analytical chemistry

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Analysis of Metabolic Pathways by ^{13}C-Labeled Molecular Probes and HRMAS Nuclear Magnetic Resonance Spectroscopy: Isotopologue Identification and Quantification Methods for Medical Applications

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Cite This: *Anal. Chem.* 2022, 94, 8226−8233 Read Online

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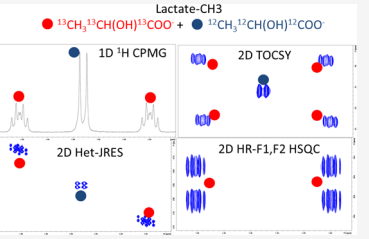
ABSTRACT: The use of ^{13}C-labeled molecular probes is essential to explore altered metabolic pathways in human pathologies. The analysis of the different ^{13}C isotopologues resulting from these changes in metabolic pathways is essential to understand the different biological processes involved. We propose an NMR methodology consisting of eight different NMR experiments performed under HRMAS conditions to explore metabolic pathways in unprocessed pathological cells and tissues. This methodology has the potential to study human pathologies in the medical field and to enable the analysis of the mode of action of therapeutic treatments.



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Over the past two decades, metabolomics has evolved into an important field of research in medicine. Metabolomics provides a direct link between the genotype and the metabotype which can lead to a better understanding of cellular metabolism adaptation in cancer. Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the two main analytical techniques that can profile the metabolome of biological fluids, cells, and tissues. In addition to the identification and quantification of metabolites, changes in metabolic pathways resulting from various pathologies can be more accurately characterized by using ^{13}C-labeled molecular probes.[1,2] Nuclear magnetic resonance (NMR) and mass spectrometry (MS) can both be used to determine the isotopic abundance (IA) $\frac{^{13}C}{^{13}C+^{12}C}$ in various metabolites. However, despite its tremendous sensitivity, MS can only provide routinely the global IA of a molecule, whereas NMR can potentially measure the IA at site-specific locations. Isotopic NMR has been used for now three decades in food science, forensics, and in the pharmaceutical industry.[3]

The use of stable isotope tracers[4] like ^{13}C-labeled substrates[5−7] is essential to probe cellular metabolism and discover new therapeutic targets and new biomarkers. In the medical field, the study of isotopologues can be crucial to elucidate the metabolic networks affected by a given pathology. In this type of studies, isotopically enriched nutrients, like [U-^{13}C]-glucose, are administrated to a biological system, and the resulting metabolic conversions are subsequently monitored.[8] Consequently, ^{13}C metabolic flux analysis has developed considerably over the past few years, enabling the quantification of intracellular fluxes in a variety of cancer cells.[9] Sampol et al.[10] have studied brain metabolism using [U-^{13}C]-glucose and [U-^{13}C]-lactate probes, and they have found that intracerebral lactate was produced during brain activation in vivo and that it could be used as a supplementary fuel for neurons. Only an isotopologue study can give access to this type of information, which is crucial to understand cancer metabolism and to develop new therapeutics in the brain and other organs. Abrantes et al.[11] showed the utility of isotopologues study by ^{13}C NMR to characterize the intermediary metabolism in three colorectal cancer cell lines that occur under hypoxia. Bruntz et al.[8] described how they studied up- and downregulation in oncogenic lesions using ^{13}C-metabolic probes. Either in cell cultures or in xenografts, molecular probes can be used to analyze energy pathways and to identify and quantify metabolites.

In this manuscript, we present an NMR methodology consisting of eight different NMR experiments to detect ^{13}C-labeled metabolites, analyze their different isotopologues, and determine their IA in complex biological systems like cells and unprocessed biopsy specimens. We present a detailed and critical comparison of these eight NMR pulse sequences to determine both the isotopologue distribution and the site-specific IA in product metabolites that derive from a stable isotope-labeled precursor molecule. The accuracy of the different NMR sequences for IA determination is first determined using standard solutions of pure compounds of known IA. Because inhomogeneous samples like cells and

Received: January 14, 2022
Accepted: May 25, 2022
Published: June 3, 2022

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https://doi.org/10.1021/acs.analchem.2c00214
Anal. Chem. 2022, 94, 8226−8233

biopsy specimens cannot be studied using traditional high-resolution NMR methods, they will be analyzed using HRMAS NMR spectroscopy,[12] a technique that allows to remove magnetic susceptibility effects by spinning the sample at the magic angle (54.7°). To our knowledge, this study is the first to present a complete panorama of isotopologue identification and quantification methods by HRMAS NMR spectroscopy.[10,13]

EXPERIMENTAL SECTION

Standard Solutions for the Evaluation of the Measurement of the Isotopic Abundance. The following eight solutions of a pure metabolite containing a mixture of natural abundance and [U-^{13}C]-labeled molecules were prepared for the evaluation of the different NMR pulse sequences: alanine, glycine, acetate, glucose, glutamine, glutamate, aspartate, and lactate (Table 1). All the solutions were prepared in triplicate in order to check the reproducibility of the measurement. The metabolite solutions were prepared in D_2O, and 24 μL of this solution was placed in a 25 μL disposable Kel-F insert. Shortly before the HRMAS analysis, the insert was placed into a standard 4 mm ZrO2 rotor.

Cell Cultures. The cell culture study was performed on osteosarcoma cells (U2OS line) that were fed with [U-^{13}C]-glucose under normoxia conditions. The number of cells used per 25 μL disposable insert was approximately 5 × 10.[6] The final insert used for the experiment contained 15.5 mg of cell culture and 5 μL of D_2O.

Mouse Mammary Biopsy Specimens. The following tumor xenograft model was used in mice: the metastasis model of PyMT-MMTV (polyomavirus middle T-antigen—mouse mammary tumor virus)[14] mammary tumors that spontaneously develop mammary adenocarcinomas. MMTV-LTR (mouse mammary tumor virus—terminal repeat) is used to stimulate the mammary gland-specific polyomavirus middle T-antigen, which leads to the rapid development of metastatic tumors.[14] Mice were subjected to the intraperitoneal injection of [U-^{13}C]-glucose for 48 h before being sacrificed. This work was done as part of a study on the impact of MMP11 on tumor progression in the PyMT-MMTV mouse model of mammary tumors.[15] The final insert used for the experiment contained 14 mg of biopsy specimen and 5 μL of D_2O.

NMR Pulse Sequences for the Detection of ^{13}C-Labeled Molecules, the Determination of Isotopologues, and Isotopic Abundance. In this work, the following four NMR experiments were used to study the isotopologue distribution and to determine isotopic abundances: 1D ^{1}H CPMG, 1D ^{1}H POCE, 2D ^{1}H-^{13}C Het-JRES, and 2D TOCSY. These NMR experiments are to our knowledge the only ones that can provide site-specific IA in solutions, cells, and biopsy specimens. To confirm the topology of the ^{13}C-network of the molecule, the complementary four experiments were also used: 1D ^{1}H/13C HSQC, 2D ^{1}H-^{13}C HSQC with high resolution in F1, F2-undecoupled 2D ^{1}H-^{13}C HSQC with high resolution in both F1 and F2, and 2D ^{1}H-^{13}C HMBC with high resolution in both F1 and F2.

1D ^{1}H CPMG spectrum using water presaturation during the relaxation delay is the simplest and the most accurate method to measure the IA in a molecule. Among the numerous existing analytical methods, it is regarded as the reference method for IA measurements. Provided the relaxation delay is long enough (5 times T1), the result of the experiment is fully quantitative and provides immediately the IA of the different

Table 1. IA Determination on Standard Solutions[a]

metabolite mixture (U-^{13}C-labeled/unlabeled)	concentrations (mM)	group	^{1}H/^{13}C chemical shift (ppm)	1D ^{1}H reference IA ^{13}C (%)	1D POCE IA ^{13}C (%)	2D JRES IA ^{13}C (%)	2D TOCSY IA ^{13}C (%)
acetate (60/40)	4,65/3,1	CH_3	1,92/25,9	60(1)	60(1)	61(1)	60(1)
glycine (50/50)	3,1/3,1	αCH_2	3,55/43,99	49(1)	48(2)	52(5)	51(5)
aspartate (40/60)	3,1/4,65	αCH	3,89/54,66	40(1)	38(2)	38(2)	39(2)
β-glucose (80/20)	12,4/3,1	C1H	4,65/98,56	81(1)	76(4)	78(2)	79(2)
alanine (70/30)	7,23/3,1	βCH_3	1,48/18,70	68(1)	68(2)	67(2)	67(3)
lactate (60/40)	4,65/3,1	CH_3	1,33/22,66	61 (1)	58(2)	61(2)	60(2)
glutamate (20/80)	3,1/12,4	γCH_2	2,34/35,99	20(1)	19(1)	19(2)	20(2)
glutamine (30/70)	3,1/7,23	γCH_2	2,45/33,29	31(2)	31(4)	29(3)	31(3)

[a]The standard deviation obtained on three replicates is indicated in parentheses.

https://doi.org/10.1021/acs.analchem.2c00214
Anal. Chem. 2022, 94, 8226−8233

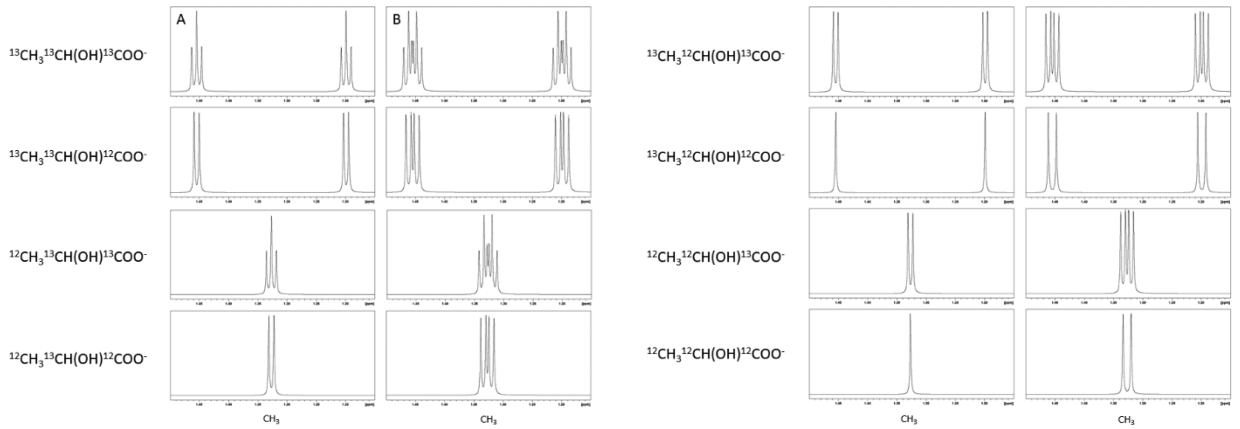

Figure 1. Theoretical 1D ^{1}H patterns for the methyl group of lactate for the 8 possible isotopologues. The following parameters were used for the simulation: CH_3 chemical shift 1.33 ppm, $^1J_{1H13C}$ = 128 Hz, $^2J_{1H13C}$ = 4 Hz, $^3J_{1H13C}$ = 4 Hz, $^3J_{1H1H}$ = 6.9 Hz, $^1J_{13C13CO}$ = 56 Hz, and $^1J_{13C13C}$ = 36 Hz. (A) Considering only heteronuclear coupling constants and (B) considering both hetero and homonuclear coupling constants.

sites of the molecule by simple integration of the central resonance (^{1}H-^{12}C) and of the two satellite resonances (^{1}H-^{13}C). Unfortunately, the method cannot be used for complex mixtures as the amount of signal overlap becomes unmanageable.

1D ^{1}H/^{13}C HSQC experiment. This experiment corresponds to the first increment of the 2D HSQC experiment described in (f). The main difference with the 2D is that a larger number of scans is used. No IA determination is possible with this experiment.

1D ^{1}H POCE experiment[16] is based on the acquisition of two experiments. The first experiment is a homonuclear spin echo sequence with ^{13}C-decoupling during ^{1}H acquisition, and the second experiment is a heteronuclear spin echo sequence also with ^{13}C-decoupling during ^{1}H acquisition. The first experiment contains the sum of the ^{1}H-^{12}C + ^{1}H-^{13}C protons, whereas the second one contains only ^{1}H-^{13}C protons. By integrating the same signal in the two experiments and taking the proper ratio, the IA is obtained.

2D heteronuclear J-resolved (Het-JRES) experiment allows to separate the ^{1}H chemical shift in one dimension and the ($J_{1H\text{-}1H}$ + $^1J_{1H\text{-}13C}$) (in Hz) in the second dimension. The standard version of the experiment generates a twisted lineshape and can only be processed in the magnitude mode, which degrades both resolution and sensitivity. Moreover, the quantitative properties of the magnitude version are extremely poor, and the determination of the IA is not accurate. A modification of this experiment recently introduced by Sinnaeve et al. (Figure S1 of ref 17) allows to obtain phase-sensitive spectra, which lead to a higher resolution and a much improved quantitativity. The IA is obtained by integrating the central cross peaks at J = 0 Hz and the two satellites at $\pm{}^1J_{CH}/2$ Hz, and by taking the ratio of the two.

2D TOCSY experiment uses the Keeler ZQC filter in order to suppress peak distortions due to zero quantum coherences.[18] The nature of the different isotopologues can be obtained directly from the cross-peak pattern.[5] By integrating the central resonance of a cross-peak ($J_{1H\text{-}1H}$) and the peripheral signals ($^1J_{1H\text{-}13C}$), the IA can be obtained.

2D HR-F1-HSQC (high-resolution HSQC in F1)[19] allows to obtain the pattern of the isotopologues present in the mixture. High resolution is needed in the F1 ^{13}C dimension in order to resolve the $^J{}_{13C\text{-}13C}$ coupling constants. No IA determination is possible with this experiment.

2D F2-Undecoupled HR-F1,F2-HSQC (high-resolution HSQC in both F1 and F2) allows to obtain the pattern of the isotopologues present in the mixture. High resolution is needed in the F1 ^{13}C dimension in order to resolve the $^1J_{13C\text{-}13C}$ coupling constants while the high resolution in F2 allows to resolve only $^1J_{CH}$,$^2J_{CH}$,$^3J_{CH}$, and$^3J_{HH}$. No IA determination is possible with this experiment.

2D HR-F1,F2-HMBC (high-resolution HMQC in both F1 and F2) allows to obtain the pattern of the isotopologues present in the mixture, notably quaternary and carbonyl carbons. These carbons can also be detected in the HSQC experiment via their coupling to the adjacent ^{13}C. High resolution is needed in the F1 ^{13}C dimension in order to resolve the $^1J_{13C\text{-}13C}$ coupling constants. No IA determination is possible with this experiment.

NMR Data Acquisition and Processing. HRMAS NMR 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 triple-resonance (^{1}H, ^{13}C, ^{31}P) HRMAS probe. The temperature was set to 277 K for all the acquisitions. All NMR experiments were conducted on samples spinning at 3502 Hz to keep the rotation sidebands out of the spectral region of interest and to minimize sample degradation. All 1H spectra were referenced by setting the lactate doublet chemical shift to 1.33 ppm.

The experimental details of the eight pulse sequences used in this study are presented in the Supporting Information.

RESULTS AND DISCUSSION

NMR Patterns Observed for Different Isotopologues. The peak pattern of the different NMR experiments is characteristic of the isotopologues present in the sample. As an example, the different patterns observed in a 1D ^{1}H spectrum for the CH_3 group of lactate in the eight possible isotopologues are shown in Figure 1. The simulated spectra (NMRSIM, Bruker BioSpin) obtained considering only the $^1J_{CH}$, $^2J_{CH}$, and $^3J_{CH}$ heteronuclear coupling constants are shown in Figure 1A, whereas the simulated spectra taking into account the full coupling network including the $^3J_{HH}$ coupling constant are shown in Figure 1B. For example, the isotopologue $^{13}CH_3{}^{13}CH(OH)^{13}COO^-$ exhibits a pattern where the signal is a doublet of a triplet. The doublet is due to the $^1J_{CH}$ coupling constant of 128 Hz, whereas the triplet is due to the $^2J_{CH}$ and $^3J_{CH}$ coupling constants, both equal to 4

https://doi.org/10.1021/acs.analchem.2c00214
Anal. Chem. 2022, 94, 8226−8233

Figure 2. Theoretical pattern obtained for the CH_3 group and the CH group of lactate for the set of NMR experiments used. In this schematic representation, only the $^1J_{CH}$, $^2J_{CH}$, and $^3J_{CH}$ heteronuclear coupling constants are displayed.

Hz. Only six different patterns are observed for the 8 isotopologues because $^2J_{CH}$ and $^3J_{CH}$ have similar values. The isotopologue $^{13}CH_3{}^{12}CH(OH)^{13}COO^-$ is indistinguishable from $^{13}CH_3{}^{13}CH(OH)^{12}COO^-$ and the isotopologue $^{12}CH_3{}^{12}CH(OH)^{13}COO^-$ is indistinguishable from $^{12}CH_3{}^{13}CH(OH)^{12}COO^-$ if only the CH_3 group is considered. Considering the pattern of the lactate, CH resonance allows to differentiate the eight isotopologues.

The patterns observed for the CH_3 and CH groups of lactate for the same eight isotopologues for the full set of NMR experiments are presented in Figure 2.

In this schematic representation, only the $^1J_{CH}$, $^2J_{CH}$, and $^3J_{CH}$ heteronuclear coupling constants are displayed in order to make the isotopologues patterns clearer. The $^{13}CH_3{}^{13}CH(OH)^{13}COO^-$ isotopologue exhibit the pattern seen previously in 1D ^1H spectrum while only a single peak is observed in the 1D POCE/HSQC spectrum because the 1H signals are acquired under ^{13}C-decoupling. The 2D Het-JRES experiment exhibits a 128 Hz ($^1J_{CH}$) doublet in the F1 dimension (in Hz) whose fine structure is a triplet similar to the one observed in the 1D ^1H spectrum. The CH_3-CH cross-peak observed in the 2D TOCSY spectrum is particularly informative as it shows a 128 Hz ($^1J_{CH}$) doublet in both dimensions, proving therefore that both CH_3 and CH are ^{13}C-labeled. The fine structure observed for the four components of the cross-peak is also identical to the 1D ^1H spectrum. The CH_3 cross-peak observed in the 2D HSQC exhibit a 36 Hz ($^1J_{CC}$) doublet in the F1 dimension, which also proves unambiguously that the CH_3 and the CH groups are both ^{13}C-labeled and therefore coupled. A powerful experiment for the determination of isotopologues is the 2D HSQC (heteronuclear single quantum coherence spectroscopy) recorded in high-resolution mode in both F1 and F2. The high resolution in F2 is obtained by removing ^{13}C-decoupling during signal acquisition and therefore allowing for longer signal acquisition. The extra resolution provided by the ^{13}C-dimension allows to resolve cross-peaks that would otherwise overlap in the 1D spectrum. The CH_3 cross-peak exhibits a pattern, which is split into four components separated by the 128 Hz ($^1J_{CH}$) in the F2 dimension and 36 Hz ($^1J_{CC}$) in the F1 dimension. Remarkably, the high resolution achieved in F2 allows to resolve the fine structure of the multiplets and to obtain a pattern identical to the 1D spectrum. As mentioned above, this 1D pattern can also be observed in the TOCSY (TOtal Correlated SpectroscopY) spectrum but in a slightly distorted manner. The 2D HMBC resolution recorded in high-resolution mode in F1 is used mainly to confirm the presence of ^{13}C-labeled quaternary carbons like carbonyls. The full set of experiments allows to obtain a unique pattern for the eight isotopologues. The complete information is obtained by considering simultaneously the patterns of the CH_3 and CH signals.

Analysis of the Isotopologues and Measurement of the IA on the Standard Solutions of the Pure Metabolites. The eight standard solutions of pure metabo-

https://doi.org/10.1021/acs.analchem.2c00214
Anal. Chem. 2022, 94, 8226−8233

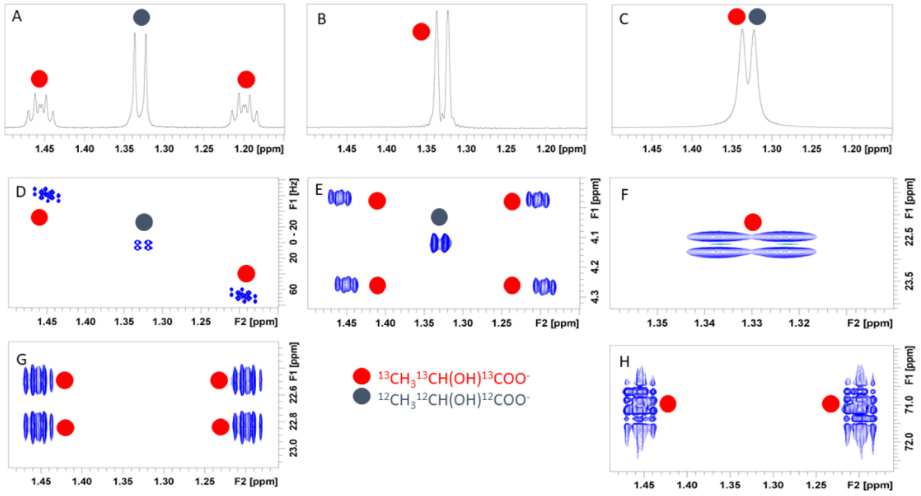

Figure 3. Standard lactate solution and pattern observed for the CH_3 group for the set of NMR experiments used. (A) 1D ^{1}H CPMG, (B) 1D ^{1}H HSQC, (C) 1D ^{1}H POCE, (D) 2D Het-JRES, (E) 2D TOCSY, (F) 2D HR-F1-HSQC, (G) 2D HR-F1,F2-HSQC, and (H) 2D HR-F1-HMBC.

lites were analyzed using the full set of eight NMR experiments. The results obtained with these experiments on the CH_3 group of the lactate sample are shown in Figure 3.

The 1D ^{1}H spectrum shows the typical pattern expected for a mixture of 40% natural abundance lactate and 60% [U-13C]-lactate. The central line at 1.33 ppm corresponds to the unlabeled molecule, whereas the satellites at $\pm{}^1J_{CH}/2 = \pm 64$ Hz display the complex structure generated by the three ^{13}C labels (cf. Figure 1). The 1D POCE/HSQC sequences that are recorded under ^{13}C-decoupling shows only a signal at 1.33 ppm, and all information related to the isotopologues is lost. This information is present in the 2D Het-JRES experiment in the fine structure of the cross peaks located at ca. ±64 Hz in the F1 dimension. The TOCSY experiment is particularly powerful, and the cross peak between the CH_3 group and the CH group in the 2D TOCSY experiment immediately proves the presence of the two isotopologues present in the samples $^{13}CH_3{}^{13}CH(OH){}^{13}COO^-$ and $^{12}CH_3{}^{12}CH(OH){}^{12}COO^-$. A particularly powerful experiment is the 2D HR-F1,F2 HSQC experiment, which displays a similar pattern for the satellites peaks as the 1D experiment but with the added resolution of the 2D experiment. This feature is essential when studying isotopologues. The results of the HMBC experiment confirm the presence of a ^{13}C-labeled carbonyl group. With respect to IA determination, this set of spectra contains no signal overlap and can be analyzed in a straightforward manner by simple peak integration. The IA values obtained for the 8 standard solutions of pure metabolites with the 1D ^{1}H, 1D ^{1}H POCE, 2D Het-JRES, and 2D TOCSY spectra are presented in Table 1.

The four experiments provide very similar IA values that shows that in the absence of the signal overlap, they all perform equally well.

Analysis of Isotopologues and Measurement of the IA in Osteosarcoma Cell Cultures. These same NMR experiments were used to study osteosarcoma cell cultures that were previously fed with [U-^{13}C]-glucose. The goal was to check their ability to detect ^{13}C-labeled metabolites, identify isotopologues, and determine IA values in more complex samples. The detection of ^{13}C-labeled metabolites is most readily achieved by looking simultaneously at the 1D ^{1}H HSQC and the 1D ^{1}H POCE experiments. In these experiments, both intra and extracellular metabolites are detected and are not discriminated. The 1D ^{1}H CPMG experiment is also shown in Figure 4 as a reference spectrum.

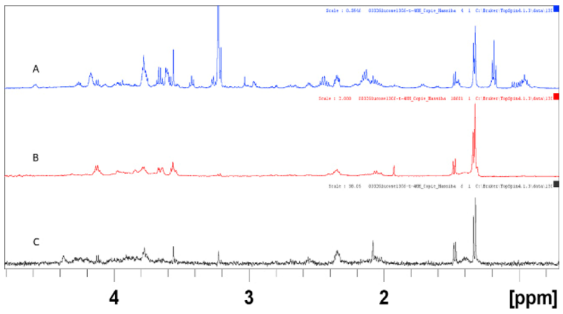

Figure 4. Osteosarcoma cell cultures, (A) 1D ^{1}H CPMG, (B) 1D ^{1}H POCE, and (C) 1D ^{1}H HSQC spectra.

These two experiments provide similar results and only contain signals originating from ^{13}C-bound protons. The main advantage of the 1D ^{1}H HSQC is that the gradient selection of the signals insures a very low level of artifacts at a cost of a lower signal-to-noise. Inspection of these two spectra immediately reveals the presence of the following ^{13}C-labeled molecules: lactate, alanine, acetate, glutamate, aspartate, glutathione, glycine, serine, and UXP (UMP, UDP, UTP...). The main difference between the two spectra is due to the partial degradation of NAA into acetate and aspartate. NAA is clearly visible in the 1D HSQC spectrum, but its intensity decreases significantly in the 1D POCE spectrum that was recorded a few weeks later. Another effect of prolonged spinning is a slight increase in the level of the metabolites detected.[20] Once the presence of these ^{13}C-labeled metabolites has been ascertained, one can then turn to the identification of isotopologues and the determination of IA.

In order to establish the isotopologue distribution, the full set of NMR experiments was recorded. The results obtained are shown in Figure S2 for the CH_3 group of lactate. In this more complex sample, the 1D ^{1}H spectrum becomes overcrowded and detecting the ^{13}C satellites becomes tedious. Remarkably, the patterns of the2D Het-JRES and TOCSY experiments are identical to the ones observed on the lactate

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Anal. Chem. 2022, 94, 8226−8233

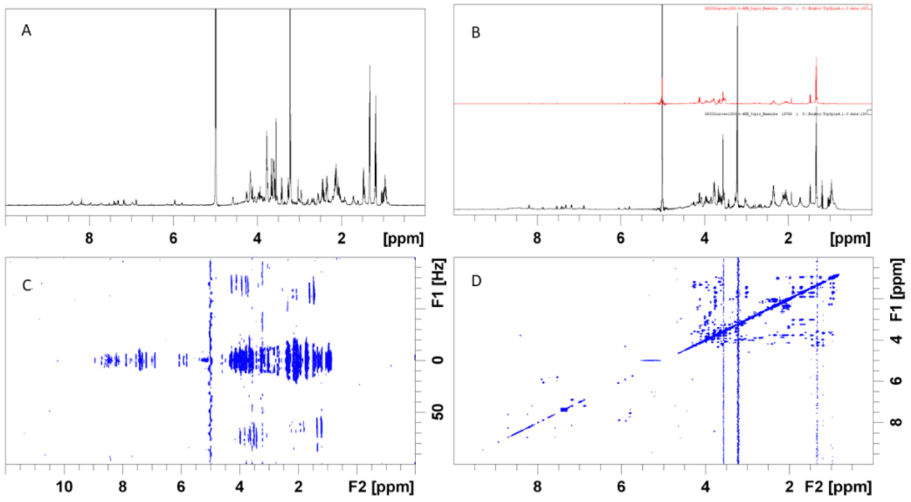

Figure 5. Representative spectra of osteosarcoma cells, (A) 1D ^{1}H CPMG, (B) 1D ^{1}H POCE (Bottom spectrum ^{1}H-^{12}C + ^{1}H-^{13}C protons, Top spectrum ^{1}H-^{13}C protons), (C) 2D Het-JRES, and (D) 2D TOCSY.

Table 2. Isotopic Abundance (IA) Determination of the most Abundant Metabolites Found in Osteosarcoma Cell Cultures[a]

metabolite	group	^{1}H/^{13}C chemical shift (ppm)	1D POCE IA ^{13}C (%)	2D JRES IA ^{13}C (%)	2D TOCSY IA ^{13}C (%)	concentrations (nmoles)	concentrations (mmoles/kg)
lactate	CH_3	1,33/22,66	49.1(0.5)	48.3(0.2)	47.1(0.3)	38.7(0.3)	2.50(0.02)
lactate	CH	4,12/71,05			47.0(1.4)		
alanine	βCH_3	1,48/18,70	40.3(1.2)	34.2(1.2)	39.2(0.9)	12.4(0.3)	0.80(0.02)
acetate	CH_3	1,92/25,9	45.1(0.5)	44.3(0.7)		0.40(0.02)	0.03(0.001)
glutamate	γCH_2	2,34/35,99	17.2(0.3)	17.1(0.3)		51.4(0.2)	3.30(0.01)
aspartate	βCH_2 d	2,81/39,1	18.3(1.3)			9.7(0.2)	0.62(0.01)
glutathione	γCH_2	2,56/33,89	15.1(1.5)			16.8(0.2)	1.08(0.01)
glycine	αCH_2	3,55/43,99	14.2(0.7)	19.2(1.1)		15.3(0.2)	0.98(0.01)
serine	βCH_2	3,96/62,84	23.1(1.2)	20.2(1.0)		13.9(0.5)	0.89(0.03)
UXP	CH	5,80/103,84	55.2(2.4)	54.1(3.0)	55.2(1.8)	1.9(0.1)	0.13(0.01)

[a]The standard deviation obtained on three technical replicates is indicated in parentheses.

solution. Overall, the general pattern in these experiments is very similar to the pattern observed on the solution containing 40% natural abundance lactate and 60% [U-^{13}C]-lactate, proving the presence of the two isotopologues $^{13}CH_3{}^{13}CH(OH)^{13}COO^-$ and $^{12}CH_3{}^{12}CH(OH)^{12}COO^-$. All the data shown in Figure 5, in particular, the undecoupled 2D HR-HSQC, are in agreement with the presence of these two isotopologues.

An interesting observation is the fact that UXP appears as multiple isotopologues when analyzing the TOCSY cross-peak between the two aromatic protons H5 and H6 (Figure S3). The nine peaks present in this cross-peak prove the presence of the four isotopologues: $^{12}CH5-^{12}CH6$, $^{13}CH5-^{13}CH6$, $^{12}CH5-^{13}CH6$, and $^{13}CH5-^{12}CH6$. Using the results of the 1D ^{1}H POCE, 2D Het-JRES, and 2D TOCSY spectra, the IA value was computed for several metabolites. If we consider the case of lactate, the main difficulty for this metabolite is that in the POCE and the 2D Het-JRES sequences, the threonine and the lactate signals overlap at 1.33 ppm. A deconvolution of the 1D spectrum at this frequency reveals that the peak is composed of 24% threonine and 76% lactate. Based on these data, a correction factor was used to compute the results of the POCE and JRES experiments. The TOCSY experiment does not suffer from this overlap, and the IA can be computed directly from the CH_3-CH cross-peak. In the case of a metabolite exhibiting a cross-peak in the TOCSY, this experiment is always the one that gives the most reliable results. The IA value obtained for the Lac-CH3 was found to be quite consistent among the three experiments: 1D POCE (49.1%), 2D Het-JRES (48.3%), and 2D TOCSY (47.1%). The isotopologue distribution of lactate is therefore the following: $^{13}CH_3{}^{13}CH(OH)^{13}COO^-$ (48.2%) and $^{12}CH_3{}^{12}CH(OH)^{12}COO^-$ (51.8%). The isotopic abundance (IA) and the concentration[21] values obtained for the most abundant ^{13}C-labeled metabolites present in osteosarcoma cells are reported in Table 2.

Analysis of Isotopologues and Measurement of the IA on Biopsy Specimens of Mammary Xenografts in Mice. The same technique was used to study biopsy specimens of a mouse mammary xenograft. The mouse had previously been fed intravenously with [U-^{13}C]-glucose for 48 h before being euthanized. The inspection of the 1D ^{1}H HSQC and of the 1D ^{1}H POCE experiments (Figure 6) immediately reveals the presence of mostly ^{13}C-labeled lactate (IA 21%) but also compounds that are ^{13}C-labeled at a lower level: creatine (IA 11%), glucose (IA 5%), and alanine (IA 3.7%). Signals from ^{13}C-labeled lipids are also visible in these experiments at 0.9 ppm ($-CH_3$ group) and 1.3 ppm ($-CH_2$ group). These two signals exhibit a clear correlation in the TOCSY experiment. The IA measured for these two signals in the POCE experiment is equal to 1.1%, which corresponds to the natural abundance.

The full set of the NMR experiments, recorded on this sample, is shown for the lactate-CH_3 in Figure S4. The patterns

https://doi.org/10.1021/acs.analchem.2c00214
Anal. Chem. 2022, 94, 8226−8233

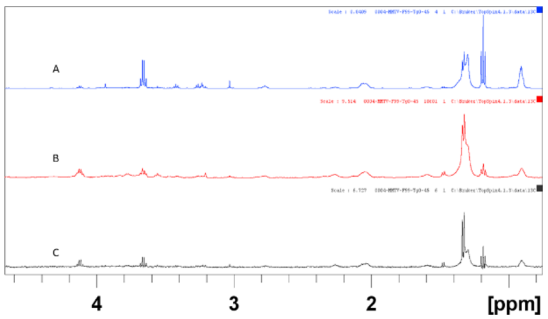

Figure 6. Xenografts biopsies, (A) 1D ^{1}H CPMG, (B) 1D ^{1}H POCE, and (C) 1D ^{1}H HSQC spectra.

observed in the TOCSY experiment for the CH_3−CH cross-peak and the HMBC experiment for the CH_3−CO and CH−CO cross-peaks reveal the presence of the two isotopologues of lactate ($^{13}CH_3{}^{13}CH(OH)^{13}COO^-$ and $^{12}CH_3{}^{12}CH(OH)^{12}COO^-$) but also at a lower concentration $^{12}CH_3{}^{13}CH(OH)^{12/13}COO^-$ and $^{13}CH_3{}^{12}CH(OH)^{12/13}COO^-$. In this manuscript, the notation $^{12/13}COO^-$ means that the determination of the carbonyl label was no possible. Integration of the different multiplets of the lactate CH3-CH TOCSY cross peak leads to the following results for the IA: $^{13}CH_3{}^{13}CH(OH)^{13}COO^-$ 13.5%,$^{12}CH_3{}^{12}CH(OH)^{12}COO^-$79.1%, $^{12}CH_3{}^{13}CH(OH)^{12/13}COO^-$ 4.8%, and $^{13}CH_3{}^{12}CH(OH)^{12/13}COO^-$ 2.7%.

Specificity of the Pulse Sequences Used. The NMR pulse sequences presented in this manuscript for the purpose of isotopologues and IA determination have their own characteristics. The 1D ^{1}H CPMG experiment is the simplest and the most accurate method to measure the site-specific IA in a molecule when studying a sample with limited signal overlap. Among all the analytical methods, it is usually regarded as the reference method for IA measurements. Unfortunately, this experiment cannot be used for complex solutions as the amount of signal overlap quickly becomes unmanageable. Sophisticated deconvolution procedures or pattern recognition techniques based on spectral bases of simulated isotopologues spectra have, however, the potential to resolve this overlap issue and make the method more readily applicable for medical applications. The 1D ^{1}H HSQC/POCE experiment allows to study more complex systems by removing all the heteronuclear J_{CH} heteronuclear coupling constants in the final ^{1}H spectrum. The spectral resolution in these spectra is however limited as the ^{13}C-decoupling required during the ^{1}H acquisition limits the acquisition time. This is nevertheless a powerful experiment that allows a rapid and efficient detection of ^{13}C labels in metabolites. The IA values obtained from the POCE experiment are usually extremely accurate in case of limited spectral overlap. The 2D Het-JRES experiment is a powerful experiment to study the incorporation of ^{13}C-labels in metabolites. A simple inspection of the experiment allows to quickly identify the metabolites that are labeled as a doublet at $\pm^{1}J_{CH}/2$ clearly appears in the F1 dimension. An additional advantage of the experiment is that the fine structure of the cross peak can be used to identify the presence of neighboring ^{13}C-labels. The phase sensitive version of the Het-JRES experiment used in this study was proposed by Sinnaeve et al.[17] In their study, they also propose a pure shift version of the experiment that offers a significantly improved resolution by removing homonuclear coupling constants. Unfortunately, this improvement comes at the cost of roughly a factor of 10 in *S*/*N*. The pure shift version of the Het-JRES was therefore not used in this work because of the limited amount of sample available. The 2D TOCSY experiment allows to go one step further. The pattern of the cross peaks obtained for the different isotopologues in the two- or three-carbon system is highly specific and allows to differentiate the different isotopologues unambiguously. The resolution provided by the additional dimension also makes the overlap problem much less severe. The determination of IA is also particularly straightforward and robust. Its main limitation is that it is unfortunately not applicable to isolated spin systems like the CH_3 group of NAA. The 2D HR-HSQC experiment is essential to observe the isotopologues present in the sample by using the $^{1}J_{CC}$ coupling in the F1 dimension. A complementary powerful alternative to the HR-HSQC experiment is the 2D HR-F1,F2 HSQC experiment. By removing the ^{13}C-decoupling element during signal acquisition, long acquisition time can be used, and the fine structure of multiplets can be revealed. This fine structure can be used to detect precisely which isotopologues are present in the sample. A striking example is provided by the lactate spectrum shown in Figure 3G. The fine structure of the satellites peaks is identical to the one observed in the 1D spectrum.

We therefore propose the following strategy to study ^{13}C-labeled metabolites in both cells and biopsy specimens by HRMAS NMR and in biological fluids by classical high resolution NMR:

The 1D ^{1}H HSQC and the 1D ^{1}H POCE experiments can both be used to identify rapidly and reliably the presence of ^{13}C-labeled metabolites in the sample. These experiments can be recorded in less than 10 min and are ideally suited to follow dynamic metabolic processes. The identity of the metabolites can be further confirmed by recording a 2D HR-F1-HSQC experiment. If a detailed characterization of the isotopologues and a determination of the IA is required, further experiments need to be carried out. The two most powerful experiments for that purpose are the 2D HR-TOCSY and the 2D Het-JRES. The TOCSY experiment is however limited to the chemical groups of the metabolite that are J_{1H1H}-coupled because the analysis of the fine structure of the cross peaks is required.

CONCLUSIONS

We have proposed a HRMAS NMR strategy consisting of eight different NMR experiments to perform a combined analysis of ^{13}C-labeled metabolites, including isotopologues and IA determination, in complex mixtures like unprocessed cells and biopsy specimens. These experiments have the potential to be used in the medical field to study altered metabolic pathways in human pathologies. By extension, this strategy will also enable the analysis of the metabolic modifications induced by therapeutic treatment and therefore allow a better understanding of their mode of action.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c00214.

NMR experimental parameters; theoretical ^{1}D ^{1}H patterns of acetate; NMR spectra of osteosarcomas; TOCSY cross peak of UXP in osteosarcomas; and NMR spectra of xenograft biopsies (PDF)

https://doi.org/10.1021/acs.analchem.2c00214
Anal. Chem. 2022, 94, 8226−8233

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Author Contributions

I.J.N. designed the project. H.O. and M.P. performed the experiments and the data analysis. M.P, H.O., C.B, I.J.N. wrote the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We would like to thank Dr. Monique Dontewill and Pr Natacha Entz-Werlé (Laboratory of Bioimaging and Pathologies, UMR CNRS 7021, 67405 Illkirch, France) for the laboratory equipment for cell cultures. We also would like to thank Dr. Nacim Dali-Youcef (Institut de Génétique et de Biologie Moléculaire et Cellulaire Illkirch, 1 rue Laurent Fries, BP 10142, 67404 Illkirch CEDEX, France) for the biopsy specimens in xenograft mice.

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