Metabolic Profiling and Quantitative Analysis of Cerebrospinal Fluid Using Gas Chromatography–Mass Spectrometry: Current Methods and Future Perspectives

Alisa Pautova 1,*, Natalia Burnakova 2 and Alexander Revelsky 2

1 Federal Research and Clinical Center of Intensive Care Medicine and Rehabilitology, Laboratory of Human Metabolism in Critical States, Negovsky Research Institute of General Reanimatology, Petrovka str., 25-2, 107031 Moscow, Russia
2 Laboratory of Mass Spectrometry, Chemistry Department, Lomonosov Moscow State University, CSF-1, Leninskie gory, 1-3, 119991 Moscow, Russia; nburnakova@me.com (N.B.); sorbent@yandex.ru (A.R.)
* Correspondence: aliceau@mail.ru; Tel.: +79-057-738-981

Abstract: Cerebrospinal fluid is a key biological fluid for the investigation of new potential biomarkers of central nervous system diseases. Gas chromatography coupled to mass-selective detectors can be used for this investigation at the stages of metabolic profiling and method development. Different sample preparation conditions, including extraction and derivatization, can be applied for the analysis of the most of low-molecular-weight compounds of the cerebrospinal fluid, including metabolites of tryptophan, arachidonic acid, glucose; amino, polyunsaturated fatty and other organic acids; neuroactive steroids; drugs; and toxic metabolites. The literature data analysis revealed the absence of fully validated methods for cerebrospinal fluid analysis, and it presents opportunities for scientists to develop and validate analytical protocols using modern sample preparation techniques, such as microextraction by packed sorbent, dispersive liquid–liquid microextraction, and other potentially applicable techniques.

Keywords: metabolomics; targeted analysis; nontargeted analysis; sample preparation; derivatization; validation; biomarkers

1. Introduction

Modern differential diagnostics of a wide variety of diseases and pathologies is not complete without analyzing the composition of biological fluids of the body. The most available and studied fluids are blood and urine. Less studied, as well as less available, is cerebrospinal fluid (CSF). CSF performs a number of important physiological functions in the brain and spinal cord, providing metabolic processes between the blood and the brain. Chemical compounds of different structures can penetrate from the blood through the blood–brain barrier (BBB) into the CSF, and then into the brain cells, subsequently affecting the functioning of the central nervous system (CNS) [1].

The routine laboratory study of the CSF composition is usually aimed at the diagnosis of acute infectious diseases of the CNS such as meningitis and encephalitis. The current interest in the study of the CSF composition is due to the rapidly increasing number of neurodegenerative, mental, and other slowly progressive—and in most cases, incurable—diseases. Despite the advances in science and medicine, treatment of these diseases is directed at reducing the symptoms, but not at eliminating the cause of the disease, since the etiologies of most of the common diseases, such as multiple sclerosis [2–7], Parkinson’s disease [8], Alzheimer’s disease [9–14], and others, remain not fully understood.
One of the modern concepts in the medical community is the concept of “microbiota–gut–brain” connection. Previously, it was thought that the brain affects the functioning of the body “unidirectionally”, but recent research studies clearly indicate that the composition and function of the gut microbiota have an equal impact on the brain and the CNS. Numerous studies have shown that the composition of the microbiota in healthy volunteers differs from that in patients with various mental, neurodegenerative, and chronic diseases [15,16]. The gut microbiota synthesize and utilize a large number of biologically active substances, including fatty acids, amino acids, neurotransmitters, and others that can penetrate the BBB; thus, the microbiota are becoming a new potential target for the monitoring and treatment of various CNS diseases [17–19].

The main fact that limits the active study of the composition of the CSF is its inaccessibility. A lumbar puncture is required to obtain the CSF that carries the risk of side complications [20]. The most common one is a postdural puncture syndrome, which is accompanied by headaches and symptoms of meningism, as well as less common complications (spinal or epidural bleeding, adhesive arachnoiditis, and trauma to the spinal cord) [21]. The technique of lumbar puncture requires professionalism and experience from the specialist who performs it; this determines its nonprevalence along with the above-mentioned side effects. Lumbar puncture for diagnosis of subarachnoid hemorrhage, hydrocephalus, or infectious diseases of the CNS is frequently carried out in cases in which computed tomography or magnetic resonance imaging is not possible (with the exception of meningitis) [20].

During the diagnostic lumbar puncture, the volume of the sampled CSF is 2.0–5.0 mL (protocol approved by the regional guidelines approved by the Ministry of Health of the Russian Federation, 4 March 2004, #4.2.1887-04). Subsequent routine analysis includes the determination of cytosis, microbiological studies, and biochemical analysis (determination of the level of glucose, chlorides, etc.). Since the total volume of the CSF in an adult is 125–150 mL, any actions aimed at the additional CSF collection or procedure must be justified and approved by the local ethics committee of the institution. For scientific purposes, carrying the CSF analysis out in the residual volume of the sample after clinical and laboratory studies, which is usually about 0.5–1 mL, is highly desirable. Despite all mentioned limitations, the investigation of the CSF composition—in particular, of that of the healthy donors—is an important and crucial issue for the discovering of the new biomarkers.

The limited available sample volume requires the use of modern efficient sample preparation techniques and sensitive analytical methods for the determination of metabolites, most often at the trace level. Such methods are chromatographic ones, namely gas (GC) and high-performance liquid chromatography (HPLC) in combination with different mass-selective (MS) detectors (single quadrupole (Q), time-of-flight (TOF), including high resolution equipment and tandem MS/MS or Q-TOF) [22].

Nontargeted metabolic profiling analysis is the initial stage in the search for metabolites that distinguish patients with pathology from patients of the control group/healthy volunteers. The most common task is to identify as many chromatographic peaks in the sample as possible using the total ion current mode [8, 10, 23, 24]. The subsequent steps should be directed at clearly establishing the formulas of the most promising markers, and statistically processing the quantitative data, which will answer the question of whether the found metabolite or several metabolites are promising biomarkers [25–27]. An important stage is the validation of the method for determining the target components, which currently needs to be carried out in accordance with the Food and Drug Administration (FDA) or the European Medicines Agency (EMA) guidelines [28]. Since the volume of biological samples for full validation is about 40–50 mL (for plasma samples), CSF validation requirements can be reduced because of its specificity. However, validation for the CSF metabolites is justified for several compounds simultaneously.

Based on the information provided, the following requirements for the CSF analysis method can be formulated:
- the use of the minimum (less than 0.5 mL) volume of the CSF for one analysis;
- the quantitative analysis of several target components at the trace level in one analysis.

Gas chromatography is the most common method for the analysis of volatile compounds in biological samples. Correctly selected sample preparation conditions lead to the quantitative determination of different classes of chemical compounds in one chromatographic analysis, which meets the above requirements. When comparing the capabilities of GC and HPLC, the lower cost of the GC equipment and the simpler selection of the parameters of the analysis are worth noting. Despite the wider possibilities of the HPLC in the analysis of nonvolatile high-molecular-weight compounds, gas chromatography is the “gold standard” for the tasks of metabolomics—in particular, the determination of low-molecular-weight compounds [29]. Thus, the aim of this review was to analyze the original articles published since 2000 on the study of the CSF composition using GC–MS and to describe the most promising modern methods of sample preparation, which are potentially suitable for studying CSF composition. The PubMed, Science Direct, and Google Scholar database platforms were used for the search. The keywords “cerebrospinal fluid” and “gas chromatography–mass spectrometry” or “GC–
MS” were used in combination in the search list.

2. The Human Cerebrospinal Fluid Metabolome

A large-scale study of the CSF composition (Table 1) was carried out by a group of scientists who are the creators of the Human Metabolome Database. The main publication describing the result of their research provided a link to the created resource www.csfmetabolome.ca. From a total of 308 metabolites detected, 53 were identified using nuclear magnetic resonance (NMR) spectroscopy, 41 using GC–MS, and 17 using LC–MS [22]. Later work by the same authors described 476 metabolites [30]. At the time of preparing the present review, there were 445 metabolites in the metabolite catalog, 443 of which were listed as qualitatively and quantitatively measured. The main classes of low molecular weight metabolites found in CSF are the compounds that can be determined by GC–MS, namely amino acids, fatty acids, including short-chain ones, steroids and their derivatives, hydroxy acids, dicarboxylic acids, and nucleosides. Moreover, most of the identified compounds are neurotransmitters or their metabolites [22]. As noted by the authors, despite the greater number of compounds determined by the NMR, the potential remains with GC–MS when using selective methods of sample preparation and derivatization, as well as when using TOF mass spectrometry.
| Aim                                                                 | GC–MS Method, Capillary Column | CSF Sampling               | Compounds                               | Sample Volume | Sample Preparation                                                                 | Reference |
|-------------------------------------------------------------------|--------------------------------|---------------------------|-----------------------------------------|---------------|------------------------------------------------------------------------------------|-----------|
| Presenting a catalog of detectable metabolites (including their  | GC–MS: DB-5 column            | Patients screened for     | 41 metabolites: amino acids, fatty      | 200 μL        | CSF + 800 μL 8:1 HPLC-grade MeOH-deionized water + vortexing + centrifugation +    | [22]      |
| concentrations and disease associations) that can be found in human |                                | meningitis (n = 50).      | acids, steroids, carbohydrates, et al.  |               | 200 μL of the supernatant was evaporated to dryness + 40 μL methoxyamine hydrochloride + incubation 90 min at 30 °C + 40 μL MSTFA + 20 μL proline IS + incubation at 30 °C for 45 min. |           |
| CSF. This catalog was assembled using a combination of both     |                                |                           |                                         |               |                                                                                   |           |
| experimental (NMR, GC–MS, LC–FTMS) and literature-based         |                                |                           |                                         |               |                                                                                   |           |
| research.                                                        |                                |                           |                                         |               |                                                                                   |           |
| Update of the CSF metabolome database. Determination of         | GC–MS: DB-5 column            | Patients screened for     | 93 metabolites: amino acids, organic    | 60–100 μL     | 60 μL CSF + 250 μL MeOH + centrifugation. 100 μL CSF samples from the validation  | [30]      |
| metabolites using different methods, including NMR, GC–MS, LC– |                                | meningitis (n = 7).       | acids, nucleosides, fatty acids,        |               | sample set + 400 μL MeOH + drying under N:\- derivatization with MSTFA in pyridine. The final volume was 45 μL for the original sample set and 135 μL for the validation sample set. |           |
| FTMS, direct flow injection–MS/MS and ICP–MS.                   |                                |                           | mono- and disaccharides, et al.         |               |                                                                                   |           |
| Analysis of protein and metabolite abundances in CSF by multiple | GC–MS: 30 m × 0.25 mm × 0.25  | Subjects (n = 9), the    |                                         |               |                                                                                   | [31]      |
| analytical platforms. Integration of metabolomics and proteomics | μm, HP5-MS                     | validation sample set (n = | 150 metabolites: amino acids, fatty      | 500 μL        | CSF + 9 volumes MeOH + centrifugation + IS + drying under N:\- oximation and       |           |
| to present biological variations in metabolite and protein       |                                | 28), and the experimental | acids, inosines, carbohydrates, et al.  |               | trimethylsilylation.                                                                 |           |
| abundances and compare these with technical variations with the  |                                | sample sets (n = 36 for   |                                         |               |                                                                                   |           |
| currently used analytical methods.                               |                                | proteomics and n = 42 for  |                                         |               |                                                                                   |           |
|                                                                   |                                | metabolomics).             |                                         |               |                                                                                   |           |
| To conduct a global metabolomics analysis to provide an overview  | GC–MS/MS: 30 m × 0.25 mm × 0.25 | Healthy subjects (n = 9). |                                         |               |                                                                                   | [32]      |
| of the postprandial alterations in CSF and plasma metabolites and | μm, DB-5MS-DG.                 | CSF collected both preprandial | 49 metabolites: amino acids, sugars,    | 100 μL        | CSF + 9 volumes MeOH + centrifugation + IS + drying under N:\- oximation and       |           |
| to facilitate the application of CSF for biomarker screening (    |                                | and postprandial CSF. The  | hydroxy acids, et al.                    |               | trimethylsilylation.                                                                 |           |
| using metabolomics).                                              |                                | postprandial time was set  |                                         |               |                                                                                   |           |
|                                                                   |                                | at 1.5 h (n = 3), 3 h (n = 3), and 6 h (n = 3). | | |                                                                                   |           |
| Study on the effects of preanalytical factors on the porcine     | GC–MS: 30 mm × 0.25 mm × 0.25  | Conventional pigs (n = 5). |                                         |               |                                                                                   | [33]      |
| CSF proteome and metabolome using a variety of techniques        | μm, HP5-MS                     |                           | 1280 metabolites, quantitatively        | 25 μL         | Lyophilization + derivatization with methoxyamine hydrochloride in pyridine + derivatization with MSTFA. |           |
| comprising LC–MS, GC–MS, and MALDI-FT-ICP-MS.                   |                                |                           | determined 21 compounds                 |               |                                                                                   |           |
| Characterization of the metabolites present in CSF and comparison| GC × GC–TOFMS: 10 m × 0.18 mm | Healthy subjects (n = 53). |                                         |               |                                                                                   | [34]      |
| of metabolite levels in patient-matched setting to those found in | 0.18 μm × 0.18 μm, Rxi-5ms:   |                           | 1280 metabolites, quanitatively         | 25 μL         | CSF + 10 μL IS + 400 μL MeOH + vortexing + centrifugation + 30 min at ~20 °C + drying under N:\- derivatization 25 μL O-methylhydroxylamine hydrochloride + incubation 60 min, 45 °C + 25 μL |           |
| serum.                                                           | 1.5 m × 0.1 mm × 0.1 μm.       |                           | determined 21 compounds                 |               |                                                                                   |           |
| BPX-50.                                                          |                                |                           |                                         |               |                                                                                   |           |
| Molecules 2021, 26, 3597 | MSTFA + incubation 60 min at 45 °C + hexane. |
|-------------------------|---------------------------------------------|
| A GC–MS-based metabolomic analysis of CSF samples from glioma patients. | MSTFA + incubation 60 min at 45 °C + hexane. |
| GC–MS: 30 m × 0.25 mm × 1.0 μm, DB-5, 30 m × 0.25 mm × 0.25 μm, CP-SIL 8 CB low bleed/MS. | GC–MS: 30 m × 0.25 mm × 1.0 μm, DB-5, 30 m × 0.25 mm × 0.25 μm, CP-SIL 8 CB low bleed/MS. |
| 50 metabolites, quantitatively determined 16 compounds. | 50 μL MSTFA + 250 μL MeOH–water–chloroform (2.5:1:1) + IS + vortexing + centrifugation + 250 μL of supernatant + 200 μL distilled water + vortexing + centrifugation + 250 μL of supernatant sample volume was lyophilized + 40 μL 20 mg/mL methoxamine hydrochloride in pyridine + 20 μL MSTFA + centrifugation. |
| Patients with intracranial glial tumors (n = 32). | Patients with intracranial glial tumors (n = 32). |
| Patients suspected to have inflammatory demyelinating diseases (n = 145), control subjects without medical or neurological illness (n = 12) | Patients suspected to have inflammatory demyelinating diseases (n = 145), control subjects without medical or neurological illness (n = 12) |
| 962 metabolic signatures, quantitatively determined 85: sugars and sugar alcohols (24%), amino acids (28%), fatty acids (15%), organic acids (15%), amines (2%) et al. | 962 metabolic signatures, quantitatively determined 85: sugars and sugar alcohols (24%), amino acids (28%), fatty acids (15%), organic acids (15%), amines (2%) et al. |
| Exploring potential biomarkers and improving understanding of biochemical features of CSF-mediated autoimmune inflammatory diseases of the CNS. | Exploring potential biomarkers and improving understanding of biochemical features of CSF-mediated autoimmune inflammatory diseases of the CNS. |
| GC–TOFMS: RTX-5Sil MS. | GC–TOFMS: RTX-5Sil MS. |
| 100 μL MSTFA + 250 μL MeOH–water–chloroform (2.5:1:1) + IS + vortexing + centrifugation + 250 μL of supernatant + 200 μL distilled water + vortexing + centrifugation + 250 μL of supernatant sample volume was lyophilized + 40 μL 20 mg/mL methoxamine hydrochloride in pyridine + 20 μL MSTFA + centrifugation. | 100 μL MSTFA + 250 μL MeOH–water–chloroform (2.5:1:1) + IS + vortexing + centrifugation + 250 μL of supernatant + 200 μL distilled water + vortexing + centrifugation + 250 μL of supernatant sample volume was lyophilized + 40 μL 20 mg/mL methoxamine hydrochloride in pyridine + 20 μL MSTFA + centrifugation. |
| 100 μL MSTFA + 900 μL IS and MeOH–water, 1.9 + 11 IS + beadmill for 1 min (90 Hz), 2 h on ice + centrifugation + 200 μL evaporized + storage at –80 °C + 100 μL 40 mg/mL methoxamine hydrochloride in pyridine + incubation 90 min at 200 rpm, 30 °C + 2 μL IS + 45 μL MSTFA+1% TMCS + incubation 1 h at 200 rpm, 37 °C. | 100 μL MSTFA + 900 μL IS and MeOH–water, 1.9 + 11 IS + beadmill for 1 min (90 Hz), 2 h on ice + centrifugation + 200 μL evaporized + storage at –80 °C + 100 μL 40 mg/mL methoxamine hydrochloride in pyridine + incubation 90 min at 200 rpm, 30 °C + 2 μL IS + 45 μL MSTFA+1% TMCS + incubation 1 h at 200 rpm, 37 °C. |
| CSF metabolome study in a group of patients with different clinical and genetic subtypes of amyotrophic lateral sclerosis using GC–TOFMS. | CSF metabolome study in a group of patients with different clinical and genetic subtypes of amyotrophic lateral sclerosis using GC–TOFMS. |
| GC–TOFMS: 10 m × 0.18 mm × 0.18 μm DB 5-MS. | GC–TOFMS: 10 m × 0.18 mm × 0.18 μm DB 5-MS. |
| amyotrophic lateral sclerosis patients (n = 78), healthy subjects (for control) | amyotrophic lateral sclerosis patients (n = 78), healthy subjects (for control) |
| 120 peaks, 40 identified. | 120 peaks, 40 identified. |
| 100 μL MSTFA + 400 μL MeOH–water, 1.9 + 11 IS + beadmill for 1 min (90 Hz), 2 h on ice + centrifugation + 200 μL evaporized + storage at –80 °C + 30 μL (15 μg/μL) methylhydroxylamine hydrochloride 98% in pyridine + vortexing + heating 70 °C, 1 h + 16 h at room temperature + 30 μL MSTFA+1% TMCS, room temperature, 1 h + 30 μL heptane. | 100 μL MSTFA + 400 μL MeOH–water, 1.9 + 11 IS + beadmill for 1 min (90 Hz), 2 h on ice + centrifugation + 200 μL evaporized + storage at –80 °C + 30 μL (15 μg/μL) methylhydroxylamine hydrochloride 98% in pyridine + vortexing + heating 70 °C, 1 h + 16 h at room temperature + 30 μL MSTFA+1% TMCS, room temperature, 1 h + 30 μL heptane. |
| A detailed analytical evaluation of GC–APCI–TOFMS. In addition to the detailed examination of the analytical performance (repeatability, reproducibility, linearity, and detection limits), the applicability of this technique for metabolic profiling of CSF was demonstrated. | A detailed analytical evaluation of GC–APCI–TOFMS. In addition to the detailed examination of the analytical performance (repeatability, reproducibility, linearity, and detection limits), the applicability of this technique for metabolic profiling of CSF was demonstrated. |
| GC–APCI–TOFMS 30 m × 0.25 mm × 0.25 μm HP-S-MS. | GC–APCI–TOFMS 30 m × 0.25 mm × 0.25 μm HP-S-MS. |
| Human CSF | Human CSF |
| 300 compounds, 21 identified | 300 compounds, 21 identified |
| 250 μL MSTFA + 600 μL MeOH + centrifugation + evaporation + 100 μL methoxamine:pyridine mixture, 40 °C, 60 min + 100 μL BSTFA or MSTFA containing 1% TMCS, 40 °C, 30 min + 2 h equilibration. | 250 μL MSTFA + 600 μL MeOH + centrifugation + evaporation + 100 μL methoxamine:pyridine mixture, 40 °C, 60 min + 100 μL BSTFA or MSTFA containing 1% TMCS, 40 °C, 30 min + 2 h equilibration. |
| Study Description                                                                 | Methodology                                                                 | Participants                                                                 | Metabolites | Extraction Details                                                                 |
|---------------------------------------------------------------------------------|------------------------------------------------------------------------------|-------------------------------------------------------------------------------|-------------|-----------------------------------------------------------------------------------|
| GC–MS/MS-based metabolome analysis of the CSF in pediatric patients with and without epilepsy. | GC–MS/MS                                                                    | Patients with epilepsy ($n = 34$), patients without epilepsy ($n = 30$)       | 180 metabolites | 50 μL (from reference): 50 μL serum + 250 μL MeOH–water–chloroform (2.5:1:1) + shaking, 30 min at 37 °C + centrifugation + 225 μL supernatant + 200 μL distilled water + centrifugation + 250 μL + 40 μL 20 mg/mL methoxyamine hydrochloride in pyridine + shaking + 20 μL MSTFA + incubation 30 min, 1200 rpm at 37 °C + centrifugation. |
| Testing the hypothesis that fatty acid metabolism in Alzheimer’s disease or mild cognitive impairment is altered compared to cognitively healthy study participants, and that details of the changes could be revealed by study of the brain-derived nanoparticles and supernatant fluid fractions of CSF. | GC–MS: 30 m × 0.25 mm × 0.50 μm, Phenomenex Zebron ZB-1MS.                  | Total participants ($n = 139$): cognitively healthy ($n = 70$), mild cognitive impairment ($n = 40$), Alzheimer’s disease ($n = 29$). 20 fatty acids (6 saturated, 6 monounsaturated, 8 polyunsaturated fatty acids) | 1 mL         | 1 mL CSF + 100 ng IS + formic acid (0.9%, 3 drops) + lipid extraction + 0.5 mL chloroform:MeOH solution (1:1, v/v), 0.5 mg/mL butylated hydroxytoluene + vortexing + storage −40 °C + PFBBr in MeCN solution (1:19 v/v, 50 μL) and disopropyl ether in MeCN solution (1:9 v/v, 50 μL), 20 min at 45 °C + drying under N₂ + 1 mL hexane. |
| Investigation of CSF and plasma metabolomic profiles for prediction of Parkinson’s disease progression. | GC–MS                                                                        | Participants with relatively mild Parkinsonism. Donors ($n = 49$) were randomly selected placebo-treated participants. | 383 biochemicals | No data. Brief information describes extraction and derivatization with BSTFA. |
| Investigation of the metabolomics profile of patients affected by relapsing–remitting multiple sclerosis and primary progressive multiple sclerosis, in order to find potential biomarkers to distinguish between the two forms. | GC–MS: 30 m × 0.25 mm × 0.25 μm, TG-5MS.                                    | Patients (relapsing–remitting multiple sclerosis $n = 22$, primary progressive multiple sclerosis $n = 12$) | Different classes of compounds (data most discussed in this article were obtained from LC–MS and flow injection–MS analysis). | 200 μL 200 μL lyophilization + drying + 50 μL methoxyamine in pyridine (10 mg/mL), 70 °C + 100 μL MSTFA, room temperature, 1 h + 100 μL hexane. |
| Comprehensive analysis of the absorbed constituents in the plasma and CSF of rabbits after intranasal administration of Asari Radix et Rhizoma by headspace solid-phase microextraction–GC–MS and HPLC-atmospheric pressure chemical ionization–ion trap-time of flight-multistage mass spectrometry (HPLC–APCI–ion-trap-TOF–MSn). | GC–MS: 30 m × 0.25 mm × 0.25 μm, Rxi-5MS.                                   | Rabbits ($n = 15$) | 25 metabolites | 500 μL CSF in 10 mL headspace vial + 0.10 g NaCl + polydimethylsiloxane/divinylbenzene fiber was exposed to the headspace at 70 °C, 40 min + fiber was withdrawn. |
| Study                                                                 | Methodology                                                                 | Samples | Metabolites/Variables | Derivatization | Conditions                                                                 | References |
|----------------------------------------------------------------------|-----------------------------------------------------------------------------|---------|-----------------------|----------------|-----------------------------------------------------------------------------|------------|
| Investigation of CSF metabolomics in an acute experimental autoimmune encephalomyelitis rat model using targeted LC–MS and GC–MS. | GC–MS: 30 m × 0.25 mm × 0.25 μm, HP5-MS.                                    | Rats (n = 84) | 14 amino acids and related compounds | MSTFA | 30 μL CSF + 250 μL MeOH + centrifugation + drying under N₂ + derivatization with MSTFA in pyridine. The end volume was 45 μL | [2]        |
| A nontargeted metabolomic analysis using GC–MS was conducted to identify differentially expressed metabolites between naturally occurring depressive and control macaques. | GC–MS: 30 m × 0.25 mm × 0.25 μm, HP5-MS.                                    | Naturally occurring depressive female macaques (n = 10) and age- and gender-matched healthy controls (n = 12) | 663 variables, 37 metabolites | BSTFA+TMCS | ~15 μL CSF + 10 μL IS + vortexing + 90 μL MeOH + centrifugation + 95 μL of supernatant + drying under N₂ + 30 μL methoxamine hydrochloride (20 mg/ml pyridine) + incubation 37 °C, 90 min + 30 μL of BSTFA + 1% TMCS 70 °C, 60 min + cooling to room temperature. | [38]       |
| Report on an analytical method that can be used for metabolomics studies when only a limited amount of sample volume is available. | GC–MS: 30 m × 0.25 mm × 0.25 μm, HP5-MS.                                    | Rats (n = 60, total number of tricarboxylic cycle acids, carbohydrates, polyols, purine/pyrimidine bases, et al.) | 93 metabolites, 73 identified: fatty acids, amino acids, sugars, et al. | MSTFA+TMCS | 10 μL CSF + 40 μL MeOH + centrifugation 10 min, 11,800 rpm + drying under N₂ + 10 μL ethoxylamine-HCl (c = 56 mg/ml (0.58M) in pyridine), 90 min, 40 °C + 20 μL MSTFA, 50 min, 40 °C. The final volume was 50 μL | [39]       |
| Presentation of an analytical method using in-liner silylation coupled to GC–MS that is suitable for metabolic profiling in ultrasmall sample volumes of 2 μL down to 10 nL. | GC–TOFMS: 30 m × 0.25 mm × 0.25 μm HP5-MS.                                 | Mouse and human CSF samples. | 342 peaks, 52 identified in human CSF: amino acids, organic acids, fatty acids, sugars, et al. | BSTFA+TMCS | 2 μL The microvials containing the dried sample were placed inside the PTV injection liner + 1 μL IS + 3 μL MSTFA | [40]       |
| Application of untargeted metabolomics using GC–TOFMS to the CSF of aneurysmal subarachnoid hemorrhage patients to determine global metabolic changes and metabolite predictors of long-term outcome that are independent of vasospasm status. | GC–MS: 30 m × 0.25 mm × 0.25 μm, Rtx-5Sil MS.                             | Patients with aneurysmal subarachnoid hemorrhage (n = 15). | 97 metabolites | BSTFA+TMCS | 5 μL of CSF + 1.0 mL MeCN, isopropanol, and water in proportion 3:3:2 + vortexing + centrifugation + drying + 450 μL degassed 50% MeCN + centrifugation + derivatization. | [41]       |
The authors also noted the presence of biological variability in the concentration of metabolites between individuals in average ±50%, and in some cases ±100%. This phenomenon should be considered by researchers who study potential biomarkers in the CSF [22]. A large-scale metabolomic–proteomic investigation was directed to the study of the biological variability of the concentrations of the CSF metabolites using normal human CSF from patients undergoing routine, non-neurological surgical procedures. As a result of the metabolomic study, which included nontargeted GC–MS analysis, 93 of 108 detected metabolites were identified, including amino acids, organic and fatty acids, nucleosides, mono- and disaccharides, the biological variability of which was 15–85% (analytical variability less than 20%) [31]. Another study was devoted to the characterization of postprandial effects on the CSF metabolic profile of healthy volunteers (n = 9), which was performed using GC–MS/MS. Individual plots of postprandial samples of 150 CSF hydrophilic metabolites were positioned similar to the corresponding plots of preprandial samples. The postprandial effects had a far lower impact compared with interindividual variations [32]. Thus, biological interindivudual variations appear to have more significant impact on the CSF metabolite profile than food intake.

Important results were obtained describing different effects of preanalytical factors on stability of the proteomic and metabolomics profiles of the CSF. These factors were a 30/120 min delayed storage after the CSF collection at room temperature as the potential delays in the clinic, storage at 4 °C as the time that samples remain in the cooled autosampler, and repeated freeze–thaw cycles. The delayed storage factor led to the increased levels of 49 metabolites, which were analyzed using nontargeted GC–MS, and explained by metabolic processes that occurred because of the remaining white blood cells. The author’s recommendations are to remove white blood cells by the CSF centrifugation immediately after collection, use liquid nitrogen for the snap-freeze of the supernatant for storage at −80°C, and avoid freeze/thaw cycles. Samples should not be left in the autosampler for more than 24 h [33].

The chemical composition of low-molecular-weight CSF metabolites justifies its study using GC–MS in case of compliance with the necessary requirements for sampling, storage, and selection of appropriate and sensitive methods of sample preparation, which will provide quantitative determination of the target components at the required level, taking their potential biological variability among individuals into account.

3. Metabolic Profiling of the Cerebrospinal Fluid Using Advanced GC–MS Technologies

Different groups of authors have made attempts for the metabolic profiling of the CSF using more sensitive types of MS detectors than those described in Section 2 (Table 1). The most abundant types of mass analyzers are scanning single and triple-quadrupoles (MS/MS) and time-of-flight (TOF). TOF analyzers are more suitable for metabolic profiling because of their high speed, resolving power, sensitivity, and high quality of identification achieved by retention time, combination of accurate mass, and isotopic distribution. At the same time, the sample preparation approach for the metabolic profiling remains nonselective and includes liquid–liquid extraction (most often using methanol for protein precipitation and as extraction solvent) and widespread two-step derivatization using oxymation (the first step) and silylation (the second step) with different types of reagents to form the volatile derivatives. The most abundant type of the capillary column for the GC is a 30 m × 0.25 mm × 0.25 μm column with a phase of 5% phenyl/95% dimethyl polysiloxane crosslinked polymer, which is characterized by low bleed of the stationary phase, resistance to active compounds, and high temperature stability. This column is suitable for the determination of a wide range of compounds and produced as HP-5MS, TR-5MS, and under other trade names. However, more specific types of columns are also applied.

Application of two-dimensional GC–TOFMS led to the identification of 91 metabolites out of over 1200 detected. Sensitivity was achieved using cryogenic modulation, which concentrated analyte fractions transferred from the first (a 10 m × 0.18
mm i.D. RxI-5ms column with film thickness of 0.18 μm) to the second column (a 1.5 m × 0.1 mm i.d. BPX-50 column with film thickness of 0.1 μm) [34]. The method was based on two types of capillary columns and two types of mass-selective detectors (DB-5 column (30 mm × 0.25 mm i.d., film thickness 1.0 μm) and GCMS-QP2010 Plus; a fused silica capillary column CP-SIL 8 CB low bleed/MS (30 m × 0.25 mm i.d., film thickness 0.25 μm) and GCMS-QP 2010 Ultra) for targeted and nontargeted analysis led to the detection of 61 metabolites, where 45 metabolites were identified with a nontargeted semiquantitative analysis. Sixteen metabolites involved in the tricarboxylic acid cycle, glycolysis, and amino acids were identified quantitatively: succinic acid, fumaric acid, malic acid, aconitic acid, isocitric acid, citric acid, alanine, valine, leucine, isoleucine, proline, serine, threonine, methionine, phenylalanine, and tyrosine [23].

Two groups of authors explored a similar multicomponent biomarker approach [25,26] using classical two-step derivatization and GC–TOFMS metabolite profiling coupled to a multiplex bioinformatics approach. The first research resulted in 40 identified compounds of 120 peaks; the second resulted in 85 structurally identified and quantified compounds of 962 metabolic signatures. The identified metabolites in the second publication were classified as sugars and sugar alcohols (24%), amino acids (28%), fatty acids (15%), organic acids (15%), and amines (2%) [26].

Careful attention should be paid to the results of the study, in which metabolic profiling was performed using not common configuration of GC with an orthogonal-accelerated TOFMS with atmospheric pressure chemical ionization interface. A distinctive feature of the chemical ionization (CI) is a softer ionization at the energy not exceeding 5 eV, compared to the electron ionization at 70 eV commonly used in GC–MS systems, which provides less fragmentation of the precursor ion. Evaluation of the analytical parameters (repeatability, reproducibility, linearity, and detection limits) using model solutions led to the successful determination of the 25 different compounds (valine, alanine, sarcosine, leucine, proline, isoleucine, benzoic acid, glycine, serine, threonine, methionine, aspartic acid, glutamic acid, phenylalanine, Phenyl-Gly, hippuric acid, caffeine, theophylline, lysine, tyrosine, 4-methylisopropamine, dopamine, uric acid, 5-hydroxyindole-3-acetic, and nortriptyline). The applicability of this technique for the CSF metabolic profiling was demonstrated and resulted in 21 identified compounds from more than 300 detected [35].

Tandem GC–MS/MS was applied for the metabolic analysis in pediatric patients and revealed 180 metabolite derivatives in the CSF samples. The main metabolites were 2-ketoglutaric acid, pyridoxamine, tyrosine, 2-propyl-5-hydroxypentanoic acid, 1,5-anhydroglucitol, 2-aminoacetonic acid, 2-ketoisocaproic acid, 4-hydroxyproline, acetylglucine, methionine, N-acetylserycine, and serine [36]. Fatty acid analysis was performed using GC–MS/MS and resulted in identification of 20 compounds (6 saturated, 6 monounsaturated, and 8 polyunsaturated fatty acids) [10].

Metabolomic research studies are also in demand—specifically, studies in animals. A highly sensitive headspace solid-phase microextraction–GC–MS technique was successfully put into practice for the detection of 25 volatile constituents in rabbit CSF after intranasal administration of Asari Radix et Rhizoma frequently used in traditional Chinese medicine [37]. Numerous amino acids and related compounds (glycine, L-alanine, L-asparagine, L-glutamic acid, L-glutamine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, and O-phosphoethanolamine) were detected using GC–MS in the rat CSF [2]. GC–MS metabolomics profiling for the macaque CSF samples produced 663 variables across the two different groups of animals with depression, which were used in the subsequent multivariate analysis. In total, 37 metabolites responsible for discriminating these two groups were identified (propanoic acid, acetic acid, hydroxyamine, propanedioic acid, butanoic acid, proline, methanamine, glycine, isothiourea, nonanoic acid, carbamic acid, threonine, β-alanine, threitol, erythronic acid, L-aspartic acid, xylitol, ribitol, 2-keto-D-gluconic acid, L-arginine, L-proline, D-fructose, myoinositol, glucaric acid, hexadecanoic
acid, scyllitol, gulose, heptadecanoic acid, linoleaidic acid, trans-9-octadecenoic acid, oleic acid, octadecanoic acid, N-acetyl-d-glucosamine, D-glycero-D-galactoheptitol, galactitol, 5-phenyllvaleric acid, benzenecacetic acid, and 1H-indole-2-carboxylic acid) [38].

Most of the described investigations required from 25–50 μL [2,23,34,36] to 100–500 μL [25,26,37] of the CSF (even 1 mL [10]). After different steps of sample preparation, especially during derivatization and subsequent dilution with organic solvent, the final volume of the mixture is usually 100–200 μL. As the volume of CSF is usually limited, especially in cases of experimental rat or mouse models of diseases, approaches requiring extremely small amounts of the CSF and sample for the GC–MS analysis are of great interest.

Three investigations appeared to be promising in solving these issues. The analysis with modified vial design and sample workup procedure became applicable to small volume of the CSF (10 μL), and 50 μL of the final mixture was used for GC–MS analysis. The modified vial design reduced the required volume of the insert from 500 to 200 μL, and the smaller amount of derivatizing agent resulted in a reduction to 50 μL of the total volume of the mixture for the analysis. This approach had similar number of metabolites as in the analysis of >100 μL of the CSF, i.e., 73 identified compounds from 93 detected peaks, and was successfully applied for the metabolic profiling of the rat CSF [39]. Application of nontargeted metabolomics using 5 μL of the CSF for GC–TOFMS seemed to be interesting for investigation, as it resulted in 97 metabolites (including phenylalanine, leucine, threonine, valine, tryptophan, serine, glycero, 1,5-anhydroglucitol, methionine, β-mannosylglycerate, asparagine, tyrosine, lysine, glutamine, isoleucine, proline, 2-hydroxyglutarate, tryptophan, glycine, proline, isoleucine, and alanine) being identified. Unfortunately, there was no complete information about sample preparation, particularly on derivatization and total sample volume for the analysis [41]. An analytical method based on in-liner silylation in the programmed temperature vaporizer (PTV) injector at 70 °C coupled to GC–TOFMS used only 0.01–2 μL of the CSF and was subsequently applied for metabolic profiling of the human and mouse CSF. A total of 342 peaks were found in both human and mouse profiles and 52 metabolites were identified in the human CSF (amino acids, organic acids, fatty acids, sugars, and others) [40]. The described methods for 0.01–2 and 10 μL of CSF demonstrated promising results and could be recommended for the metabolomics studies, although the method for 0.01–2 μL of CSF requires more expensive equipment (PTV injector and TOFMS) compared to those for 10 μL (GC–MS).

Metabolic profiling resulted in most cases in a number of compounds or groups of compounds that successfully distinguished the compared groups of patients or patients and healthy donors. These compounds are candidate biomarkers for a various type of diseases, including inflammatory demyelinating, neurodegenerative, oncological, infectious, mental, genetic, vascular, and epilepsies (Table 2).

| Disease Classification | Diagnosis | Candidate Biomarkers | Reference |
|------------------------|-----------|----------------------|-----------|
| Inflammatory demyelinating | Multiple sclerosis | 5 amino acids, O-phosphoethanolamin | [2] |
| | | sorbitol, fructose | [3] |
| | | homocysteine | [4] |
| | | N-acetylaspartic acid | [5] |
| | | 2 metabolites of arachidonic acid | [6] |
| | | quinolinic acid, picolinic acid | [7] |
| | | 3 neuroactive steroids | [42] |
| | | 4 endocannabinoids | [43] |
| | | 2 monoglycerides, salicylaldehyde, 4 organic acids, inosine, threon, butane-2,3-diol, hypoxanthine, glutamine | [26] |
| Neurodegenerative | Amyotrophic lateral sclerosis | amino acids, organic acids | [25] |
| | Alzheimer's disease | 2 steroids | [9] |
| | | fatty acids | [10] |
| Disease                                      | Metabolites                                                                 |
|----------------------------------------------|-----------------------------------------------------------------------------|
| Multiple system atrophy                      | 8,12-iso-iPF2α-VI, total isoprostane iPF2α-VI, polyunsaturated fatty acids  |
|                                              | F2-IsoPs                                                                   |
| Glioma                                       | 7 polyamines                                                               |
| Glioma                                       | eicosapentaenoic acid                                                      |
| Leukemia                                     | citric and iso-citric acid                                                 |
| Meningitis                                   | 5-amino acids                                                              |
|                                              | prostaglandins, thromboxane B₂                                             |
| Meningitis                                   | muramic acid                                                               |
| Malaria                                      | quinolinic acid, picolinic acid                                            |
| HIV-associated impaired prospective memory   | quinolinic acid                                                            |
| Subacute sclerosing panencephalitis          | sorbitol                                                                   |
| Mental                                       | fatty acids, amino acids                                                   |
| Major depressive disorder                    | nervonic acid                                                              |
| Post-traumatic stress disorder               | allopregnanolone, pregnanolone                                             |
| Diagnosis of suicidal behavior               | allopregnanolone, pregnanolone                                             |
| Pyruvate carboxylase deficiency              | 5-hydroxyindolacetic acid                                                  |
|                                               | homovanillic acid                                                          |
|                                               | 5-hydroxyindolacetic acid, homovanillic acid                               |
| Genetic                                      | free-gamma-aminobutyric acid, glutamine, C5 ketone bodies                  |
| Combined sepiapterin reductase and           | 2 polyunsaturated fatty acids                                              |
| methyrimony-CoA epimerase deficiency         |                                                                           |
| Guanidinoacetate methyltransferase (GAMT)    | guanidinoacetate                                                           |
| (GAMT) and creatine transporter deficiency   |                                                                           |
| Vascular                                     | anhydroglucitol                                                           |
| Aneurysmal subarachnoid hemorrhage           | free amino acids                                                           |
| Epilepsy                                     | 2-ketoglutaric acid, pyridoxamine, tyrosine, L,5-anhydro-                    |
|                                              | glucitol                                                                   |

4. Quantitative Analysis of Different Groups of the Cerebrospinal Fluid Metabolites Using GC–MS

As mentioned in the Introduction section, after metabolic profiling and various statistical and bioinformatics approaches, it is necessary to develop a selective method for the detection of a specific compound or group of related compounds. At this stage, modifications of sample preparation techniques are required to reduce the matrix effect and coextraction of nontargeted compounds.

Validation of the developed analytical methods for the quantitative evaluation of the potential biomarkers in a biological matrix is critical for the successful conduct of nonclinical and clinical studies, and it ensures that the obtained data are reliable. Validation includes the evaluation of a number of parameters, such as linearity, lower limit of detection and quantitation (LLLOD and LLOQ), selectivity and specificity, sensitivity, accuracy, precision, recovery, and stability of the analyte in the matrix. However, full validation requires a large amount of the biological matrix. In the case of rare matrices, the validation can be performed on a pooled sample from several persons or a model solution that has a chemical composition similar to that of the matrix. We did not manage to find fully validated methods for the determination of the analytes in the CSF, which were performed according to the FDA or EMA guidelines. Several methods, such as recovery, LOQ and LOD values, and linearity will be discussed in this section (Table 3). However, most of the published methods do not provide enough validation data, and they will be mentioned in passing.
Table 3. The analytical methods for the determination of the low-molecular-weight compounds in the CSF using GC–MS methods.

| Compounds | GC–MS Method, Capillary Column | CSF Sampling | Sample Volume | Sample Preparation | Method Validation | Concentration | Reference |
|-----------|--------------------------------|--------------|---------------|-------------------|------------------|---------------|-----------|
| Glycine, sarcosine, l-forms: alanine, valine, leucine, isoleucine, serine, threonine, methionine, aspartic acid, proline, cysteine, glutamic acid, phenylalanine, asparagine, lysine. | GC–MS: 30 m × 0.25 mm × 0.25 μm Rtx-5MS. | Artificial CSF, patients (n = 16). | 200 μL | 200 μL CSF + 800 μL MeOH at −10 °C + vortexing + centrifugation + 200 μL supernatant + evaporation to dryness at room temperature under N2 + 15 μL methoxamine in pyridine (20 mg/mL) + 35 μL BSTFA+TMCS (99:1 v/v) + vortexing + derivatization under microwave irradiation, 210 W, 3 min. | Recovery: 88–129%. LOD: 0.01–4.24 μM. LOQ: 0.02–7.07 μM. Intraday (RSD): 4.1–15.6%. Interday (RSD): 6.4–18.7%. Lin. 0.1–133.0 μM (R² = 0.99 for amino acids except cysteine). | Median (n = 16), μM: 6.9/4.9/19.9/3.6/7.4/2.7/9.7/6.4/10.6/2/375.0/1046.7/13.0/4.8/11.0/4. | [64] |
| 5-hydroxyindole ethanol (5-HTOL), 5-hydroxyindole acetic acid (5-HIAA), 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT). | GC–MS: 30 m × 0.25 mm × 0.25 μm DB-5. | Children with acute lymphoblastic leukemia without chemotherapy (n = 36), control group (n = 24) | 3 mL | 3 mL CSF + SPE + washing + elution 1 mL MeOH + 0.5% formic acid + drying under N2 + 70 μL BSTFA+1% TMCS + 30 μL pyridine + 2.5 μL MeOH + incubating for 1 h at 95 °C. | Matrix effect: 92.3–106.2% (no significant ME). Lin. 0.5–2000.0 μg/L (5-HTOL, 5-HIAA, R² ≥ 0.9924) and 2.0–800.0 μg/L (5-HTP, 5-HT, R² ≥ 0.9918). LOD: 0.1–0.4 μg/L. LOQ: 0.5 (5-HTOL, 5-HIAA) and 2.0 (5-HTP, 5-HT) μg/L. Intraday recovery: 94.6–105.6% (RSD): 6.4–18.7%. Interday recovery: 93.0–106.9% (CV 1.4–4.5%). | Children with acute lymphoblastic leukemia without chemotherapy [46] |
| Indole-3-carboxylic (3ICA), indole-3-acetic (3IAA), indole-3-propionic (3IPA), indole-3-lactic (3ILA), 5-hydroxyindole-3-acetic (5-HIAA) acids. | GC–MS: 30 m × 0.25 mm × 0.25 μm TR-5ms. | CSF (n = 3) samples of different patients with CNS diseases. | 40 μL | 40 μL CSF + 40 μL distilled water + MEPS + elution with diethyl ether + drying + 40 μL BSTFA/MTBSTFA + incubation 30 min at 90 °C + cooling 30 min at 4 °C + 350 μL of hexane. | Recovery: 40–80% (for pooled CSF). LOD: 0.2–0.4 μM. LOQ: 0.4–0.5 μM. Precision (RSD): <20%. Accuracy (the relative error, RE): <±20% (at the LOQ concentrations). Lin.: 0.4–7 μM (R² ≥ 0.9949). | 3IAA, μM: 0.42 ± 0.08; 0.6 ± 0.1; 0.43 ± 0.03 | [65] |
| Quinolinic, picolinic, nicotinic acids. | GC–ECNI-MS: 30 m × 0.25 mm, HP–5MS (i) 0.25 μm or (ii) 1.0 μm stationary-phase film thickness. | Human CSF samples, artificial CSF | 20–50 μL | CSF + evaporation to dryness + 100 μL trifluoroacetic anhydride + 100 μL hexafluoroisopropanol + heating at 60 °C for 30 min + dissolving in 1 mL toluene + washing with 1 mL 5% NaHCO3 + 1 mL water + −500 mg anhydrous Na2SO4. | On-column LOQ: < 1 fmol (S/N 10:1). Lin.: 0–5 pmol on column. Slope: for nicotinic acid 5.8; for picolinic acid 25.8 (R² > 0.996). Precision (RSD): 0.5–4.3%. Accuracy: 94.0–105.5%. Interday precision: 1.0–8.9%. Interday accuracy: 96.7–104.0%. | Nicotinic acid: 2.0 μM (prehydrolysis and 56.2 after hydrolysis). | [66] |
| Benzoic (BA), phenylpropanoic (PhPA), phenylactic (PhLA), 4-hydroxybenzoic (p-HBA), 4-hydroxyphenylacetic (p-HPhAA), 4- | GC–MS: 30 m × 0.25 mm × 0.25 μm TR-5ms. | CSF samples (n = 138) from neurosurgical patients (n = 84), | 40 μL (MEPS) 200 μL BSTFA, 30 min at 90 °C + cooling 30 min at 4 °C + 350 μL of hexane. LLE: 200 μL CSF + 800 μL distilled water + 0.3–0.5 g solid NaCl + 15 μL | Recovery: 40–90%. LOD: 0.1–0.3 μM. LOQ: 0.4–0.7 μM. Precision (the reproducibility, RSD): <20%. Accuracy (the relative error, RE): <±20%. Lin.: over 0.4–10 μM (R² ≥ 0.99). | Median (BA/PhPA/PhLA/p-HBA/p-HPhAA/HVA/p-HPhLA), μM: 0.7/LOQ/0.1/nd/LOQ/0.3/0.72/5. | [67] |
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|--------------------------|--------------------------|

| **hydroxyphenylpropionic (p-HPhPA), homovanillic (HVA), 4-hydroxyphenyllactic (p-HPhLA).** | Stable isotope dilution GC–MS: SGE BPX-70. | 100 µL | 100 µL CSF + 50 µL NaHCO₃ + 50 µL hexafluoroacetylelaceton + 500 µL toluene + heating 2 h to 80 °C + 300 µL toluene phase + drying under N₂ + 10 µl triethylamine + 100 µL 7% PFBBr in MeCN (v/v), 15 min + 200 µL 0.5N HCl + 1 mL hexane + extraction. | Linearity: 0.5–10 nmol and 0.05–0.5 nmol LOD (S/N = 5): 0.01 and 0.0012 µg. LOQ (S/N = 10): 0.02 and 0.0024 µg. Intra-assay (n = 10): 0.25 ± 0.02 (CV 6.0%) and 57 ± 3 (CV 6.0%) µg. Interassay (n = 5): 0.25 ± 0.01(CV 4.0%) and 62 ± 3.7(CV 6.0%) µM. Control (n = 25): 0.036—0.22 µM and 24–66 µM GAMT deficient: 14–15 µM and not detected SLC6A8 deficient: 56–62 µM. |

| **Guanidinoacetate, creatine** | GAMT-deficient patients (n = 8) and SLC6A8-deficient patients (n = 8) | 100 µL | | |

| **Gamma-hydroxybutyric acid (GHB)** | GC–MS: 30 m × 0.25 mm × 0.25 µm, VF-5 ms. From autopsy cases (n = 21) | 50 µL | 50 µL CSF + 15 + 200 µL 0.1M HCl + 1 mL ethyl acetate + centrifugation + evaporation to dryness at 20 °C, 2 mbar in a vacuum centrifuge + 50 µL MeCN + 25 µL BTFSA+1% TMCS + mixing. | Inter- and intraday accuracy: ≥91%. Imprecision: ≥9%. LOD: 0.5 mg/L. LOQ: 0.6 mg/L. Cal. curve: 1.0 mg/L, 10 mg/L, 40 mg/L, 80 mg/L and 100 mg/L. Range concentrations after immediate analysis/after storage for 14 days at 4 °C/20 °C, mg/L: 1.1–10.4/0.6–13.2/≤0.5–21.6. |

| **Gamma-aminobutyric acid (GABA)** | Isotope-dilution GC–ECNI–MS: 25 m × 0.32 mm, CPSII 88. CSF samples of a patient before and during Vigabatrin treatment, control samples. | 50, 500 µL | Free GABA: 500 µL CSF + 800 µL 1M phosphate buffer, pH 11.5 + 50 µL methylchloroformate + 150 µL 6 M HCl + 4 mL ethyl acetate + drying under N₂ at 40 °C + 100% 7% PFBBR in MeCN + 10 µl triethylamine + 150 µL 0.5 M HCl + 1 mL hexane + drying under N₂ at 40 °C + 50 µL hexane. Total GABA: 50 µL CSF + 450 µL water + 250 µL 20% sulphosalicylic acid + hydrolysis 24 h at 110 °C. LOD: <0.005 µM. Free GABA. Intra-assay: 0.188 ± 0.004 µM (1.9% SD). Interassay: 0.177 ± 0.013 µM (7.3% SD). Total GABA. Intra-assay: 3.00 ± 0.05 µM (1.8% SD). Interassay: 3.57 ± 0.33 µM (9.2% SD). Free/total GABA, µM. Control: 0.029–0.127/4.72–11.8. Before therapy: 0.153/13.2. During therapy: 0.274/24.1. |

| **Pipelic acid** | Isotope-dilution GC–ECNI–MS: 30 m × 0.25 mm, DB-19. Pediatric CSF samples | 500 µL | CSF/aqueous standard solution + 1.0 mL 1 M potassium phosphate–sodium carbonate buffer, pH 11.5 + 50 µL methylchloroformate + 0.15 mL 6 M HCl, pH 2 + 4 mL ethyl acetate + drying under N₂ + 5 µL PFBBR + 10 µl triethylamine in 50 µL MeCN + 1 mL hexane + washing with 0.5 mL 100 mM HCl + 50 µL hexane. | Lin. 0.05–5 nmol (R > 0.999). LOD: 1.6E-6 nmol of PA (~0.5 nM). Recovery (CV): 97.3%–101.2% (4.0%–7.2%) Patients: 0.93–4.53 µM. Control: 0.010–0.120 µM. |

| **Androsterone, dihydrotestosterone, testosterone, allopregnanolone, isopregnanolone, pregnenolone.** | GC–ECNI–MS: 15 m × 0.25 mm × 0.05 µm, HP 5890. Normal volunteers, cisternal monkey. | 1–2 mL | 1–2 mL CSF + 100 mg C18 SPE + 50 µL 0.2% carboxymethylxylamine hemihydroloride in pyridine + incubation 45 min at 60 °C + drying in N₂+ 100 µL 1.25% pentafluorobenzyl bromide + 2.5% di-isopropylethylamine in MeCN + drying in N₂+ 100 µL 50% BTFSA in MeCN + drying + 5 µL hexane. Recovery: 78.2–99.5%. Reproducibility (RSD): 4.6–35.0%. Lin.: 10–1000 pg/mL (R > 0.996). Two-month variation <10%. Human/monkey CSF, pg/ml.: androsterone—52.8/24.7; testosterone—158.3/73.7; allopregnanolone—44.1/6.3; pregnenolone—52.8/16.7. |

| **Pregnenolone, dehydroepiandrosterone.** | GC–MS: 15 m × 0.25 | 1 mL | 1 mL CSF + 3 mL of diethyl ether + drying at 37 Lin.: 10–1000 pg Slope: 0.96–1.33. R: >0.995. CV: 1.0–5.1%. LOD: 0.04–11.3 PM. Recovery: 75–104%. Median, nM: 0.060/0.078/0.235/0.208/0.231/ [72] |
| Compound                  | Method                  | Description                                                                                      | LOQ            | Accuracy          |
|---------------------------|-------------------------|--------------------------------------------------------------------------------------------------|-----------------|-------------------|
| Progesterone, androstenedione, testosterone, allopregnanolone, isopregnanolone, androsterone, epiandrosterone, 7α-hydroxy-dehydroepiandrosterone, 7β-Hydroxy-dehydroepiandrosterone, 5-androstene-3β,7α,17β-triol, 5-androstene-3β,7β,17β-triol, 16α-hydroxy-pregnenolone, 16α-hydroxy-dehydroepiandrosterone, 16α-hydroxy-progesterone. | GC-NICI-MS: 30 m × 0.25 mm × 0.25 μm, HP-5MS. | 250 μL CSF + acidification + C18 SPE + evaporation + 200 μL PFBB (3.5% v/v in MeCN) + 50 μL di-isopropylethylamine + extraction (water and toluene). | LOQ: 0.1 ng/sample. Accuracy: 98–122%. Recovery: 85–87%. Intraday (RSD, n = 3): 3–34%. Lin.: 0.1–5 ng/sample. | 0.2–5.0 ng/mL (median, 1.4 ng/mL) |
| Indomethacin              | GC-MS                   | Extraction from human CSF diluted 1:1 with blank human plasma by protein precipitation/derivatization/LLE. | LLOQ: 0.4 μg/mL. Linearity 0.4–80 μg/mL. Precision for QC samples: 1.7–2.3%. Accuracy at all concentrations: 0.5% to +3.0%. | Prior to administration of ELND005 1.4–1.5 μg/mL. |
| Scylo-Inositol (Elnd005)  | GC-MS                   | Healthy adults (n = 8). No data.                                                                 | 6.0 + SPE + 100 μL toluene + analysis of codeine + toluene evaporation + 50 μL BSTFA + 1% TMCS 10 min at 70 °C. | Linearly: to 1.7 mg/L. LOQ: 0.002 mg/L for morphine and 0.001 mg/L for codeine and 6-monooacetyl morphine. Precision: 0.115–0.121 mg/L (CV 4.4–7.2%). | 0.001–0.406/0.01–0.38/<0.01–0.04 mg/L |
| 6-monooacetyl morphine, morphine, codeine | GC-MS: 30 m × 0.25 mm × 0.25 μm, HP-5. | 3 mL CSF + 18 mL 0.1M phosphate buffer, pH 6.0 + SPE + 100 μL toluene + analysis of codeine + toluene evaporation + 50 μL BSTFA + 1% TMCS 10 min at 70 °C. | Linearity: to 1.7 mg/L. LOQ: 0.002 mg/L for morphine and 0.001 mg/L for codeine and 6-monooacetyl morphine. Precision: 0.115–0.121 mg/L (CV 4.4–7.2%). | 0.001–0.406/0.01–0.38/<0.01–0.04 mg/L |
| Diethylene glycol, ethylene glycol, glycolic, oxalic, diglycolic, hydroxymethoxy acetic acids. | GC-NICI-MS: 30 m × 0.25 mm ID × 0.50 μm, ZB-5 ms. | 250 μL CSF + 1.0 mL water + 100 μL 5 N NaOH + 500 μL toluene + 50 μL PFBCI | LOQ: 0.05–1.0 μg/mL. Lin.: for diethylene glycol and ethylene glycol 0.02–2 (R² 0.9984–0.9989) μg/mL; for other 0.05–51 (0.9990), 0.5–25 (0.9907), 0.5–100 (0.9985), 1–100 (0.9922) μg/mL. Accuracy: ≤15%. | No data |
4.1. Amino Acids

The presence and levels of free amino acids in the CSF can be indicators of neurological diseases [4,47,64,77]. Silylation is commonly used for the amino acids and an alternative derivatization using a microwave-assisted derivatization was described [64]. A 200 μL aliquot of the artificial CSF (contains 127 μM NaCl, 2 μM KCl, 1.2 μM KH₂PO₄, 26 μM NaHCO₃, 2 μM MgSO₄, 2 μM CaCl₂, 10 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 mM glucose and bubbled with a carbogenic mixture (95% v/v O₂ and 5% v/v CO₂)) was used for the validation of this method for 16 amino acids (glycine, sarcosine, L-forms: alanine, valine, leucine, isoleucine, serine, threonine, methionine, aspartic acid, proline, cysteine, glutamic acid, phenylalanine, aspartagine, and lysine) according to the Eurachem guidelines. Arginine and histidine were not analyzed because of the thermal instability of their derivatives. The evaluated analytical parameters, such as LOD 0.01–4.24 μM, LOQ 0.02–7.07 μM, intraday and interday precision values, recoveries, and linearity allowed the authors to determine all 16 amino acids in the human CSF samples (n = 16) at the level higher than LOD values, which indicated that the developed analytical method is applicable to solving the task of the quantitative determination of free amino acids in the CSF.

4.2. Tryptophan Metabolites

Tryptophan is one of the most important amino acid for CNS function. Its metabolism occurs in two main ways: the indole and kynurenine pathways. The indole metabolism is divided into the serotonin (5-hydroxytryptamine, 5-HT) via 5-hydroxytryptophan (5-HTP) and the microbial pathways. 5-Hydroxyindole-3-acetic acid (5-HIAA) or 5-hydroxyindole-3-ethanol (5-HTOL) appears as a result of the serotonin metabolism. The microbial pathway leads to the formation of metabolites containing an indole ring, for example, tryptamine and several indole-containing acids (indole-3-acetic (3IAA), indole-3-propanoic (3IPA), indole-3-carboxylic (3ICA), and indole-3-lactic (3ILA) acids).

5-HT is a neurotransmitter, and its related indole derivatives from serotonin pathway are involved in physiological and pathological responses, which are associated with many neurological diseases [46,58–60]. A method for detecting 5-HTOL, 5-HIAA, 5-HTP and 5-HT using solid-phase extraction (SPE) with Cleanert PEP-2 column was developed. This method required 3 mL of CSF, which seemed to be too much in the case of children, who were the participants of the study. However, many important analytical parameters (without reference to the FDA or EMA guidelines) were evaluated (matrix effect, linearity, LOD 0.1–0.4 μg/L and LOQ 0.5–2.0 μg/L, intraday and interday precision values, recovery, and coefficient of variation); these allowed the authors to obtain the statistically significant data about the changes in the concentration of the target compounds between children with acute lymphoblastic leukemia and the control group [46].

In the described method with classical SPE, a modern microextraction by packed sorbent (MEPS) with C18 was applied for the determination of the indole-containing acids (5-HIAA, 3IAA, 3IPA, 3ICA, and 3ILA) using only 40 μL of the CSF. The pooled CSF samples were used for the validation and the following parameters were evaluated (according to the FDA guidelines): linearity, recovery, LOD 0.2–0.4 μM and LOQ 0.4–0.5 μM, accuracy, precision, selectivity, and carryover effects. Despite the satisfactory results, only 3 IAA was detected in CSF samples of the patients with the CNS diseases [65].

Several studies describe an importance of the changes in the concentration of the kynurenine pathway metabolites, particularly pyridine-containing quinolinic, picolinic, and nicotinic acids, which are involved in the inflammatory and apoptotic processes associated with the CNS neuronal cell damage and death [50–53,66]. These metabolites were detected in the CSF after derivatization with trifluoroacetic anhydride and hexafluoroisopropanol, and electron-capture negative-ion chemical ionization (ECNI) GC–MS. One of these studies describes an almost fully validated method (LOQ less than 1 fmol for each of the analytes, linearity, precision, and accuracy) using for the concurrent
quantification of quinolinic, picolinic, and nicotinic acids in 20–50 μL of model solutions and an artificial CSF [66].

4.3. Organic Acids

Phenyl-containing acids (benzoic, 3-phenylpropionic, 3-phenyllactic, 4-hydroxybenzoic, 2-(4-hydroxyphenyl)acetic, homovanillic, and 3-(4-hydroxyphenyl)lactic acids), which are mostly microbial metabolites of the tyrosine and phenylalanine, were detected in the CSF samples (n = 138) from neurosurgical patients (n = 84) with different CNS pathology using MEPS and traditional liquid–liquid extraction (LLE). The validation (linearity, recovery, LOD 0.1–0.3 μM and LOQ 0.4–0.7 μM, accuracy, precision, selectivity, and carryover effects) according to FDA guidelines was performed for both MEPS and LLE, demonstrating the equal possibilities of these sample preparation techniques. Similar results were achieved using 40 μL of the CSF sample for MEPS instead of 200 μL for LLE [67].

The detection of creatine, an N-containing acid, and its precursor guanidinoacetate is crucial in cases of creatine deficiency syndromes, a group of inherited metabolic disorders that are caused by abnormalities in creatine biosynthesis and/or transport [78]. A sample preparation technique included LLE from 100 μL of the CSF and derivatization with subsequent stable isotope dilution (SID) GC–MS. This method provides LOD and LOQ 0.0012 and 0.0024 μM for creatine, and 0.01 and 0.02 μM for guanidinoacetate, respectively. Linearity, interassay, and intra-assay variability were also evaluated. The reference values for creatine and guanidinoacetate were revealed and ranged from 17 to 78 μM and 0.02 to 0.56 for μM, respectively [63].

Gamma-hydroxybutyric acid (GHB) is a naturally occurring neurotransmitter and a precursor to gamma-aminobutyric acid (GABA), glutamate, and glycine in certain brain areas. The postmortem examination of the influence of temperature and time storage to in vivo production of GHB was evaluated using traditional LLE and silylation. The validation was performed using 50 μL of the CSF and resulted in LOD 0.5 mg/L, LOQ 0.6 mg/L, and interday and intraday accuracy ≥91%. GHB concentration changes were affected both during postmortem interval in the dead body and during in vitro storage [68].

GABA is the chief inhibitory neurotransmitter and plays an important role in various neurological and mental disorders, in which both elevated and decreased concentrations in CSF may occur. A sensitive, selective, and accurate SID GC-ECNI-MS method for the determination of free and total GABA was developed using 500 μL of the CSF, derivatization in aqueous solution with methylchloroformate, extraction with ethyl acetate, and derivatization of the dried residue with pentafluorobenzylbromide in acetonitrile and triethylamine (for free GABA). Total GABA determination included hydrolysis with sulphosalicylic acid during 24 h. The following analytical parameters were evaluated: LOD <0.005 μM and interassay and intra-assay variability for both free and total GABA. The applicability of the method was successfully demonstrated for the determination of free and total GABA in patients suffering from succinic semialdehyde dehydrogenase deficiency before and during specific treatment [69]. This method was used for the evaluation of free GABA in the CSF samples in a patient with pyruvate carboxylase deficiency [61] and for the determination of the picrolic acid [70], a carboxylic acid of piperidine and one of the biomarkers of the pyridine dependent epilepsy [79].

Different polyunsaturated fatty acids are the components of neuronal and glial membrane phospholipids and participate in the development of Parkinson’s [45] and Alzheimer’s diseases [13]. Although the determination of these compounds includes traditional LLE and silylation, there are no validated methods for the CSF [13,45,62]. Furthermore, there are no validated methods for the determination of the nervonic acid, a candidate biomarker for depressive and manic symptoms [55], and N-acetylaspartic acid, a neuron-specific marker that is identified in multiple sclerosis [5].
4.4. Neuroactive Steroids

Neuroactive steroids are steroids synthesized de novo in the CNS and play a central role in neuronal processes [80]. Allopregnanolone and related neurosteroids (androsterone, dihydrotestosterone, testosterone, isopregnanolone, and pregnanolone) were detected as carboxymethoxime, pentafluorobenzyl, and trimethylsilyl derivatives using GC–ECNI-MS. The sample preparation included SPE with C18 sorbent from 1–2 mL of the CSF. Linearity, LOD 0.2–1.2 μg/L, recovery, and reproducibility were evaluated. This method was successfully applied for the analysis of the human and monkey CSF [56]. Another study was devoted to the evaluation of the correlations between peripheral and CSF steroids using a wide spectrum of bioactive steroids, their precursors and metabolites. Unconjugated steroids (pregnenolone, dehydroepiandrosterone, progesterone, androstenedione, testosterone, allopregnanolone, isopregnanolone, androsterone, epiandrosterone, 7α-hydroxy-dehydroepiandrosterone, 7β-Hydroxy-dehydroepiandrosterone, 5-androstene-3β, 7α, 17β-triol, 5-androstene-3β, 7β, 17β-triol, 16α-hydroxy-pregnenolone, 16α-hydroxy-dehydroepiandrosterone, and 16α-hydroxy-progesterone) were extracted from 1 mL of the CSF and derivatized in a common two-step procedure. LOD from 0.04 (for 5-androstene-3β, 7β, 17β-triol) to 11.3 pM (for androstenediene) were measured together with other analytical parameters. Significant correlations between some steroids in serum and CSF were revealed, particularly between the 7α/β-hydroxy-metabolites of dehydroepiandrosterone and androstenediol [72]. Another study applied similar sample preparation for the detection of free dehydroepiandrosterone and its 7-hydroxylated derivatives: 7α-hydroxy-dehydroepiandrosterone, 7β-hydroxy-dehydroepiandrosterone, 5-androstene-3β, 7α, 17β-triol, and 5-androstene-3β, 7β, 17β-triol [81]. Different neuroactive steroids were evaluated using GC–MS preceded by HPLC purification in Alzheimer’s disease [9], relapsing–remitting multiple sclerosis [42], and post-traumatic stress disorder [56].

4.5. Arachidonic Acid Metabolites

F2-isoprostanes (F2-IsopPs) and F4-neuroprotanes (F4-NPs) are compounds formed in vivo from the nonenzymatic free-radical-catalyzed peroxidation of essential fatty acids, primarily arachidonic and docosahexaenoic acids, respectively. Since CNS is characterized by a high level of polyunsaturated fatty acids and significant oxygen demand, considering its weak antioxidant defenses, it is also rather liable to oxidative damage caused by reactive oxygen or nitrogen species. Imbalance between free radicals and antioxidants, so-called “oxidative stress”, plays a crucial role in neurodegenerative disorders. F2-IsopPs and F4-NPs, being products of lipid peroxidation, can be biomarkers of oxidative stress and neurodegenerative diseases. The literature indicates that levels of F2-IsopPs and F4-NPs in CSF and brain tissue are elevated in case of such disorders as Alzheimer’s disease [11,14,82–84] and equine neuroaxonal dystrophy [85]. For sensitive quantification of these compounds, which are present in the CSF samples in low concentrations, GC–MS methods are applied [86], and their validation is required not only for urine and serum [87], but for CSF as well.

Prostanoids, which include prostaglandins and thromboxanes, are the metabolites of the enzymatic pathways of arachidonic acid. These compounds have similar chemical structures but different biological and therapeutic effects. Simultaneous assay of these compounds in the CSF was developed, which included extraction with octadecyl silica gel and two-step purification with silicic acid gel chromatography [48]. Some representatives of this class of compounds were elevated in patients with multiple sclerosis [6].

The validated analytical procedures for different metabolites of arachidonic acid are required because of the high demand in their evaluation in different neurodegenerative disorders.
4.6. Glucose Metabolites

Abnormalities in carbohydrate metabolism are of interest in mood disorders studies, as a possible relationship between diabetes and major depression has been shown. Glucose, which serves as an energy source for cells, is converted to fructose via the polyol pathway with sorbitol being an intermediate compound formed during this two-step process. The CSF sorbitol levels were investigated in patients with bipolar and unipolar mood disorder, and sorbitol concentrations were higher in the CSF of depressed subjects compared to normal controls [54]. Sorbitol levels along with fructose levels, both being glucose metabolites, were found to be elevated in the CSF of multiple sclerosis patients as well, while concentrations of myoinositol that is not produced via the polyol pathway did not differ significantly from its concentrations in the CSF of control subjects [3]. No validation data were demonstrated; thus, the development of the validated analytical method for the determination of the glucose metabolites is required.

4.7. Drugs and Toxic Metabolites

Indomethacin is a nonsteroidal anti-inflammatory drug, mainly known for its ability to inhibit cyclooxygenase, which is responsible for the prostaglandins production catalysis. Indomethacin is often prescribed to treat inflammation and pain caused by rheumatic and orthopedic diseases or surgery. To determine its concentrations in 250 μL of CSF, a SPE sample preparation technique was applied followed by derivatization with pentafluorobenzylbromide and GC–NICI–MS. The method provides moderate analytical characteristics (recoveries, accuracy, intraday precision) with LOQ 0.1 ng/sample. The CSF indomethacin levels in healthy children were found to be 0.2 and 5.0 ng/mL after administering it intravenously [73].

GC–MS was also used for determination of another drug: ELND005 (scyllo-inositol), an endogenous inositol stereoisomer. This drug could be used for Alzheimer's disease treatment, and its pharmacokinetic behavior in the CSF after oral administration was of interest. A traditional combination of protein precipitation, LLE, and derivatization was used for sample treatment, and validation resulted in LLOQ 0.4 μg/mL (linearity, precision, and accuracy were also evaluated) [74].

Analysis of the CSF could also be beneficial for toxicology studies. For instance, morphine can be found in human biological samples and tissues due to the ingestion of heroin or codeine, since these compounds are both metabolized to morphine, or because of exposure to morphine itself. To distinguish whether it was heroin or morphine administering, determination of 6-monoacetylmorphine is often used. However, it converses to morphine rather rapidly, and its concentrations in blood may be lower than the LOD of the method used for 6-monoacetylmorphine determination. Several studies suggested that 6-monoacetylmorphine persists in the CSF and some other human biological samples when compared to blood. 6-Monoacetylmorphine, free morphine, and free codeine levels were investigated in the CSF samples in 25 heroin deaths. The sample pretreatment procedure included such steps as SPE and derivatization and was combined with GC–MS analysis. The method's LOQ for 6-monoacetylmorphine was 0.001 mg/L; linearity and precision were evaluated. 6-Monoacetylmorphine levels were 6.6 times higher on average in the CSF samples than in blood [75].

Analysis of the diethylene glycol and its potential metabolites (ethylene glycol, glycolic acid, oxalic acid, diglycolic acid, and hydroxyethoxy acetic acid) is required because of the human poisoning during misformulation into pharmaceutical products. Sample preparation for acid metabolites from 100 μL of the CSF included traditional LLE and silylation. Sample preparation for diethylene and ethylene glycol from 250 μL of the CSF included extraction and derivatization with pentafluorobenzoyl chloride with the following analysis by GC–NICI–MS. The LOQ values were 0.05–1.0 μg/mL; accuracy and linearity were evaluated [76].
Different low-molecular-weight compounds are required to be detected in the CSF for the diagnosis of the CNS diseases. The literature data analysis revealed the absence of fully validated methods, and it presents opportunities for scientists to develop and validate analytical protocols using modern sample preparation techniques.

5. Miniaturization in Sample Preparation Techniques for the GC–MS Analysis

A small sample volume is one of the main criteria for the analysis of CSF. There are interesting approaches to sample preparation of biological fluids for GC–MS analysis, which use the principle of miniaturization.

The method of homogenous liquid–liquid microextraction (HLLME), compared to the classical LLE using significant volumes of organic solvents, is based on the extraction of polar organic compounds from aqueous matrices, including biofluids, with small volumes (microliters) of water-miscible organic solvents. For the phase separation, the salting-out effect is often used, followed by centrifugation. The volumes of biological fluids are usually up to hundreds of microliters and the volumes of polar solvents are often several times less; the weighed portion of the salting-out agent is tenths of a gram. The achievable LOD of analytes are nanograms and tenths of nanograms per milliliter [88]. The method could be combined with HPLC–MS [89], but a combination with GC–MS is also possible. To determine volatile analytes without derivatization, it is necessary to dry the extract by adding anhydrous sodium sulfate. Analytes can be derivatized both directly in the extract (addition of chloroformates) and in the dried extract (silylation in acetonitrile).

Dispersive liquid–liquid microextraction (DLLME) is based on the extraction of analytes with a microemulsion followed by the phase separation. Usually, the volume of a mixture of an extracting solvent and a dispersing solvent is hundreds of times less than the volume of the analyzed solution. Analytes are extracted quantitatively at high preconcentration factors. Extraction equilibrium is established in minutes [90]. Various methods of dispersion have been proposed and various extracting solvents have been studied for a large number of different matrices, including biological ones [91,92]. The DLLME method is applicable for the analysis of small volumes of analyzed solutions and could be combined with derivatization procedure for polar and/or nonvolatile analytes with small volumes of reagents. Different derivatizing agents are used, e.g., for analysis of human urine, ethyl chloroformate in pyridine was utilized to convert 20 amino acids into their volatile carbamate esters, which were further analyzed using GC–MS. The derivatization process was carried out simultaneously with DLLME using trichloroethylene and acetonitrile as extracting and dispersing solvent, respectively. The range of LOD was 0.4–3.7 µg/L [93].

An interesting solution is to combine the capabilities of DLLME and injector port silylation technique for the determination of polar analytes in biological matrices. Aliquots of the extract and derivatizing reagent are injected simultaneously or sequentially into a heated GC–MS injector. A gas phase reaction occurs between the silylating reagent and polar analytes at the injector temperature. This approach reduces the derivatization time (less than a minute), the possibility of derivatives decomposition, and the amount of toxic reagent and solvents used for the process is smaller [94].

QuEChERS (the name is formed from “quick, easy, cheap, effective, rugged, and safe”) is a two-step process involving liquid extraction (usually using acetonitrile) and dispersive SPE (dSPE) using (more commonly) primary secondary amines, C18, and/or graphitized carbon black sorbents to eliminate significantly interfering matrix components (for example, humic acids, lipids, etc.). QuEChERS, originally developed for the extraction of acidic and basic pesticides from food [95], is also used for analysis of blood plasma samples. A method for the simultaneous extraction of acidic, basic, neutral, and amphiphilic analytes from blood plasma using a micro version of QuEChERS (micro-QuEChERS) is proposed. It reduces the volumes of samples (200 µL of plasma compared to 1.5 mL required in the nonmicro version) and reagents by 8 times. The method allowed for the extraction of analytes from blood plasma with high (65 to 80%) recoveries and low
matrix effect. The developed approach is considered to be a fast and clean alternative to “dilute and shoot” approaches or the protein precipitation procedure, which are used for high-throughput clinical diagnostics (including analysis of the CSF samples), coupled with HPLC–MS. However, it is of interest to study a possible combination of micro-QuEChERS with GC–MS [96].

Micro-QuEChERS was used to analyze 148 avian blood samples collected in an environmental field study of the impact of rodenticides (applied for the treatment of common vole plague) on the wildlife. The volume of each sample was 250 µL. In combination with GC–MS/MS, this method detected the desired analytes at the level of 1.5 ng/mL [97]. Although micro-QuEChERS is not yet widely used for sample preparation of biological matrices [95–98], the ability to vary components for the extraction and extracts purification procedures applying dSPE, reduce the cost and time of analysis, and analyze relatively small sample volumes (even smaller than those described in the publications above) presents broad prospects for the analysis of biological fluids, including CSF [96–98].

Solid-phase microextraction (SPME) is a solvent-free sample preparation technique [99]. This method is widely used for analyzing the vapor phase of various biological fluids in order to determine volatile organic compounds and to extract analytes directly from the liquid phase [100–102]. Substances are absorbed by a polymer film or solid sorbent covering a piece of fiber (a piece of fused silica capillary). The capillary is placed inside a needle connected to a syringe-like device. During sorption and desorption, the capillary moves out of the needle. The metabolomic composition of the circulating blood of laboratory mice was investigated [100], and for selective recovery of hydrophilic and hydrophobic analytes with respect to high molecular weight matrix components, an SPME fiber coated with mixed-mode polymers (phenylboronic acid and polystyrenedivinylbenzene) was used. The fiber was placed in an injection needle, and it absorbed the metabolites directly from the bloodstream. The vapor phase over saliva samples (sample volume 500 µL) was investigated. The LOD for the 20 detected volatile metabolites ranged from 0.008 to 1 µM [101]. The vapor phase over urine samples was studied in order to identify biomarkers of cancer; 82 metabolites were found, and the sample volume was 4 mL [102]. The SPME capabilities allow the researchers to vary the volumes of the studied samples, depending on the aims of the research.

Stir bar sorptive extraction was also developed as another solvent-free sample pretreatment technique, and it is actively used for isolation of low-molecular-weight components of different polarity from biological fluids [103–105]. The device used for stir bar sorptive extraction is a glass tube with a metal rod inside (magnetic stirrer), often coated with polydimethylsiloxane. The main difference between stir bar sorptive extraction and SPME is the larger amount of stationary phase covering the surface of the mixer (up to 25 µL) compared to the capillary cut (0.5 µL), which increases the extraction efficiency. Desorption of analytes is carried out either by solvent re-extraction or thermal desorption. For polar components in biological matrices, it is possible to combine simultaneous deconjugation and extraction of analytes (in situ deconjugation) or derivatization and extraction (in situ derivatization) followed by thermal desorption into the GC injector. Derivatization could also be carried out after extraction (postextraction derivatization), both during thermal desorption of analytes and after their re-extraction. Sample volumes are typically on the order of 1 mL, with LOD being attainable from ng/mL to pg/mL.

A method of amino acids microextraction from biological fluids (including CSF), based on a combination of hollow fiber SPME and extraction with stir bar sorptive extraction—hollow fiber–stir bar sorptive extraction—was proposed [106]. Hollow polymer fibers are obtained using the coaxial electrospinning technology when an electrostatic field acts on an electrically charged jet of a polymer solution or its melt. It is also possible to obtain a hollow fiber membrane with specified properties (average pore diameter, membrane thickness), providing a semipermeable barrier (analytes pass
through the pores of the membrane, the matrix components remain in solution). For the first time, the hollow fiber membrane was used as a SPME fiber coating for the extraction of BTEX (benzene, toluene, ethylbenzene, and xylenes) from aqueous matrices, and was based on a polypropylene coated copper wire [107].

To extract amino acids, hollow polyvinylidene fluoride fiber was used. A piece of polyvinylidene fluoride hollow fiber was sealed on the one side, and a steel rod was placed inside the fiber. A dispersion system of 0.1 g of silica microspheres in ethanol was introduced into the fiber. After removal (including evaporation) of ethanol, the fiber was sealed on the other side. Before use, the resulting hollow fiber–stir bar sorptive extraction device was washed with acetone and dried. An aliquot of the biological fluid was mixed with ethanol in a 3:1 ratio in order to reduce the surface tension of the sample to facilitate the penetration of analytes into the membrane micro pores. A hollow fiber–stir bar was placed in a vial with the sample and extraction was performed while stirring under the influence of a magnetic field for a chosen time. During this time, amino acids selectively with respect to biological macromolecules penetrated from the solution of biological fluid through the pores of the membrane and were absorbed on the surface of silica microspheres due to the formation of hydrogen bonds. Next, the hollow fiber–stir bar was removed from the sample vial, dried until moisture was completely evaporated, placed in another vial, and 0.1 mL of BSTFA and 0.9 μL of a nonpolar solvent were added to extract amino acid derivatives, which should have been formed as a result of silylation. BSTFA molecules also penetrated the pores of the membrane and interacted with amino acid molecules. Derivatization was carried out in a microwave field. The resulting derivatives were extracted with a nonpolar solvent and analyzed using GC–MS. Before the next extraction, the hollow fiber–stir bar was conditioned in distilled water and acetone. The LOD of the studied amino acids ranged from $3 \times 10^{-4}$ to $6 \times 10^{-3}$ μg/mL. Recoveries from the rat CSF samples ranged from 71.8 to 101.2%. The resulting extraction device could be used 30 times without loss of analyte sensitivity [106]. Due to the miniaturization of the device, it can be used for small volumes of biological fluids. One of the advantages of this method is that there is no need in sample cleanup.

One of the perspective sample preparation techniques is a MEPS [108] that is also aimed at miniaturizing SPE. The method is based on the use of a small amount of sorbent (1–4 mg) and multiple passage of the test sample through this sorbent layer located in the extension of the syringe needle. The method requires small amounts of sample (tens, hundreds of microliters), and solvent volumes (up to tens of microliters) which elute analytes. This technique minimizes the dead volume as well. The syringe is placed in an automatic dispenser, and the speed and number of cycles of passing the sample through the sorbent could be programmed. It is also possible to connect the dispenser to an HPLC or GC system. A large number of needles with sorbents, which are common for classical SPE (C$_{18}$, C$_{8}$, silica gel), have been developed and are commercially available. A method for the determination of low-molecular-weight metabolites using a hand-operated device with hypercrosslinked polystyrene was described [109]. MEPS has found wide application in analytical chemistry, including the field of biological fluids analysis [110–113]—in particular, for the determination of low-molecular-weight microbial metabolites in the CSF (see Section 4) [65,67].

The CSF samples are characterized not only by low levels of the analytes of interest, but also by small sample volumes available for analysis. Miniaturization of classic extraction methods, such as LLE and SPE which are often used for analysis of biological fluids, coupled with GC–MS presents new perspectives in the metabolic analysis of the CSF.

6. Conclusions

GC–MS plays an important role in the development of the metabolic analysis of biological fluid samples. High separation efficiency and detection sensitivity, stable retention times, and reproducible mass spectra of analytes make it possible to analyze multicomponent mixtures of low-molecular-weight organic compounds of complex
composition and to perform nontargeted and targeted analysis. Sample preparation based on the selective extraction of analytes with respect to interfering matrix components is often required to detect a wide range of components by this method. The complexity of the CSF analysis is caused not only by the low content of the target analytes, but also by the small volume of samples available for analysis. The miniaturization of the traditional LLE and SPE methods in combination with various options for the derivatization of polar analytes presents new possibilities in the metabolic analysis of the CSF using GC–MS.

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| APCI         | atmospheric pressure chemical ionization |
| BA           | benzoic acid |
| BBB          | blood-brain barrier |
| BSTFA        | N, O-bis(trimethylsilyl)trifluoroacetamide |
| CI           | chemical ionization |
| CNS          | central nervous system |
| CSF          | cerebrospinal fluid |
| CV           | coefficient of variation |
| DLLME        | dispersive liquid–liquid microextraction |
| dSPE         | dispersive solid-phase extraction |
| ECNI         | electron-capture negative-ion chemical ionization |
| EMA          | European Medicines Agency |
| FDA          | Food and Drug Administration |
| FTMS         | Fourier transform mass spectrometry |
| F-IsoPs      | F-isoprostanes |
| F-NPs        | F-neuroprostanes |
| GABA         | gamma-aminobutyric acid |
| GAMT         | guanidinoacetate methyltransferase |
| GC           | gas chromatography |
| GHB          | gamma-hydroxybutyric acid |
| HEPES        | 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid |
| HIV          | human immunodeficiency virus |
| HLLME        | homogenous liquid–liquid microextraction |
| HPLC         | high-performance liquid chromatography |
| 5-HT         | 5-hydroxytryptamine |
| 5-HTP        | 5-hydroxytryptophan |
| HVA          | homovanillic acid |
| 5-HIAA       | 5-hydroxyindole-3-acetic acid |
| 5-HTOL       | 5-hydroxyindole-3-ethanol |
| ICP          | inductively coupled plasma |
| IS           | internal standard |
| 3IAA         | indole-3-acetic acid |
| 3ICA         | indole-3-carboxylic acid |
| 3ILA         | indole-3-lactic acid |
| 3IPA         | indole-3-propionic acid |
| Abbreviation | Description |
|--------------|-------------|
| LC           | liquid chromatography |
| LLE          | Liquid–liquid extraction |
| LOD          | limit of detection |
| LLOD         | lower limit of detection |
| LLOQ         | lower limit of quantitation |
| LOQ          | limit of quantitation |
| MALDI        | matrix-assisted laser desorption/ionization |
| ME           | matrix effect |
| MEPS         | microextraction by packed sorbent |
| MS           | mass spectrometry |
| MS/MS        | tandem mass spectrometry |
| MSn          | multistage mass spectrometry |
| MSTFA        | N-methyl-N-(trimethylsilyl)trifluoroacetamide |
| MTBSTFA      | N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide |
| NMR          | nuclear magnetic resonance |
| PFBr         | 2,3,4,5,6-pentafluorobenzyl bromide |
| PFBCl        | 2,3,4,5,6-pentafluorobenzoyl chloride |
| p-HBA        | 4-hydroxybenzoic acid |
| PhLA         | phenyllactic acid |
| PhPA         | phenylpropionic acid |
| p-HPPhAA     | 4-hydroxyphenylacetic acid |
| p-HpLA       | 4-hydroxyphenyllactic acid |
| p-HpPA       | 4-hydroxyphenylpropionic acid |
| PTV          | programmed temperature vaporizer |
| Q            | single quadrupole |
| QuEChERS     | “quick, easy, cheap, effective, rugged, and safe” |
| RE           | relative error |
| RSD          | relative standard deviation |
| SID          | stable isotope dilution |
| SPE          | solid-phase extraction |
| SPME         | solid-phase microextraction |
| TBDMS        | tert-butyldimethylsilyl |
| TMCS         | trimethylchlorosilane |
| TOF          | time-of-flight |

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