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Direct profiling of endogenous metabolites in rat brain microdialysis samples by capillary electrophoresis-mass spectrometry with on-line preconcentration

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ABSTRACT

Metabolic profiling of body fluids from small animal models is often used in (translational) biological studies in order to obtain insight into the underlying molecular mechanisms of (complex) diseases. An example is the use of brain microdialysis samples from rats to study neurological disorders by means of a metabolomics approach. From an analytical point of view, the profiling of (endogenous) metabolites in rat brain microdialysates is challenging because of the limited sample volume for both sample preparation and injection, notably in longitudinal studies. In this work, we have assessed the analytical performance of capillary electrophoresis-mass spectrometry (CE-MS) for the direct profiling of endogenous metabolites in rat brain microdialysates, i.e. without using any sample preparation or derivatization. An on-line preconcentration procedure with sample stacking, which was fully compatible with the high-salt concentration in microdialysates, was used to significantly improve the detection sensitivity of the CE-MS method for metabolic profiling. A response surface methodology, applying a Box-Behnken design, was considered to determine the optimal conditions for preconcentration. A linear response ($R^2 > 0.99$) for selected metabolites in the concentration range from 0.05 to 10 μM was obtained in perfusate samples. Interday RSD values for peak area and migration time were 2.6-19% and below 3.8%, respectively. Limits of detection ranged from 11 to 284 nM when employing an injection volume of about 291 nL, corresponding to 17% of the total capillary volume. The utility of the CE-MS approach was demonstrated by the direct profiling of endogenous metabolites in rat brain microdialysates. At least 48 compounds could be analyzed of which 25 were provisionally identified and quantified.

Introduction

Metabolomics, i.e. the global profiling in biological samples of (endogenous) molecules with a molecular mass below 1000 Dalton, represents one of the most recent introduced 'omics' techniques. Elucidating the structure and dynamics of metabolic pathways provides essential information regarding the underlying mechanisms of the biological system concerned [1]. In the central nervous system (CNS), the exact pathological mechanisms for many neurological diseases have not yet been completely elucidated. In addition to genomics and transcriptomics, brain metabolomics offers an alternative tool for unveiling new insights into the complex structure and function of the brain and thereby plays an important role in the discovery of new therapeutics and treatments for neurological diseases [2-4].

Brain microdialysis is a widely used approach in neuroscience for the dynamic monitoring of brain neurochemistry via the collection of both endogenous and exogenous small molecules in freely moving animals. Rodents, like mice or rats, are often used for this purpose [5]. Until now, reversed-phase liquid chromatography (RPLC) in combination with classical detection methods or mass spectrometry (MS), has been most often used for the analysis of compounds in rat or mouse brain microdialysates. The analysis of low-molecular mass biomolecules in microdialysates by standard RPLC-based methods has shown to be analytically challenging, mainly due to the intrinsically low sample

Abbreviations: **BBD**, Box-Behnken Design

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volumes of rat and mouse brain microdialysis samples, but also due to the low concentrations of bioactive compounds in the CNS. Therefore, the collection times of microdialysis samples often have to be prolonged in order to produce enough sample material for analysis by the conventional analytical techniques, resulting in poor temporal resolution of microdialysate sampling [6,7]. Instead of conventional RPLC columns, miniaturized (nano, capillary or microbore) RPLC-based approaches in combination with fluorescence or electrochemical detection can be considered for the selective analysis of neurotransmitters in volume-limited samples. However, when using RPLC-based approaches, a derivatization step is generally needed in order to obtain sufficient retention for polar and charged metabolites, in particular for amino acids and monoamine neurotransmitters [8-13].

Capillary zone electrophoresis (CE) is a very strong analytical tool for the highly efficient analysis of polar and charged compounds in biological samples without using any derivatization, as the separation mechanism of CE is based on charge-to-size ratios. Another advantage of CE is that only small sample amounts are required for both the injection (nL-range) and for the sample vial (i.e., <10 μL is sufficient to perform multiple injections), which makes it an attractive tool for the analysis of volume-limited or scarcely available samples, such as microdialysates from animal models. Especially, CE employing laser-induced-fluorescence (LIF) detection has greatly improved the possibility for high-temporal resolution monitoring of neurotransmitters in microdialysates [14-16]. For example, CE-LIF has shown to be a very useful tool for on-line high-speed separations of amino acids and amines in rat brain microdialysates [17,18]. However, when employing CE-LIF, derivatization is required in order to introduce a fluorophore moiety to the compounds, an approach which is not amenable to all metabolites. Moreover, when it comes to identification of compounds, the selectivity of LIF is limited.

In this work, the aim was to assess for the first time the performance of CE coupled to MS via a sheath-liquid interface for the profiling of endogenous metabolites in rat brain microdialysis samples without performing any derivatization or sample preparation. In order to enable the detection of as many endogenous metabolites as possible in rat brain microdialysis samples, an on-line preconcentration procedure was applied. For this purpose, various sample preconcentration techniques can be used, such as pH-mediated stacking, dynamic pH junction, transient-isotachophoresis, counterflow focusing and sweeping [19]. Especially, dynamic pH junction has shown to be very effective for the preconcentration of zwitter-ionic and basic compounds, such as amino acids and amines, in human urine and cerebrospinal fluid (CSF) using minimal sample preparation, allowing their analysis in the nanomolar range by CE-MS, thereby showing an about 10-fold sensitivity improvement [20-22]. Therefore, this strategy was also considered in this study and further optimized employing a Box-Behnken Design (BBD). The final CE-MS method allowed the reliable quantification of endogenous metabolites in rat brain microdialysate using direct sample injection. Moreover, the developed approach permits multiple injections from the same volume-restricted microdialysis sample, thereby allowing repeatability studies with scarcely available samples.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were of analytical grade or higher purity. Acetic acid (99-100% m/m), methanol and isopropanol were purchased from Biosolve (Valkenswaard, The Netherlands). Hydrochloric acid (37% m/m) was from Thermo Fisher Scientific (Waltham, MA, USA). Ammonium hydroxide (28–30%) was acquired from Acros Organics (Amsterdam, the Netherlands). Amino acids standards, such as glycine, serine, leucine, isoleucine, glutamine, aspartic acid, glutamic acid, methionine, phenylalanine, arginine, tyrosine and γ-amino butyric acid (GABA), were purchased from Sigma-Aldrich (Steinheim, Germany).

Table 1
Factors and their levels in Box-Behnken Design

Independent factors	Levels		
	−1	0	+1
A: Sample injection volume (nL)	89	265	442
B: Ammonium hydroxide pre-injection volume (nL)	0.5	10.5	20.5
C: Ammonium hydroxide concentration (%)	5	12.5	20

^{13}C and/or ^{15}N stable-isotope labeled (SIL) internal standards were purchased from Cambridge Isotope Laboratories (Apeldoorn, the Netherlands). A Milli-Q Advantage A10 water purification system (Merck, Darmstadt, Germany) was used to obtain pure water. Background electrolyte (BGE) solution was prepared by diluting acetic acid in water (10% v/v). As sheath-liquid, a mixture of water and isopropanol (50:50, v/v), containing 0.03% acetic acid was used [23]. Ammonium hydroxide (28–30%) was diluted in water to the desired concentrations (5%, 12.5% and 20%).

2.2. Sample solutions

Metabolite standards (10 mM) were dissolved in a mixture of water:acetonitrile (95:5, v/v) containing 0.5% formic acid. Standard metabolite mixtures of 27 compounds (see S-1) were prepared in water as 50 μM stock solutions, aliquoted and stored at -80 °C. Internal standards ([$^{13}C_2$]-glutamine, [$^{13}C_6$]-lysine, [$^{13}C_2$]-aspartic acid, [$^{13}C_5$]-valine, [$^{13}C_2$]-glutamic acid) were prepared in BGE as 50 μM stock solutions, aliquoted and stored at -80°C. An internal standard stock aliquot was thawed the day of use, and diluted in BGE to the desired concentration (10 μM). Before injection, samples in water, perfusate or microdialysate were diluted at a 1:1 (v/v) ratio in BGE containing internal standards. The final concentration of internal standards was 5 μM.

2.3. Microdialysis sample collection

Intracerebral baseline samples from male albino Wistar rats (Charles River Laboratories, L'Arbresle, France) were collected for 2 hours. The perfusion fluid was composed of modified Ringer's solution (147 mM NaCl, 2.3 mM $CaCl_2$, 4 mM KCl) at a flow rate of 2 μL/min. Samples were collected with a temporal resolution of 20 min and split into two aliquots of 15 μL. All rats had a weight between 250-300 g. The animals were acclimatized for at least 1 week to the animal facility before implanting the microdialysis probe (CMA12/1 mm) in the nucleus tractus solitarii, as described previously [24]. Their housing was at a constant temperature (24°C), 12-hr light-dark cycle and with free access to food and water. All procedures are according to the National and European guidelines for animal experimental research and were approved by the Ethical committee for Animal Experiments of the Vrije Universiteit Brussel, Belgium (project 174-213-9: 01/02/2014-01/02/2018 and project 18-213-1: 01/02/2018-01/02/2020) [24-26].

2.4. Instrumentation and procedures

The CE-MS experiments were carried out on a 7100-capillary electrophoresis system from Agilent Technologies (Waldbronn, Germany) hyphenated with a 6230 QTOF, which was also from Agilent (Santa Clara, CA, USA). Fused-silica capillaries with an internal diameter of 50 μm were purchased from BGB Analytik (Harderwijk, The Netherlands). They had a total length of 90 cm for analysis. New capillaries were conditioned by subsequently rinsing, at 5 bar for 1 minute, with methanol, water, sodium hydroxide 1M, water, hydrochloric acid 1M, water, hydrochloric acid 0.1 M, water and background electrolyte (BGE). Injections were performed hydrodynamically. Injected volumes were calculated with Zeecalc v1.0b (https://epgl.unige.ch/labs/fanal/zeecalc). CE-MS coupling was realized via a co-axial sheath-liquid ESI

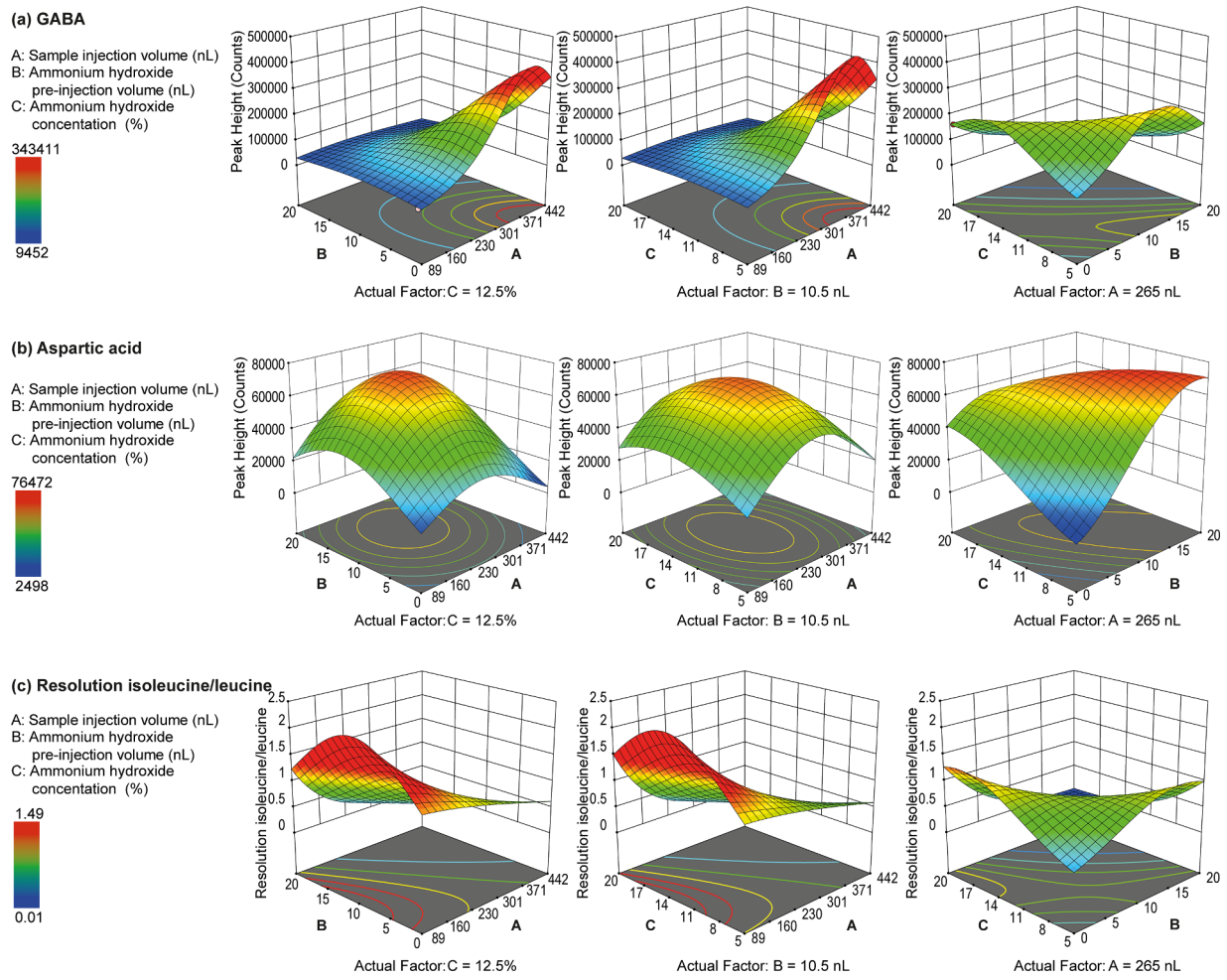

Figure 1. 3D response surface plots showing the signal intensity as determined by CE-MS for (a) GABA, (b) aspartic acid and (c) the resolution of the isomers isoleucine/leucine as a function of (left) sample injection volume (A), and ammonium hydroxide pre-injection volume (B), (middle) sample injection volume (A) and ammonium hydroxide concentration (C) and (right) ammonium hydroxide concentration (C) and ammonium hydroxide pre-injection volume (B). The third factor is kept constant at the value given below the plot.

interface equipped with a standard triple-tube sprayer. Sheath-liquid was delivered at a flow rate of 3 μL/min by an Agilent 1260 Infinity Isocratic Pump (Agilent Technologies) with a flow splitter that splits the sheath liquid in the ratio 1:100. MS experiments were acquired in positive mode between 65 and 1000 *m/z* with an acquisition rate of 1.5 spectra/s. The nebulizer gas was set to 0 psi, while the sheath gas flow rate and temperature were set at 11 L/min and 100°C, respectively and both were nitrogen. The ESI capillary voltage was adjusted to 5500 V. Fragmentor and skimmer voltages were set at 150 V and 50 V, respectively. MassHunter version B.06.00 (Agilent, Santa Clara, CA, USA) was used for data acquisition, instrument control and data treatment [23,27]. From the sheath-liquid, isopropanol ($C_3H_8OH^+$) and its clusters ($[(C_3H_8O)_2 + H]^+$ and ($[(C_3H_8O)_3 + H]^+$) with corresponding m/z values of 61.06479, 121.12231 and 181.17982, respectively, were used as lock masses. Data treatment was performed using MassHunter Workstation Quantitative Analysis. Peak extraction was performed with a mass error of 20 ppm and peak integrations were visually inspected to ensure correct integration.

2.5. Determination of analytical performance characteristics

The response function of the measured peak area for 28 metabolites was examined using 3 replicates at 7 different concentrations over a concentration range from 0.05 to 10 μM. As internal standard, SIL [$^{13}C_2$]-glutamine (5μM) was used. The limits of detection (LOD) and quantification (LOQ) were determined by the standard deviation of y-residuals (*s*) and the slope (*b*) of the calibration curve as 3*s*/*b* and 10*s*/*b*, respectively [28]. Repeatability (expressed as percentage relative standard deviation, % RSD) for peak area and migration time (with and without normalization to an internal standard) were determined by analyzing in triplicate amino acids standard mixtures and perfusate samples spiked with amino acids. The last experiment has been repeated on three consecutive days to determine interday precision. Matrix effects were assessed using the standard addition method, by comparing the peak areas of amino acids standards prepared in modified Ringer's solution to amino acid standards prepared in water [29].

2.6. Experimental design

The on-line preconcentration procedure was optimized using response surface methodology (RSM). Preconcentration was based on a pre-injection of ammonium hydroxide prior to the sample injection. Therefore, sample injection volume (A), ammonium hydroxide pre-injection volume (B) and ammonium hydroxide concentration (C) were investigated at three levels (-1, 0, +1) using a BBD (Table 1). A total of 15 experiments, i.e. 13 design experiments with three replicate runs at the center point, were executed to estimate the response surface using Design Expert Software (S-2) (version 12, Stat Ease Inc., MN, USA). The experiments were carried out in a random sequence to minimize bias and to reduce the outcomes of unpredicted variability in the responses.

Table 2
Summary of linear range, the F-value of the Lack-of-fit (LOF) test (Fcrit, 95% = 3.26), limit of detection (LOD) and limit of quantification (LOQ), of a six-point calibration for metabolite standards and metabolites spiked in perfusate as obtained by CE-MS. See section 2 for experimental conditions.

Analyte		Water			Perfusate		
	Range (nM)	*LOF*	*LOD (nM)*	*LOQ (nM)*	*LOF*	*LOD (nM)*	*LOQ (nM)*
Glycine	50-10000	0.242	11	37	0.151	42	142
Alanine	50-10000	0.113	22	75	0.445	35	116
Sarcosine	50-10000	0.760	16	53	0.372	19	63
GABA	50-10000	0.142	19	64	0.120	49	164
Serine	50-10000	0.103	15	51	0.267	33	109
Cytosine	50-10000	0.167	25	85	0.001	27	89
Creatinine	50-10000	0.098	22	72	0.042	35	116
Proline	50-10000	0.607	70	234	0.352	24	79
Valine	50-10000	0.159	49	162	0.900	15	51
Threonine	100-10000	1.066	31	103	0.464	87	290
Homoserine	50-10000	0.510	42	139	0.247	29	97
Hydroxyproline	50-10000	0.325	44	148	0.167	11	37
Creatine	50-10000	0.035	20	66	0.188	18	59
Isoleucine*	50-10000	0.002	33	110	0.906	29	96
Leucine*	50-10000	0.034	28	92	0.438	25	82
Asparagine	50-10000	0.953	26	87	0.589	50	166
Ornithine	50-10000	0.078	25	83	0.049	45	149
Aspartic acid	100-10000	0.986	56	188	0.267	61	202
Anthranilic acid	100-10000	0.074	35	117	0.621	77	256
Glutamine	50-10000	1.176	18	58	1.924	14	46
Lysine	100-10000	1.384	15	51	0.127	84	282
Glutamic acid	50-10000	2.044	16	54	0.298	20	67
Methionine	50-10000	0.310	7.5	25	0.009	17	58
Histidine	50-10000	0.012	8.9	30	0.047	12	39
Phenylalanine	50-10000	0.233	20	68	0.513	16	54
Arginine	50-10000	0.166	6.2	21	0.158	55	184
Tyrosine	50-10000	2.962	16	54	0.256	36	120
Tryptophan	500-10000	0.481	33	110	0.057	284	946

* isoleucine and leucine were only partially separated

Table 3
Intra- and interday precision (expressed as %RSD values) of migration time (MT) and peak area (Area) obtained for metabolite standards and for metabolites spiked in perfusate (5μM).

Analyte	Water				Perfusate			
	RSD % MT Intraday (n = 3)	Interday (n = 3)	*RSD % Area* Intraday (n = 3)	Interday (n = 3)	*RSD % MT* Intraday (n = 3	Interday (n = 3)	*RSD % Area* Intraday (n = 3)	Interday (n = 3)
Glycine	2.9	3.3	5.4	9.6	0.91	2.8	4.7	5.3
Alanine	3.0	3.4	4.3	6.0	0.83	2.9	3.3	5.4
Sarcosine	3.2	3.6	2.7	5.1	0.73	3.1	2.8	6.5
GABA	2.5	3.2	2.8	3.3	1.3	2.6	22	17
Serine	3.2	3.6	3.7	6.7	0.67	3.2	3.2	4.4
Cytosine	2.4	3.1	4.6	4.4	1.3	2.6	13	14
Creatinine	2.5	3.2	5.7	4.6	1.2	2.6	13	11
Proline	3.4	3.8	2.4	5.8	0.58	3.6	1.2	6.5
Valine	3.2	3.6	4.5	6.0	0.76	3.1	0.9	6.6
Threonine	3.3	3.6	3.2	6.1	0.65	3.3	1.2	3.9
Homoserine	3.2	3.5	1.8	6.1	0.68	3.2	1.7	4.5
Hydroxyproline	2.8	3.2	5.3	6.4	0.89	2.7	3.0	2.6
Creatine	2.8	3.2	6.4	7.0	0.89	2.7	3.0	3.4
Isoleucine*	3.1	3.5	5.8	6.1	0.71	3.1	3.2	9.7
Leucine*	3.1	3.5	5.2	6.2	0.71	3.1	4.5	9.9
Asparagine	3.3	3.7	2.5	6.0	0.59	3.3	2.0	4.1
Ornithine	2.5	3.1	5.3	5.8	1.2	2.7	11	16
Aspartic acid	3.5	3.9	2.5	6.0	0.52	3.8	8.8	8.1
Anthranilic acid	3.3	3.7	4.1	3.7	0.52	3.5	1.1	8.1
Glutamine	3.3	3.7	2.8	7.2	0.61	3.4	2.3	4.5
Lysine	2.5	3.1	2.8	5.3	1.3	2.6	5.5	15
Glutamic acid	3.3	3.7	5.6	7.0	0.61	3.4	2.1	4.7
Methionine	3.3	3.7	3.8	4.9	0.62	3.4	1.3	6.4
Histidine	2.5	3.1	2.1	5.3	1.8	2.6	7.5	19
Phenylalanine	3.3	3.7	3.4	4.8	0.58	3.4	2.3	8.6
Arginine	2.5	3.1	2.0	4.2	1.2	2.6	8.5	12
Tyrosine	3.3	3.7	2.3	4.9	0.59	3.5	2.0	4.7
Tryptophan	3.2	3.6	1.3	3.8	0.59	3.3	0.68	5.1

* isoleucine and leucine were only partially separated

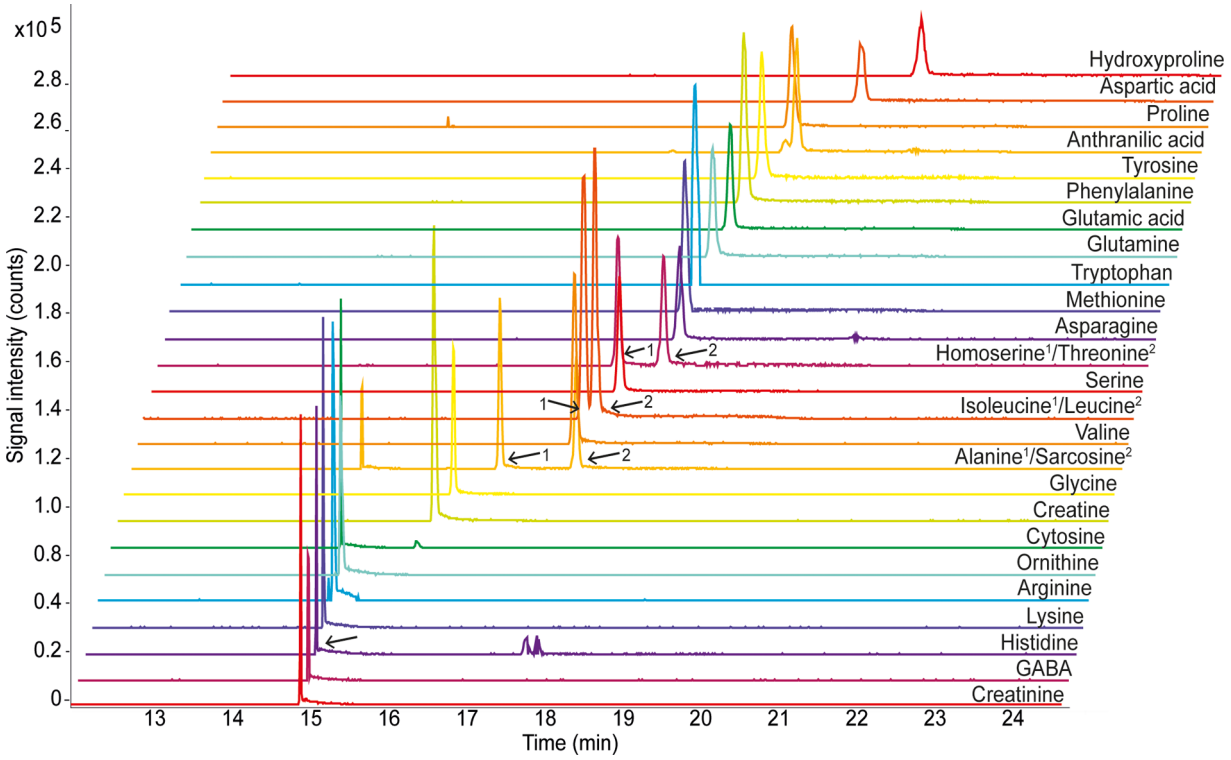

Figure 2. Extracted-ion electropherograms obtained by CE-MS from the analysis of 28 metabolites (5 μM) spiked in perfusate. Separation conditions: BGE, 10% acetic acid; sample injection volume 291 nL; ammonium hydroxide (concentration: 5%) pre-injection volume, 12 nL.

The responses considered signal intensity (y_1), peak width (y_2) migration time (y_3) and resolution between isoleucine and leucine (y_4) were fitted into second order polynomial equation (1), given below:

$$y = \beta_0 + \sum_{i=1}^{f} \beta_i x_i + \sum_{1 \le i \le j}^{f} \beta_{ij} x_i x_j + \sum_{i=1}^{f} \beta_{ii} x_i^2 \quad (1)$$

where y is the response; βo, βi, βij and βii are the regression coefficients of the intercept, linear, two factor and quadratic terms, respectively; xi and xj represent the independent variables and f the number of factors.

Interpretation of the effects was performed by both statistical and graphical evaluation using the Design Expert software. The significance of the model equations, individual factors and factor interactions were evaluated by the analysis of variance (ANOVA) at a confidence interval (CI) of 95% in order to determine the most important factors for sample preconcentration and determine potential interaction effects between factors. Graphical evaluation of the developed quadratic models was obtained by construction of two-dimensional (2D) contour plots and three-dimensional (3D) response surface plots. The optimal values of the variables for sample stacking using ammonium hydroxide were obtained using Derringer's desirability (D) function shown in equation (2). Responses signal intensity (y_1), peak width (y_2), migration time (y_3) and resolution between isoleucine and leucine (y_4) were transformed by a linear transformation onto a desirability scale (between 0 and 1); y_1 and y_4 had to be maximized, while y_2 and y_3 had to be minimized. The global desirability value was determined using the Design-Expert software by modelling D in a polynomial model resulting in representative 2D contour plots.

$$D = (d_1 \cdot d_2 \cdot d_3 \cdot d_4)^{1/4} \quad (2)$$

Results and discussion

The analysis of low-volume biological samples, such as body fluids from small animal models, may provide valuable insight into complex diseases. CE-MS has shown to be a very useful technique for the profiling of metabolites in volume-restricted biological samples, such as urine from mice and sweat from infants requiring minimal sample preparation [30,31]. Therefore, in this work, our aim was to assess for the first time the performance of CE-MS with a sheath-liquid interface for the efficient and sensitive profiling of endogenous metabolites in rat brain microdialysis samples without employing any sample preparation or derivatization.

CE-MS method development

For direct metabolic profiling of rat brain microdialysates by CE-MS, an on-line preconcentration procedure would be highly advantageous as many metabolites could be present at very low concentrations, often depending on the perfusion flowrate and sampling time employed for microdialysis. Dynamic pH junction showed to be an effective way for the preconcentration of amino acids and related compounds in urine samples [21,32,33]. Therefore, this on-line preconcentration technique was optimized in a systematic way in this work using a multivariate optimization approach based on BBD results. BBD was specifically selected since it requires fewer experiments than a central composite design (CCD) (i.e. 13 different experiments for BBD vs 15 for CCD to examine 3 factors), and it does not include runs where all factors are at their extreme settings [34].

Earlier studies that evaluated this type of on-line sample stacking in CE for the analysis of high-salt biological matrices revealed that three experimental parameters have a significant effect on the efficiency of the sample preconcentration, i.e., sample injection volume, ammonium hydroxide pre-injection volume and ammonium hydroxide concentration [22,32,33]. Therefore, these parameters were selected as factors in the BBD. Factor levels, as listed in Table 1, were also selected in accordance to these previous studies.

As a starting point, aspartic acid and GABA were selected, as they are a slow and fast migrating compound, respectively, under the employed CE separation conditions. The structural isomers isoleucine and

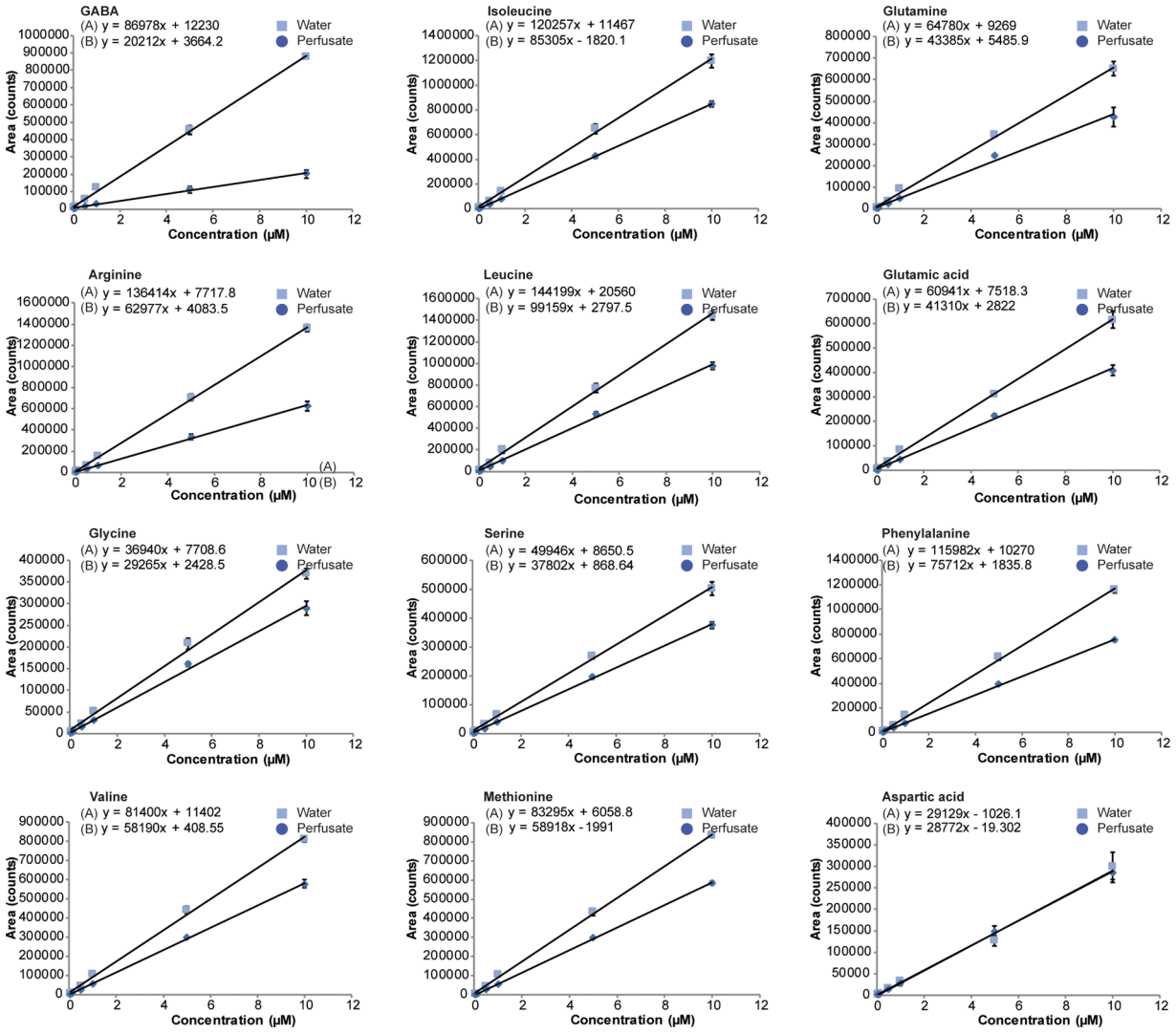

Figure 3. Calibration curves for (A) amino acid standards in water and (B) amino acids spiked in perfusate. The standard concerned is indicated above the figure. Separation conditions: BGE, 10% acetic acid; sample injection volume 291 nL; ammonium hydroxide (concentration: 5%) pre-injection volume, 12 nL.

leucine were also selected in order to determine whether their electrophoretic separation could be maintained. Since the goal was to develop a rather fast CE-MS method with high detection sensitivity, without losing separation efficiency, selected responses were signal intensity, peak width and migration time. Additionally, the separation efficiency of the method was evaluated by determining the resolution between isoleucine and leucine.

Earlier studies revealed that the amount of ammonium hydroxide necessary for efficient sample preconcentration differed between water-dissolved samples and spiked urine samples [32]. This is most probably due to the difference in salt concentration (i.e. conductivity) and pH between the two sample types. Therefore, the effect of the on-line preconcentration parameters was examined for two sample types: 1) amino acids standards in water, and 2) amino acids spiked into microdialysis matrix. As microdialysis matrix, perfusate, or (modified) Ringers solution, was used, mainly because of the scarce availability of the valuable/precious rat brain microdialysis samples. Before injection, samples in water or perfusate were diluted in BGE containing internal standards at a 1:1 (v/v) ratio. To check whether the amino acid profiles were comparable between the two sample types, CE-MS results of three BBD runs (3, 5 and 12) were evaluated (S-3). The effect of sample preconcentration was comparable in terms of peak shape and migration order for amino acids either dissolved in water or spiked into perfusate. However, sample preconcentration was somewhat more efficient for perfusate, as the resolution between isoleucine and leucine was 5-70% higher and the separation window was 23-72% shorter, which could be due to the high salt concentration in perfusate, potentially resulting in salt-induced transient isotachophoresis in the sample zone [35] and a slightly more effective dynamic pH junction as the pH of perfusate is higher than that of water. Overall, comparable amino acid profiles were obtained in both cases and, consequently, for simplicity, amino acids standards in water were used for the BBD analyses

BBD experiments were carried out and experimental data is listed in S-4. Model coefficients for all responses were determined by regression analysis and shown as equations 3–9 (S-5). ANOVA results (S-6) show that the ammonium hydroxide pre-injection volume significantly affects all responses, and thus can be considered as the most important factor for the efficiency of sample stacking. In previous research, Tak *et al.* [29] observed a dependence of sample injection volume on ammonium hydroxide volume for the peak shape and separation efficiency of different amino acids. When using multi-factor optimization such as response surface modelling, possible interaction effects are taken into account [36,37]. The 3D surface plots (Figure 1) show interaction effects for signal intensity of a) GABA and b) aspartic acid and of c) resolution between isoleucine and leucine. Two-factor interaction effects can be assumed from these plots. Two-factor interaction effects can be

Table 4
An overview of compounds detected by CE–MS in a rat brain microdialysis sample. Separation conditions: BGE, 10% acetic acid; sample injection volume, 291 nL; ammonium hydroxide (5%) pre-injection: 12 nL.

Identified compounds				
Detected *m/z*	**Analyte**	**RSD % MT**	**RSD % Area**	**Average concentration ± %RSD (nM) (n = 12)**
76.0393	Glycine	1.1	8.0	2984 ± 8
90.0550	Alanine	1.1	16	1837 ± 15
104.0706	GABA	1.3	26	59 ± 14*
106.0499	Serine	1.0	3.8	2693 ± 5
112.0505	Cytosine	1.2	26	28 ± 10*
114.0662	Creatinine	1.4	20	351 ± 24
116.0706	Proline	1.0	3.2	1257 ± 4
118.0863	Valine	1.1	2.9	1422 ± 3
120.0655	Threonine	1.1	4.5	2151 ± 4
132.0655	Hydroxyproline	1.1	4.4	277 ± 7
132.0768	Creatine	1.1	7.5	28 ± 10
132.1019	Isoleucine	1.1	4.2	620 ± 5
132.1019	Leucine	1.1	4.3	976 ± 5
133.0608	Asparagine	1.1	6.7	228 ± 10
133.0972	Ornithine	1.3	12	1618 ± 13
134.0448	Aspartic acid	1.1	9.9	430 ± 4
138.0550	Anthranilic acid	1.0	8.6	153 ± 11
147.0764	Glutamine	1.1	4.0	5744 ± 2
147.1128	Lysine	1.3	13	2817 ± 3
148.0604	Glutamic acid	1.1	2.8	449 ± 2
150.0583	Methionine	1.1	17	285 ± 15
156.0768	Histidine	1.3	8.3	967 ± 10
166.0863	Phenylalanine	1.1	3.2	635 ± 5
175.1190	Arginine	1.3	7.4	927 ± 8
182.0812	Tyrosine	1.0	5.3	757 ± 5

Unidentified compounds				
Detected *m/z*				
89.107	*130.086*	*169.057*	*178.065*	*209.092*
106.086	*134.101*	*170.092*	*180.027*	*228.098*
118.061	*136.063*	*170.121*	*184.073*	*423.166*
118.085	*139.050*	*171.063*	*186.016*	
130.050	*154.086*	*176.102*	*194.156*	

Asterisks (*) denote that value is below limit of quantification, but above limit of detection

seen in response surface plots if the behavior (effect) of the first factor is different at the low and high levels of the second (and vice versa). For instance, in the first two GABA plots this clearly can be observed. 3D surface plots for peak width and migration time are shown in S-7A and S7-B, respectively. As can be seen in both the ANOVA results and the 3D surface plots, indeed interactions seem to play a role for peak shape and separation efficiency, which is in agreement with earlier findings.

To identify the optimal conditions for sample stacking, Derringer`s desirability multi-criteria decision making was applied (as shown in equation 2) [38]. The goal of this optimization was to find a set of conditions with the best compromise between the individual goals, i.e. maximize signal intensity, minimize peak width, minimize migration time and maximize resolution. Desirability plots combining these goals are shown in S-8, where a higher D-value denotes a more favorable option. An optimal response was achieved with a sample injection volume of 270-300 nL, an ammonium hydroxide pre-injection volume of 12-18 nL, and an ammonium hydroxide concentration of about 5%.

Two optimal parameter settings (option 1. sample injection volume: 291 nL, ammonium hydroxide pre-injection volume: 12 nL, ammonium hydroxide percentage: 5%; and option 2. sample injection volume: 291 nL, ammonium hydroxide pre-injection volume: 16 nL, ammonium hydroxide percentage: 5%) were tested for the amino acid test mixtures (5 μM) comprised of 12 amino acids (including GABA, glycine, arginine, valine, aspartic acid, serine, glutamine, phenylalanine, methionine, glutamic acid, isoleucine and leucine) with different physico-chemical properties. Only the ammonium hydroxide volume was varied, since this factor should have the largest impact on stacking performance as was determined earlier. The analysis was performed for both amino acids standard mixtures and perfusate samples spiked with amino acids, and the corresponding extracted ion electropherograms (EIEs) are shown in S-9 for options 1 and 2. When looking at the amino acid profiles, it can be stated that for both sample types a larger pre-injection volume (solution 2) shows slightly higher signal intensities (about 0.2-14%) in perfusate. However, the resolution between isoleucine and leucine is 43% lower compared to a lower pre-injection volume (i.e., 0.9 vs. 1.4). For this reason, solution 1 was considered as best and thus used for follow-up studies.

3.2. Analytical performance evaluation

After determining the optimal conditions for on-line preconcentration with sample stacking, the analytical performance of the CE-MS method was further evaluated using amino acids standard mixtures and perfusate samples spiked with amino acids (10-5-1-0.5-0.1-0.05 μM) by establishing calibration curves in order to assess the linearity range, LODs, LOQs in the different matrices, as well as migration time and peak area precision. A metabolite mixture composed of 28 compounds was spiked into perfusate and analyzed (see S-1). SIL glutamine (5μM) was used as internal standard.

A linear response (and with $R^2 > 0.99$) for selected metabolites in the concentration range from 0.05 to 10 μM was obtained in perfusate samples. Linearity was evaluated by a lack-of-fit (LOF) test [39,40]. The method yields linearity for all test compounds. Detection limits ranged from 6.2 to 70 nM for amino acid standards dissolved in water and from 11 to 284 nM for amino acids spiked into perfusate (Table 2). In general, higher LODs and LOQs were obtained for the compounds spiked into perfusate samples, which is due to matrix effects as explained in the final part of this section. Precision of the CE-MS method for direct profiling of metabolites was assessed based on the repeated analyses of perfusate samples spiked with amino acids at one concentration level (5 μM). Intraday RSD values ($n = 3$) for peak areas and migration times of all analytes were better than 22% and 1.3%, respectively, while interday RSDs ($n = 9$) were below 19% and 3.8%, respectively (Table 3). By using relative migration times (RMT) instead of migration times, interday RSD values for RMTs were below 1.5% (see S-10). Given that perfusate was analyzed directly by CE-MS, the obtained figures of merits for repeatability could be considered as highly favorable and acceptable for comparative metabolic profiling studies. Figure 2 clearly shows that all compounds in perfusate could be analyzed with a high separation efficiency (i.e., plate numbers per meter were above 300,000 for all compounds) and a good detection sensitivity. Under these conditions, the structural isomers isoleucine and leucine were separated with a resolution of 1.4.

Subsequently, the effect of matrix interferences was evaluated. Especially, when injecting biological sample without using any sample pretreatment, ion-suppression can occur when matrix substances co-migrate with the analytes of interest. In order to assess matrix effects, the standard addition method was applied. The amino acid standards spiked into perfusate were analyzed and the results were compared to the standard solutions in water. Results for 12 amino acids with varying physico-chemical properties are shown in Figure 3. Both the calibration curves of amino acids in standard solutions in water and in perfusate were linear in the concentration range up to 10 μM. The slopes were different for all compounds, except for aspartic acid. The slopes in perfusate are lower than in water, resulting in higher LOD and LOQ values, as already mentioned higher.

This clearly indicates that all metabolites (apart from aspartic acid) experience a matrix effect, which was more pronounced for fast migrating compounds as they migrate close to the salt plug (MT~14.0-15.5 min), which can be seen in S-11, where a TIE is shown. Therefore,

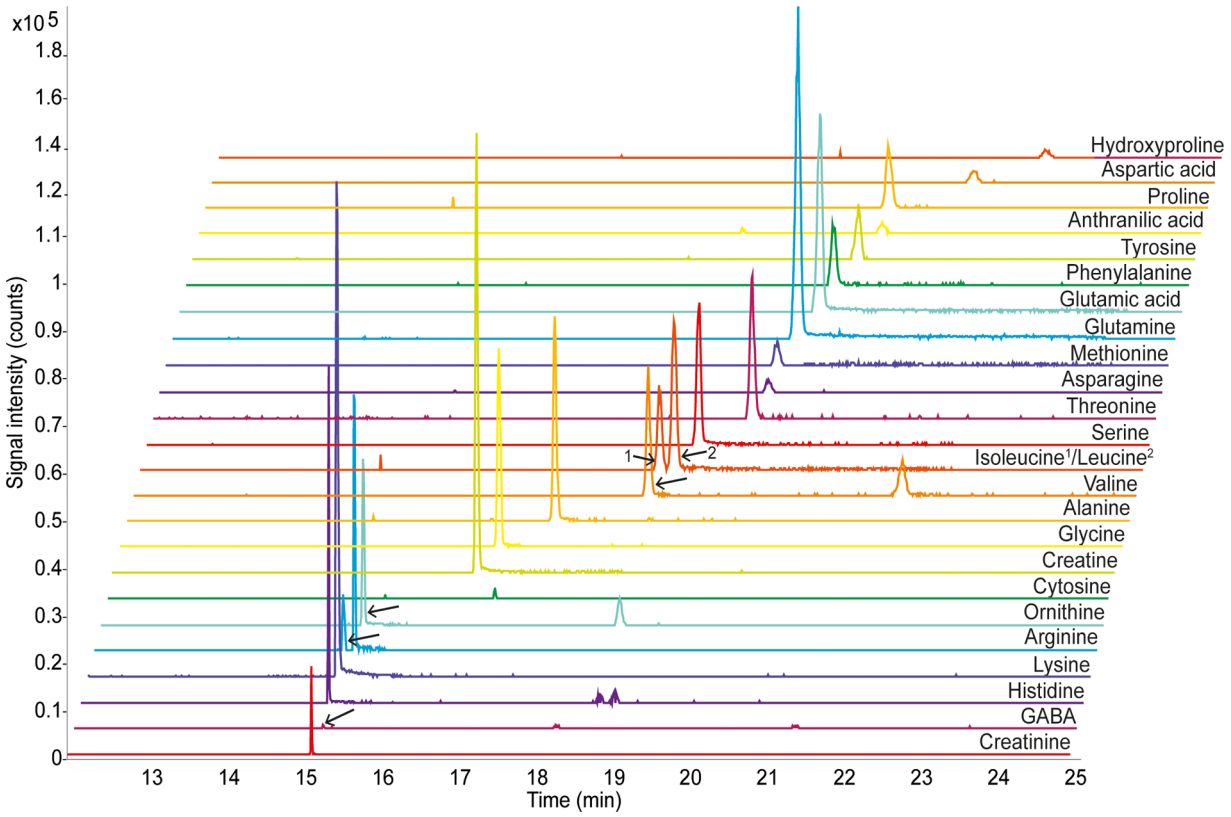

Figure 4. Extracted-ion electropherograms obtained by CE-MS from the analysis of endogenous metabolites in basal rat brain microdialysate. Separation conditions: BGE, 10% acetic acid; sample injection volume 291 nL; ammonium hydroxide (concentration: 5%) pre-injection volume, 12 nL.

in order to allow a reliable quantification of metabolites in microdialysis samples, calibration curves need to be constructed in perfusate to account for matrix effects.

3.3. Metabolic profiling of rat brain microdialysates

The utility of the CE-MS method for the direct profiling of endogenous metabolites in rat brain microdialysates was demonstrated by the analysis of a basal rat brain microdialysis sample of about 10 μL, which was diluted with BGE (1:1, v/v) and stable isotope labeled (SIL) internal standards were added ([$^{13}C_6$]-lysine, [$^{13}C_2$]-aspartic acid, [$^{13}C_5$]-valine, [$^{13}C_2$]-glutamine, [$^{13}C_2$]-glutamic acid). The total sample volume was divided in three equal portions and each portion was analyzed 4 times, where injection from the same sample vial was performed. Before and after the microdialysis sample analysis, a calibration curve in perfusate was run for quantification purposes and also to check the performance of the CE-MS method.

The number of detected compounds was manually determined in the *m/z* range from 65 to 1000, where only peaks with a detection response above 500 counts were included (except for GABA and cytosine). In total, 48 compounds were detected in rat brain microdialysis (Table 4). Peak identification in microdialysis samples was carried out by comparing migration times and *m/z* values with those of the metabolite standards, and 25 compounds could be identified (Figure 4). The repeatability was determined for all identified compounds in microdialysis samples using twelve repeated analyses. For all analytes, RSD values for peak areas (not corrected by an internal standard) and migration times were better than 20% and 1.4%, respectively.

Comparing concentrations of metabolites determined in microdialysis samples with literature values is difficult as (small) variations in the microdialysis process between labs may have an impact on the actual metabolite concentrations present. Still, for the compounds GABA and glutamate we could perform such a comparison with one previous study in which a microdialysis probe was employed for the collection of sample from the same brain region [21]. This study provided a concentration of about 300 nM for glutamate and 12 nM for GABA in rat brain microdialysis sample, whereas in our study we have found a concentration of about 449 nM for glutamate and 59 nM for GABA. Though, rather comparable results were obtained, we still decided to test whether our CE-MS method can be used for the reliable quantification of metabolites in microdialysis samples. For this purpose both perfusate and actual microdialysis samples were spiked with isotope-labeled amino acids (5 μM) and the peak areas obtained by CE-MS were compared. The results of this experiment (see S-12) show that comparable peak areas were obtained for SIL glutamine, valine and aspartic acid (student's t-test at 95% CI, paired). For SIL glutamic acid and lysine, peak areas were 9% lower and 15% higher, respectively (see S-12), which we could attribute to the analytical variation. On the basis of these findings, we are confident that our CE-MS method can be used for the reliable quantification of metabolites in microdialysis samples when employing standard curves of metabolites prepared in perfusate. Table 4 gives an overview of the compounds detected and quantified and also of compounds detected but not identified and quantified in a rat brain microdialysis sample by CE–MS. For quantification, isotope-labeled aspartic acid, glutamic acid, glutamine, lysine and valine were used as internal standards for their corresponding amino acids, whereas isotope-labeled glutamine was used as internal standard for the other compounds, as this internal standard gave the most repeatable relative peak area values.

Concluding Remarks

In this work, we have evaluated for the first time the analytical performance of CE-MS for the direct analysis of endogenous metabolites in rat brain microdialysis samples. This method allows to obtain LODs for amino acids in rat brain microdialysates in the range which is rather

comparable to LC-MS approaches [11], with the main differences that in CE only an injection volume of about 300 nL is utilized, whereas typically 5000 nL is injected in LC-MS, and no derivatization is needed. Therefore, the proposed CE-MS method is highly suitable for metabolic profiling of volume-limited biological samples. Even though lower detection limits can be obtained using electrokinetic injections [41], an advantage of our method is that it allows performing multiple injections from the same sample, whereas only a single injection can be performed with electrokinetic injection. Another strong point of our method is that highly repeatable migration times are obtained for metabolites in rat brain microdialysis samples without using any sample pretreatment. This approach offers potential for comparative metabolomics studies using microdialysis samples, that may potentially lead to the discovery of novel metabolic markers for neurological diseases. It is anticipated that a further improvement in detection sensitivity could be achieved by employing a sheathless instead of a sheath-liquid interface for coupling CE to MS, potentially enabling the evaluation of biochemical changes in the brain at a very short temporal resolution. Moreover, such approach could be further extended to mouse brain microdialysis studies, where volumes are even smaller.

CRediT authorship contribution statement

Marlien van Mever: Conceptualization, Data curation, Methodology, Software, Writing - original draft, Writing - review & editing. **Karen Segers:** Data curation, Methodology, Software, Writing - review & editing. **Nicolas Drouin:** Methodology, Writing - review & editing. **Faisa Guled:** Methodology, Writing - review & editing. **Yvan Vander Heyden:** Writing - review & editing. **Ann Van Eeckhaut:** Writing - review & editing. **Thomas Hankemeier:** Writing - review & editing. **Rawi Ramautar:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors have declared no conflict of interest.

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Supplementary materials

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