence or absence of a metal/metalloid in the molecule? Is the use of the metal/metalloid as a tag in the analysis? Then, when an organism is exposed to arsenic and we measure iAs^{V} and MA^{III} (monomethylarsonous acid) for example in a tissue, they are chemical species or metabolites? What is unquestionable is that sometimes the objective of these approaches is the same.

Thus, metabolomics, as technology catching the phenotypic change at molecular level, allows the characterization of the impact of an agent in living organisms. In particular, toxicometabolomics, is a promising tool in the search for the action mechanisms of chemical species in living organisms. On the other hand, toxicometabolomics presents

actually some pitfalls that are in part the same in the case of metabolomics and other specific ones. For example the absence of unified analytical protocols for sample preparation to give really a comparable list of metabolites and/or responses (over/down-regulation). These protocols are related to sample preparation, analysis or statistical treatments.

Moreover, toxicometabolomics present several inherent pitfalls, which are mainly related with the use of animals in the experiments. Likewise, some important decisions should be made before performing the experiments, for example the definition of the model organism to be used, the selected xenobiotic dose, the chemical form, the exposure route selected, the time of exposure, the biological sample used and other ones, which can drastically affect the metabolism generating incomparable results.

In this context, the present review is focused on the typical workflow for toxicometabolomics with a special emphasis on arsenic and the analytical platforms used, including a discussion about strengths, weaknesses, new trends and future directions in toxicometabolomics as well as the main metabolic pathways altered by arsenic exposure in mammals.

2. Analytical platforms for toxicometabolomics applied to arsenic: the metabolomic workflow

To assess the multiple and intricately intertwined metabolic changes involved in arsenic and other toxic elements interactions in tissues and biological fluids after element intake, powerful analytical platforms, capable to study simultaneously hundreds or thousands of

molecules with molecular mass less than 1500 Da are mandatory [23]. Usually, these molecules are intermediate metabolites and end products of cellular functions that are over/down-expressed as a consequence of biological response to metals or other toxic substances. The metabolome is very diverse including soluble lipids usually associated to cell membranes, polar and non-polar metabolites from the cell, as well as acid and basic ions. Therefore, analytical approaches for non-targeted metabolomics have to be comprehensive and reproducible, in order to include so wide variety of substances in the complex biological matrices in which proteins, nucleic acid, salts and many other molecules can adversely affect the analysis.

The basic workflow used in non-targeted toxicometabolomics is outlined in Figure 3 and consisted of several general steps: (i) Selection of model organisms, (ii) Selection of an appropriate xenobiotic and biofluid/tissue for analysis: Experimental design, (iii) Sample preparation, (iv) Data acquisition and analysis, (v) Metabolites identification/quantification and (vi) Biological interpretation. This metabolomic workflow comprises several well established steps. Firstly, experimental design involves the selection of a model organism or bioindicator (e.g. rats, rabbit, mice, monkey, cell cultures), the appropriate xenobiotic (e.g. metals) which should be administrated in a correct dose as well as the route of exposure. The correct selection of the tissue or biofluid is of pivotal importance since the metabolic information is strongly related with the sample used and they can offer complementary information. At this stage, the experiment can be designed. In addition, exposure experiments for toxicometabolomics involve several steps: sample preparation, direct detection of metabolites by organic/inorganic mass spectrometry or after a separation step, data analysis by means of complex bioinformatics tools and finally a biological interpretation of the results obtained. As

important as the chosen analytical technique is the selection of suitable pre-analysis procedures, involving collection of organisms and sample treatment. Moreover, data analysis requires sophisticated techniques, in which multivariate statistical analysis and bioinformatics tools play fundamental roles.

3. Selection of model organisms for toxicometabolomic studies

As described in Table 1, several model organisms have been studied for toxicometabolomics of arsenic like *mice* [24–32], *rats* [33–37] and *pigs* [38]. Several important studies with *humans* are also described [39–41]. The first question is if *free-living animals* will be used or instead, *controlled exposure experiments* will be undertaken. One disadvantage of using free-living organisms in environmental metabolomics is the difficulty of isolate the metabolic changes observed to establish direct connection with a particular xenobiotic, since the organisms may be affected by a huge quantity of environmental factors of diverse nature [42] and by different routes in a more similar fashion to humans compared to laboratory-based studies (e.g. subcutaneous injection). Thus, since the xenobiotics are all together, some elements or their species can counteract the action of others through cooperation or availability mechanisms [43]. In this sense, the interaction of arsenic in mammals has been well documented with selenium, cadmium, zinc and phosphorous [43]. On the other hand, the collection of free-living animals and the subsequent analysis is relatively cheap and easy, which is not the case of the controlled exposure experiments to several cocktails of contaminants that need an expert technician to administrate the xenobiotics, synthetic diets, etc. Finally, the studies with free-living animals to obtain correlations between the response of the

bioindicator at the laboratory and in the environment is crucial to validate the results [20,44].

Moreover, care should be taken in controlled exposure experiments to generate living conditions for the animals that do not generate stress (e.g. number of animals per cage, administration of xenobiotics), as this can directly influence the obtained results. In relation to this, some species has been described as difficult to manipulate in exposure experiments like is the case of the mice *Mus spretus* which is easily stressed when xenobiotics are administrated and can practice puerperal cannibalism with its newborns.

Another important question is if the animal should be a good *model for humans*. In this context rats, mice and rabbits belong to the same evolutionary class than humans, so in principle, they should generate data that are relevant to humans [45]. However, in the particular case of arsenic, the hemoglobin (Hb) in rats present 3-16 times higher affinity for As^{III} than human Hb [46], but they exhibit many physiological characteristics closer to humans [45] in comparison with mice. In addition, while the effect of arsenic on cell membrane breakdown has been demonstrated in mice by the increase of LPCs (lysophosphatidylcholines), other authors reported that some LPCs decreased in rats, which can also be explained by the distinct responses of arsenic toxicity of rats in different life stages [36,47].

In summary, mice present several advantages when used as model organisms to humans: (i) mice and humans share 95 percent of the same genes, being our immune systems even more compatible and consequently, they get many of the same diseases [48], (ii) mice can be genetically manipulated to mimic any human disease or condition, (iii) they can be

inbred to yield genetically identical strains, which are very useful for more accurate and repeatable experiments, (iv) mice have an accelerated lifecycle, with one mouse year equaling about 30 human years and therefore, their entire life cycle can be studied within only two or three years, (v) xenobiotic-induced depletion of an essential trace element in a particular organ is detected sooner in smaller animals because of their correspondingly reduced organ storage capacity and their faster metabolic rate [49] (vi) mice are well understood because they have been used in biomedical research for nearly a century, (vii) mice are a cost-effective and efficient research tool because they are small, they reproduce quickly, and they are relatively easy to handle and transport.

The Metabolomics Society's Model Organism Metabolomes (MOM) task group has proposed a prioritized list of model organisms that they recommend for deep investigations of their metabolomes, which includes zebrafish and *Mus musculus* for vertebrate animals. However, some important drawbacks derived from the use of *Mus musculus* mice as a model organism have also been outlined, for example, the immune system is not so similar to humans, mainly due to the fact that they have been long time under controlled conditions and loss an important part of their responses [50,51]. On the other hand, the sequenced *Mus musculus* specie (classical inbred laboratory specie) is genetically homologous to *Mus* spretus, which diverged from *Mus musculus* one to three million years ago [52,53] and therefore, it can be used instead of *M. spretus* in exposure experiments in order to know the metabolic response [54].

The *size of animals* is also crucial in metabolomics since sample consumption is relatively high, even more if metabolomics is combined with total metals content determination and chemical speciation. As an example, about 30 mg are required for tissue metabolomics,

0.1 g of tissue for total metals content analysis and 50 mg for arsenic speciation in tissue, while for serum/plasma analysis about 100 µl are usually consumed for total metals content analysis, 100 µl for non-targeted metabolomics and 100 µl for arsenic speciation [29,55]. In relation to this, if bile, for example, is the fluid of interest to detect an endogenously formed metabolite/detoxification product, it would be more appropriate to select rabbits because more bile can be obtained from this species and in a much easier manner than from mice from a surgical point of view [56].

Finally, when selecting animal models, the *sex and age* are also very important factors. Usually, males are selected because they are less affected by hormonal fluctuations than females which can be important to eliminate a potentially confounding factor to obtain clear results. Furthermore, testicles represent an additional organ in which toxicologically relevant observations can be made [57]. The age is important since the metabolism of the species can be different in some aspects. As an example of this, some detoxification mechanisms, such as the biliary excretion of GS-$HgCH_3$ conjugates, are not yet fully operational in juvenile animals as compared to adults [58] and other authors found that selenomethionine and methylmercury interact in different way in young and adults specimens of *Anas platyrhynchos* [59].

4. Selection of an appropriate xenobiotic dose, administration route and biofluid/tissue for analysis: The experimental design

4.1. Selection of an appropriate xenobiotic dose and administration route

One of the most important issues in toxicometabolomics is the dose, the exposure time and the chemical form selected (e.g. arsenic specie), since the metabolism can be greatly affected (section 7.1.). The selection of the administration route is also important since the absorption of the xenobiotic can be slow (e.g. subcutaneous), thus the exposure times should be adjusted.

Regarding the selection of an appropriate *xenobiotic dose*, several questions should be taken into account: (i) If we are interested in a *single exposure* during short periods (acute toxicity, < 24 h) or *repeated exposures*: subacute (≤ 1 month), subchronic (1-3 months) or chronic toxicity (> 3 months) [60]. It is important to point out that some effects can only be demonstrated by using chronic doses like the arsenic influence in purine metabolic process by a decrease in uric levels in plasma and urine in rats after chronic arsenic exposure [35,36].; (ii) *large doses* to study acute toxicity or *smaller ones* for chronic toxicity. In this sense, As^{III} for example, inhibits several enzymes [61] in the acute toxicity, whereas its chronic toxicity involves the inhibition of selenoprotein synthesis [62]. (iii) the *environmentally relevant dose*, if the studied animal is a model organism to study a polluted area; (iv) the *lethal doses*; (v) the *half-life of the xenobiotic in the body*, is important to set the final time of the experiment and (v) the *chemical species*, because the metabolism and toxicity mechanisms are strongly related to that [63].

The LD_{50} (mg As/kg bw) of sodium arsenite, arsenic trioxide and calcium arsenate in mice are 15-22, 34 and 20-800 in mice, respectively, considering oral administration [64]. A good criterion for repeated exposure experiments can be to administrate the LD_{50} divided by the number of days of the whole experiment [26,27,29,65]. Other authors select 1/5 of the LD_{50} for the high exposed group and 1/5 of this for the low exposed one,

both administered in tap water [47], while others considers several dosages to be administered in tap water taking into account environmentally relevant doses [36].

Regarding the environmentally relevant doses, a comparative metallomic study of *Mus musculus* (exposed mice) and *Mus spretus* (free-living mice, genetically homologous) responses under the action of metal contaminants is discussed in a previously published paper [20].

Another important question is the *administration route* of the xenobiotic which usually can be introduced by *oral*, *cutaneous*, *parenteral* (*intraperitoneal*, *subcutaneous*, *intramuscular* or *intravenous*) or *respiratory absorption* [66]. Regarding the *oral administration*, the introduction of a xenobiotic by the diet has the disadvantage of calculating how much was ingested by the animal, but usually, gastric probes overcome the problem (less than <10 mL/kg of the animal weight should be introduced). However, the reduced solubility of some xenobiotics convert the oral administration by the diet in a good alternative [66]. The *subcutaneous injection* also allows introducing fatty solutions that cannot be administrated *intravenously*, but solutions at not physiological pH produce more irritation when administrated subcutaneously and the absorption is slow. *Intramuscular absorption* is fast. *Intraperitoneal* is as fast as intravenous administration and allows introducing not hydrosoluble mixtures, but is only recommended when a reduced number of applications are performed due to the aggression that it can produce. The parenteral administration should be limited to 0.5 mL volumes for rodents and if higher volumes have to be used, several injections by different routes should be performed. In addition, a control group should be studied after the administration of the solution without the xenobiotic in identical food and ambient conditions [66]. Finally,

another important question is if several xenobiotics have to be introduced simultaneously to study their interaction in the body. In this case, if some of them can react before enter into the body, they should be introduced by different routes. In this sense, a combination of metallomic and metabolomic study has been performed in mice after the exposure of arsenic (As_2O_3) and cadmium ($CdCl_2$) using an oral gavage since both chemicals do not react [30], but mercury and selenium should be administrated by different routes [67] or by the same at different time [68], since they can react to form a mercury selenide which is part of the detoxification mechanism of the later against the toxicity of the former.

4.2. Selection of an appropriate biofluid/tissue for analysis

The selection of an appropriate *cell type, tissue, biological fluid or cellular compartment* is crucial since different biological responses can be detected in a particular biological matrix. Table 1 shows the different tissues or biofluids that have been used in arsenic toxicometabolomics.

Urinary biomonitoring provides the most accurate arsenic exposure assessment and it is a non-invasive sample. Urinary metabolomics allows to identify arsenic-related metabolic biomarkers which are required to understand the internal processes that may be perturbed [69]. In controlled exposure experiments with animals special metabolic cages are required for this purpose [70]. Likewise, several papers describe the use of urine in arsenic toxicometabolomics in mice [24, 32] rats [33] and humans [40, 41]. As we can see in Table 1, while plasma and serum present an important number of common metabolites, urine is an interesting biofluid since it allows identifying a great number of urine specific metabolites (almost until now) after arsenic exposure in mammals (Table 1) that have not been measured (or the response is identical in controls and exposed organisms) in other

biofluid. These metabolites (Table 1) are mainly related with pathways that only have been shown to be altered until now in urine such as Niacin metabolism, Porphyrin metabolism, glucuronidation, Betaine metabolism, Purine metabolism, Protein catabolism, RNA biosynthesis, Pantothenate and Coenzyme A biosynthesis, Transfer RNA degradation, Folate metabolism and Fatty alcohol metabolism.

On the other hand, *serum and plasma* are undoubtedly the most studied biofluids. Plasma can be obtained from whole blood by addition of an appropriate anti-coagulant (heparin, citrate, EDTA) to prevent the fibrinogen-clotting cascade, followed by centrifugation for separation into plasma (supernatant) and red blood cells (RBC), which are later washed with a phosphate buffer solution (PBS). Blood samples should be maintained at room temperature to allow natural clot to obtain sera after centrifugation (supernatant) [71]. Then, plasma has the advantage that the samples can be put once into ice water which avoids effects on the metabolome that could take place at room temperature needed to obtain serum samples (enzymatic conversions, losses or degradation of metabolites), but in contrast, in the serum samples, activated platelets (essential for coagulation processes) release a variety of metabolites, lipids and proteases during the coagulation processes [72,73]. On the other hand, serum samples are easier to collect and do not require a refrigerated centrifugation step, but plasma samples results in a greater sample volume per volume of blood withdrawn. Therefore, in general, both biofluids are adequate for metabolomics and maybe they are complementary.

Serum samples have been analyzed for arsenic toxicometabolomics in humans [39], mice [25] and rats [33,34,35], while plasma has been studied in rats [35], mice [29–32] and the whole blood in pigs [38] (Table 1). In humans [39], the use of serum samples is of pivotal importance since it is a sample easy to obtain, to conserve and allows identifying

intermediate biomarkers of response to toxicants at environmental/occupational concentrations. Regarding the response of the metabolites after arsenic exposure in serum, plasma or urine, there are some biofluid-specific metabolites (until now) after arsenic exposure in mammals (Table 1) that have not been measured (or the response is identical in controls and exposed organisms) in other biofluid. Likewise, "serum specific metabolites" are: pyroglutamic acid [36], creatinine [33], indolacetaldehyde [36], cytosine [36], cholesterol, ceramides, sphingosine, phytosphingosine [36] and luteinizinghormone [25]. Surprisingly, in spite of creatine is excreted as creatinine in the urine under oxidative stress [29], a differential response of creatinine in arsenic exposed mammals has only been measured in serum and kidney (not in urine or plasma). The serum levels of *total cholesterol* might be associated with realgar induced apoptosis [33]. On the other hand, increased rat serum ceramide (d18:0/16:0, d18:0/14:0) and sphingosine under arsenite exposure, might indicate also a disruption of membrane distribution. Increased levels of ceramide has been associated to cell apoptosis, oxidative stress and proteolysis [74,75].

There are "plasma specific metabolites" in mammals after arsenic exposure that are not detectable until now in other bio-fluid (or the response is identical in controls and exposed organisms) are: glutamine [30], serine, threonine [30], isoleucine [30], fructose, galactose [30], urea [30], choline [30], lysophosphatidylcholines [30], phosphatidylcholines [30], triglycerides [30], diglycerides [30], fatty acid beta-oxidation (palmitic acid [30], pipecolic acid [30], arachidonic acid [30]), isocitric acid [30]. A decrease of the glutamate level (or increase of its precursor glutamine) can be explained by the increased activity of the transporter glutamate antiporter, which exchanges extracellular cysteine for intracellular glutamate [76] to finally increase GSH synthesis. The expression of this

transporter is induced by oxidants, electrophiles, and inflammatory mediators [76]. On the other hand, the LPCs, and PCs are related with the lipid metabolism and an increase of choline and LPC with a correlative decrease of PC can be associated with drug induced disruption of cell membrane [77] since the majority of phospholipids (PL) in cellular membranes consist of glycerophospholipids such as PC.

A toxicometabolomic study has also been carried out in *red blood cells (RBC)* after mercury exposure. RBC are transporter cells, but also possess effective anti-oxidative system, providing antioxidant protection not only to themselves, but also to other tissues and organs [78]. Moreover, RBC are highly susceptible to oxidative damage due to the high concentration of oxygen and hemoglobin, which are powerful promoters of the oxidative damage [79]. However, precautions have to be taken during the storage of erythrocytes since alterations of their metabolome has been described [80]. The quantitative analysis and simultaneous activity measurement of Cu,Zn-superoxide dismutase has been accomplished in RBC by liquid chromatography coupled to inductively coupled plasma mass spectrometry and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [81].

On the other hand, to study *tissues* for metabolomics, they should be disrupted by cryomogenic homogenization and then, metabolites can be directly extracted as described in the next section. Liver is one the most metabolically active organ; it is the central organ of the homeostasis of carbohydrates, lipids and proteins metabolism [27]. It is also a key organ in maintaining lipid homeostasis being the main site of fatty acid oxidation together with the muscle (mainly beta-oxidation taking place into the mitochondria) and it is the sole organ able to de novo synthesizes of fatty acids by lipogenesis [82]. Liver tissue has

been extensively analyzed for arsenic toxicometabolomics in mice [26–28], rats [33] and pigs [38]. Kidney is also an interesting organ since contaminants are excrete by urine and it has been studied for arsenic toxicometabolomics in mice [29]. As we can see in Table 1, there are not any kidney specific metabolites. It is remarkable that practically all the organs have been studied from the metabolomics point of view and offer interesting and complementary information. Table 1, shows some liver specific metabolites detected after arsenic exposure like homocystein [26] involved in the glutathione metabolism [26] and that it is not obtained from the diet, but it is biosynthesized from methionine (detectable in liver, kidney and plasma of mice) that is converted to S-adenosyl-methionine (SAM) which donates a methyl group to arsenic to form methyl and dimethyl arsenic [83]. Other metabolites are *lysine* and *glyceraldehide-3-phosphate*. The former is metabolized to acetyl-CoA through an initial transamination with α-ketoglutarate and increase in the mice fed high fat diet and arsenic relative to the high fat diet group [28]. *Glyceraldehide-3-phosphate* can be related to alteration in carbohydrate metabolism, since Szinicz and Forth (1988) demonstrated the occurrence of carbohydrate depletion and inhibition of glucogenesis caused by the toxic action of arsenic in rats [84].

Moreover, the separation of *organelles* is crucial to look for specific metabolic alterations induced by xenobiotics. In this sense, a shotgun metabolomic approach based on mass spectrometry has been applied to mice hepatic mitochondria after arsenic exposure [27]. This organelle plays essential roles in several cell processes since its dysfunction causes a decrease in adenine triphosphate (ATP) production, oxidative damage and apoptosis [85]. In addition, hepatocyte mitochondria are essential in regulating the flux of metabolites into the cell in order to adjust energetic demand, ammonia detoxification, or anabolic pathways [86]. An example of this is the decreased levels of creatine that has

been measured in mitochondria of hepatic cells of As-treated mice [27], which represent a possible marker of bioenergetics mitochondria dysfunction, since creatine is excreted as creatinine by urine under oxidative stress situations. Table 1 shown some metabolites that have only been detected in hepatic mitochondria mainly related with the fatty acid beta-oxidation pathway (free fatty acids) [27]. The metabolism of arsenic have been shown to deplete methyl donor pools which causes hepatic steatosis by impairing normal lipid metabolism [87].

At the laboratory, once that the hepatic tissue has been disrupted by cryogenic homogenization, the mitochondria can be isolated using a Mitochondria Isolation Kit commercially available based on the sequentially addition of different reagents and centrifugation, followed by the mitochondria lysis with a methanol/acetonitrile mixture. The isolation of this organelle and the application of a metabolomics approach directly reveals important metabolic changes. For example, the detection of an increase of creatinine in mouse kidney after arsenic exposure, represents a possible biomarker of bioenergetics mitochondria dysfunction since creatine is excreted as creatinine in the urine under oxidative stress [29], but with this methodology, decreased levels of creatine can be directly detected in mitochondria. Moreover, superoxide dismutase (Cu,Zn-SOD) has also been absolutely quantified in mitochondria of mice hepatic cells using orthogonal chromatographic systems (size exclusion and anion exchange chromatography) coupled to inductively coupled plasma mass spectrometry (ICP-MS) and organic mass spectrometry after tryptic digestion [88]. Mitochondria has also been isolated for investigation of arsenic toxicity by several authors [89,90].

5. Sample preparation procedures and precautions in toxicometabolomics

5.1. Preparation of biofluids for toxicometabolomics

As in any analytical approach, preanalytical errors during sample collection and storage are crucial to reach analytical objectives. This risk is particularly high in bioanalysis in general, and not only in toxicometabolomics. For this reason, the discussion in this section is general for metabolomics and at the end, we focus on toxicometabolomics. For example, an important question when collecting serum and plasma is the *type of tubes* used. In this sense, polymeric gel tubes (which accelerate the separation of serum and plasma) and conventional ones have been compared in a recent paper in which the authors conclude that when these biofluids are collected in conventional tubes, critical pathways are affected such as the citric acid cycle, metabolism of amino acids, fructose and manose and that of glycerolipids, as well as pentose and glucuronate interconversion. In addition, polymeric gel tubes only promote differences at the metabolite level in serum samples, mainly related to the metabolism of amino acids, glycerolipids and two primary metabolites such as aconitic and lactic acids [91]. These alterations in the metabolic cycles can be, almost in part, overcome with the use of control samples since they will be altered in the same extension than samples. However, some metabolites than could be used as potential biomarkers will not be detected due to the alteration of the pathways in which they are involved. Other authors also studied this issue as well as the *influence of hemolysis* on the metabolome which is one of the major risks during blood drawing [92–94]. The collection of a dried blood spots on a filter paper is a standard sample matrix in newborn screening for example and, followed by solvent extraction has been regarded as one of the most efficient strategies in whole-blood sample analysis [72]. A drawback of

this technique is the stability of analytes, background interferences from the paper, difficulties in extracting metabolites that interact strongly with the paper and the effect of storage in this matrix [95]. In addition, the volume of sample that can be collected with the paper is not enough for the majority of procedures commonly used in metabolomics or in particular in toxicometabolomics and some interesting metabolites could be lost.

The *type of anticoagulant* used during blood collection is also important especially in connection with the analytical technique that will be later applied [72,96,97]. The anticoagulants commonly used to obtain plasma samples (heparin, citrate, EDTA) have been compared in terms of metabolite coverage and precision and did not give major differences [95], but there is no consensus and other authors report important effects. In this sense, the cations can provoke matrix effects in liquid chromatography coupled to mass spectrometry (LC-MS) such as Li^+-heparin which may increase the ionization efficiency of phospholipids and tryacylglycerols, but may also increase the signals of plastic polymers [72]. On the other hand, a citrate peak can be found in LC-MS chromatograms obtained by both, reversed phase and hydrophilic interaction liquid chromatography (HILIC) and causes significant ionization suppression of metabolites at the same retention time [98]. Other authors find similar effects with using EDTA as anticoagulant [99]. In addition, in the particular case of toxicometabolomics related with metals, metallomics or speciation techniques are combined as above commented to obtain more information, and the use of complexing agents will represent a problem because they can sequester the metal and avoid identifying metal containing metabolites, species or macromolecules that can be of interest.

For the preparation of *urine samples* prior to metabolomics mass-spectrometry analysis important sample-pretratation steps have been extensively discussed [100]. Briefly, these steps are related with: (i) *sampling time*: e.g. random, timed or 24h samples and collection: additives to avoid precipitations or metabolite interaction with the container surface, (ii) *quenching*: cold solvent addition [100], freezing in liquid nitrogen [100], addition of acid [73] or fast heating [73], (iii) *preservative addition*: i.e. sodium azide addition to prevent bacterial contamination has been used in a mercury [70] and arsenic [37] toxicometabolomic study of urine or *filtration*: 0.20 μm filtration was found better for removal bacteria [94], (iv) *volume correction normalization*: creatinine concentration [55,101], osmolality, urine volume or components that are common to all samples, (v) *pH adjustment*, (vi) *deproteinization*: dilution with acetonitrile, centrifugation or filtration and (vii) *freezing/thawing cycles*: storage up to 6 months and less to 9 freeze-thaw cycles. In general, several overlapping peaks can be a problem especially when they are at high concentrations. This is the case of urea in urine, which elutes in a peak of 2-3 minutes in GC-MS and can be eliminated to avoid this problem [102].

The quenching procedure, which is important to preserve the true metabolome at the time of sampling, is usually routinely incorporated in cell and plant metabolomics, but its suitability is discussed in biosamples since the consequences of omitting or introducing this step are not fully understood [73]. In addition, the quenching step is difficult to implement in biological samples on an appropriate time and it could cause degradation or loss of some metabolites [73]. The common strategies for quenching are based on pH (either to high alkali or acid) or temperature (usually $T < 20^{o}C$, e.g. with cold methanol, liquid nitrogen) modifications [103].

Finally, in relation with the storage conditions, storage at -80ºC or below is regarded as the preferred condition [104]. In this sense, it is important to conserve samples in different aliquots in order to avoid cycles of freezing-thaw for different analysis.

After an adequate sample collection, storage and pretratation steps, the selection of a suitable sample treatment procedure is very crucial because it affects the correct metabolite identification and/or quantification and consequently, the biological interpretation of active metabolite pathways [105]. In untargeted metabolomics, extraction procedures must be non-selective, simple and fast with a minimum number of steps [73], reproducible [73] and they should incorporate a metabolism quenching step [73,106]. Usually, salts and macromolecules are the most important interferences in untargeted metabolomics, especially when mass spectrometry is later used since they usually provokes ionization suppression or peak overlapping.

The most simple sample preparation procedures are dilution or solvent precipitation, while other selective procedures are avoided in global metabolomics. The reproducibility is mandatory since metabolomics usually compares the relative levels of metabolites in a large number of samples [73].

Thus, analysis of biofluids is normally preceded by *protein precipitation* and/or *sample dilution (dilute-and shoot)* to avoid matrix effect [107] but, there is not a universal extraction method for global analysis of metabolome. Approaches based on dilution and precipitation allows high metabolite coverage with main disadvantages related with ionization suppression and co-precipitation of metabolites with proteins or due to the poor solubility in the solvent used for dilution/extraction [108]. The precipitation is especially

recommended in plasma and serum samples, which contain more proteins than urine. Usually, the simple addition of an organic solvent precipitates the proteins and disrupts any metabolite-protein binding. The most used organic solvents are acetonitrile, acetone, pure ethanol and pure methanol, as well as some mixtures of them. Heating and the addition of acid (e.g. trichloroacetic acid) or metals (e.g. zinc sulphate) [73], a cut-off molecular mass filter [109] and lowering pH (less metabolite coverage) have also been used for protein removal.

Sometimes, the use of *evaporation and later reconstitution* is used for pre-concentration, to improve stability of dried extracts during storage or to change the solvent to be compatible with the subsequent analytical technique used. On the other hand, the benefits of this procedure have not been well established because hydrophobic and volatile metabolites can be lost and increased ionization suppression may give lower metabolite coverage [73].

Ultrafiltration is other interesting alternative in metabolomics in which a special filter is used to separate molecules of different molecular weights. The disadvantage is the loss of hydrophobic species compared with solvent precipitation and solid-phase microextraction, but ultrafiltration has the advantage of ensuring better metabolome preservation [73]. Alternatively, *solid phase extraction (SPE)* has been reported for simultaneous extraction and purification of biological fluids, using different types of adsorbent materials; such as reverse/normal phase and ion exchange [110,111]. However, mixed mode extraction using different retention mechanisms on the basis of several ligands or active sites on the solid phase shows a higher potential in metabolomics, due to the most efficient extraction of metabolites [112]. The main advantage is that samples

are cleaned-up before analysis producing the enrichment of low-abundant metabolites and removing several interferences on analyte signal, but on the other hand, the selectivity is higher, and then, the metabolite coverage lower. This technique is therefore preferred for targeted metabolomics. *Solid-phase microextraction (SPME)* present other interesting alternative, but in this case, the extraction is based on an equilibrium and not in an exhaustive extraction and then, sorbent amount is much lower than in SPE. SPME also present the same pros and cons than SPE, but an additional and promising advantage is the *in vivo* use for the extraction of metabolites (see section 5.3.), since it can provide an intact snapshot of the true metabolome at the sampling time.

Quality control strategies and method validation in metabolomics for sample preparation approaches to be used before organic mass spectrometry have been reviewed [113]. The MIAMET (Minimum Information About a METabolomic experiment) program is also interesting for metabolomics analysis standardization [114].

Regarding arsenic toxicometabolomics by organic mass spectrometry, mice plasma has been prepared for electrospray ionization mass spectrometry (ESI-MS) by liquid-liquid extraction (LLE) with a methanol/ethanol mixture (4:1, v/v) followed by vigorous vortex shaking and centrifugation (elimination of proteins). After that, metabolites are extracted in two steps depending on their polarity. After the extraction, the supernatant (polar metabolites) is taken to dryness and reconstituted with the same extraction solvent. The lipophilic metabolites were extracted from the pellet after homogenization with a chloroform/methanol mixture (2:1, v/v), centrifuged and evaporated/reconstituted with a (1:1, v/v) chloroform/water mixture [115]. Metabolites related to arsenic toxicity have also been extracted with methanol from rat serum using a simple procedure (extraction,

centrifugation, filtration and analysis) prior to their introduction into an ultra-performance liquid chromatography (UPLC) orbitrap mass spectrometry [36]. Other authors extracted metabolites after mice exposure to arsenic from urine and plasma with a procedure based on cold methanol addition, centrifugation, evaporation and reconstitution with a mixture of water:acetonitrile for MS analysis. Metabolite extraction from fecal pellets was also conducted in a similar manner [24]. Metabolites from rat serum have also been extracted after arsenic exposure after deproteinization with methanol (1:5), evaporation and reconstitution with acetonitrile and water (3:1) before UPLC coupled to quadrupole time of flight (QTOF) analysis [47]. On the other hand, rat urinary metabolites have been analyzed by LC-MS after thawing, centrifugation and dilution with methanol, vortexing and filtering [37,116]. For NMR (Nuclear Magnetic Resonance) analysis, the sample preparation is much simpler (e.g. buffering with deuterated solutions) as described in several papers related to arsenic metabolomics [33,37].

The urine of arsenic exposed rats have also been analyzed by ultrafast liquid chromatography (UFLC) coupled with ion trap time-of-flight mass spectrometry (IT-TOF/MS) and ^{1}H NMR spectroscopy after a simple dilution with methanol for LC-MS or with a phosphate buffer in deuterium oxide containing 3-trimethylsilylpropionic acid as a shift-lock reagent for NMR [37].

In addition, depending on the analytical technique to be latterly used, the addition of some solvents/reagents is mandatory. For example, for data acquisitions with direct infusion ESI-MS in positive ionization mode, 0.1 % (v/v) formic acid can be added to polar extracts and 30mM of ammonium acetate to lipophilic extracts, but for negative ionization, intact extracts can be directly infused to the mass spectrometer [29]. On the

other hand, if gas chromatography mass spectrometry (GC-MS) is used, a previous derivatization step is mandatory because the majority of metabolites are not volatile. To this end, a methoxylamine hydrochloride solution has been used for protection of carbonyl groups by methoximation with methoxiamine in pyridine, followed by treatment with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) with Trimethylchlorosilane (TMCS). In addition, TMCS aids in the derivatization of amides and secondary amines and hindered hydroxy groups. After vortex and centrifugation, the samples can be directly analyzed [29,117].

5.2. Preparation of tissue samples for toxicometabolomics

As in the case of biofluids, some special precautions have to be taken before sample preparation. For example, several organs are heterogeneous (i.e. liver, kidney and brain have regions with different enzymatic activities), so the tissues from them could give rise to region specific results. The presence of blood can also affect the results since it circulates through different organs and carry other metabolites not specific of the tissue [118].

Then, unlike metabolomics analysis of biofluids, extraction of tissues requires a previous homogenization and lysis of cells, combining a cryhomogenization step with simple extraction protocols based on the use of polar/lipophilic solvent mixtures [119]. The quenching step usually can be performed by freezing the excised organs in liquid nitrogen, after cleaning with a 0.9 (w/w) NaCl solution as preservative which have also been used for toxicometabolomics of arsenic [26,29,65], but also denaturing enzymes with the addition of acid or solvents after tissue collection [118].

The most commonly used analytical procedure to extract metabolites from biological tissues for untargeted metabolomics is solid-liquid extraction, which present high efficiency and minimum degradation. The contact with the appropriate solvent can be favored by shaking, vortexing or magnetic stirring [103]. Like in the case of biofluids, alcohols (especially methanol) and acetonitrile are the best extractants since they precipitate proteins, effectively permeate the cell, are easy to evaporate-concentrate and do not add salts, which are in turn incompatible with MS analysis. The two – step (biphasic) extraction, first proposed by Bligh and Dyer [120], combining methanol-water phase and chloroform or chloroform/methanol phase is one of the most popular procedure to extract polar and lipophilic metabolites from biological tissues, respectively [103] which has also been used for toxicometabolomics of arsenic with some modifications (Figure 4) [27,29]. Several papers have probe that a single phase extraction (all-in-one) based on methanol/chloroform/water, methanol/water/ethanol and other phases provides a wider metabolite coverage. However, stepwise extraction provides more acute snapshot of the tissue metabolome and could enhance solvent specific metabolite extraction [118].

Other analytical procedures for the extraction of metabolites (e.g. microwave, ultrasound, supercritical fluids, SPE, Soxhlet) have also been used, but usually for targeted metabolomics [103]. However, several interesting procedures are described for untargeted metabolomics, such as an on-line solid-phase extraction capillary electrophoresis mass spectrometry (SPE-CE-MS) which has been proposed for untargeted metabolomics in mice plasma [121]. On the other hand, not only polarity based extraction methods have been used in bioanalytical and clinical applications of metabolomics, but also vector energy based techniques (e.g. ultrasound, microwave,

pressurized extraction techniques). The ultrasound probe has also been proposed to improve sample preparation steps in metabolomics, like freeze-drying, metabolites leaching by solid-phase extraction, biochemical reactions (e.g. hydrolysis, enzymatic reactions) [114]. On the other hand, microwave assisted extraction (MAE) has been successfully used in lipidomics applied to skin samples [122].

Figure 4 shows the most commonly used procedures for tissue arsenic toxicometabolomics. Regarding arsenic metabolomics, for ESI-MS analysis, frozen organs of arsenic exposed mice have been disrupted by cryogenic homogenization, polar metabolites extracted twice with a (1:1) methanol-water phase (vortex + pellet mixer), centrifuged and then, lipophilic metabolites extracted from the pellet with a chloroform/methanol (2:1) mixture (vortex + pellet mixer) [26,29,65]. To extract metabolites from hepatic mitochondria, after the isolation of the organelle a 2:1 mixture of methanol/acetonitrile can be used for lysis and metabolite extraction prior to ESI-MS or GC-MS analysis [27]. As in the case of biofluids, formic acid or ammonium acetate are added before positive ESI-MS to the polar or lipophilic extracts, respectively, but for negative ionization, intact extracts can be directly infused to the mass spectrometer [29].

As in the case of biofluids, if GC-MS will be used, a derivatization step is mandatory to analyze non-volatile metabolites. In this sense, arsenic metabolites have been extracted from liver tissues of exposed mice after homogenization with methanol, then evaporated and redissolved in ethoxyamine hydrochloride solution for methoximation and derivatized with N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) mixed with tert-butyldimethylchlorosilane (TBDMSCI) [28]. A similar derivatization step has been used for arsenic metabolites of mice liver mitochondria, in this case using

N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) with trimethylchlorosilane (TMCS) [27].

When NMR is used for tissue metabolomics, arsenic metabolites from frozen and homogenized liver can be extracted after homogenization with a methanol/water mixture with a chloroform (discarded)/water mixture. Finally, the aqueous layer is lyophilized, resuspended in buffered D_2O and analyzed [33].

5.3. *In vivo* metabolites extraction

As commented before, the rationale for using this approach is that it can provide an intact snapshot of the true metabolome at the sampling time, eliminating the sample exposure to oxygen, solvents or pH changes. However, in spite of the important advantages of this approach and the significant information that can be obtained, only few references related with two analytical techniques have been published.

Microdialysis is a suitable technique for *in vivo* sampling of low molecular weight metabolites from blood and tissue samples, without the need for biopsies. It is important to highlight that anesthesia was found to significantly affect metabolite profiles and the combination with liquid chromatography mass spectrometry is difficult due to the ionization suppression caused by the commonly used buffers in microdialysis and the limited extraction of highly bound and hydrophobic species [73].

With the introduction of new biocompatible coatings, which avoid adverse reactions, protein adsorption and/or clotting, SPME can also be applied to *in vivo* metabolomics.

The main advantage of this technique is the elimination of the metabolism quenching step (proteins and other macromolecules cannot diffuse into the coating) and sample contamination. Moreover, *in vivo* applications represent a step forward in biomarker discovery and open new possibilities for clinical diagnosis [123]. In addition, the method is directly compatible with LC-MS, ionization suppression is lower when reversed-phase LC-MS is latterly used and it is suitable for hydrophilic and hydrophobic metabolites, which overcome the most important limitations of microdialysis. Moreover, SPME allows detecting unstable metabolites at low levels *in vivo* [73].

SPME has been used for *ex-vivo* and *in vivo* extraction of metabolites from breath, plasma, serum, urine, saliva, tissues, hair, skin and cells and the approach has been extensively reviewed in recent papers [124–128]. Finally, an interesting technique for *in vivo* metabolites identification is NMR [129].

6. Analytical instrumentation for arsenic metabolomics in mammals

Figure 5 shows an scheme of the most widely used analytical instruments in metabolomics along with their pros and cons. As can be seen, there is no analytical technique to characterize the entire metabolome simultaneously, which makes necessary the complementary use of different analytical platforms in order to get the widest analytical coverage. High throughput techniques for a comprehensive metabolic screening such as mass spectrometry and nuclear magnetic resonance (NMR) [130] have emerged as the main instrumental approaches in metabolomics. NMR exhibits high reproducibility and fast analysis. In addition, it does not require extensive sample

preparation and the comprehensive databases available facilitate identification of metabolites [131]. The use of a buffer is advantageous because it maintains a constant pH, which avoids interconversion for chemical shift changes due to pH differences, but aqueous buffer extraction methods only extract polar metabolites [132]. The potential of NMR for high throughput analysis, along with rapid and straightforward sample preparation, its non-destructive character, small sample consumption, the possibility to use for *in vivo* metabolite detection [129] and no previous separation step requirement have made this technique the most traditionally used in metabolomics; but, inherent limitation of NMR is its poor sensitivity, which hampers the detection of important metabolites present at low concentrations. For this reason, MS is gaining importance due to its higher sensitivity and specificity, as well as the possible application to complex samples. Moreover, mass spectrometry is able to perform both qualitative and quantitative analysis, providing metabolomic fingerprints for samples classification and identification of altered metabolites.

Mass spectrometry has a great analytical potential due to the different instrumental arrangements available, which include diverse systems for sample introduction, ionization source and mass analyzers. The most widely used interface is the electrospray ionization source (ESI), and more scarcely, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) sources [133]. Nowadays, the availability of a great number of mass spectrometry analyzers and hybrids offers the possibility of using different acquisition modes as full scan (ion traps, IT and hybrids), single ion monitoring (quadrupole, Q), MS/MS (e.g. IT, QQQ, QTOF), single/multiple reaction monitoring (SRM, MRM), neutral loss and precursor ion scan (e.g. QQQ). Neutral loss and precursor ion scan have been mostly used in lipidomics [134].

The extracts or diluted samples can be introduce directly (direct infusion or flow injection) or using a separation step based on GC, CE or HPLC. Direct infusion, especially when using high resolution mass spectrometers like triple quadrupole time of flight mass spectrometry (QqQ-TOF) or Orbitrap [135,136], present high metabolite coverage, high-throughput screening capability, no sample carryover and high reproducibility and has been successfully applied in several applications related to arsenic toxicometabolomics [26,27,29,30]. The main drawback is the ionization suppression, which makes difficult to detect metabolites with low ionization efficiency, and the presence of isobaric interferences [137].

Another possibility to introduce the sample without the chromatographic step is the imaging mass spectrometry (IMS), which allows determining hundreds of metabolites in complex samples showing their distribution and relative abundance *in situ* in a single analysis, preserving sample and cell integrities [138]. In this sense, matrix assisted laser desorption ionization (MALDI), desorption electrospray/plasma assisted desorption electrospray ionisation (DESI/PADI) and secondary ion mass spectrometry (SIMS, no matrix is needed, provides distribution of metabolites in three dimensions) have been successfully applied in direct tissue analysis [138,139].

On the other hand, mass spectrometry is also coupled to liquid (LC) [118,140], gas chromatography (GC) [118,141,142], or capillary electrophoresis (CE) [118,143,144], in order to reduce the complexity of mass spectra, avoiding isobaric interferences and ion suppression caused by the matrix in DIMS.

The coupling GC–MS is one of the approaches most widely used in metabolomics since it offers very high chromatographic resolution, and high reproducibility among different instruments and laboratories, which makes possible to use databases [137], but requires chemical derivatization for many metabolites that are not volatile as described previously (2.3. Sample preparation procedures and precautions in toxicometabolomics) and it is not valid for thermally unstable compounds. The derivatization is usually based in a metoximation, usually with methoxyamine in pyridine, followed by silylation, or solely silylation with N-methyl-N(trimetilsilyl)-trifluoroacetamide (MSTFA), N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with trimethylchlorosilane (TMCS, added as a catalyst for secondary alcohols and amines) or more scarcely, ethylchloroformate [115] or N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) mixed with tert-butyldimethylchlorosilane (TBDMSCI) [28]. An interesting revision of the most widely used derivatizing reagents and their pros and cons has been previously published [145]. Finally, The most used stationary phases are the 5% phenyl group or 100% polydimethylsiloxane [118].

On the other hand, HPLC has lower chromatographic resolution, but the number of metabolites able to be analyzed is greater [146]. The use of UPLC represents also important advantages related to the higher sample throughput, sensitivity and resolution. On the other hand, reversed phase (RP) and hydrophilic interaction liquid chromatography (HILC) are the main stationary phases used in metabolomics, but there is not a unique column capable of retain all metabolites. In this sense, although RP present high sensitivity and high metabolite coverage, it does not retain polar metabolites and HILIC can be used for this purpose, but these columns have lower capacity which result in broader peak shapes [137].

On the other hand, CE is most appropriate for polar and charged analytes [147]. The most important advantages of CE against LC and GC are the higher resolution for ionic compounds and their isomers since CE separates according to the charge to mass ratio of analytes and that the resolution is high, the analysis is faster and requires low sample volume and simpler sample preparation procedures. However, the sensitivity is lower, mainly due to the low sample volume required [118]. Comparing CE with LC and GC, the interaction with a stationary phase is not required for CE, which makes this technique an interesting complementary tool in metabolomics.

Finally, Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) offers very high resolution and the highest currently available mass accuracy and tandem mass spectrometry capabilities, but it presents important drawbacks as the less information offered (only mass to charge ratio), the cost, lower reproducibility, sensibility and less quantitative capability [118,145].

7. Data acquisition and treatment. Identification of metabolites

Metabolomic procedures generate large data sets of great complexity that have to be organized in bidimensional matrices to make possible the subsequent treatment and interpretation. Actually, there are several free software tools (XCMS, MZmine, R commander) or specific of the mass spectrometer (MassHunter, Agilent Technologies; MarkerLynx, Waters; MarkerView, Sciex).

The pre-processing of data provided by the different instrumental coupling previously described involves several steps [148] as follows: (i) peak detection, that is the first step and involve the identification of all the signals eliminating noise with filters and signal to noise thresholds (e.g. vectorized methods, Gaussian polynomial adjustments); (ii) alignment, that is the correction of the inter-sample variability in the retention times and (iii) normalization, which means the elimination of systemic variability sources among the samples (e.g. changes in the instrumental sensibility, differences in dilution factors of the samples) to guarantee the comparability of chromatograms or electropherograms.
On the other hand, data treatment in direct infusion or flow injection analysis is much simpler, since the absence of chromatographic separation makes unnecessary to perform the alignment step. Thus, preprocessing is limited to finding the peaks present in the mass spectra above the threshold of instrumental noise.

After pre-processing, the interpretation of analytical data from metabolomic studies requires advanced statistical tools due to the high number of samples and variables involved in the experiments. To this end, multivariate analysis is applied to reduce the complex data matrices associated to metabolomics, although, the results have to be latterly validated by other classical univariate techniques.

Data has to be pretreated to minimize potential sources of inter-sampling variability. The most important steps in data pretreatment are transforming, scaling and imputation of missing values [149,150]. Data transformation, usually logarithmic makes possible heteroscedasticity correction, the conversion of multiplicative relationships into additive and the approximation of asymmetric distributions to normality [149]. On the contrary, scaling methods allows reducing the relative importance of majority variables against

minority ones, that are based on the division of each variable by a correction factor called scaling factor. The most frequently used method in metabolomics is the Pareto scaling in which the scaling factor used is the square root of the standard deviation of each variable. Finally, regarding the imputation of missing values, which can be related with the heterogeneity of samples, minor metabolites or technical problems and can affect the correct data treatment and interpretation, several methods have been proposed [150].

After this data pre-treatment, several multivariate techniques can be used to extract biological information like projection techniques, based on the conversion of a bidimensional data matrix into a simplified model by the reduction of a great number of variables after the combination of the original variables to generate new components [151]. These models allows identifying outliers and visualizing groups and tendencies among the different groups under study, making easier the interpretation of results. Usually, unsupervised techniques like Principal Components Analysis (PCA), in which the statistical model is constructed without a previous knowledge about the relations between samples and classes, is used at an initial exploratory step, but after that, supervised techniques, like Partial Least Squares Discriminant Analysis (PLS-DA) should be applied for a better separation [152]. On the other hand, a key point in metabolomics is the great number of variables usually generated (e.g. m/z peaks of the mass spectra), that are not compatible with the Linear Discriminant Analysis (LDA) in which the number of samples/number of variables should be ≥ 3 [153] being a great limitation when analyzing biological samples. This problem is overcome by PLS-DA in which a previous reduction of variables is performed by PLS. However, a good practice is the previous reduction of the number of variables by using a Student *t*-test before the PLS-DA [154].

As previously commented, the results have to be validated after multivariate techniques by classical univariate techniques to classify groups and select possible discriminant metabolites. To this end, univariate parametric statistical tools like Student *t*-test or analysis of variance (ANOVA) or non-parametric tools (e.g. Mann-Whitney, Kruskal-Wallis) can be applied.

Once, that the variables (e.g. m/z of the mass spectra) responsible of the classification of samples among the groups have been selected, the next step is the identification of the metabolites. When using mass spectrometry, metabolites can be identified by MS/MS with the help of mass spectra databases which is easy in GC-MS (e.g. NIST, National Institute of Standards and Technology), but difficult in LC-MS due to the variability of ionization sources. However, there are several free databases available (Human Metabolome DataBase-HMDB, http://www.hmdb.ca; Metlin, http://metlin.scripps.edu; Kyoto Encyclopedia of Genes and Genomes-KEGG, http://www.genome.jp/kegg; Lipid Maps, http://www.lipidmaps.org).

In addition, to prove the discriminant character of the metabolites the most powerful technique is the receiver operating characteristic (ROC) curves, which represent graphically the sensitivity and specificity of a biomarker, so that the area under the curve (AUC) can be used to evaluate the potential of the biomarker for diagnosis [155]. These curves can be obtained with the free tool MetaboAnalyst (http://www.metaboanalyst.ca/) as well as the metabolite pathway impact graphs to determine the impact of the metabolite in the pathway in comparison with all the metabolites involved in it.

Finally, the robustness of the analytical procedure is the pivotal importance in metabolomics to assure that the inter-sample variability is higher than those generated by the approach. To this end, the most common strategy is the use of quality control samples (QCs) which can be obtained by mixing aliquots of each sample and analyzing them at the beginning of the sequence to equilibrate the system as well as along the sequence to monitor the instrumental stability [156]. Since the biological variability of QCs is null (their composition is an average of all the samples) and any variability is due to the approach, if the method is robust, these samples should be tightly grouped in a PCA or in the center in a scores plot obtained by a supervised technique like PLS-DA. Another strategy to evaluate the robustness of the procedure is the determination of a variation coefficient of each metabolite in the QCs, which should be less than 30% in non-targeted procedures as established by the US Food and Drug Administration, FDA [157].

7.1. Metabolites identification/quantification in the context of known metabolomic pathways: Biological interpretation and arsenometabolomics

Toxicometabolomics has the capability to illustrate the toxicity in systems as well as the pathways involved in the toxicology. Likewise, table 1 shows the different metabolites over/down-expressed in mammals after arsenic exposure, which will be discussed in this section and the analytical method used in each case since some metabolic markers are techniques dependent.

The strong conditioning of arsenic toxicity with its chemical form is well known. In this way, the most toxic forms are inorganic arsenic, being iAs^{III} more toxic than iAs^{V} most

probably due to enhanced cellular uptake and accumulation of the former [158,159] and followed by methylated forms, MA^V (monomethylarsonate) and DMA^V (dimethylarsinate) which are considered moderately toxic like trimethyl-arsine oxide (TMAO) and tetra-methyl-arsonium (TETRA), and finally, arsenobetaine (AB), arsenocholine (AsC) and other arsenosugars (AsS), which show no toxicity [160]. Trivalent methylated metabolites are considered more toxic due to their high binding affinity for sulfhydryl and thiol groups of proteins in various organs as compared with iAs^V [161].

In mammals, the metabolism of arsenite and arsenate has been well documented [61]. Briefly, arsenite is absorbed from the gastrointestinal tract into the bloodstream (> 80%) and mainly bound to L-glutathione (GSH) and L-Cysteine (Cys), due to its high affinity for the thiol group, as well as to plasma proteins. Several major As^{III}-binding proteins have been identified in rabbit and rat liver cytosols as the iron storage protein ferritin [162], ^{III}As-methyltransferase and pyruvate dehydrogenase [84,163,164] and several metabolites have been identified in bile, like $(GS)_3As$, $(Cys)_3As$, MA^{III} (monomethylarsonous acid) and MA^V (monomethylarsonic acid) [165–167]. As^{III} inhibits oxidases and dehydrogenases due to the above commented affinity for the thiol group [168].

Arsenate is also efficiently absorbed from the gastrointestinal tract into the bloodstream (>90%) via ^{V}P uptake machanisms [169] to be mainly bound to transferrin. As^V inhibits pyruvate dehydrogenase and uncouples oxidative phosphorylation by competing with phosphate, thus inhibiting energy-linked reduction of NAD^+, mitochondrial respiration, and ATP synthesis [170–172].

In humans, AB and AsC are not bioavailable and directly excreted in urine. On the other hand, it has been reported that DMA^V is the major metabolite produced from iAs, and the intermediates produced *in route* to DMA^V (trivalent forms) are thought to be responsible of the toxicity of arsenic [173] (Figure 6). In humans, arsenosugars, arsenolipids and iAs are converted to DMA^V [174]. Inorganic arsenic and other arsenic species (MA^V, DMA^V or TMAO) led also to the formation of thiolated metabolites [175,176].

7.2. Arsenometabolomics

Exposure experiments of arsenic to mammals led to the identification of alterations in biochemical pathways, such as amino acid, nucleotide, lipid (lysolipid/sphingolipid and fatty acid beta-oxidation metabolisms) and androgen metabolisms as well as the glycolysis and Kreb´s cycle. Figure 6 shows the metabolic pathways altered by arsenic exposure and Table 1 summarizes the altered metabolites after arsenic exposure in mammals with the analytical method used to measure them.

Disruption of the *amino acid metabolism* seems to be a common response to many toxins in humans and animals, and it suggests a general response to various toxins rather than a specific biological response to a particular one [177]. In relation to the *glutathione metabolism* (Table 1), arsenic induces decreased levels of GSH in liver [26, 33] and plasma [29] that can be explained by its important role as antioxidant protecting cell from fee radicals and in arsenic methylation (Figure 6) [178] as a response to the oxidative stress, as well as a correlative increase of Cys and L-glutamic acid [26,29] involved in the production of this metabolite. By contrast, other authors reported a compensatory

increase of GSH synthesis in liver in response to relatively short exposures [33,179]. This example illustrates the important role of doses and exposure time used in experiments to compare metabolomes and to avoid misinterpretations.

Also in relation to the amino acid metabolism, *methionine*, increases in liver, kidney and plasma [26,29] of mice exposed to arsenic (Table 1). Methionine is converted to S-adenosyl-methionine (SAM) by methionine adenosyltransferase, being SAM involved in arsenic metabolism in mammals, in which SAM donates a methyl group to arsenic to form methyl and dimethyl arsenic [83]. In addition, Table 1 shows decreasing levels of *homocysteine* in a dose-dependent manner in mice liver [26] and plasma [29] after arsenite exposure. This amino acid is not obtained from the diet, but it is biosynthesized from methionine. *Arginine*, required to remove urea though the urea cycle for detoxification of nitrogen compounds synthesised in liver and with protective effect against oxidative stress [180] also decreases after arsenic exposure [26, 27, 29].

Regarding the *nucleotide metabolism* it has been reported that *uric acid* and *cytosine* decreased when rats are exposed to high arsenic concentrations (Table 1). Uric acid is the final oxidative product of purine nucleotide metabolism [35,36].

An important effect of arsenic exposure to mammals is related to the cell apoptosis (Figure 6) in connection with the *lysolipid and sphingolipid metabolisms*. Likewise, it is interesting to consider the increasing levels of *choline* and *choline containing metabolites* in liver [26] and kidney [29] of mice after arsenite exposure, such as phosphorylcholine, l*ysophosphatidylcholines* (LPC), and free fatty acids, with a correlative decrease in phosphocholine and phosphatidylcholines (PC) (Table 1, figure 6).

Lysophosphatidylcholines (LPC) derive from PCs by the enzymatic action of phospholipase A2 that removes one fatty acid from PC and LPC are precursors of phosphorylcholine and choline, this last is a constituent of cell membranes and lipoprotein phospholipids, and plays an important role in the integrity of cell membranes and lipid metabolism [181]. Thus, an increase of choline and LPC with a correlative decrease of PC can be associated with drug induced disruption of cell membrane [77] since the majority of phospholipids (PL) in cellular membranes consist of glycerophospholipids such as PC.

The *fatty acid beta-oxidation metabolism* can also be affected in mammals after arsenic exposure. Increased concentrations (~60%) of free fatty acids (FFAs) were measured in mitochondria of hepatic cells under arsenite exposure (*myristic acid, palmitoleic acid, palmitic acid, piranaric acid, linilenic acid, oleic acid, stearic acid, arachidonic acid, eicosatrienoic acid, eicosenoic acid* and *DHA*) [27] and in whole hepatic cells (*pipecolic acid, arachidonic acid, oleic acid, linoleic acid, gamma-linolenic acid, DHA, piranaric acid and lauric acid*) (Table 1).

Decreasing levels of *carnitine* have been reported in mice liver along the exposure time of arsenic (Table 1) as a consequence of lipids increase, previously described, since carnitine is required for the transport of fatty acids from the cytosol into the mitochondria during the breakdown of lipids in the generation of metabolic energy by β-oxidation [26].

In relation with the *glycolysis and Kreb´s cycle*, a decrease of the levels of *glucose* and *glyceraldehide-3-phosphate* in mice liver tissue can be observed in a dose-dependent

manner, which can be related to alteration in carbohydrate metabolism, since Szinicz and Forth (1988) demonstrated the occurrence of carbohydrate depletion and inhibition of glucogenesis caused by the toxic action of arsenic in rats [178]. The levels of glucose and *glycogen* decreased in liver tissue and the former also in serum and urine when rats were exposed to realgar [33], representing accelerated glycolysis and glycogenolysis. A significant depletion in blood glucose and liver glycogen and glucose, have also been reported in pignea pig and mice exposed to arsenic [38] (Table 1).

Finally, It has been reported that arsenic exposure resulted in lowered levels of *testosterone* and *luteinizing hormone* levels in the serum of arsenic exposed mice, which in turn led to the inhibition of the testosterone synthesis pathway and resulted in infertility in male mice [25]. This fact demonstrate the influence of arsenic in the androgen metabolism. By contrast, other authors observed increased urinary testosterone levels in humans [182], but there is also a work on humans which reports lowered levels of this metabolite in blood which suggests that there are important dosing and timing differences in the effects of arsenic as well as important gene-environment and coexposure interactions [183,184].

8. Gaps and future

Nowadays, there are powerful analytical techniques for metabolomics and particularly toxicometabolomics. While NMR has some specific advantages, the higher reproducibility, non-destructive character, small sample consumption and simpler sample treatments, the possible application to complex samples, higher sensitivity, specificity and the possibility to perform quantitative analysis offered by MS is crucial to probe xenobiotic induced metabolic changes to evaluate the stress responses. On the other hand,

DIMS had comparable classification and prediction capability to GC/LC-MS, but the latter is necessary when comprehensive screening of biomarkers is required.

A remaining problem in toxicometabolomics is still the absence of unified analytical protocols for sample preparation (e.g. quenching, protein precipitation, extraction with solvents), analysis (e.g. direct infusion *vs* GC/LC/CE) or statistical treatments and future efforts should be made in this way. In connection with this, an important effort should also be made in the future to simplify the work with animals defining the model organism to be used (i.e. the As metabolism in mice and rats are different), the selected xenobiotic dose (i.e. GSH expression of exposed animals against the control can be inverted), the chemical form used (i.e. the toxicity of arsenite is not the same that arsenite), the exposure route selected (i.e. subcutaneous absorption is slow), the time of exposure (i.e. sometimes the metabolic changes are reverted or inverted along the time), the biological sample used (i.e. plasma *vs* serum) and other factors can drastically affect the metabolism generating incomparable results.

From the point of toxicology (i.e. understanding how a toxicant act on its targets), toxicometabolomics plays the role to illustrate the human observations (i.e. support the epidemiology results), it also has the chance to illustrate the toxicity in systems but meet the challenge to illustrate the pathways involved in the toxicology. Likewise, the pathways related to arsenic toxicity in mammals that have been probed to be altered includes the energy metabolism (e.g. glycolysis, Kreb´s cycle), amino acid metabolism, choline metabolism, methionine cycle (transmethylation), purine metabolism and degradation of membrane phospholipids (cell apoptosis).

In conclusion, although metabolomics is much less extended in environmental monitoring of metal toxicity due to the complexity of working with animals along with the absence

of unified protocols, a few works have demonstrated its potential in toxicological assessment, evaluation of health risks and understanding of biochemical basis of toxicity.

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Figure Legends

Figure 1. The omics cascade.

Figure 2. Scheme about the different types of metabolomic studies.

Figure 3. The metabolomics workflow in toxicometabolomics.

Figure 4. Typical analytical procedures for sample preparation in arsenic tissue metabolomics.

Figure 5. Pros and cons of the analytical instrumentation for toxicometabolomics.

Figure 6. Metabolic pathways altered by arsenic exposure.

Table Legends

Table 1. Altered metabolites after arsenic exposure in mammals and the analytical method used in each case. The metabolites are listed in alphabetical order of the pathway.

Metabolite	Analytical technique	Effect	Pathway	Sample type	Organism	Arsenic species	Concentration	Exposition time (days)	Reference
1-Methyladenosine	UFLC-IT-TOF/MS	↑	Adenine metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Anserine	UFLC-IT-TOF/MS	↓	Alanine metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Luteinizinghormone	ELISA	↓	Androgen metabolism	Serum	Mice	As_2O_3	3 and 4 mg/ kg bw	56	[25]
Testosterone	ELISA	↓	Androgen metabolism	Serum	Mice	As_2O_3	3 and 4 mg/ kg bw	56	[25]
	HPLC-QTOF-MS	↑	Androgen metabolism	Urine	Human	-	-	-	[40]
Arginine	DI-ESI-QTOF-MS	↓	Arginine and proline metabolism (amino acid metabolism)	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
	DI-ESI-QTOF-MS	↓	Arginine and proline metabolism (amino acid metabolism)	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
	DI-ESI-QTOF-MS	↓	Arginine and proline metabolism (amino acid metabolism)	Kidney	Mice	As_2O_3	3 mg As/ kg day	12	[29]
Proline	UHPLC-MS	↓	Arginine and proline metabolism (amino acid metabolism)	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
	GCxGC-TOF-MS	↑	Arginine and proline metabolism (amino acid metabolism)	Liver tissue	Mice	$NaAsO_2$	5 ppm in tap water	70	[28]
	GC-MS	↓	Arginine and proline metabolism (amino acid metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Arginine and proline metabolism (amino acid metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Creatine	UHPLC-MS	↓	Arginine and proline metabolism (amino acid metabolism)	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
	DI-ESI-QTOF-MS	↓	Arginine and proline metabolism (amino acid metabolism)	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
	1H NMR	↑	Arginine and proline metabolism (amino acid metabolism)	Serum	Rats	As_4S_4	1.0 g/kg bw	14	[33]
	UFLC-IT-TOF/MS	↑	Arginine and proline metabolism (amino acid metabolism)	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Creatinine	DI-ESI-QTOF-MS	↑	Arginine and proline metabolism (amino acid metabolism)	Kidney	Mice	As_2O_3	3 mg As/ kg day	12	[29]
	1H NMR	↑	Arginine and proline metabolism (amino acid metabolism)	Serum	Rats	As_4S_4	1.0 g/kg bw	14	[33]
Betaine	1H-NMR	↓	Betaine metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Dimethylamine	1H-NMR	↓	Choline metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Dimethylglycine	1H-NMR	↓	Choline metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]

Myristic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Palmitoleic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Palmitic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Piranaric acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Linolenic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Oleic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Stearic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Arachidonic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Eicosatrienoic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Ecosenoic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
DHA	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Pipecolic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic cells	Mice	As_2O_3	3 mg As/ kg day	7	[27]
	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Arachidonic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic cells	Mice	As_2O_3	3 mg As/ kg day	7	[27]
	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Oleic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic cells	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Linoleic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic cells	Mice	As_2O_3	3 mg As/ kg day	7	[27]
γ-Linolenic acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic cells	Mice	As_2O_3	3 mg As/ kg day	7	[27]

DHA	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic cells	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Piranaric acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic cells	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Lauric acid	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic cells	Mice	As_2O_3	3 mg As/ kg day	7	[27]
2-hydroxybutyrate	GCxGC-TOF-MS	↓	Fatty acids beta-oxidation	Liver tissue	Mice	$NaAsO_2$	5 ppm in tap water	70	[28]
2-aminobutyrate	GCxGC-TOF-MS	↓	Fatty acids beta-oxidation	Liver tissue	Mice	$NaAsO_2$	5 ppm in tap water	70	[28]
Carnitine	DI-ESI-QTOF-MS	↓	Fatty acids beta-oxidation	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
	DI-ESI-QTOF-MS	↑	Fatty acids beta-oxidation	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Octadecenylcarnitine	UHPLC-MS	↑	Fatty acids beta-oxidation	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
Palmitoylcarnitine	UHPLC-MS	↑	Fatty acids beta-oxidation	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
Mannitol	UFLC-IT-TOF/MS	↓	Fatty alcohol metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Formic acid	1H-NMR	↑	Folate metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Fructose	GC-MS	↑	Fructose metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↓	Fructose metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Galactose	GC-MS	↓	Galactose metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↓	Galactose metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Alanine	1H NMR	↓	Glucose-Alanine Cycle	Serum/ Liver tissue	Rats	As_4S_4	1.0 g/kg bw	7 and 14	[33]
	GC-MS	↓	Glucose-Alanine Cycle	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↓	Glucose-Alanine Cycle	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	1H-NMR	↓	Glucose-Alanine Cycle	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
D-Glucuronic acid 1-phosphate	UFLC-IT-TOF/MS	↓	Glucuronidation	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
1-(beta-D-ribofuranosyl)-1,4-dihidronicotinamide	UPLC/ Q-TOF MS	↓	Glucuronidation	Urine	Human	Environmental As exposure	50 ng/g creatinine (High Arsenic Exposure)	First trimester of gestation	[41]
Gluconic acid	UFLC-IT-TOF/MS	↓	Glucuronidation	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]

Phenyl glucuronide	UFLC-IT-TOF/MS	↑	Glucuronidation	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
p-cresol glucuronide	UPLC/Q-TOF MS	↑	**Glucuronidation**	Urine	Human	Environmental As exposure	50 ng/g creatinine (High Arsenic Exposure)	First trimester of gestation	[41]
Cytidine	LC-MS/GC-MS	↑	Glutamate metabolism	Urine	Mice	$NaAsO_2$	1 ppm in drinking water	28	[32]
Cysteine	DI-ESI-QTOF-MS	↑	Glutathione metabolism (amino acid metabolism)	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
	GCxGC-TOF-MS	↑	Glutathione metabolism (amino acid metabolism)	Liver tissue	Mice	$NaAsO_2$	5 ppm in tap water	70	[28]
Thiocysteine	UPLC/Q-TOF MS	↑	Glutathione metabolism (amino acid metabolism)	Urine	Human	Environmental As exposure	50 ng/g creatinine (High Arsenic Exposure)	First trimester of gestation	[41]
Glutamic acid	DI-ESI-QTOF-MS	↑	Glutathione metabolism (amino acid metabolism)	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
	DI-ESI-QTOF-MS	↑	Glutathione metabolism (amino acid metabolism)	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Glutathione metabolism (amino acid metabolism)	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	GC-MS	↑	Glutathione metabolism (amino acid metabolism)	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Glutathione metabolism (amino acid metabolism)	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Glutathione metabolism (amino acid metabolism)	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[27]
	1H NMR	↓	Glutathione metabolism (amino acid metabolism)	Serum	Human	As, Cd, Pb	-	-	[39]
	GCxGC-TOF-MS	↓	Glutathione metabolism (amino acid metabolism)	Liver tissue	Mice	$NaAsO_2$	5 ppm in tap water	70	[28]
Glutamine	DI-ESI-QTOF-MS	↓	Glutathione metabolism (amino acid metabolism)	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	12	[27]
	GC-MS	↑	Glutathione metabolism (amino acid metabolism)	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Glutathione metabolism (amino acid metabolism)	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
GSH	DI-ESI-QTOF-MS	↓	Glutathione metabolism (amino acid metabolism)	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
				Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[29]
				Serum	Rats	As_4S_4	1.0 g/kg bw	14	[33]
	UPLC/Q-TOF MS	↓		Urine	Human	Environmental	50 ng/g creatinine (High	First trimester	[41]

						As exposure	Arsenic Exposure)	of gestation	
Pyroglutamic acid	UHPLC-MS	↑	Glutathione metabolism (amino acid metabolism)	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
Methionine	DI-ESI-QTOF-MS	↑	Glutathione metabolism (amino acid metabolism)	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[27]
	DI-ESI-QTOF-MS	↑	Glutathione metabolism (amino acid metabolism)	Kidney	Mice	As_2O_3	3 mg As/ kg day	12	[29]
	UFLC-IT-TOF/MS	↓	Glutathione metabolism (amino acid metabolism)	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Homocysteinein	DI-ESI-QTOF-MS	↓	Glutathione metabolism (amino acid metabolism)	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
Glycine	GCxGC-TOF-MS	↓	Glycine, threonine and serine metabolism	Liver tissue	Mice	$NaAsO_2$	5 ppm in tap water	70	[28]
	1H NMR	↑	Glycine, threonine and serine metabolism	Serum	Rats	As_4S_4	1.0 g/kg bw	14	[33]
	GC-MS	↑	Glycine, threonine and serine metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Glycine, threonine and serine metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	1H-NMR	↑	Glycine, threonine and serine metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Serine	GC-MS	↑	Glycine, threonine and serine metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Glycine, threonine and serine metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Threonine	GC-MS	↑	Glycine, threonine and serine metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↓	Glycine, threonine and serine metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Hippuric acid	LC-QTOF-MS	↑	Glycine metabolism	Urine	Mice	$NaAsO_2$	10 ppm	28	[24]
	1H NMR	↓	Glycine metabolism	Urine	Rats	As_4S_4	1.0 g/kg bw	18	[33]
	HPLC-QTOF-MS	↑	Glycine metabolism	Urine	Human	-	-	-	[40]
	LC-MS/ GC-MS	↑	Glycine metabolism	Urine	Mice	$NaAsO_2$	1 ppm in drinking water	28	[32]
	UFLC-IT-TOF/MS	↑	Glycine metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
	1H-NMR	↓	Glycine metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Indoxyl	LC-QTOF-MS	↑	Glycolysis and Kreb's cycle	Urine	Mice	$NaAsO_2$	10 ppm	28	[24]

Glucose	1H NMR	↓	Glycolysis and Kreb's cycle	Serum/ Liver tissue	Rats	As_4S_4	1.0 g/kg bw	14	[33]
	DI-ESI-QTOF-MS	↓	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	1H NMR	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_4S_4	1.35 g/kg	1/2	[31]
	DI-ESI-QTOF-MS	↓	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	GC-MS	↓	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↓	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	-	↓	Glycolysis and Kreb's cycle	Liver tissue	Mice	As_2O_3	10 mg /kg s.c.	14	[27]
	1H NMR	↓	Glycolysis and Kreb's cycle	Liver tissue	Rats	As_4S_4	1.0 g/kg bw	7 and 14	[33]
	AAS	↓	Glycolysis and Kreb's cycle	Blood	Pigs	As_2O_3	10 mg /kg s.c.	5	[38]
	AAS	↓	Glycolysis and Kreb's cycle	Liver tissue	Pigs	As_2O_3	10 mg /kg s.c.	5	[38]
Glyceraldehide-3-phosphate	-	↓	Glycolysis and Kreb's cycle	Liver tissue	Mice	As_2O_3	10 mg /kg s.c.	14	[27]
Glycogen	1H NMR	↓	Glycolysis and Kreb's cycle	Liver tissue	Rats	As_4S_4	1.0 g/kg bw	7 and 14	[33]
	AAS	↓	Glycolysis and Kreb's cycle	Blood	Pigs	As_2O_3	10 mg /kg s.c.	5	[38]
	AAS	↓	Glycolysis and Kreb's cycle	Liver tissue	Pigs	As_2O_3	10 mg /kg s.c.	5	[38]
Pyruvic acid	DI-ESI-QTOF-MS	↓	Glycolysis and Kreb's cycle	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
	1H NMR	↓	Glycolysis and Kreb's cycle	Urine	Rats	As_4S_4	1.0 g/kg bw	2 and 7	[33]
	1H NMR	↓	Glycolysis and Kreb's cycle	Serum	Rats	As_4S_4	1.0 g/kg bw	7	[33]
	1H-NMR	↓	Glycolysis and Kreb's cycle	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
3-hydroxybutyrate	1H-NMR	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_4S_4	1.35 g/kg	1/2	[31]
Succinic acid	1H-NMR	↑	Glycolysis and Kreb's cycle	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Citric acid	GCxGC-TOF-MS	↑	Glycolysis and Kreb's cycle	Liver tissue	Mice	$NaAsO_2$	5 ppm in tap water	70	[28]
	DI-ESI-QTOF-MS	↑	Glycolysis and Kreb's cycle	Kidney	Mice	As_2O_3	3 mg As/ kg day	12	[29]
	DI-ESI-QTOF-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	GC-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]

	UFLC-IT-TOF/MS	↓	Glycolysis and Kreb's cycle	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
	1H-NMR	↑	Glycolysis and Kreb's cycle	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Citrate	DI-ESI-QTOF-MS	↑	Glycolysis and Kreb's cycle	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
Isocitric acid	GC-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Glycolysis and Kreb's cycle	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
α-Ketoglutarate	DI-ESI-QTOF-MS	↑	Glycolysis and Kreb's cycle	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]
	GC-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	UFLC-IT-TOF/MS	↑	Glycolysis and Kreb's cycle	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
	1H-NMR	↑	Glycolysis and Kreb's cycle	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Lactic acid	1H NMR	↓	Glycolysis and Kreb's cycle	Serum	Rats	As_4S_4	1.0 g/kg bw	7	[33]
	1H NMR	↓	Glycolysis and Kreb's cycle	Liver tissue	Rats	As_4S_4	1.0 g/kg bw	7 and 14	[33]
	1H NMR	↑	Glycolysis and Kreb's cycle	Urine	Rats	As_4S_4	1.0 g/kg bw	7 and 14	[33]
	GC-MS	↓	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	1H NMR	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_4S_4	1.35 g/kg	1/2	[31]
	UFLC-IT-TOF/MS	↓	Glycolysis and Kreb's cycle	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
	1H-NMR	↓	Glycolysis and Kreb's cycle	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
L-aspartatic acid	GCxGC-TOF-MS	↓	Glycolysis and Kreb's cycle	Liver tissue	Mice	$NaAsO_2$	5 ppm in tap water	70	[28]
	GC-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Glycolysis and Kreb's cycle	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Hystidine	DI-ESI-QTOF-MS	↓	Hystidine and Lysine metabolisms	Hepatic mitochondria	Mice	As_2O_3	3 mg As/ kg day	7	[27]

Lysine	GCxGC-TOF-MS	↑	Hystidine and Lysine metabolisms	Liver tissue	Mice	$NaAsO_2$	5 ppm in tap water	70	[28]
18-carboxy-dinorLT4	UPLC/Q-TOF MS	↑	Leukotrienes metabolism	Urine	Human	Environmental As exposure	50 ng/g creatinine (High Arsenic Exposure)	First trimester of gestation	[41]
20-COOH-LTE4	UPLC/Q-TOF MS	↑	Leukotrienes metabolism	Urine	Human	Environmental As exposure	50 ng/g creatinine (High Arsenic Exposure)	First trimester of gestation	[41]
7-α-hydroxy-3-oxo-4-cholestenoate	LC-QTOF-MS	↑	Lipid metabolism	Urine	Mice	$NaAsO_2$	10 ppm	28	[24]
Dodecanoic acid	GCxGC-TOF-MS	↑	Lipid metabolism	Liver tissue	Mice	$NaAsO_2$	5 ppm in tap water	70	[28]
Choline	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Liver tissue	Mice	As_2O_3	3 mg As, / kg day	7	[26]
	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Kidney	Mice	As_2O_3	3 mg As/ kg day	12	[29]
	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	1H NMR	↑	Lysolipid metabolism	Plasma	Mice	As_4S_4	1.35 g/kg	1/2	[31]
Lyso-phosphatidylcholines	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	UPLC/Q-TOF MS	↓	Lysolipid metabolism	Urine	Human	Environmental As exposure	50 ng/g creatinine (High Arsenic Exposure)	First trimester of gestation	[41]
Phosphatidylcholines	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
	DI-ESI-QTOF-MS	↓	Lysolipid metabolism	Kidney	Mice	As_2O_3	3 mg As/ kg day	12	[29]
	DI-ESI-QTOF-MS	↓	Lysolipid metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↓	Lysolipid metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	LC-MS/GC-MS	↑	Lysolipid metabolism	Plasma/ Urine	Mice	$NaAsO_2$	1 ppm in drinking water	28	[32]

Triglycerides	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Diglycerides	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Lysolipid metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Cholesterol	UHPLC-MS	↑	Lysolipid metabolism	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
VLDL/LDL (verylowdensity/lowdensity lipoprotein)	1H-NMR	↑	Lysolipid metabolism	Plasma	Mice	As_4S_4	1.35 g/kg	1/2	[31]
N-Acetyl-L-methionine	UFLC-IT-TOF/MS	↓	Methionine metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Trimethylamine N-oxide	1H-NMR	↓	Niacin metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Trigonelline	UFLC-IT-TOF/MS	↓	Niacin metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
	1H-NMR	↑	Niacin metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Uric acid	HPLC	↓	Nitrogen metabolism	Plasma/ Urine	Rats	As_2O_3/ $Na_2HAsO_4.7H_2O$	1200 µg As / Kg	42	[35]
	DI-ESI-QTOF-MS	↑	Nitrogen metabolism	Kidney	Mice	As_2O_3	3 mg As/ kg day	12	[29]
	GC-MS	↓	Nitrogen metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↓	Nitrogen metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Urea	GC-MS	↑	Nitrogen metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↓	Nitrogen metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Cytosine	UPLC-MS	↓	Nucleotide metabolism	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
Adenine in ATP/ADP and NAD/NADH	1H NMR	↑	Nucleotide metabolism	Liver tissue	Rats	As_4S_4	1.0 g/kg bw	7 and 14	[33]
Ribose in ATP/ADP and NAD/NADH	1H NMR	↑	Nucleotide metabolism	Liver tissue	Rats	As_4S_4	1.0 g/kg bw	7 and 14	[33]
Guanine	HPLC-QTOF-MS	↑	Nucleotide metabolism	Urine	Human	-	-	-	[40]

Pantothenic acid	UFLC-IT-TOF/MS	↓	Pantothenate and coenzyme A biosynthesis	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Tyrosine	UPLC-QTOF-MS	↓	Phenylalanine and tyrosine metabolism/ catecholamine boisynthesis	Serum	Rats	As_2O_3/ $Na_2HAsO_4.7H_2O$	1200 µg As / Kg	42	[34, 35]
	1H NMR	↑	Phenylalanine and tyrosine metabolism/ catecholamine boisynthesis	Serum	Human	As, Cd, Pb	-	-	[39]
	GC-MS	↓	Phenylalanine and tyrosine metabolism/ catecholamine boisynthesis	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↓	Phenylalanine and tyrosine metabolism/ catecholamine boisynthesis	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Phenylalanine	1H NMR	↑	Phenylalanine and tyrosine metabolism/ catecholamine boisynthesis	Serum	Human	As, Cd, Pb	-	-	[39]
	GC-MS	↓	Phenylalanine and tyrosine metabolism/ catecholamine boisynthesis	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Phenylalanine and tyrosine metabolism/ catecholamine boisynthesis	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
Kynurenic acid	UFLC-IT-TOF/MS	↓	Porphyrin metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Decenedioic acid	UFLC-IT-TOF/MS	↓	Porphyrin metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Coproporphyrin	UFLC-IT-TOF/MS	↑	Porphyrin metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Methylguanidine	1H-NMR	↓	Protein catabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Inosine	UFLC-IT-TOF/MS	↑	Purine metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Acetate	1H-NMR	↑	Pyruvate metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Acetone	1H-NMR	↑	Pyruvate metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
3-Methyluridine	UFLC-IT-TOF/MS	↑	RNA biosynthesis	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Ceramide(d18:0/16:0)	UHPLC-MS	↑	Sphingolipid metabolism	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
Ceramide(d18:0/14:0)	UHPLC-MS	↑	Sphingolipid metabolism	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
Sphingosine	UHPLC-MS	↑	Sphingolipid metabolism	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
Phytosphingosine	UHPLC-MS	↑	Sphingolipid metabolism	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]

Vanillactic acid	UPLC/ Q-TOF MS	↑	Sulfotransferase metabolism	Urine	Human	Environmental As exposure	50 ng/g creatinine (High Arsenic Exposure)	First trimester of gestation	[41]
Taurine	1H NMR	↑	Taurine and hypotaurine metabolism	Urine	Rats	As_4S_4	1.0 g/kg bw	18	[33]
	DI-ESI-QTOF-MS	↑	Taurine and hypotaurine metabolism	Liver tissue	Mice	As_2O_3	3 mg As/ kg day	7	[26]
	DI-ESI-QTOF-MS	↑	Taurine and hypotaurine metabolism	Kidney	Mice	As_2O_3	3 mg As/ kg day	12	[29]
	DI-ESI-QTOF-MS	↑	Taurine and hypotaurine metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	DI-ESI-QTOF-MS	↑	Taurine and hypotaurine metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	UFLC-IT-TOF/MS	↑	Taurine and hypotaurine metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
	1H-NMR	↑	Taurine and hypotaurine metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
N^4-Acetylcytidine	UFLC-IT-TOF/MS	↓	Transfer RNA degradation	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Tryptophan	GC-MS	↑	Tryptophan metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Tryptophan metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]
	UFLC-IT-TOF/MS	↓	Tryptophan metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Xanthurenic acid	UFLC-IT-TOF/MS	↑	Tryptophan metabolism	Urine	Rats	As_4S_4	20, 100, and 500 mg/kg	21	[37]
Indolacetaldehyde	UHPLC-MS	↓	Tryptophan metabolism	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
3-indolepropionic acid	LC-QTOF-MS	↑	Tryptophan metabolism	Urine	Mice	$NaAsO_2$	10 ppm	28	[24]
indole-3carboxilic acid	LC-QTOF-MS	↑	Tryptophan metabolism	Urine	Mice	$NaAsO_2$	10 ppm	28	[24]
indoleavrylic acid	LC-QTOF-MS	↑	Tryptophan metabolism	Urine	Mice	$NaAsO_2$	10 ppm	28	[24]
IPA	LC-QTOF-MS	↑	Tryptophan metabolism	Urine	Mice	$NaAsO_2$	10 ppm	28	[24]
Valine	UHPLC-MS	↓	Valine, leucine and isoleucine degradation/propanoate metabolism	Serum	Rats	$NaAsO_2$	0.5, 2 or 10 ppm	90	[36]
	GC-MS	↑	Valine, leucine and isoleucine degradation/propanoate metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↑	Valine, leucine and isoleucine	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day	12	[30]

			degradation/propanoate metabolism				0,1 mg Cd/ kg day		
Isoleucine	GC-MS	↓	Valine, leucine and isoleucine degradation/propanoate metabolism	Plasma	Mice	As_2O_3	3 mg As/ kg day	12	[30]
	GC-MS	↓	Valine, leucine and isoleucine degradation/propanoate metabolism	Plasma	Mice	As_2O_3 / $CdCl_2$	3 mg As/ kg day 0,1 mg Cd/ kg day	12	[30]

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