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# Workflow methodology for rat brain metabolome exploration using NMR, LC-MS and GC-MS analytical platforms

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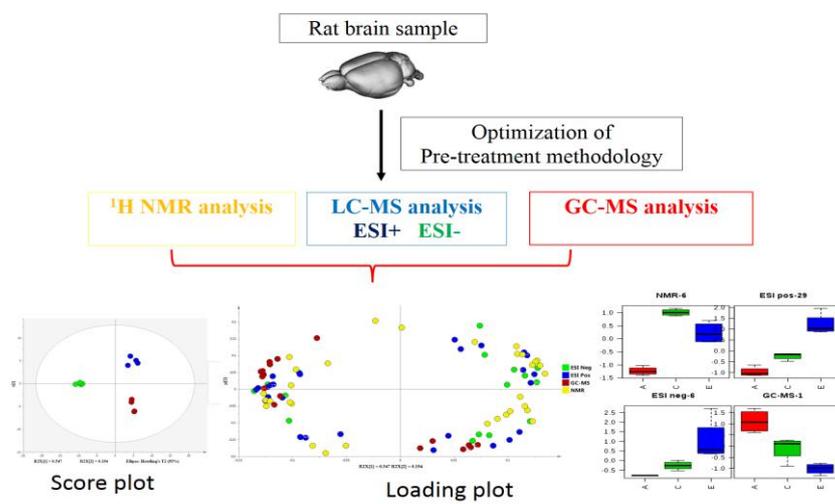
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## Graphical Abstract



## Highlights

- Metabolomics approach requires to be optimized for each biological matrix used
- At present, no method standardization for rodent brain metabolomics
- Designing of methodology for each pre-analytical step (tissue lysis, extraction...)
- Complementary information on rat brain metabolome using NMR, LC-MS, GC-MS

**ABSTRACT**

We developed a multi-platform approach for the metabolome exploration of rat brain tissue, using liquid chromatography coupled with mass spectrometry (LC-MS), nuclear magnetic resonance spectroscopy (NMR) and gas-chromatography coupled with mass spectrometry (GC-MS). The critical steps for metabolite exploration of cerebral tissues are tissue lysis and metabolites extraction. We first evaluated the impact of freeze-drying compared to wet tissue metabolites extraction using NMR and LC-MS with a reversed phase liquid chromatography. Then, we compared four metabolite extraction methods Based on the number of metabolites extracted, their intensity and their coefficient of variation (%CV), the most reproducible protocol (one-step extraction with acetonitrile on lyophilized material) was chosen to further evaluate the impact of sample mass on method performance (3, 6, and 9 mg were assayed). GC-MS analysis was also investigated by analyzing four different methoximation/silylation derivatization combinations. The optimal analytical protocols were proposed to establish the reliability required to realize untargeted brain tissue metabolomics exploration. The most reliable workflow was then exemplified by analyzing three rat brain regions (cerebellum, frontal and parietal cortices, n=12) by  $^1\text{H}$  NMR, LC-MS and GC-MS , allowing their clustering based on their metabolic profiles. We present here an example of development of methodology that should be done before running analysis campaigns.

**Keywords** tissular metabolomics; metabolic fingerprinting; untargeted methodology; optimization

**Abbreviations**

CV %, coefficient of variation; LC-HRMS, liquid chromatography coupled with high resolution mass spectrometry; OPLS-DA, Orthogonal partial least squares discriminant analysis; TSP, 3-trimethylsilylpropionic acid

## 1. Introduction

Metabolomics aims to detect, identify and quantify all metabolites in a biological sample, using chemometric and statistics tools in order to compare the metabolic signatures of different physiological conditions which depend on genetic and environmental factors [1]. A metabolic study can be conducted using two approaches: targeted and untargeted. Targeted studies focus on a panel of identified molecules, whereas untargeted studies seek to find as much information as possible by performing spectral metabolic profiles (metabolic fingerprinting). Metabolomics are applied to very different samples *e.g.* plants, biofluids, tissue or cell extracts. The chemistry of different metabolites in these complex biological systems is heterogeneous. As the chemistry families of the extracted metabolites from these different media could be very different (*e.g.* more or less macro molecules), they could interfere in different ways during the analytical processes (*e.g.* overlap or ion competition). Therefore, it is so necessary to develop a methodology for each matrix studied.

The metabolomics approach on tissue [2, 3] is widely used to study the pathophysiology of a disease in animal models [4, 5], to search for biomarkers as a diagnostic tool [6-8], or to assess the impact of a new therapeutic [9]. The development of such approaches present several pre-analytical challenges, such as a rapid quenching of enzyme activity, extraction procedures and the development of robust analytical methods [10]. The most exhaustive detection is usually achieved by analytical platforms such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled with gas chromatography (GC-MS) or liquid

chromatography (LC-MS) [11]. Combining the data acquired from multiple analytical platforms is a plus, but it requires the optimization of sample pre-treatment, separation condition, and instrumental parameters [12].

To date, there is no standardized effective protocol for tissue metabolomics study [13], a lot of research teams follow their in-house methodology, not always given with the validation of results [14, 15]. Few multi-platform metabolomics methodologies have been published so far for tissue [16-18]. We have previously described a workflow for cell-based metabolomics [19] and we want now to optimize the brain rodent tissue analysis, taking into account the analytical steps which are matrix dependent. We developed and suggest a fingerprinting methodology using three complementary analytical techniques, LC-HRMS (high resolution mass spectrometry), NMR and GC-MS, taking into account pretreatment and analytical optimization (tissue lysis, metabolite extraction and analytical process) in order to obtain a metabolome coverage as extensive as possible. This paper is an example of development of methodology that should be develop in laboratories before metabolomics study. The application of the three platforms to untargeted metabolomics of brain tissues was done *via* the analysis of three different rat brain regions.

*Please insert Fig. 1*

*Please insert Fig. 2*

## 2. Materials and methods

### 2.1. Brain tissues

All the experiments were conducted on brain tissue extracted from spare adult female Wistar rats from other research protocols. Animals were sacrificed by decapitation and their whole brain, or cerebellum, frontal and parietal cortices dissected. Then, brain tissues were placed in tared tubes, that were reweighed and frozen at -80°C.

### 2.2. Brain tissue preparation

#### 2.2.1. To study wet tissue versus lyophilized tissue

Frozen brain tissue samples (around 2 g) were homogenized with an Ultra-Turrax (Ika) at 20000 tr/min for 1min x3. The homogenate was divided into Eppendorf vials with 10x50 mg of the ground sample and then extracted in two steps with 1.5 mL of acetonitrile:milliQ water (1/1) as detailed bellow for lyophilized tissue.

The remaining mass was lyophilized during 48h in a FreeZone® Freeze Dry Systems 4.5 Liter Benchtop lyophilizer (Labconco®, USA) and milled to a fine powder (from 50 to 60 mg of fresh tissues, around 10 mg of powder were obtained, and each weight, for fresh and lyophilized tissue, was noted and taking into account) and stored at -80°C until extraction process.

#### 2.2.2. To study the impact of mass of lyophilized tissue extracted, on LC-HRMS analysis

Frozen rat brain tissue sample (around 0.6 g) was homogenized with an Ultra-Turrax and lyophilized during 48h. The obtained powder was homogenized and split

into Eppendorf vials with 3 mg, 6 mg or 9 mg of powder, extracted with acetonitrile/water as detailed below.

### 2.2.3. Optimization of extraction methods from lyophilized tissues.

Three different extraction methods were evaluated:

Metabolites were extracted in two steps from lyophilized sample using either 2 x 0.7 mL of a mixture of: (1) methanol:milliQ water (1/1), or (2) acetonitrile:milliQ water (1/1) or (3) dichloromethane:methanol:milliQ water (2/2/1.4) in 50 mL centrifuge tubes. Samples were vortexed for 30 s, sonicated at 30 W for 10 min on ice using a Sonicator (Bransonic 12, Ultrasonic Cleaner, USA) and centrifuged at 15000g at 4°C for 10 min after each extraction. The supernatant/ upper phase (0.5 mL) was isolated. The extraction was done again, and the combined supernatants/ upper phases were evaporated using a SpeedVac Concentrator (Savant SPD 111v SpeedVac, Thermo Scientific, USA).

### 2.3. <sup>1</sup>H-NMR analysis

Analyses were done to compare results from freeze-drying and fresh tissues (from 6 mg of lyophilized sample or from 30-40 mg of fresh tissues n=5 replicates) and to study impact of extracting solvent (n=3 replicates). Each extracted sample was reconstituted in 200 µL of 0.2 M potassium phosphate buffered deuterium oxide (D<sub>2</sub>O) (pH 7.4 ± 0.5) and 8 µL of D<sub>2</sub>O with external reference [3-trimethylsilylpropionic acid (TSP), 0.05 wt% in D<sub>2</sub>O], vortexed briefly, and centrifuged at 3000 g for 10 min at 4°C. The resulting supernatant was then transferred to conventional 3-mm NMR tubes. <sup>1</sup>H NMR spectra were acquired as previously described [20] (see supporting information). Briefly, the analyses were done on a Bruker DRX-600 Avance III HD

(Bruker SADIS, France) equipped with a TCI cryoprobe. Spectra were acquired using a “noesypr1d” pulse sequence with a relaxation delay of 20 s (n=64 scans).

#### 2.4. LC-HRMS analysis

The impact of pre separation by solid phase extraction (SPE) by pipette tip packed with C18 (Strata C18-E, 100 mg/3 mL, Phenomenex) was compared to single extracted extracts (n=3 replicates). The dry supernatant/upper phase was reconstituted in 150  $\mu$ L MeOH:H<sub>2</sub>O (1/1) followed by 5 min centrifugation at 4°C.

LC-HRMS analysis was performed as previously described [20] (see supporting information). Briefly, the analyses were done on a UPLC Ultimate 3000 system (Dionex), coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Germany) and operated in positive (ESI+) and negative (ESI-) electrospray ionization modes. Typical total ion current chromatograms (TIC) were presented in supporting information (Fig. A.1). Chromatography was carried out with a Phenomenex Kinetex 1.7  $\mu$ m XB – C18 (150 mm x 2.10 mm) and 100 Å UHPLC column. The solvent system comprised mobile phase A [0.1% (vol/vol) formic acid in water], and mobile phase B [0.1% (vol/vol) formic acid in methanol] (see supporting information, Table A.1). Instrumental chromatography stability was evaluated by multiple injections (x = 15) of a quality control (QC) sample obtained from a pool of 10  $\mu$ L of all samples analyzed. This QC sample was injected once at the beginning of the analysis, every 10 sample injections, and at the end of the run.

#### 2.5. GC-MS experiment

Lyophilized tissues (6 mg) were extracted with ACN:H<sub>2</sub>O (1/1). After evaporation using a SpeedVac Concentrator (2.5h), the residue was lyophilized for one night to remove the trace amount of water residue for the derivatization step.

Metabolite extracts were suspended in methoxyamine hydrochloride in pyridine (20 mg/mL; 60  $\mu$ L) and incubated at (1) at 60°C for 1h, (2) 25°C for 2h, (3) 60°C for 2h or (4) 25°C for 16h. BSTFA:pyridine (40/60) (100  $\mu$ L) was then added, the mixture was vortexed for 1 min and incubated for (1) and (2) at 37°C for 4h or for (3) and (4) at 70°C for 1 hour (n=4 replicates).

GC-MS analysis was adapted from our previously published method [19] (see supporting information). Briefly, a Shimadzu GC-MS system (Kyoto, Japan) was used, a gas chromatograph 2010 and a QP-2010-Plus mass spectrometer. The derivatized samples (1  $\mu$ L) were separated on a capillary CG column (Phenomenex, Zebron ZB-5).

## 2.6. *Data processing*

### 2.6.1. *NMR*

Spectra were processed as previously described using Topspin version 3.2 software (Bruker Daltonik, Karlsruhe, Germany). Each spectrum was reduced to buckets of various widths (from 0.01 to 0.15 ppm) using AMIX software (Analysis of MIXture, version 3.9.14, Bruker, Karlsruhe, Germany) and normalized to the total area, after normalization to the TSP [20]. Spectra annotations were performed using Chenomx 7.1 software (Chenomx Inc, Edmonton, Canada), in-house data base and HMDB (<http://www.hmdb.ca/>).

### 2.6.2. *LC-HRMS*

XCMS software[21] implanted in the Galaxy platform was used to process raw data for peak alignments and framing in one batch. Peaks with greater than 30% variance (CV%) in QC samples were removed [11]. The normalization was done to the weight of sample and the total area.

### 2.6.3. GC-MS

The GC/MS raw files were converted to mzXML format using the GC-MS Solution Postrun Analysis® software (Shimadzu, Japan), and the converted files were imported into the XCMS software (Galaxy project Metabolomics, France) for feature detection, and retention time (RT). All chromatograms were analyzed simultaneously and normalized to the weight of sample. Spectral identification was done when the spectra and the NIST spectral mass library (NIST 05) combined with our in-house library matched with a spectral similarity >90 %.

### 2.7. Statistical analysis

The multivariate analysis [22] was performed using Simca-P<sup>+</sup>-13 software (Umetrics, Sweden). The data analyses were conducted using a method developed in our laboratory [23] (see supporting information). In parallel with multivariate analysis, the reliability of the results was assessed with the coefficient of variation (%CV), defined as the ratio of the standard deviation to the mean, and multiplied by 100 [12]. Non-parametric tests (Wilcoxon rank-sum test) have been done using the web free server Metaboanalyst ([www.metaboanalyst.ca](http://www.metaboanalyst.ca)), for significance, the critical *p*-value was set to 0.05 [24].

## 3. Results and discussion

We have evaluated the different tested conditions based on metabolites extraction yield (signal intensities, number of features) and reproducibility (coefficient of variation, %CV) while accuracy for untargeted method was not applicable [25].

### 3.1. Pretreatment optimization

#### 3.1.1. Lyophilization versus fresh tissue

For tissues homogenization, studies which used lyophilized (freeze-dried) brain tissues did not justify their choice of pre analytical treatment [7, 26], and so, we wanted to compare these two preparations using biological replicate samples (n=5) (Fig. 2a). One mg of lyophilized powder corresponded to  $5.5 \pm 0.5$  mg of wet tissue (n=10).

The workflow for untargeted LC-HRMS metabolomics was conducted as previously described [8]. The number of reproducible features (with a CV<30%) from lyophilized brain tissue compared to wet tissue was basically alike, with 94% of common features (Table 1) (see supplementary data, Fig. A.3). The reproducibility [12] was slightly improved for the lyophilization technique.

**Table 1**

Reproducibility assessment of detected features (ions annotated) in the reverse phase (RP) C18 LC-HRMS analysis in ESI+ and ESI- mode for wet rat brain tissue compared to the same lyophilized tissue (n=5 replicates).

	ESI +		ESI -	
	Wet Tissue	Lyophilized Tissue	Wet Tissue	Lyophilized Tissue
Mean total intensity <sup>a</sup>	$8.9 \cdot 10^{10}$ ( $\pm 0.4 \cdot 10^{10}$ )	$19 \cdot 10^{10}$ ( $\pm 0.2 \cdot 10^{10}$ )	$4.2 \cdot 10^{10}$ ( $\pm 0.09 \cdot 10^{10}$ )	$1.5 \cdot 10^{10}$ ( $\pm 0.5 \cdot 10^{10}$ )

Features with CV<30%	1753	1770	685	695
Mean of CV from ions with CV<30%	14.9%	12.7%	13.7%	9.3%

<sup>a</sup>Total intensity of all features chosen after normalization to the weight of tissue extracted, standard deviation in brackets.

We have also evaluated the impact of lyophilization by NMR analysis (Table 2). As previously described on muscle tissues [10], we found a slightly better NMR baseline on spectra obtained from lyophilized brain extracts compared to wet tissue, as well as a very slight improvement in the reproducibility for buckets under 3 ppm. M.R. Viant and coll. explained that lyophilized samples yielded flatter baselines by the fact that the part of extracted unwanted macromolecules “did not re-solubilize efficiently following lyophilization” [10]. The reproducibility and the intensity of all signals were comparable between these two homogenizations.

**Table 2**

Comparison of wet tissues analysis *versus* lyophilized ones by analysis of <sup>1</sup>H NMR buckets (n=5 replicates)

Extraction method:	Wet tissues	Lyophilized tissues
Total intensity <sup>a</sup>	39.5 (38.4-40.8)	37.5 (36.5-38.5)
Mean CV	3.21%	3.35%
CV% for the 23 buckets <3ppm (8 different buckets */23 buckets) <sup>b</sup>	2.38%	2.13%

<sup>a</sup> Total intensity corresponded to the sum of the integral of all buckets, when the integral of the external reference TSP was fixed at one. <sup>b</sup> non-parametric test (Wilcoxon rank-sum test), \* *p* value < 0.03

Since lyophilized tissues present a slightly better reproducible way of analyzing by LC-HRMS and NMR, and mainly for an easy handling and homogenization

especially for low sample amounts, we conclude that lyophilization is also an efficient way to homogenize and break up brain tissues and cells and made the choice to lyophilize all along this protocol.

### 3.2. Optimization of extraction methods from lyophilized tissues

Tissue samples for a given experiment were always taken from the same rat brain. The reproducibility was tested in independent extractions using lyophilized tissues (Fig. 2b). Several publications have studied several extraction protocol for metabolomics studies of tissue samples [10, 15]. Extraction is frequently done with perchloric acid, with one or two liquid phases [10]. In our case (data not shown), extraction in acidic conditions did not produced reliable results as acidic treatments may damage the structure of metabolites [10], as some metabolites were not described in the studies with perchloric acid extraction procedure [13]. A simple methanolic extraction has been described to be the best method for metabolomics of different animal tissue types [27], while a 2 phases extraction method (methanol/water chloroform) has been suggested by another publication [28]. In this study, we focused on comparing the metabolite extraction efficiency with a liquid/liquid extraction [methanol:dichloromethane:water (2/2/1.4)] to that of two other solvents, *i.e.* methanol:water (1/1) and acetonitrile:water (1/1). We used dichloromethane ( $\text{CH}_2\text{Cl}_2$ ) instead of chloroform for its lower toxicity, and because it is a lipid extracting solvent as good as chloroform [29, 30].

#### 3.2.1. Optimization of extraction methods by LC-HRMS analysis

Chromatographic separation limits the ion suppression effects (*i.e.* changes in analyte ionization due to interfering substances present in biological matrices) [25, 31]. ]. Biphasic condition provided the lower signal intensity, recovered a lower number of metabolites, and was less reproducible (Table 3). This could be explained as proposed by Wang *et al.*, a solvent mixture is not always stable during filtration or centrifugation compared to monophasic solvent leading to an uneven distribution of some compounds [13, 32]. These mean values were obtained on the totality of the features, because for individual observations depending of metabolites classes, results could be inversed, as previously observed [25] (see supporting information, Fig. A.2). For ESI+, each solvent gave only  $50 \pm 10$  specific reproducible features, and 1091 features with a  $CV < 30\%$  (which represents 66% of total features) were recovered by the 3 solvent extractions (see supporting information, Fig. A.3). The same trend was also observed in negative ionization mode (Table 3 and Fig. A.3).

Literature gave a coefficient of variation (for a lower number of features) around 11% with methanolic extraction [27], comparable to what we found. The efficiency of methanol and acetonitrile was almost similar, with a slightly improvement with acetonitrile in negative ionization which provided more reproducible ions with an excellent CV (lower than 5%) compared to methanol (Table 3).

**Table 3**

Reproducibility assessment of detected features (ions annotated) in RP analysis in ESI+ and ESI- mode with three extraction solvents (n=5 replicates). A/W: ACN:H<sub>2</sub>O (1/1); M/W: MeOH:H<sub>2</sub>O (1/1); and M/D/W: MeOH:CH<sub>2</sub>Cl<sub>2</sub>:H<sub>2</sub>O (2/2/1.4).

	ESI +			ESI -		
Extraction method:	A/W	M/W	M/D/W	A/W	M/W	M/D/W

Mean total intensity <sup>a</sup> (standard deviation)	2.0.10 <sup>11</sup> (±3.10 <sup>9</sup> )	15.10 <sup>11</sup> (±4.10 <sup>9</sup> )	1.3.10 <sup>11</sup> (±6.10 <sup>9</sup> )	16.10 <sup>9</sup> (±5.10 <sup>9</sup> )	3.0.10 <sup>9</sup> (±3.10 <sup>8</sup> )	3.1.10 <sup>9</sup> (±0.6.10 <sup>8</sup> )
Features with CV < 5%	230	160	74	282	111	13
Features with CV < 10%	726	709	455	500	442	67
Mean of CV from the (x) features with CV<30%	12.7% (1652)	12.8% (1619)	15.5% (1347)	9.3% (717)	10.6% (721)	17% (445)

<sup>a</sup> Total intensity of all features chosen after normalization to the weight of lyophilized tissue extracted.

### 3.2.2. Optimization of extraction methods by NMR

*Please insert Fig. 3*

We analyzed the total intensity for all buckets for the replicates for each NMR spectra (a typical spectrum is reported in Fig. 3), to compare the experimental reproducibility. Acetonitrile extraction seems to be the more efficient, based on the highest quantity and the highest reproducibility of metabolites extracted (Table 4). Two signals were missing by MeOH:H<sub>2</sub>O compared to the 80 buckets observed with the two others extractions. Almost 67% of all signals presented an excellent reproducibility with a CV<5% with ACN:H<sub>2</sub>O extraction method. The two phases system (CH<sub>2</sub>Cl<sub>2</sub>:MeOH:H<sub>2</sub>O) seemed reproducible as well with a mean CV=5.2%, but this technique recovered around 21% less signal intensity compared to ACN/H<sub>2</sub>O.

These results are similar with those published where it has been shown that ACN gave a better yield extraction on salmon muscle tissue compared to MeOH and a two-phase extraction, and, a lower reproducibility was found when using MeOH [10].

**Table 4**

Analysis of  $^1\text{H}$  NMR buckets for acetonitrile:water (1/1), methanol:water (1/1) and MeOH:CH<sub>2</sub>Cl<sub>2</sub>:H<sub>2</sub>O (2/2/1.4) extraction methods (n=3 replicates)

Extraction method	ACN:H <sub>2</sub> O	MeOH:H <sub>2</sub> O	MeOH:CH <sub>2</sub> Cl <sub>2</sub> :H <sub>2</sub> O
Total intensity <sup>a</sup>	37.0 (36.1-38.0)	33.9 (28.3-37.1)	30.5 (27.4-33.1)
CV < 5%	54/80	45/78	52/80
5<CV% < 10	16/80	10/78	18/80
CV < 15%	79/80	61/78	76/80
Mean CV	4.2%	8.9%	5.2%

<sup>a</sup>Total intensity corresponded to the sum of the integral of all buckets, when the integral of the external reference TSP was fixed at one.

From all results obtained on NMR and LC-HRMS, we chose the acetonitrile extraction method to the final protocol.

### 3.2. Analytical optimization

#### 3.2.1. Impact of weight of lyophilized tissue on LC-HRMS analysis

In order to assess the effect of the mass of lyophilized brain tissue extracted on LC-HRMS sensitivity and precision, samples ranging from 3 to 9 mg were analyzed. Furthermore, brain tissue is a complex matrix which produces high chemical background noise, leading to a significant problem of ion suppression that reduces the sensitivity of LC-HRMS analysis. Because macromolecules (proteins/lipids) create a significant matrix effect, we compared the impact of a pre separation using pipette tip solid-phase extraction (SPE) after an extraction with acetonitrile *versus* liquid extraction alone. Somehow, the signal was not improved by a solid-phase extraction step (mean CV were higher for all masses tested after SPE, Table 5), and intensity of signals were significantly lower (Fig. 4).

Please insert Fig. 4

When experiment could not be obtained by pooled quantity sample, it was necessary to validate if small amount of samples imposed by some experiments (*i.e.* rodent brain region-specific studies and more especially with embryonic studies, given separately for each animal) are relevant and reproducible. The sample weight had no impact on the number of detected features, which remained almost the same (Table 5), but it had an impact on the intensity of these features (Fig. 4) which was higher with more material (around 30% more between 3 mg and 6 mg in ESI+ and ESI- and around 15% more between 6 mg and 9 mg in ESI+ and 20% more in ESI-). In ESI+, the results obtained with 3 mg were similar to those with 6 mg, but for sampling with higher weights (9 mg), we observed an effect of analyte ion suppression/ionization competition with a lower number of features detected (Table 5).

**Table 5**

Reproducibility assessment of detected features (ions annotated) in reverse phase (RP) LC-HRMS analysis in ESI+ and ESI- mode with three different mass of tissue (3, 6, and 9 mg of lyophilized rat brain tissue) and reproducibility for samples using SPE treatment (n=3 replicates).

		ESI +			ESI -		
Without SPE	Weight of tissue	3 mg	6 mg	9 mg	3 mg	6 mg	9 mg
	Features with CV < 5%	1029	1153	507	169	124	250
	Mean of CV from the (x) features with CV < 30%	11.3% (2002)	10.9% (1948)	13.8% (1975)	11.7% (947)	12.7% (931)	9.8% (928)

SPE	Mean of CV (from ions with CV < 30%) for samples using SPE	19.9%	16.1%	15.7%	14.1%	16.0%	8.3%
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With regards to the number of features and the reproducibility, the weight of 3 mg of rat brain lyophilized was enough to make metabolomics in RP LC-HRMS.

### 3.2.3. GC-MS experiment: optimization of methoximation and silylation process

Lyophilized tissues were extracted with ACN:H<sub>2</sub>O. We have done a two-step derivatization. The methoximation step aims at stabilizing molecules that contain ketone and keto-acid groups (protection against decarboxylation). Silylation acts on the molecules that contain labile hydrogen. A typical annotated GC-MS total ion current (TIC) chromatogram of cerebral rat brain tissue was presented in Fig.5. We focused on four conditions depending on reaction temperature (25, 37, 60, or 70°C) and several durations (1, 2, 4, 16h) for the methoximation and silylation processes.

*Please insert Fig. 5*

**Table 6**

Analysis of chosen metabolites for four different two-step GC-MS derivatization methods (n=3 replicates).

	Process 1 <sup>a</sup>	Process 2 <sup>a</sup>	Process 3 <sup>a</sup>	Process 4 <sup>a</sup>
	60°C 1h; 37°C 4h	25°C 2h; 37°C 4h	60°C 2h; 70°C 1h	25°C 2h; 70°C 1h
Total intensity <sup>b</sup>	25 e <sup>06</sup> (± 1 e <sup>06</sup> )	18 e <sup>06</sup> (± 2 e <sup>06</sup> )	16 e <sup>06</sup> (± 6.8 e <sup>06</sup> )	13 e <sup>06</sup> (± 2.4 e <sup>06</sup> )
Mean %CV	18.5%	14.9%	37.0%	30.3%

CV<5%	15/122 <sup>c</sup>	16/127 <sup>c</sup>	-	1/123 <sup>c</sup>
5<CV%<10	37/122 <sup>c</sup>	38/127 <sup>b</sup>	2/115 <sup>c</sup>	5/123 <sup>c</sup>
CV<20% <sup>d</sup>	79/122 <sup>c</sup> (65%)	94/127 <sup>c</sup> (74%)	15/115 <sup>b</sup> (13%)	19/123 <sup>c</sup> (15%)

<sup>a</sup> experimental conditions for methoximation step followed by silylation step. <sup>b</sup> total intensity of all signals chosen after normalization to the weight of lyophilized tissue extracted. <sup>c</sup> number of annotated metabolites and corresponding percentage in brackets. <sup>d</sup> percentage (in brackets) of annotated metabolites

For brain tissue metabolites derivatization, a methoximation at 60°C during 2h followed by a silylation at 70°C (process 3, Table 6) was not the right method, since it provided the highest mean CV (37%) and the lower number of metabolites with a CV lower than 20% (15/115 metabolites). Another silylation at 70°C (process 4, methoximation at 25°C during 2h; silylation 70°C for 1h) seemed deleterious as process 3 and provided the same low reproducibility (CV=30%). Low temperature (37°C) for silylation led to good results. Methoximation at room temperature for 2h followed by a silylation at 37°C for 4h seemed like a suitable protocol for brain tissue analysis with regards to the higher number of metabolites with a CV<20% (94/127, so 74%) leading to a better repeatability (corresponding to the lower mean CV of 15%). The repeatability of the sample workup and analysis should typically be between 7 and 15% for GC-MS-based protocols as mentioned in the literature [33]. Process 2 fulfilled this recommendation.

### 3.3. Application

As a proof of concept, we have studied the global metabolic profile of extracts (6 mg) from three rat brain regions (parietal cortex, frontal cortex and cerebellum) combining complementary analytical platforms for untargeted metabolomics

application. As previously described on rat brain region studied by NMR [34, 35] or LC-MS [36], we found discriminating metabolic profiles depending of the brain region as showed by the non-supervised PCA score plot (Fig. 6). These region-specific clusters were observed by the 3 platforms. The quality of this samples classification was given by the PCA built for 2 components, where the first component (PC1: 53%) explained the separation between cerebellum and the two cortices, while their separation was explained by the second component (PC2: 22%).

The significant metabolic variation between the different rat brain regions was clearly demonstrated when the three analytical platforms were studied together (see on Fig. 6 the loading plot, where each metabolites identified by the different methods contributes to the cluster separation), with a fusion of data [20].

Table 7 summarizes the multivariate analysis features of the different analytical platforms. The processed variables are represented by X (metabolites), Y variables represent the different cerebral samples. The quality of the models was expressed by the cumulative modeled variation in the Y matrix  $R^2Y(\text{cum})$  and the cross validated predictive ability  $Q^2(\text{cum})$  values. Statistical permutation test validations CV-ANOVA (ANalysis Of VAriance testing of Cross-Validated predictive residuals obtained by dividing the data into calibration group and test group) were performed as diagnostic tool for assessing the reliability of OPLS models to select the most reliable method. From Table 7, the fusion of all analytical data show an improvement in the multivariate metabolic discrimination of the three cerebral regions, with a  $p$  value of the CV ANOVA equal to  $5 \times 10^{-09}$ , compared to  $5.0 \times 10^{-07}$  for the ESI+ analysis, the others analytical technics leading to the same  $p = 5.0 \times 10^{-05}$ .

**Table 7**

Summary of statistical values of OPLS-DA of  $^1\text{H-NMR}$ , LC-HRMS ESI+, ESI-, and GC-MS analyses. The number of variables (X), cumulated modelled variations in Y [ $R^2Y(\text{cum})$ ] matrix on analytical datasets, predictability of the model ( $Q^2$ ) and  $p$  value of CV ANOVA as statistical test are given [observations  $N=12$ ].

	Variables (X) <sup>a</sup>	$R^2Y$ (cum)	$Q^2$ (cum)	CV ANOVA $p$ value
Model $^1\text{H NMR}^b$	32	0.88	0.81	5.0 e-05
Model ESI+ <sup>b</sup> :	29	0.96	0.92	5.0 e-07
Model ESI- <sup>c</sup>	15	0.99	0.96	2.0 e-05
Model GC-MS <sup>b</sup>	18	0.88	0.81	5.0 e-05
Model Data Fusion <sup>b</sup> NMR+(ESI+)+(ESI-)+GC-MS	63	0.98	0.96	5.0 e-09

<sup>a</sup> number of variables in the optimized model <sup>b</sup> number of components 2+0+0<sup>c</sup>  
number of components 2+2+0

The complementarity of these analytical platforms is given in Figure 6 in the loading plot where ions or metabolites analyzed by all platforms were necessary to discriminate the three cerebral regions, LC-HRMS, in the two ionization modes, gave 45% of these ions, while NMR and GC-MS represent 28 and 27%, respectively.

*Please insert Fig. 6*

#### 4. Conclusions

Since gathering together data from analytical multiplatform is a recommended approach for metabolomics, sample preparation, metabolite extraction, and

instrumental parameters need to be optimized. The tissue extraction method, which delivered the best compromise for the 3 analytical techniques in terms of number of metabolites observed and their reliability, was acetonitrile on lyophilized tissue. We show that 3 mg of brain extract samples was sufficient for LC-HRMS analysis, with 2002 and 947 features from RP ESI+ and ESI-, respectively, and 51% and 18% of them, respectively, showed a CV < 5%. By <sup>1</sup>H-NMR, 67% of the 80 selected signals detected presented an excellent reproducibility with a CV < 5%. For GC-MS analysis, methoximation at 25°C for 2h followed by a silylation derivatization at 37°C for 4h led to the best results with a mean CV of 15% for 127 features.

This strategy provided a reliable technique for a multiplatform untargeted or targeted metabolomics study. Using the complementary advantages of NMR, GC-MS and LC-HRMS, the proof of concept was done by the analysis of three different rat brain regions. Method standardization is needed for comparison of metabolomics data between laboratories in multi-disciplinary studies, and this, for each biological matrix.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the (doi:xxxxxxxxxxxxxxxx) with more details about Materials and Methods, Elution gradient for RP LC-HRMS; Typical TIC chromatograms obtained by LC-MS; Effects of extraction method on lyophilized rat brain tissue on recovery of some metabolites detected by LC-MS, Venn Diagram analysis. Comparison of LC-MS features in the various analyses.

## Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## List of captions

**Fig. 1.** Workflow protocol for rat brain tissue pre-treatment and optimization of analytical performance.

**Fig. 2.** Workflow protocol for rat brain tissue pre-treatment evaluated by NMR and LC-HRMS a) evaluation between wet and lyophilized tissues, b) evaluation of extraction solvent.

**Fig. 3.** A typical annotated  $^1\text{H}$ -NMR spectrum acquired from extracts of lyophilized rat brain tissue.

**Fig. 4.** Mean total intensity (for features with CV < 30%) obtained from LC-HRMS in ESI+ and ESI- from 3, 6, and 9 mg of rat brain lyophilized tissues after SPE or after extraction alone (n=3 replicates). The 2-sample Wilcoxon rank-sum test revealed that intensity differ significantly ( $*p < 0.05$ ) between SPE and without SPE treatment, except for 9 mg in ESI+.

**Fig. 5.** A typical GC-MS total ion current (TIC) chromatogram obtained from cerebral rat tissue.

**Fig. 6.** Multivariate analysis of the data fusion of 12 NMR, 12 GC-MS and 12 LC-HRMS data analyzing three rat brain regions (each region analyzed in quadruplicate). (a) Principal component analysis (PCA) scatter plot obtained from the illustrating brain region separation. PCA score plot separations of cerebellum (green dots), frontal cortex (blue dots), parietal cortex (red dots). (b) Loading plot obtained from the PCA. Discriminant metabolites coming from the four analytical analyses: ESI- (green dots), ESI+ (blue dots), GC-MS (red dots) and NMR (yellow dots).

