Journal of Neuroscience Methods 201 (2011) 89–97

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Ultra fast *in vivo* microwave irradiation for enhanced metabolic stability of brain biopsy samples during HRMAS NMR analysis

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ARTICLE INFO

Article history:
Received 29 April 2011
Received in revised form 11 July 2011
Accepted 14 July 2011

Keywords:
HRMAS
NMR
Microwaves
Metabolites
Brain
Tissue fixation

ABSTRACT

High resolution magic-angle spinning (HRMAS) NMR spectroscopy is a well established technique for *ex vivo* metabolite investigations but experimental factors such as ischemic delay or mechanical stress due to continuous spinning deserve further investigations. Cortical brain samples from rats that underwent ultrafast *in vivo* microwave irradiation (MWp group) were compared to similar samples that underwent standard nitrogen freezing with and without exposure to domestic microwaves (FN and FN + MWd groups). One dimensional ^{1}H HRMAS NMR spectra were acquired and 16 metabolites of interest were quantified. Within each group 3 samples underwent long lasting acquisition (up to 15 h). Statistically significant differences in metabolite concentrations were observed between groups for metabolites associated to post mortem biochemical changes and/or anaerobic glycolysis including several neurotransmitters. Spectral assessment over time showed a drastic reduction of biochemical variations in both MW groups. Only 2/16 metabolites exhibited significant signal variations after 15 h of continuous spinning and acquisition in the MWp group. This number increased to 10 in the FN group. We confirmed limited anaerobic metabolism and post mortem degradation after ultra fast *in vivo* MW irradiation. Furthermore, spectra obtained after MWp and MWd irradiation exhibited an extremely stable spectral pattern over extended periods of continuous acquisition.

1. Introduction

High resolution magic-angle spinning (HRMAS) NMR spectroscopy is able to provide metabolic information on biological systems using physically heterogeneous biopsy samples. In the neurometabolic field HRMAS NMR is a well established technique for *ex vivo* metabolite identification and quantification. Nonetheless, to achieve optimal experimental conditions, methodological issues such as sample collection, fixation methods, insert preparation or tissue alteration during an extended period of continuous spectral acquisition deserve further studies.

Investigations of cerebral metabolism require proper fixation procedures. Efficient freezing techniques have been described such as freeze-blowing (Veech et al., 1973) or funnel-freezing (Pontén et al., 1973). At the moment, the vast majority of laboratories uses instant freezing in liquid nitrogen to inactivate the metabolism of resected tissue prior to HRMAS acquisition while only a few teams use pulsed microwaves (Risa et al., 2009; Violante et al., 2009). Unfortunately nitrogen freezing does not completely avoid the initiation of ischemic metabolism. A short delay is required to insure freezing within the core of the sample. This delay is relatively long for whole brain fixation as usually performed for neurometabolic animal studies (decapitation immediately followed by snap freezing in liquid nitrogen). The kinetics of ischemic metabolism initiation after resection are known to be tissue dependent and represent a critical issue regarding brain metabolism (Lust et al., 1980; Waters et al., 2000). The brain has a high metabolic rate that requires a continuous supply of glucose and oxygen. Any interruption in the supply of nutriments results in rapid changes of brain metabolites. Rapid post mortem changes have been described in levels of neurotransmitters, cyclic nucleotides, amino acids and intermediary metabolites (Sharpless and Brown, 1978). Using HRMAS NMR, Opstad et al. (2008) recently reported biochemical changes occurring after various delays of ischemia compared to cerebral funnel-freezing. As expected, these authors observed variations in labile metabolite concentrations associated with anaerobic glycolysis such as alanine or lactate. The use of microwave (MW) irradiation to rapidly inactivate brain enzymes

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doi:10.1016/j.jneumeth.2011.07.014

was already reported in the 1970s (Stavinoha et al., 1973). Ultra fast *in vivo* microwave fixation preserves cellular structures with minimal alteration of cellular biochemistry (Ainley and Ironside, 1994). Nowadays microwave devices deliver pulsed microwaves (typically 5–10 kW) that are able to transiently elevate brain temperature to 80–90 °C during less than to 2 s. This leads to a permanent inactivation of enzymes, thereby minimizing enzyme-dependent post-mortem metabolic changes. This technique is now regarded as a reference method for brain metabolism inactivation by several groups in the NMR neurometabolic field (Risa et al., 2009; Violante et al., 2009; De Graaf et al., 2009). Although such issues have been extensively studied in the 1970s, they remain understudied by HRMAS NMR.

Another critical methodological issue regarding HRMAS NMR experiments deals with acquisition conditions. Schematically *in situ* biochemical variations during HRMAS NMR acquisition are dependent on tissue sample, temperature, spinning rate and experimental duration (Waters et al., 2000; Burns et al., 2005; Taylor et al., 2003; Wu et al., 2003). Opstad et al. (2008) suggested that extended spinning periods during acquisition might be the major driving factor for *in situ* biochemical variations. According to this team, tissue ischemia during biopsy excision and delay in snap-freezing may have less significant effects on metabolite profile than prolonged spinning times. The authors evoked an element of mechanical damage that may explain some of these biochemical changes. In particular, total creatine increased in an asymptotic exponential manner that suggested the release of previously NMR-invisible bound creatine due to mechanical stress. Prolonged spinning times are required for two-dimensional HRMAS NMR acquisition. Extended periods of spinning are also required when performing *ex vivo* ^{13}C HRMAS NMR spectroscopy after infusion of ^{13}C labeled substrates. Hence for HRMAS NMR, the question remains on how biochemical changes induced by extended periods of mechanical stress upon biopsy samples could be limited by the use of appropriate fixation methods.

The first objective of the present study was to compare ^{1}H NMR spectra obtained on cerebral biopsies using nitrogen freezing and ultra fast *in vivo* MW fixation to confirm the advantages of the latter technique for HRMAS NMR analysis. Such an experiment may also contribute to identify metabolite artifacts induced by the ischemic delay when freezing in liquid nitrogen is used. The second objective was to assess the NMR signal stability over an extended period of continuous HRMAS acquisition (up to 15 h) for biopsy samples obtained with these two fixation techniques. We finally added a group of animals that underwent immediate nitrogen freezing followed by microwave irradiation in a domestic microwave oven to confirm the effect of previous samples heating upon NMR signal stability during long HRMAS NMR acquisition.

2. Methods

2.1. Tissue collection and sample preparation

Twenty adults male Wistar rats were selected for the study (weight ~250–300 g). The animals were maintained at room temperature (22 °C) under a 12-h/12-h normal light/dark cycle (light on at 7:00 a.m.) with food and water *ad libitum*. All animal experimentations were performed in accordance with the rules of the European Community Council Directive of November 24, 1986 (86/609/EEC) and the French Department of Agriculture (License N° 67/97, AN). Six rats were subjected to ultra fast *in vivo* microwave fixation (MWp group) using a dedicated device with focused head beam (4 kW, 2.45 GHz, 1.8 s, Püschner Gmbh, Schwanewede, Germany). The delivery of high output and pulsed microwaves kills the animal instantaneously (MWp group). Fourteen rats underwent instant neck dislocation, followed by decapitation and the heads were immediately snap frozen in liquid nitrogen. No surgical procedure or anesthetic medications were applied before sacrifice. Then, the brains were taken out of the skull, put on ice and regions of interest (ROIs) were punched out in order to prepare inserts. In seven of these 14 rats, ROIs were punched out without any additional treatment (FN group) while the other seven rat brain samples, were placed into a domestic microwave oven during 20 s with an energy output of 850 W (Whirlpool, Benton Harbour, USA; FN + MWd group).

For each animal, two biopsy samples from the frontal cortex were introduced into a 30 μL KelF insert (weight 15–20 mg). To provide a lock frequency, 10 μL of D_2O were added to the insert. The insert insures that the entire biopsy is detected by the radio-frequency coil of the probe and that no leaks occur during the HRMAS analysis. The inserts containing the biopsy were then stored at −80 °C. Shortly before the HRMAS analysis, the insert was placed into a standard 4 mm ZrO_2 rotor and closed with a cap. The ensemble was then inserted into a HRMAS probe. The rotor preparation followed the procedure reported by our research group (Piotto et al., 2008). A total number of 14 inserts were obtained for the FN group, 14 for the FN + MWd group and 9 for the MWp group. HRMAS ^{1}H NMR spectra were acquired for these samples. Among each group 3 samples underwent a NMR signal stability assay over time.

2.2. Data acquisition

HRMAS spectra were recorded on a Bruker Avance III 500 spectrometer operating at a proton frequency of 500.13 MHz. The spectrometer is equipped with a 4 mm double resonance (^{1}H, ^{13}C) gradient HRMAS probe. A Bruker Cooling Unit is used to regulate the temperature at 4 °C by cooling down the bearing air flowing into the probe. All NMR experiments were conducted on samples spinning at 3502 Hz to keep the rotation sidebands out of the spectral region of interest.

For each biopsy sample, a one-dimensional proton spectrum using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was acquired as previously reported (Piotto et al., 2008). The inter-pulse delay between the 180° pulses of the CPMG pulse train was synchronized with the sample and set to 285 μs ($1/\omega = 1/3502 = 285$ μs) to eliminate signal losses due to B_1 inhomogeneities (Elbayed et al., 2005; Piotto et al., 2001). The number of loops was set to 328 giving the CPMG pulse train a total length of 93 ms. Parameters for the CPMG experiment were: sweep width 14.2 ppm, number of points 32 k, relaxation delay 2 s and acquisition time 2.3 s. A total of 128 FID were acquired resulting in an acquisition time of 10 min. All spectra were recorded in such a manner that only a zero phase order correction was necessary to properly phase the spectrum. The FID was multiplied by an exponential weighing function corresponding to a line broadening of 0.3 Hz prior to Fourier transformation. All spectra were processed using automated baseline correction routines. Spectra were referenced by setting the lactate doublet chemical shift to 1.33 ppm. Signal stability assay over time within each sample used the above described parameter according to the following sequence of acquisition: 1D acquisition every 10 min during the first 2 h and then at 4, 6, 12 and 15 h (16 acquisitions per sample, total of 96 spectra).

2.3. Metabolite quantification

Quantification was performed for 16 metabolites using an external reference standard of lactate. Spectra were normalized according to sample weight. Peaks of interest were automatically defined by an in house program using MATLAB 7.0 (Mathworks, Natik, United States). Peak integration was then compared to the

one obtained with the lactate reference and were corrected according to the number of protons. Results were expressed in nmol mg^{-1} of tissue. Chemical shifts for regions of interest were adapted from the work reported by Martinez-Bisbal et al. (2004). For each metabolite, the region of interest and proton number (according to the chemical function) were defined as follows: acetate (Ac; [1.918; 1.927], CH_3), alanine (Ala; [1.459; 1.497], βCH_3), aspartate (Asp; [2.760; 2.85], βCH_2), choline (Cho; [3.193; 3,207], $N(CH_3)_3$), creatine (Cr; [3.921; 3.941], CH_2), γ-aminobutyric acid (GABA; [2.268; 2.319], CH_2), glutamine (Gln; [2.418; 2.465], γCH_2), glutamate (Glu; [2.037; 2.080], βCH_2), glycine (Gly; [3.553; 3.568], αCH), lactate (Lac; [1.305; 1.350], CH_3), myo-inositol (mIns; [3.517; 3.552], C1H, C3H), N-acetylaspartate (NAA; [2.006; 2.029], CH_3), phosphocholine (PCho; [3.213; 3.228], $N(CH_3)_3$), phosphocreatine (PCr; [3.945; 3.958], CH_2), taurine (Tau; [3.401; 3.447], CH_2-SO_3), valine (Val; [1.025; 1.061], γCH_3).

2.4. Data analysis

For inter-group analysis, concentrations in nmol mg^{-1} of tissue were compared using an ANOVA across groups followed by Fischer post-hoc tests as appropriate. For intra-group analysis, values were analyzed using a non-parametric test because the number of samples (3 in each group) was low and the variances were not equal. Thus, a Friedmann test was used for within groups' comparisons, followed by a post hoc modified Mann–Whitney *U*-test.

Two types of principal component analyses (PCA) were also performed. Such analysis was already detailed by our research group (Piotto et al., 2008). For the first PCA, spectral region between 4.7 and 0.5 ppm of each 1D CMPG NMR spectrum was automatically binned into regions of 0.01 ppm using the AMIX 3.8 software (Bruker GmBH, Germany). Each 0.01 ppm region was computed and normalized according to the total integral of the spectrum. This process generated a *X*-matrix containing the chemical shifts as columns (421*X* variables) and each tissue sample as rows. This *X* matrix was analyzed using PCA. The robustness of the PCA model was assessed using R^2X (the fraction of the sum of squares of all the *X*s explained by the component) and the goodness of fit evaluated by Q^2 (the fraction of the total variation of the *X*s that can be predicted by a component as estimated by cross-validation). Q^2 is computed as (1 − PRESS/SS) where PRESS is the predicted squared sum of error and represents the squared differences between observed and predicted values and SS is the residual sum of squares. Since the number of samples is relatively small regarding the number of variables from binned spectra, an extensive cross-validation of the analysis is mandatory to avoid overfitting of the data. The maximum theoretical value for Q^2 is equal to 1 for a perfect prediction. A Q^2 value superior to 0.5 is generally considered as a good predictor. Actually parameters are estimated on one part of the matrix and the goodness of the parameters tested in terms of their success in the prediction of another part of the matrix. Data sets were analyzed within the software package SIMCA P (Umetrics AB, Umea, Sweden). A second PCA was performed the same way excepted that instead of integrating region between 4.7 and 0.5 ppm, only the 16 metabolites regions of interest were retained (regions are mentioned in Section 2.3).

3. Results

3.1. Metabolomics at the time of specimen collection (t = 10 min)

Fig. 1 reports examples of NMR spectra after different acquisition times (one sample for each group after 10 min, 1 h, 4 h and 15 h of continuous acquisition, respectively). A qualitative comparison of spectral profiles between nitrogen frozen samples (FN group) and pulsed microwave irradiated animals (MWp group) shows significant differences after 10 min of continuous acquisition. Quantitative values after the same acquisition time are summarized in Table 1. One striking result was the difference in Lac levels. Lac was largely reduced in the MWp group compared to the FN group. In the same way, PCr levels were reduced by 82% in the FN group. Concentration of these two metabolites in the FN + MWd group was not significantly different compare to the FN group. After a 10 min acquisition period, significant differences were observed for Ala, Asp, Cho, GABA, Gln, Glu, Lac, mIns, NAA, PChol, PCr between the FN and MWp groups. No significant variations were observed for Ac, Cr, Gly, Tau, Val. A principal component analysis (PCA) performed on spectra acquired after 10 min is reported in the scatter plot of Fig. 2. This figure illustrates the first two components based on the whole NMR spectra (0.5–4.7 ppm; left panel) or on specific regions corresponding to the 16 metabolites studied (right panel). Fig. 2A and B represents a PCA analysis that included all groups, Fig. 2C–H represents a group by group analysis. For each model only the first two components were included. R^2X and Q^2 values were: $R^2X = 0.504$, $Q^2 = 0.427$ for Fig. 2A; $R^2X = 0.584$, $Q^2 = 0.404$ for Fig. 2B; $R^2X = 0.83$, $Q^2 = 0.80$ for Fig. 2C; $R^2X = 0.89$, $Q^2 = 0.80$ for Fig. 2D; $R^2X = 0.524$, $Q^2 = 0.375$ for Fig. 2E; $R^2X = 0.513$, $Q^2 = 0.354$ for Fig. 2F; $R^2X = 0.440$, $Q^2 = 0.237$ for Fig. 2G; $R^2X = 0.458$, $Q^2 = 0.217$ for Fig. 2H. Results reported for Fig. 2C and D showed that these models were able to convincingly separate the FN from the MWp group ($Q^2 > 0.5$). For the other models, the first two components were barely able to separate groups but not as significantly as for Fig. 2C and D since Q^2 values were never superior to 0.5.

3.2. Signal stability over time

As depicted in Fig. 1, spectra from the MWp group were relatively constant throughout the acquisition period compared to those of the FN group even after several hours of continuous spinning (the same was observed to a lesser extend for the FN + Mwd group). For example, signals associated to Ac and NAA were much more stable in the MWp group than in the FN group after 4 h of continuous spinning. Visually, several metabolite signals seemed to increase or decrease more largely over time in the FN group, whereas a more stable pattern was observed in the MWp and FN + MWd groups (i.e. Ac, Ala, NAA, Gly, mIns, PCho).

Metabolite concentration changes over time during continuous HRMAS experiments (up to 15 h of spinning) are represented in Fig. 3. In the FN group, almost every metabolite signal tended to increase during acquisition, whereas most metabolite concentration tended to remain stable over time in the MWp and FN + MWd groups. Only PCr in the MWp group and NAA in the FN group tended to decrease. In the FN + MWd group most metabolite concentrations exhibited a pattern similar to that obtained in the MWp group (Ac, Ala, Asp, Cho, GABA, Gly, PCho, PCr, Tau and Val) although some metabolites increased over time (Cr, Gln, Glu, Lac). In addition, in the FN + MWd group, Cr, Gln, and Lac concentrations followed a pattern quite similar to that of the FN group (Fig. 3).

To assess NMR signal stability over time, we performed intra-groups comparisons. Table 2 reports the statistically significant changes within each group after 1 h, 4 h and 15 h compared to the first acquisition. The number of statistically significant changes in metabolite concentration in the FN group increased over time: 3 metabolites after 1 h (GABA, Gln, Gly), 5 after 4 h (Ala, mIns), most metabolites after 15 h (except Cho, Glu, NAA, PCr and Tau). In the MWp group, only the signal of Cr and PCr changed significantly after 1 h and 4 h, respectively. Only those two metabolites exhibited statistically significant signal changes after 15 h of continuous spinning in the MWp group. The number of significant metabolite concentrations changes in the FN + MWd group went from 2 after 1 h (Gln, Lac) to 4 after 4 h (+Cr, Glu) and 6 after 15 h (+mIns, NAA).

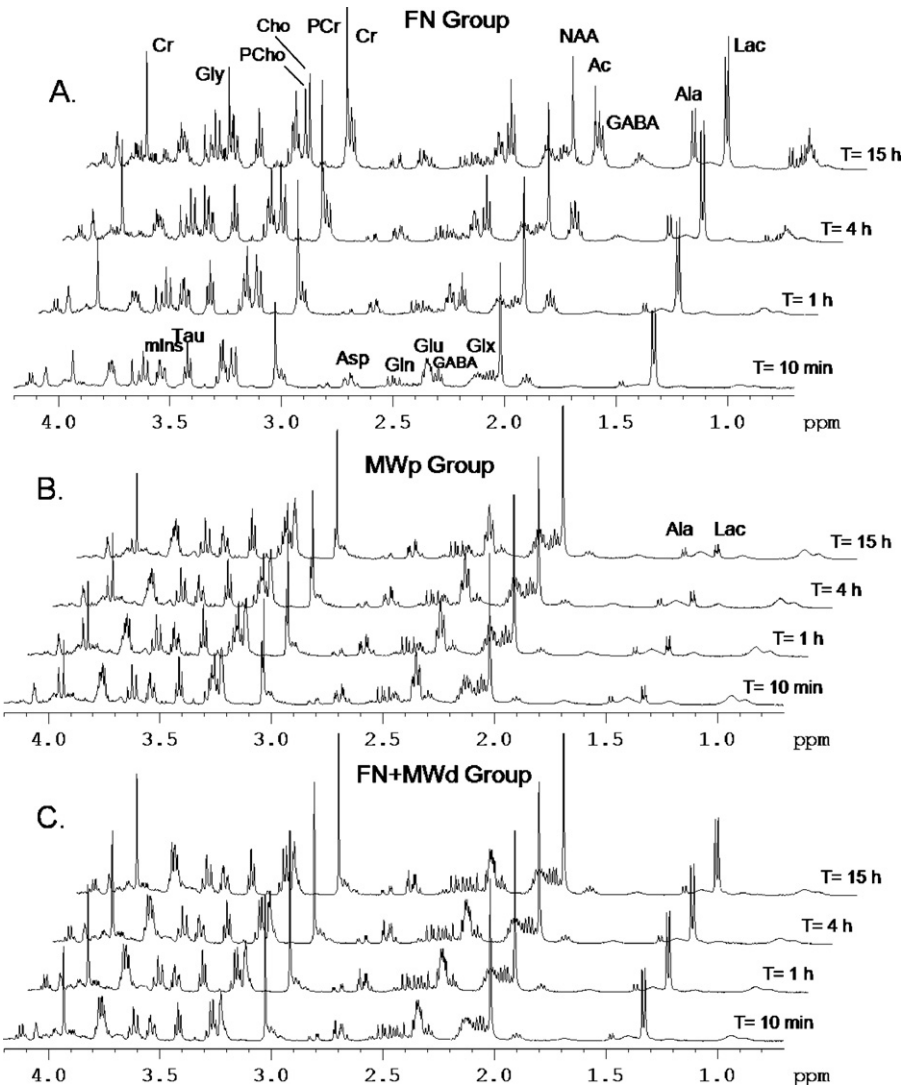

Fig. 1. Stability pattern of HRMAS ^{1}H NMR spectra over time after simple nitrogen freezing (A, FN group), after ultra fast MW irradiation (B, MWp group) and after nitrogen freezing and domestic MW irradiation (C, FN + MWd group). For each group represented spectra were acquired after 10 min, 1 h, 4 h and 15 h of continuous spinning. *Abbreviations*: Ac, acetate; Ala, alanine; Asp, aspartate; Cho, choline; Cr, creatine; GABA, γ-aminobutyric acid; Gln, glutamine; Glu, glutamate; Glx: glutamate + glutamine; Gly, glycine; Lac, lactate; mIns, myo-inositol; NAA, N-acetylaspartate; PCho, phosphorylcholine; PCr, phosphocreatine; Tau, taurine; Val, valine.

The scatter plot from the PCA analysis is represented in Fig. 4. This analysis was performed on all spectra acquired over time for each group. The left panel is based on whole NMR spectra (0.5–4.7 ppm) while the right panel is based on specific spectral regions from the 16 metabolites studied. In both cases, the first two components of the PCA were able to separate convincingly the groups over time using an unsupervised multivariate data analysis. For each model, the PCA analysis was characterized by the following parameters: (Fig. 4A) $R^2X = 0.769$, $Q^2 = 0.758$; (Fig. 4B) $R^2X = 0.693$, $Q^2 = 0.640$; (Fig. 4C) $R^2X = 0.741$, $Q^2 = 0.730$; (Fig. 4D) $R^2X = 0.654$, $Q^2 = 0.627$; (Fig. 4E) $R^2X = 0.617$, $Q^2 = 0.600$; (Fig. 4F) $R^2X = 0.570$, $Q^2 = 0.528$. PCA was able not only to convincingly separate biopsy samples according to their fixation method but also to represent the large dispersion of the signals acquired over time for a single biopsy in the FN group compared to the relatively narrow dispersion for biopsies from both MW groups.

4. Discussion

Our study assesses the efficiency of ultra fast *in vivo* MW irradiation on samples prepared for HRMAS NMR analysis. We report two major advantages of such procedure: (i) limitation of ischemic metabolism initiation; (ii) long term fixation properties of *in vivo* MW on rat brain samples even in relatively stringent conditions of spinning.

The first striking result comes from metabolite signals associated to ischemia or post mortem degradation. The concentration of some labile metabolites was much lower in the MWp group than in the FN group. This was the case of Ala, Lac and PCr that have been associated with the initiation of post mortem metabolism mainly due to anaerobic glycolysis. The low level of PCr in the FN group may confirm the breakdown of this compound during the early phase of ischemia to maintain ATP at a constant level (Lust et al., 1980). Almost no Lac could be found in the samples from the MWp group compared to the FN group. Likewise, GABA remained stable in the MWp group over time while it increased in the FN group. This is in accordance with previous data showing that GABA increases in a few minutes after rodent decapitation as a result of the activity of the glutamic acid decarboxylase pathway (Geddes et al., 1999). Moreover, the increase of GABA has been reported in autoptic brain tissues compared to fresh human biopsy samples (Knörle et al., 1997). This first comparison highlights the advantage

Table 1
Intergroups comparisons of metabolites concentrations at the time of tissue collection (after 10 min of acquisition).

Metabolite	Concentration (nmol mg^{-1} tissue) (±SD)			
	FN group (n = 13)	MWp group (n = 9)	FN + MWd group (n = 8)	MWp vs FN + MWd value
Acetate	0.24 ± 0.06	0.39 ± 0.31	0.16 ± 0.01	0.027
Alanine	0.47 ± 0.05	0.80 ± 0.27**	0.68 ± 0.03#	NS
Aspartic acid	1.58 ± 0.53	2.41 ± 0.37**	2.22 ± 0.12##	NS
Choline	0.57 ± 0.18	0.30 ± 0.10**	0.31 ± 0.01##	NS
Creatine	4.16 ± 0.56	3.93 ± 1.01	5.08 ± 0.38#	0.005
GABA	2.63 ± 0.41	3.15 ± 0.63*	3.63 ± 0.07##.	0.0036
Glutamine	2.38 ± 0.30	3.31 ± 0.68**	4.57 ± 0.27##	<0.001
Glutamate	7.21 ± 1.16	9.65 ± 1.04**	9.74 ± 0.36##	NS
Glycine	1.89 ± 0.34	1.81 ± 0.94	1.37 ± 0.09	NS
Lactate	4.85 ± 0.48	1.90 ± 0.64**	5.06 ± 0.22	<0.001
myo-Inositol	5.21 ± 0.49	4.14 ± 0.54**	4.51 ± 0.17#	NS
NAA	4.77 ± 0.52	5.42 ± 0.62*	5.74 ± 0.27##	NS
Phosphocholine	0.68 ± 0.10	0.93 ± 0.25*	1.14 ± 0.02##	0.009
Phosphocreatine	0.25 ± 0.06	1.18 ± 0.63**	0.15 ± 0.02	<0.001
Taurine	5.02 ± 1.03	4.27 ± 0.66	4.63 ± 0.26	NS
Valine	0.008 ± 0.07	0.094 ± 0.11	0.06 ± 0.01##	<0.001

Metabolite concentration (nmol mg^{-1} of tissue) measured by HR-MAS ^{1}H NMR spectroscopy in cortical biopsy samples from Wistar rats after nitrogen freezing (FN group), after prior ultra fast *in vivo* MW irradiation (MW group) and after nitrogen freezing and exposure to domestic microwaves (FN + MWd). Results are presented as means ± SD.
* Statistical significance difference between the FN group and the MWp group at $p < 0.05$ (post hoc Fischer test).
** Statistical significance difference between the FN group and the MWp group at $p < 0.001$ (post hoc Fischer test).
# Statistical significance difference between the FN group and the FN + MWd group at $p < 0.05$ (post hoc Fischer test).
Statistical significance difference between the FN group and the FN + MWd group at $p < 0.001$ (post hoc Fischer test).
The right column reports p value of the statistical results between the MWp and the FN + MWd group (post hoc Fischer test).

of ultra fast MW irradiation over simple nitrogen freezing mainly by avoiding anaerobic metabolism initiation and subsequent post mortem degradation as a result of irreversible enzyme inactivation (Medina et al., 1980). Our results are coherent with those of the recent report by De Graaf et al. (2009) who used *in situ* 3D MRI to investigate the effect of focused beam MW irradiation on post mortem modifications. This group demonstrated that MW irradiated rat brains possess the structural integrity and temporal stability allowing acquisition of high-resolution *in situ* MR spectra and images that reflect the *in vivo* condition. This has previously been suggested by histopathological studies (Login and Dvorak, 1994). Moreover, we found lower concentrations for some neurotransmitters (Asp, GABA and Glu) in the FN compared to the other groups. The cerebral metabolite concentrations assessed in this study by ^{1}H HRMAS NMR after ultra fast MW irradiation are within the range of a comparable study recently published (Risa et al., 2009). Thus, the fixation properties of pulsed high energy MW might be extremely valuable in sample preparation before HRMAS analysis. The absolute concentrations might be influenced by the amount of D_2O added which may affect metabolite extraction from the tissue sample to the water. However, the quantity of D_2O added to the samples was similar in all study conditions. Nonetheless our data confirm that, by using the technique of enzyme inactivation by pulsed MW irradiation, a far more accurate determination of endogenous cerebral metabolites should be achievable.

On the other hand, it has been suggested that temperature elevation during a few seconds may induce biomolecules trapping. In the case of HRMAS NMR analysis, metabolites trapped to adjacent proteins may modify the proportion between NMR visible and invisible parts of a given metabolite. This might explain why Cho, Lac, mIns were significantly reduced in the MWp compared to the FN group. However, this does not happen with brain prostanoids which, however, have a higher molecular weight (Golovko and Murphy, 2008). Additional investigations using for example labeled metabolites might be necessary.

Regarding the FN + MWd group, metabolite concentrations can be affected by both freezing and longer-lasting microwave irradiation and hence heating of the sample. They are more widespread

Table 2
Summary of significant statistical changes in metabolite concentration over time in cortical biopsy samples from Wistar rats after nitrogen freezing without (FN group), after ultra fast MW irradiation (MWp group) and after domestic MW irradiation (FN + MWd group). Intragroup comparison using non-parametric test for paired sample, see Section 2.

Metabolite	Nitrogen freezing (FN group, n = 3)			Pulsed Microwaves (MWp group, n = 3)			Domestic Microwaves (FN + MWd group, n = 3)		
	10 min vs 1 h	10 min vs 4 h	10 min vs 15 h	10 min vs 1 h	10 min vs 4 h	10 min vs 15 h	10 min vs 1 h	10 min vs 4 h	10 min vs 15 h
Acetate	–	–	<0.05+	–	–	–	–	–	–
Alanine	–	<0.05+	<0.005+	–	–	–	–	–	–
Aspartic Acid	–	–	<0.05+	–	–	–	–	–	–
Choline	–	–	–	–	–	–	–	–	–
Creatine	–	–	<0.05+	<0.05+	<0.05+	<0.05+	–	<0.05+	<0.05+
GABA	<0.05+	<0.05+	<0.05+	–	–	–	–	–	–
Glutamine	<0.05+	<0.05+	<0.05+	–	–	–	<0.05+	<0.005+	<0.005+
Glutamate	–	–	–	–	–	–	–	<0.05+	<0.005+
Glycine	<0.05+	<0.05+	<0.01+	–	–	–	–	–	–
Lactate	–	–	<0.05+	–	–	–	<0.05+	<0.05+	<0.05+
myo-Inositol	–	<0.05+	<0.05+	–	–	–	–	–	<0.05+
NAA	–	–	–	–	–	–	–	–	<0.005+
Phosphocholine	–	–	<0.05+	–	–	–	–	–	–
Phosphocreatine	–	–	–	–	<0.05–	<0.05–	–	–	–
Taurine	–	–	–	–	–	–	–	–	–
Valine	–	–	<0.05+	–	–	–	–	–	–

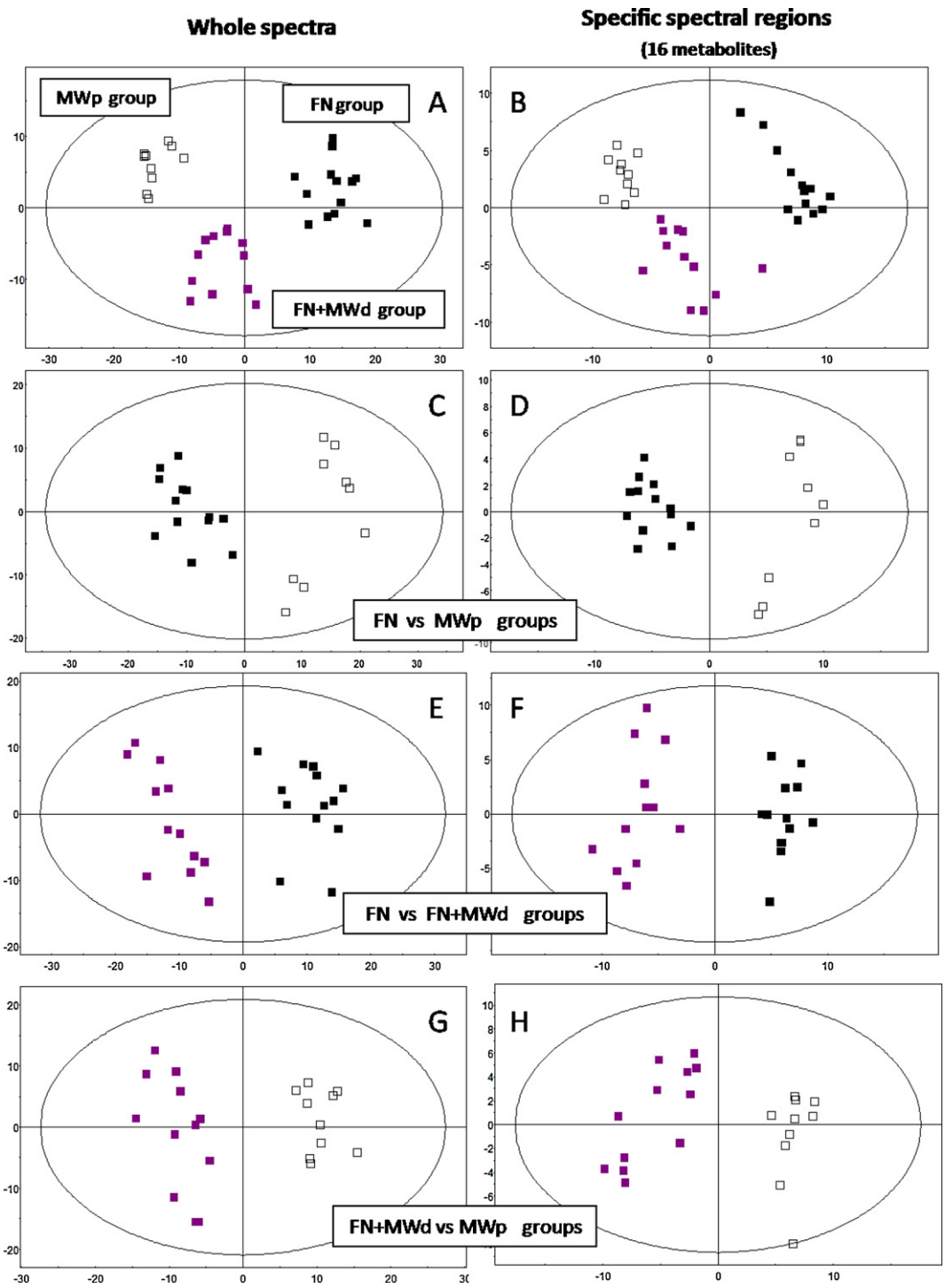

Fig. 2. PCA model based on whole ^{1}H HRMAS NMR spectra (left panel; A, C, E, G) or spectral regions from 16 metabolites (right panel; B, D, F, H) after the first acquisition (t = 10 min) of cortical cerebral samples from Wistar rats after ultra fast *in vivo* MW irradiation (MWp group, open squares), after nitrogen freezing (FN group, black squares) and after nitrogen freezing and domestic MW irradiation (FN + MWd group, purplesquares). For each model R^2X and Q^2 values are reported in Section 3.

and close to FN or MWp values, depending on the metabolite. To our best knowledge, this is the first HRMAS NMR study using combined nitrogen freezing and domestic microwave technology which needs further investigation.

Another great advantage of MW irradiated brain samples for HRMAS NMR analysis stems from their extreme spectral stability over time under long-lasting continuous spinning conditions. MW irradiated samples exhibited a marked stable metabolomic pattern even after numerous hours of continuous acquisition. Such results were observed when applying ultra fast and pulsed MW on living rodent (MWp group) but it was also achieved, to a lesser extent though, using domestic MW after nitrogen freezing (FN + MWd group). We showed that only 2 out of the 16 studied metabolites exhibited a significant signal change after a 15 h period of continuous acquisition in the MWp group, whereas this number raised up to 11 in the FN group. The combined use of nitrogen freezing and domestic microwave (FN + MWd) also showed signal stabilization over time and led to intermediate data, with 4 metabolites exhibit-

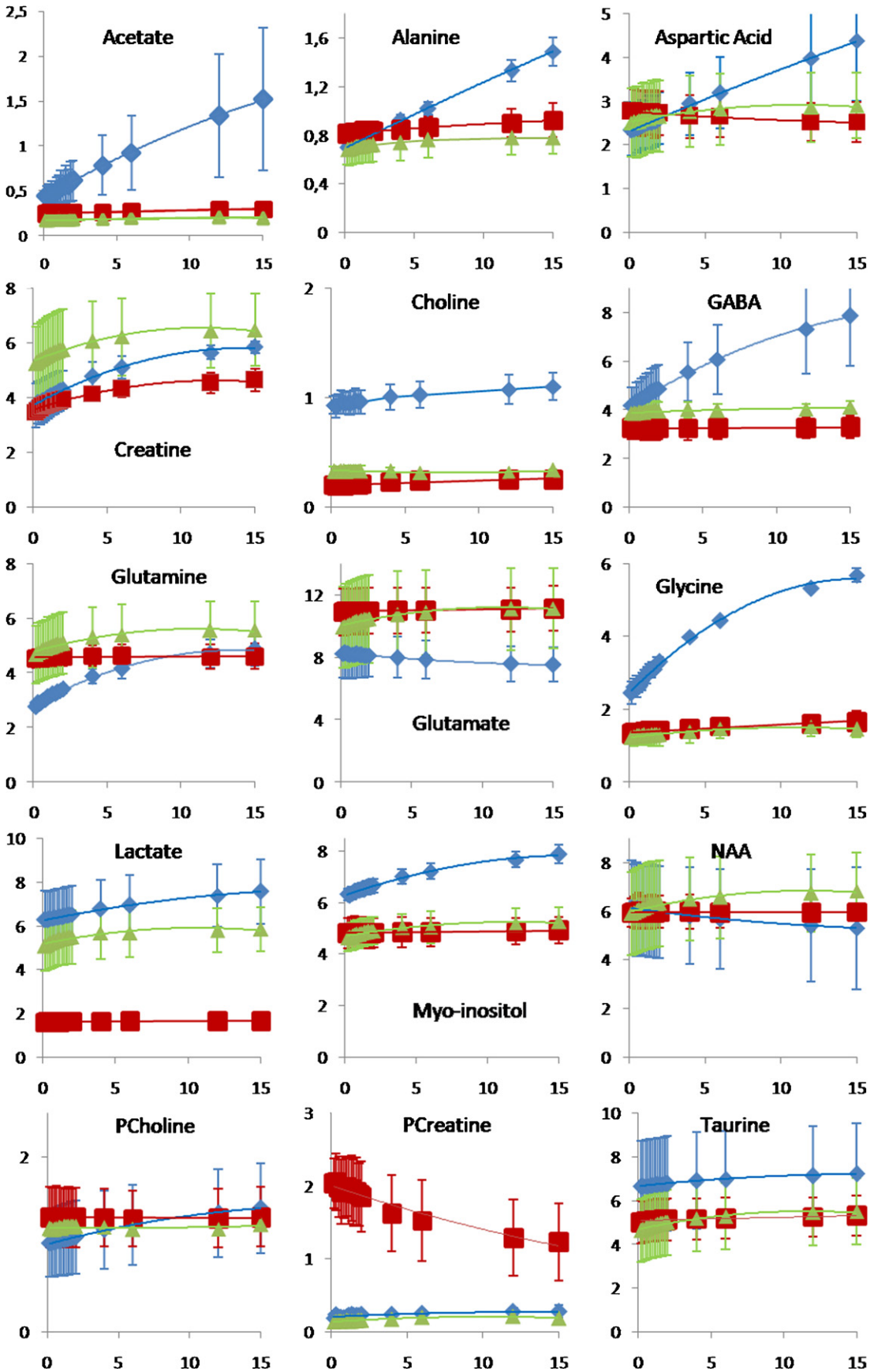

Fig. 3. Comparison of relative metabolite concentration changes over time during continous HRMAS experiments after ultra fast *in vivo* MW irradiation (MWp group, squares, $n = 3$), after nitrogen freezing (FN group, diamonds, $n = 3$) and after nitrogen freezing and domestic MW irradiation (FN + MWd group, triangles, $n = 3$). Spectra were acquired every 10 min during the first 2 h and at 4, 6, 12 and 15 h. The vertical axis represents metabolite concentration in nmol mg^{-1} of tissue. Standard deviation are represented in each group. The horizontal axis represents time (h).

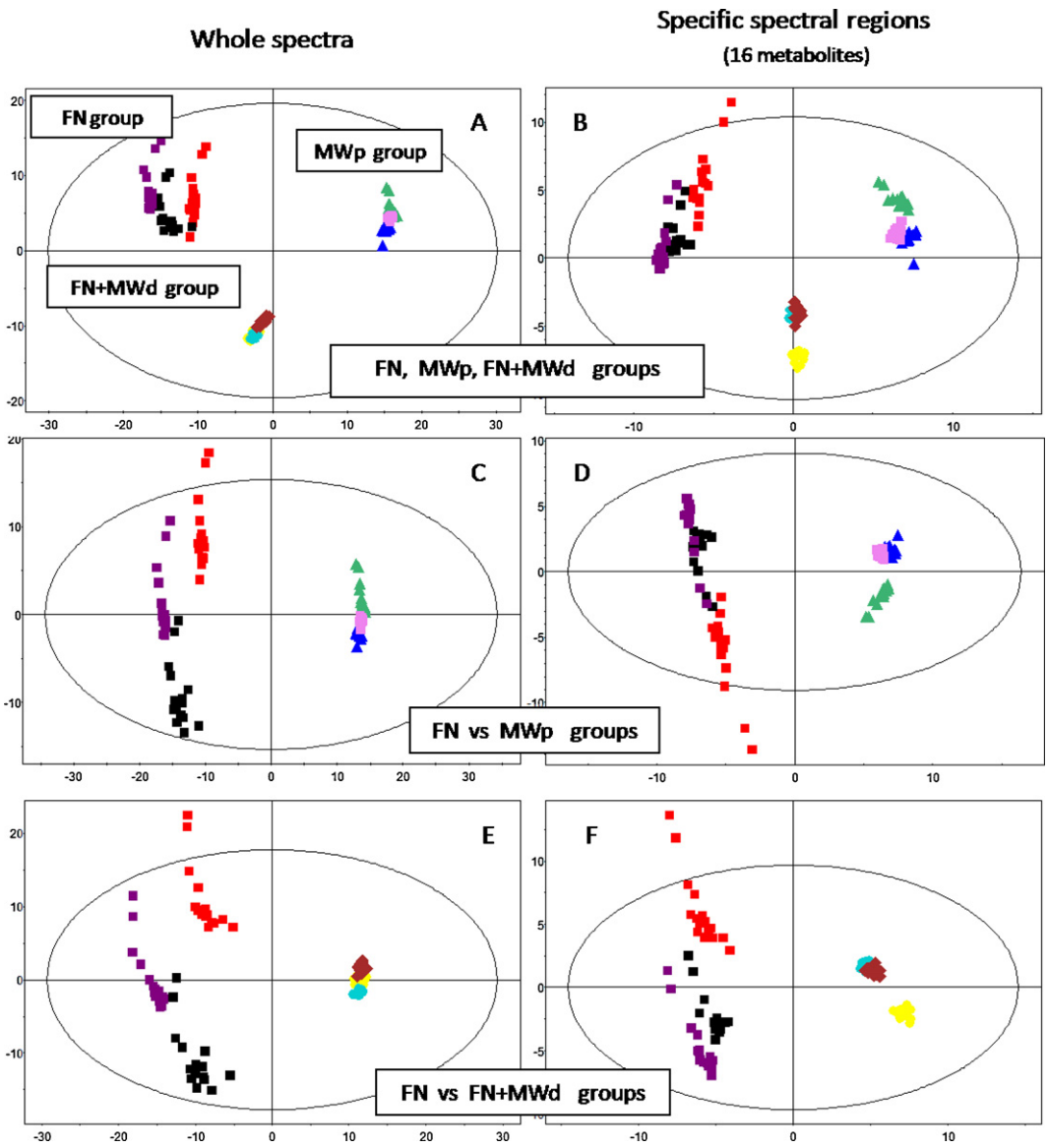

Fig. 4. PCA model based on whole ^{1}H HRMAS NMR spectra (left panel; A, C, E) or on specific spectral regions of 16 metabolites (right panel; B, D, F) from 13 consecutive acquisitions (9 during the first 2 h and then at 4, 6, 12 and 15 h) of cortical cerebral samples from Wistar rats after ultrafast *in vivo* MW irradiation (MWp group), after immediate nitrogen freezing (FN group) and after nitrogen freezing followed by domestic MW irradiation (FN + MWd group). Each color represents the 13 consecutive acquired spectra from one cerebral biopsy (3 times, 13 spectra per group).

ing signal changes after 4 h of continuous spinning. This number rose to 6 after 15 h.

The mechanism by which MW irradiations make biological samples so stable under continuous spinning has not yet been clarified. *In situ* biochemical variations observed during HRMAS acquisition may be due to several factors. One factor might be a progressive degradation of the sample. Since these samples are usually not fixed before HRMAS analysis, several enzymes might have a residual activity during acquisition even at 4 °C. Another factor concerns mechanical stress induced by continuous spinning. Deleterious effects of high spinning rate (5 kHz) on cerebral samples have already been suggested for relatively long periods of acquisition (Opstad et al., 2008). This mechanical stress may lead to biochemical changes and/or spectral variations during the experiment. Such effects might be related to the concept of NMR-visible and -invisible metabolites. Cases of NMR-invisible metabolites have been reported for Cr, Lac (De Graaf et al., 1999) and Glu (Kauppinen and Williams, 1991). This phenomenon is thought to rely, at least partly, on environment, ability to bind to adjacent proteins and magnetic transfer properties of metabolites (De Graaf et al., 1999; Middleton et al., 1998). It is known that pulsed MW irradiation elevates the temperature of the sample (near 80 °C) during a very short period (less than 2 s) and denaturates proteins in an irreversible manner. In turn, samples lose water and their mechanical properties are modified. As a consequence biological samples may be more "resistant" towards continuous spinning, and less prone to biochemical variations. This might also be true for domestic microwave although irradiation is not focused, provides less power and is 10-fold longer. Nonetheless NMR signal stabilization could also be observed in the FN + MWd group. A typical illustration is the variation of the PCho level. PCho is suggested to be a marker of cellular damage (Opstad et al., 2008). In the present study, PCho concentration remained stable over time in both MW groups even after 15 h while it increased significantly and progressively over time in the FN group. This change could reflect spinning induced mechanical cell damage and/or biochemical variations, as suggested previously (Opstad et al., 2008).

Hence pulsed high energy MW may limit cellular damage induced by spinning that in turn modifies the proportions of visible and invisible metabolites. mIns has been shown to remain stable for over 24 h postmortem in bovine brain homogenates (Michaelis et al., 1996) and rabbit brain (Petroff et al., 1988). Here, the concentration of the latter metabolite only increased in the FN group, most likely as a result of lack of tissue fixation and prolonged spinning. Another example is Gln that was reported to be stable in biopsied human brain tissue when maintained at 35 °C over a 4 h period (Knörle et al., 1997; Perry et al., 1981). In this study, in the first 4 h Gln increased in the FN group and remained stable in the MWp group. NAA decreased in the FN group and was stable in the MWp group. On the other hand, concentration of Gly has been shown to be superior in autoptic brain tissue compared to fresh cerebral biopsies samples (Knörle et al., 1997). Here, Gly concentration increased only over time in the FN group while it remained stable in the MWp and FN + MWd groups. Thus, the increase of Gly in the FN group seems to be rather linked to sample spinning. Hence the use of prior MW fixation might be helpful to disentangle NMR signal variations associated to sample degradation from those associated to visible and invisible metabolites (mainly metabolites closely tied to macromolecules). To this end, the influence of different spinning rates and durations has to be further investigated. Thus, our results are in agreement with the proposal formulated by Opstad et al. (2008) who considered that ischemia seemed to have less effect on metabolite profile than prolonged spinning time. The present data tend to indicate that such deleterious effects might be drastically reduced by prior ultra fast MW fixation.

In conclusion, we showed here that the use of HRMAS ^{1}H NMR combined to ultra fast *in vivo* MW irradiation of the rodent brain compared to the more widely used instant nitrogen freezing procedure allowed, as expected, a much better preservation of several metabolites essentially linked to post mortem degradation and anaerobic metabolism as well as less labile metabolites such as neurotransmitters. Our report indicates that MW irradiation will undoubtedly increase the reliability of metabolite assignment and quantification by powerful NMR signal stabilization (without adding NMR signals from other chemical fixation methods). Thus the build-up of dedicated MW ovens for *ex vivo* fixation should be considered giving specific attention to energy output, irradiation time, temperature elevation, sample volume, magnetron warm-up (Login et al., 1998). Finally, snap freezing remains a reliable fixation technique for HRMAS NMR analysis, mainly for short acquisition times.

Acknowledgements

This work is part of the CARMeN project and was supported by grants from Région Alsace, Oseo, Communauté Urbaine de Strasbourg, Conseil Général du Bas-Rhin, Bruker BioSpin, Université de Strasbourg, Hôpitaux Universitaires de Strasbourg and the Centre National de la Recherche Scientifique (CNRS). We thank Estelle Koning for friendly, professional and helpful technical assistance. Dr. J.C. Cassel is acknowledged for the free access to his microwave device and his assistance in its use. We also thank Pr. J.P. Bellocq and the Centre de Ressources Biologiques of the Hôpitaux Universitaires de Strasbourg.

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