FORENSIC SCIENCE JOURNAL SINCE 2002

Forensic Science Journal
2019;18(1):9-18
fsjournal.cpu.edu.tw
DOI:10.6593/FSJ.201912_18(1).0002

Evaluation of the Feasibility of Metabolome Analyses for the Identification of Body Fluids

Yi-Ching Chen [1], B.S. ; Yung-Fou Chen [1], Ph.D. ; Hsing-Mei Hsieh [1], Ph.D. ; Li-Chin Tsai [1*], Ph.D.

[1] Department of Forensic Science, Central Police University, 56 Shujen Rd., Takang Vil., Kueishan District, Taoyuan City, Taiwan 33304 R.O.C.

Received: July 19, 2019; Accepted: August 26, 2019.

Abstract

Metabolomics is regarded as a new approach for studying the composition of cells, tissues, or even the entire set of metabolic molecules *in vivo* after Genomics and Proteomics. Recently, there have been several papers analyzing specific metabolites in different body fluids by liquid or gas chromatography–mass spectrometry (LC-MS or GC-MS), nuclear magnetic resonance (NMR), and other ways. These diverse sets of metabolites may be potential as specific biomarkers for body fluid identification. This report summarized the research related to body fluid metabolites in published papers, and also reported the current MS and NMR papers, which were the current established analytical techniques for metabolome analyses of body fluids. Furthermore, the potential of these technologies for being applied to forensic body fluid differentiation was evaluated in this report.

Keywords: forensic body fluid identification, mass spectrometry, nuclear magnetic resonance, metabolites, biomarkers

Introduction

Biological evidence plays an important role in crime scene reconstruction. Not only DNA, but also the sources of body fluids can lead the direction of a criminal investigation. Forensic scientists detect key components in body fluids by targeted testing, including presumptive catalytic color tests, immunoassays, specific enzyme activity assay methods, crystal tests, and spectroscopy analyses [1-3]. With methods mentioned above, except for blood and semen, we can still only primarily infer the classes of these fluids not to confirm the sources. Moreover, if samples are mixed, it is also very difficult to identify each body fluid.

Currently, scientists are trying to find novel techniques in order to improve sensitivity and specificity, and to reduce the amounts used of the samples of body fluids in the identification, such as Raman spectroscopy, epigenetic analyses, proteomic analyses, and other techniques [1,4-9]. Currently, metabolic-related researches focus on the applications in medical biology and clinical diagnoses, further research on forensic applications of body fluid identification is needed [10-12].

This review provides the comprehensive and current overview of the feasibility of metabolic analyses for body fluid identification by mass spectrometry (MS) and nuclear magnetic resonance (NMR) for different aspects of identification.

Metabolomics

The concept of metabolic profiling was first proposed by Williams et al., attempting to find metabolic

*Corresponding author: Li-Chin Tsai, Department of Forensic Science, Central Police University, 56 Shujen Rd., Takang Vil., Kueishan District, Taoyuan City, Taiwan 33304 R.O.C. Tel: 886-3-3282321 ext 5116, Fax: 886-3-3275907
Email: lctsai@mail.cpu.edu.tw

patterns that are distinctive for alcoholics and connecting to the preliminary studies of schizophrenic individuals [13]. With the well-developed techniques, we already could understand the significance of alleles in the genome, and searched the changes in individual expression for transcripts or protein concentration in transcriptome or proteome [14]. For further information, a metabolomics analysis can directly reflect the activity of the metabolic system, and the quantity and quality of the metabolites can be treated as the ultimate reply of biological networks to genetic or environmental changes [15,16].

Metabolites, small (<1,500 Da) compounds present in a cell or organism, which take part in metabolic reactions have much greater variability in their composition of atoms and subgroups than the linear coding sequences for genes or proteins [16,17]. Therefore, different tissues have their own metabolic fingerprints providing tissue-specific profiles that offer valuable information for identifying the sources of the unknown body fluids [18-20]. Additionally, until 1998, the term, metabolome, a complete set of small-molecule chemicals, was first suggested by Oliver *et al*. while performing functional analysis on yeast [21].

With different analytical approaches for metabolites, they can solve various key problems. First, targeted analyses are mainly used for screening purposes and providing clarity by confirming a specific hypothesis with absolute quantification. Second, metabolite profiling focuses on a specific known metabolite for investigating a selected biochemical pathway. Third, a comprehensive analysis of total metabolites that reveals the metabolome of the biological system of interest is called 'metabolomics'. These approaches must include strategies to identify unknown metabolites and lead to models of theoretical biochemical networks. Furthermore, there is another method called metabolic fingerprinting, which is a rapid classification of samples by their origins or their biological relevance [22]. A rough workflow of metabolic studies is summarized in Fig. 1 [15].

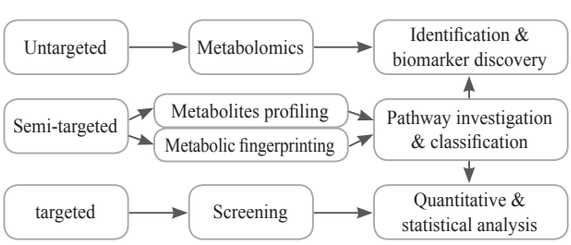

Fig. 1 General framework of different workflows available for metabolome studies [15].

Because of the rapid development of the metabolome, the requirement of metabolite identification is also raised; therefore, in 2005, METLIN, a metabolite database based on Fourier transform mass spectrometry (FTMS), tandem mass spectrometry (MS/MS), and liquid chromatography–mass spectrometry (LC-MS) analysis results, established to accelerate metabolite identification by providing known information from multiple related sources, now collects about one million molecules ranging from lipids, steroids, small peptides, carbohydrates, toxicants, and so on [23].

Moreover, the Human Metabolome Project completed another database, the Human Metabolome Database (HMDB), a comprehensive, web-accessible metabolomics database, and brought together quantitative data for extensive endogenous human metabolites from MS and NMR in 2007 [24]. However, the database has only collected and displayed metabolites found in various body fluids, including urine, serum, cerebral spinal fluid (CSF), saliva, feces, and sweat. There are still some other kinds of body fluid sources waiting to be confirmed.

So far, there are lots of devices and technologies available to analyze metabolites, including radioimmunoassay (RIA) [25], NMR [26-28], spectrometry [29-31], thin layer chromatography (TLC) [32], capillary electrophoresis (CE) or other chromatographic techniques coupled with MS [33-37], and even the recently developed complementary metal oxide semiconductor (CMOS)-chip [38] and nanopores [39]. Among them, the most widely used applications for metabolic analyses are NMR and MS. Table 1 summarized the features of several perspectives of NMR and MS. NMR can simultaneously measure the quantity and identify molecules in samples; however, you can only semi-quantify with the standard samples to get the concentration relatively with MS [15]. Additionally, one can directly use intact samples with little or even without any chemical treatments in NMR. In other words, it requires fewer sample preparation steps and there is easy recovery of the samples. However, some challenges such as larger sample volume, lower sensitivity, more interference peaks, are needed to be conquered [28]. In contrast, MS provides superior sensitivity and resolution, it is commonly used for semi-targeted or untargeted metabolomics [29]. Both techniques offer the online spectral databases available

for comparing and identifying the experimental data [40-43]. Complementarily, the mass spectrometer can also combine with separation techniques to simplify the complexity of metabolic profiling and make the identification of individual metabolites easier [44]. Despite NMR offers high reproducibility and sample recovery, MS with broader metabolites coverage and high sensitivity can detect more signals with minimal volume of samples. Therefore, researchers can choose proper analytical approaches depending on their purpose of the experiments. However, there are also some researchers using both techniques to do the analysis and compare the data with each technique, trying to get the maximum recovery of metabolites [26,36,37].

Table 1 A comparison of NMR and MS for metabolite analyses.

Characteristics	Nuclear magnetic resonance	Mass spectrometry
Metabolite coverage	narrow	broad
Sensitivity	low	high
Sample recovery	noninvasive	destructive
Reproducibility	very high	moderate
Selectivity	mostly nonselective analysis	targeted and untargeted
Pretreatment	minimal	demanded
Quantification	concentration level	semi-quantification
Databases	HMDB, BMRB	HMDB, METLIN

The Metabolome Analysis of Body Fluids by NMR

NMR detects molecular features by measuring the resonance in magnetic fields of the atomic nuclear spin that reveals the chemical environment of the compound and its molecular structure. Although we can directly analyze the sample without any pretreatment or only after dissolution and digestion, to improve the underrated metabolites' signal intensities, several chemical treatments below can be performed.

(1) Protein precipitation:

Both the existence of high molecular weight molecules and binding of the proteins can affect the quantification and the broadening of the NMR signals. In order to solve the problem, researchers usually precipitate the proteins by several organic solvents [27,28]. However, although we can efficiently increase the resolution of the signals, these treatments might alter the real spectra.

(2) Lyophilization:

Residual solvents appear in NMR analyses and may let the compounds in samples interact with the buffers or lead cross-contamination. Therefore, after chemical treatments, removing the solvents by drying and extending the storage of the samples is necessary. On the contrast, part of the compounds possibly evaporates during processing and not able to be shown in the spectra [26].

(3) Filtration:

Instead of using chemical pretreatment, more and more scientists decide to use filters with different cut-offs of molecular weights. In recent study, it showed the approach can appraise the nitrogenous bases and nucleosides in serum samples [26].

The Metabolome Analysis of Body Fluids by MS

MS is a method where ionized samples are classified by their mass-to-charge ratio through magnetic and electric fields. Combined with chromatographic separation, matrix effects and ionization suppression can be reduced, isomers can be separated, and comprehensive data valuable for metabolite annotation can also be provided [30]. Gas chromatography with mass spectrometry (GC-MS) is more suitable for low molecular weight and volatile metabolites. Moreover, most GC-MS devices are equipped with electron ionization (EI) and the energy of ionizing electrons are

usually set at the standard value of 70 eV, so the databases of GC-MS are very reliable, and researchers can identify the spectra by comparing the signal peaks [47]. On the other hand, liquid chromatography (LC) can be applied for nonvolatile and polar metabolites which compose the largest proportion of body fluids. However, the ionization modes differ significantly with devices and no standard retention index has been established, which leads to difficulties in identifying compounds in the LC-MS data and arranging a standard database [29]. An integrated comparison of these two chromatographic separations is shown as Table 2.

Table 2 A comparison between GC-MS and LC-MS for metabolite analyses [a].

	GC-MS	LC-MS
Metabolites	Volatile, nonpolar	Nonvolatile, polar
Resolution	High	Low
Reproducibility	Low	High
Standard database	Yes (70 eV)	No
Mobile phase	Noble gas, N_2	Water, MeOH, ACN

[a] The table was extracted and reorganized from the reference [29].

Nevertheless, before injecting the samples to GC-MS or LC-MS, there are some sample preparations that should be done. Variable metabolite extractions lead to different recoveries of metabolites. Simple modifications, including temperature alteration, protein precipitation with several solvents, and even sample washing will affect the detection of the metabolites [48]. Several common methods are listed below:

(1) Quenching and washing:

A review suggested that the common metabolism inactivation is usually to be carried out by modification of temperature and pH value [49]. In addition, washing is an efficient way to remove the uninterested metabolites, and also may increase the signal to noise ratio [50].

(2) Protein removal:

Organic solvent-based protein precipitation (PPT) followed by ultrafiltration is the most prevalent method for removing proteins [51]. Compared to inorganic solvents, organic ones are more suitable for denaturing those interfering proteins. Not only can the organic solvents simultaneously extract hydrophilic and hydrophobic compounds, but they also inhibit the interactions between metabolites and proteins in order to improve metabolite coverage and reproducibility. However, the method might cause the loss of some metabolites because of co-precipitation or poor solubility [52]. Until now, many researchers have already studied and compared the effect of different procedures in terms of protein removal efficiency, and the metabolite coverage and precision for MS analysis [53,54].

(3) Liquid-liquid extraction (LLE):

LLE separates polar and non-polar metabolites into two fractions (aqueous and organic) [55]. In fact, LLE is now mainly used to remove lipids [52]. Based on the reported experiments, chloroform and methanol mixed extraction can recover the broadest lipid classes [56,57]. Recently, scientists found that the methyl tert-butyl ether (MTBE) can also provide the same or even better recovery, and with less harm to human beings [58]. In 2012, Jasmina *et al.* have reported that with a chloroform: methanol: water mixture (15:59:26) is the most optimal extractive solvent [59].

The pretreatment could also affect the quality of the analysis, so setting the instrument parameters plays

an important role. In chromatography, the resolution is the key feature to examine the efficiency of separation. However, the principle of separation will occur according to the differences of sample components' rate of movement through the balance between the mobile and stationary phases. The mobile phase is either liquid or gas that moves along with the analytes, while stationary phase is solid or gel that analytes are separated in during the passage of balancing [60]. Nevertheless, many elements may influence the separating efficiency including plate numbers, capacity factor, and separation factor. Plate theory is usually used to quantify the column efficiency. By changing the column length and flow rate, the number of plates can be increased and improve the resolution of the chromatography [60,61]. Capacity factor (K) stands for the time that the analytes spend in the stationary phase. By means of changing the amounts and types of organic modifiers added, it can alter the polarity and regulate the affinity of the mobile phases. In general, the best resolution is achieved as the K value ranges from 1 to 5 [61,62]. Separation factor (α) is the ability to chemically distinguish different sample components. Many factors can be used to improve the selectivity, including the pH values of mobile phases, types of stationary phases, column temperature, and so on [61,63]. Furthermore, the flow rate, column diameter, and gradient elution are also the elements that are associated with the resolution of chromatographic separation [64, 65].

The following MS can be equipped with GC or LC for increasing the analysis efficacy of separation or broadening the sample suitability, including matrix-assisted laser desorption ionization (MALDI) [66,67], quadrupole [68-70], ion traps [71], and time of flight (TOF) [72].

Metabolites Detected in Different Kinds of Body Fluids

Body fluid identification is always a topic worth exploring for many forensic scientists. Many primary catalytic tests and confirmatory tests are developed for analyzing body fluids as crime scene evidence; however, there are some false positive or negative results that mislead the direction of the reconstruction of scenes. Fortunately, since 2009, the year the human proteome project started, many scientists have performed proteomic analyses and discovered several specific protein markers in different body fluids [73-75].

As a next step beyond proteins, Table 3 integrated published papers of metabolites that can be detected in different kinds of body fluids by NMR and MS. Based on the published researches, although there are a few metabolic analyses of body fluids, there are still some sources of body fluids lack of data, such as menstrual blood, and so on. In addition, most of the recent papers only focus on the screening of the biomarkers in body fluids for several diets or illness, such as Parkinson's disease [10] or dementia patients [82] for clinical or biological usages, and the profiling of single body fluid source. Suitable systems for the body fluid identification on forensic applications are still under development.

Table 3 The metabolome research applied to body fluids by MS and NMR.

	Mass spectrometry [a]	Nuclear Magnetic Resonance [a]
Urine	Luan et al. [10] Boizard et al. [37] Huang et al. [71] Zhou et al. [72] Bouatra et al. [45]	Shanaiah et al. [28] Bouatra et al. [45]
Venous Blood	Gupta et al. [26] Liu et al. [33] Alshammari et al. [52] Want et al. [54] Sana et al. [55] Huang et al. [71] Yang et al. [74]	Gupta et al. [26] Nagana Gowda et al. [27] Shanaiah et al. [28] Stringer et al. [76] Nagana Gowda et al. [77] Kromke et al. [78] Tiziani et al. [79]
Sweat	NA	Kutyshenko et al. [80]

	Mass spectrometry [a]	Nuclear Magnetic Resonance [a]
Saliva	Wang et al. [12] Yang et al. [74] Dame et al. [46]	Dame et al. [46] Figueira et al. [82]
Semen	Chen et al. [34] Paiva et al. [35] Yang et al. [74]	Paiva et al. [35]
Menstrual Blood	NA	NA
Vaginal Fluid	NA	Vitali et al. [81]

[a] NA, no related papers were found.

Conclusions

Recently, an increasing number of researchers attempt to figure out the mysterious and complicated biological network interactions among metabolites because of the smaller compound size and deep insight into cell regulation. For forensic science applications, a smaller molecular weight of the analyzed molecules provides the advantage of not being denatured in the external environment easily. To study metabolites, NMR and MS are now the two main detection techniques. There are also now many integrated online databases and related published papers that can help us overcome the difficulties of interpreting complex spectrum. Based on the statements above, with optimal experimental design, we conclude that there is great potential for metabolic analyses of body fluid identification in the future. However, more researches are needed to improve the identification of body fluid sources, no matter the integrity of the databases, the settings of the separating parameters, or the specificity of the biomarkers for diverse body fluids.

Because of the high resolution, high sensitivity, less sample usage, and high analytic speed of NMR and MS, with the establishment of increasing metabolic information of different body fluids, the metabolic analyses also show great forensic application potentials.

Acknowledgements

This work was supported by the grants of no. MOST 105-2320-B-015-001-MY3 from the Ministry of Science and Technology in Taiwan. We also wish to thank Scott C. Schuyler providing us critical feedback on the manuscript.

References

1. Harbison S, Fleming R. Forensic body fluid identification: state of the art. Research and Reports in Forensic Medical Science. 2016; 6:11-23.
2. Virkler K, Lednev I-K. Analysis of body fluids for forensic purposes: From laboratory testing to non-destructive rapid confirmatory identification at a crime scene. Forensic Sci Int 2009; 188(1-3):1-17.
3. An J-H, Shin K-J, Yang W-I, Lee H-Y. Body fluid identification in forensics. BMB Rep 2012; 45(10):545-53.
4. Frumkin D, Wasserstrom A, Budowle B, Davidson A. DNA methylation-based forensic tissue identification. Forensic Science International: Genetics 2011; 5(5): 517–24.
5. Madi T, Balamurugan K, Bombardi R, Duncan G, McCord B. The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing. ELECTROPHORESIS 2012; 33(12): 1736–45.
6. Lee H-Y, An J-H, Jung S-E, Oh Y-N, Lee E-Y, Choi A, Yang W-I, Shin K-J. Genome-wide methylation profiling and a multiplex construction for the identification of body fluids using epigenetic markers. Forensic Science International: Genetics 2015; 17:17–24.
7. Park J-L, Kwon O-H, Kim J-H, Yoo H-S, Lee H-C, Woo K-M, Kim S-Y, Lee S-H, Kim Y-S. Identification of body fluid-specific DNA methylation markers for use in forensic science. Forensic Sci Int Genet 2014; 13:147-53.
8. Van Steendam K, De Ceuleneer M, Dhaenens M, Van Hoofstat D, Deforce D. Mass spectrometry

based proteomics as a tool to identify biological matrices in forensic science. Int J Legal Med 2013; 127(2):287-98.
9. Legg K-M, Powell R, Reisdorph N, Reisdorph R, Danielson P-B. Discovery of highly specific protein markers for the identification of biological stains. Electrophoresis 2014; 35(21-22):3069-78.
10. Luan H, Liu L-F, Tang Z, Zhang M, Chua K-K, Song J-X, Mok V-C, Li M, Cai Z. Comprehensive urinary metabolomic profiling and identification of potential noninvasive marker for idiopathic Parkinson's disease. Sci Rep 2015; 5:13888.
11. Sekula P, Goek O-N, Quaye L, Barrios C, Levey A-S, Römisch-Margl W, Menni C, Yet I, Gieger C, Inker L-A, Adamski J, Gronwald W, Illig T, Dettmer K, Krumsiek 1, Oefner P-J, Valdes A-M, Meisinger C, Coresh J, Spector T-D, Mohney R-P, Suhre K, Kastenmüller G, Köttgen A. A metabolome-wide association study of kidney function and disease in the general population. J Am Soc Nephrol 2016; 27(4):1175-88.
12. Wang Q, Gao P, Wang X, Duan Y. The early diagnosis and monitoring of squamous cell carcinoma via saliva metabolomics. Sci Rep 2014; 4:6802.
13. Williams RJ, 1951, Introduction, general discussion and tentative conclusions. In: Univ. of Texas (eds.), Biochemical Institute Studies IV. Individual metabolic patterns and human disease: An exploratory study utilizing predominantly paper chromatographic methods. U. Texas Publication No. 5109, 204:7-21.
14. Beecher, Chris WW, 2003, The Human Metabolome. In: Harrigan, George G, Goodacre, Royston (eds.), Metabolic Profiling: Its role in biomarker discovery and gene function analysis. Springer US. 311-19.
15. Liu X, Locasale J-W. Metabolomics: A Primer. Trends Biochem Sci 2017; 42(4): 274-84.
16. Fiehn O. Metabolomics – the link between genotypes and phenotypes. Plant Mol Biol 2002; 48(1-2):155-71.
17. Samuelsson L-M, Larsson D-G. Contributions from metabolomics to fish research. Mol Biosyst 2008; 4(10):974-9.
18. Taioli E, Im A, Xu X, Veenstra T-D, Ahrendt G, Garte S. Comparison of estrogens and estrogen metabolites in human breast tissue and urine. Reprod Biol Endocrinol 2010; 8:93.
19. Rask E, Olsson T, Söderberg S, Andrew R, Livingstone D-E, Johnson O, Walker B-R. Tissue-specific dysregulation of cortisol metabolism in human obesity. J Clin Endocrinol Metab 2001; 86(3):1418-21.
20. Sung J, Yun H, Cho M, Lim J-U, Lee Si, Lee D, Oh TK. Tissue-specific response of primary metabolites in tomato plants affected by different K nutrition status. Plant Omics Journal 2017; 10(1):37-44.
21. Oliver S-G, Winson M-K, Kell D-B, Baganz F. Systematic functional analysis of the yeast genome. Trends Biotechnol 1998; 16(9):373-8.
22. Fiehn O. Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. Comp Funct Genomics 2001; 2(3):155-68.
23. Smith C-A, O'Maille G, Want E-J, Qin C, Trauger S-A, Brandon T-R, Custodio D-E, Abagyan R, Siuzdak G. METLIN: a metabolite mass spectral database. Ther Drug Monit 2005; 27(6):747-51.
24. Wishart D-S, Tzur D, Knox C, Eisner R, Guo A-C, Young N, Cheng D, Jewell K, Arndt D, Sawhney S, Fung C, Nikolai L, Lewis M, Coutouly M-A, Forsythe I, Tang P, Shrivastava S, Jeroncic K, Stothard P, Amegbey G, Block D, Hau D-D, Wagner J, Miniaci J, Clements M, Gebremedhin M, Guo N, Zhang Y, Duggan G-E, Macinnis G-D, Weljie A-M, Dowlatabadi R, Bamforth F, Clive D, Greiner R, Li L, Marrie T, Sykes BD, Vogel H-J, Querengesser L. HMDB: the human metabolome database. Nucleic Acids Res 2007; 35(Database issue):D521-6.
25. Stanczyk F-Z, Xu X, Sluss P-M, Brinton L-A, McGlynn K-A. Do metabolites account for higher serum steroid hormone levels measured by RIA compared to mass spectrometry? Clin Chim Acta 2018; 484:223-5.
26. Gupta A, Kumar D. Beyond the limit of assignment of metabolites using minimal serum samples and 1H NMR spectroscopy with cross-validation by mass spectrometry. J Pharm Biomed Anal 2018; 151:356-364.
27. Nagana Gowda G-A, Gowda Y-N, Raftery D. Expanding the limits of human blood metabolite quantitation using NMR spectroscopy. Anal Chem 2015; 87(1): 706-15.
28. Shanaiah N, Desilva M-A, Nagana Gowda G-A, Raftery M-A, Hainline B-E, Raftery D. Class selection of amino acid metabolites in body fluids using chemical derivatization and their enhanced 13C NMR. Proc Natl Acad Sci U S A 2007; 104(28):11540-4.

29. Zhang P, Zhang W, Lang Y, Qu Y, Chu F, Chen J, Cui L. Mass spectrometry-based metabolomics for tuberculosis meningitis. Clin Chim Acta 2018; 483:57-63.
30. Lei Z, Huhman D-V, Sumner L-W. Mass spectrometry strategies in metabolomics. J Biol Chem 2011; 286(29):25435-42.
31. Zapata F, Ossa A-F, Ruiz C-G. Emerging spectrometric techniques for the forensic analysis of body fluids. Trends in Analytical Chemistry 2015; 64:53-63.
32. Szultka M, Papaj K, Rusin A, Szeja W, Buszewski B. Determination of flavonoids and their metabolites by chromatographic techniques. Trends in Analytical Chemistry 2013; 47:47-67.
33. Liu R, Chou J, Hou S, Liu X, Yu J, Zhao X, Li Y, Liu L, Sun C. Evaluation of two-step liquid-liquid extraction protocol for untargeted metabolic profiling of serum samples to achieve broader metabolome coverage by UPLC-Q-TOF-MS. Anal Chim Acta 2018; 1035:96-107.
34. Chen X, Hu C, Dai J, Chen L. Metabolomics analysis of seminal plasma in infertile males with kidney-yang deficiency: a preliminary study. Evid Based Complement Alternat Med. 2015; 2015:892930.
35. Paiva C, Amaral A, Rodriguez M, Canyellas N, Correig X, Ballescà J-L, Ramalho-Santos J, Oliva R. Identification of endogenous metabolites in human sperm cells using proton nuclear magnetic resonance (1H-NMR) spectroscopy and gas chromatography-mass spectrometry (GC-MS). Andrology 2015; 3(3): 496-505.
36. Nagata Y, Hirayama A, Ikeda S, Shirahata A, Shoji F, Maruyama M, Kayano M, Bundo M, Hattori K, Yoshida S, Goto Y-I, Urakami K, Soga T, Ozaki K, Niida S. Comparative analysis of cerebrospinal fluid metabolites in Alzheimer's disease and idiopathic normal pressure hydrocephalus in a Japanese cohort. Biomark Res 2018; 6:5.
37. Boizard F, Brunchault V, Moulos P, Breuil B, Klein J, Lounis N, Caubet C, Tellier S, Bascands J-L, Decramer S, Schanstra J-P, Buffin-Meyer B. A capillary electrophoresis coupled to mass spectrometry pipeline for long term comparable assessment of the urinary metabolome. Sci Rep 2016; 6:34453.
38. Patil S-B, Dheeman D-S, Al-Rawhani M-A, Velugotla S, Nagy B, Cheah B-C, Grant J-P, Accarino C, Barrett M-P, Cumming D-R-S. An integrated portable system for single chip simultaneous measurement of multiple disease associated metabolites. Biosens Bioelectron 2018; 122:88-94.
39. Galenkamp N-S, Soskine M, Hermans J, Wloka C, Maglia G. Direct electrical quantification of glucose and asparagine from bodily fluids using nanopores. Nat Commun 2018; 9(1):4085.
40. Johnson S-R, Lange B-M. Open-access metabolomics databases for natural product research: present capabilities and future potential. Front Bioeng Biotechnol 2015; 3:22.
41. Vinaixa M, Schymanski E-L, Neumann S, Navarro M, Salek R-M & Yanes O. Mass spectral databases for LC/MS- and GC/MS-based metabolomics: State of the field and future prospects. Trends in Analytical Chemistry 2016; 78:23-35.
42. Longnecker K, Futrelle J, Coburn E, Kido Soule M-C, Kujawinski E-B. Environmental metabolomics: databases and tools for data analysis. Marine Chemistry 2015; 177:366–373.
43. The Metabolomics Society. Databases. http://metabolomicssociety.org/resources/metabolomics-databases.
44. Kuehnbaum N-L, Britz-McKibbin P. New advances in separation science for metabolomics: resolving chemical diversity in a post-genomic era. Chem Rev 2013; 113(4):2437-68.
45. Bouatra S, Aziat F, Mandal R, Guo A-C, Wilson M-R, Knox C, Bjorndahl T-C, Krishnamurthy R, Saleem F, Liu P, Dame Z-T, Poelzer J, Huynh J, Yallou F-S, Psychogios N, Dong E, Bogumil R, Roehring C, Wishart D-S. The human urine metabolome. PLoS One 2013; 8(9):e73076.
46. Dame Z-T, Aziat F, Mandal R, Krishnamurthy R, Bouatra S, Borzouie S, Guo A-C, Sajed T, Deng L, Lin H, Liu P, Dong E, Wishart D-S. The human saliva metabolome. Metabolomics 2015; 11(6):1864–83.
47. Koo I, Shi X, Kim S, Zhang X. iMatch2: compound identification using retention index for analysis of gas chromatography–mass spectrometry data. J Chromatography A 2014; 1337:202-10.
48. Dettmer K, Nürnberger N, Kaspar H, Gruber M-A, Almstetter M-F, Oefner P-J. Metabolite extraction from adherently growing mammalian cells for metabolomics studies: optimization of harvesting and extraction protocols. Anal Bioanal Chem 2011; 399(3):1127-39.

49. León Z, García-Cañaveras J-C, Donato M-T, Lahoz A. Mammalian cell metabolomics: Experimental design and sample preparation. Electrophoresis 2013; 34(19):2762-75.
50. Lorenz M-A, Burant C-F, Kennedy R-T. Reducing time and increasing sensitivity in sample preparation for adherent mammalian cell metabolomics. Anal Chem 2011; 83(9):3406-14.
51. Hyötyläinen T. Critical evaluation of sample pretreatment techniques. Anal Bioanal Chem 2009; 394(3):743-58.
52. Raterink R-J, Lindenburg P-W, Vreeken R-J, Ramautar R, Hankemeier T. Recent developments in sample-pretreatment techniques for mass spectrometry-based metabolomics. Trends in Analytical Chemistry 2014; 61:157–67.
53. Alshammari T-M, Al-Hassan A-A, Hadda T-B, Aljofan M. Comparison of different serum sample extraction methods and their suitability for mass spectrometry analysis. Saudi Pharm J 2015; 23(6):689-97.
54. Want E-J, O'Maille G, Smith C-A, Brandon T-R, Uritboonthai W, Qin C, Trauger S-A, Siuzdak G. Solvent-dependent metabolite distribution, clustering, and protein extraction for serum profiling with mass spectrometry. Anal Chem 2006; 78(3): 743-52.
55. Sana T, Fischer S. Maximizing metabolite extraction for comprehensive metabolomics studies of erythrocytes. www.agilent.com/chem/metabolomics.
56. Folch J, Lees M, Sloane Stanley G-H. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957; 226(1):497-509.
57. Bligh E-G, Dyer W-J. A rapid method of total lipid extraction and purification.Can J Biochem Physiol 1959; 37(8):911-7.
58. Matyash V, Liebisch G, Kurzchalia T-V, Shevchenko A, Schwudke D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res 2008; 49(5):1137-46.
59. Saric J, Want E-J, Duthaler U, Lewis M, Keiser J, Shockcor J-P, Ross G-A, Nicholson J-K, Holmes E, Tavares M-F. Systematic evaluation of extraction methods for multiplatform based metabotyping: application to the Fasciola hepatica metabolome. Anal Chem 2012; 84(16):6963-72.
60. Skoog DA, Holler FJ, Crouch SR, 2017. An introduction to chromatographic separations. In: Principles of instrumental analysis, 7th Edition. Cengage Learning. 696-719.
61. Instrumentation of HPLC Mobile Phase Considerations. Crawford Scientific. CHROMacademy. https://pdfs.semanticscholar.org/04b2/2934d16b88625acaa439f119bddd303451ad.pdf.
62. The Theory of HPLC Chromatographic Parameters. Crawford Scientific. CHROMacademy. https://www.chromacademy.com/lms/sco2/Theory_Of_HPLC_Chromatographic_Parameters.pdf.
63. Hua Y, Jenke D. Increasing the sensitivity of an LC–MS method for screening material extracts for organic extractables via mobile phase optimization. J Chromatogr Sci 2012; 50(3):213-27.
64. Hopfgartner G, Bean K, Henion J, Henry R. Ion spray mass spectrometric detection for liquid chromatography: A concentration- or a mass-flow-sensitive device? Journal of Chromatography A 1993; 647(1):51-61.
65. The Theory of HPLC: Gradient HPLC. Crawford Scientific. CHROMacademy. https://www.chromacademy.com/lms/sco8/theory_of_hplc_gradient_hplc.pdf.
66. Sturtevant D, Lee Y-J, Chapman K-D. Matrix assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) for direct visualization of plant metabolites in situ. Curr Opin Biotechnol 2016; 37:53-60.
67. Rzagalinski I, Volmer D-A. Quantification of low molecular weight compounds by MALDI imaging mass spectrometry – A tutorial review. Biochim Biophys Acta Proteins Proteom 2017; 1865(7):726-39.
68. Carry E, Zhao D, Mogno I, Faith J, Ho L, Villani T, Patel H, Pasinetti G-M, Simon J-E, Wu Q. Targeted analysis of microbial-generated phenolic acid metabolites derived from grape flavanols by gas chromatography-triple quadrupole mass spectrometry. J Pharm Biomed Anal 2018; 159:374-83.
69. Zhao D, Yuan B, Carry E, Pasinetti GM, Ho L, Faith J, Mogno I, Simon J, Wu Q. Development and validation of an ultra-high performance liquid chromatography/triple quadrupole mass spectrometry method for analyzing microbial-derived grape polyphenol metabolites. J Chromatogr B Analyt Technol Biomed Life Sci 2018; 1099:34-45.

70. Jia W, Shi L, Chu X, Chang J, Chen Y, Zhang F. A strategy for untargeted screening of macrolides and metabolites in bass by liquid chromatography coupled to quadrupole orbitrap mass spectrometry. Food Chem 2018; 262: 110-17.
71. Huang M, Cheng Z, Wang L, Feng Y, Huang J, Du Z, Jiang H. A targeted strategy to identify untargeted metabolites from in vitro to in vivo: Rapid and sensitive metabolites profiling of licorice in rats using ultra-high performance liquid chromatography coupled with triple quadrupole-linear ion trap mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2018; 1092:40-50.
72. Zhao H, Liu Y, Li Z, Song Y, Cai X, Liu Y, Zhang T, Yang L, Li L, Gao S, Li Y, Yu C. Identification of essential hypertension biomarkers in human urine by nontargeted metabolomics based on UPLC-Q-TOF/MS. Clin Chim Acta 2018; 486:192-8.
73. Van Steendam K, De Ceuleneer M, Dhaenens M, Van Hoofstat D, Deforce D. Mass spectrometry based proteomics as a tool to identify biological matrices in forensic science. Int J Legal Med 2013; 127(2):287-98.
74. Yang H, Zhou B, Deng H, Prinz M, Siegel D. Body fluid identification by mass spectrometry. Int J Legal Med 2013; 127(6):1065-77.
75. Legg K-M, Powell R, Reisdorph N, Reisdorph R, Danielson P-B. Discovery of highly specific protein markers for the identification of biological stains. Electrophoresis 2014; 35(21-22):3069-78.
76. Stringer K-A, Younger J-G, McHugh C, Yeomans L, Finkel M-A, Puskarich M-A, Jones A-E, Trexel J, Karnovsky. A whole blood reveals more metabolic detail of the human metabolome than serum as measured by 1H-NMR spectroscopy: implications for sepsis metabolomics. Shock. 2015; 44(3):200-8.
77. Nagana Gowda G-A, Raftery D. Whole blood metabolomics by 1H NMR spectroscopy provides a new opportunity to evaluate coenzymes and Antioxidants. Anal Chem 2017; 89(8):4620-7.
78. Kromke M, Palomino-Schätzlein M, Mayer H, Pfeffer S, Pineda-Lucena A, Luy B, Hausberg M, Muhle-Goll C. Profiling human blood serum metabolites by nuclear magnetic resonance spectroscopy: a comprehensive tool for the evaluation of hemodialysis efficiency. Transl Res 2016; 171:71-82.
79. Tiziani S, Emwas A-H, Lodi A, Ludwig C, Bunce C-M, Viant M-R, Günther U-L. Optimized metabolite extraction from blood serum for 1H nuclear magnetic resonance spectroscopy. Anal Biochem 2008; 377(1):16-23.
80. Kutyshenko V-P, Molchanov M, Beskaravayny P, Uversky V-N, Timchenko M-A. Analyzing and mapping sweat metabolomics by high-resolution NMR spectroscopy. PLoS One 2011; 6(12):e28824.
81. Vitali B, Cruciani F, Picone G, Parolin C, Donders G, Laghi L. Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis. Eur J Clin Microbiol Infect Dis 2015; 34(12):2367-76.
82. Figueira J, Jonsson P, Nordin Adolfsson A, Adolfsson R, Nyberg L, Öhman A. NMR analysis of the human saliva metabolome distinguishes dementia patients from matched controls. Mol Biosyst 2016; 12(8):2562-71.