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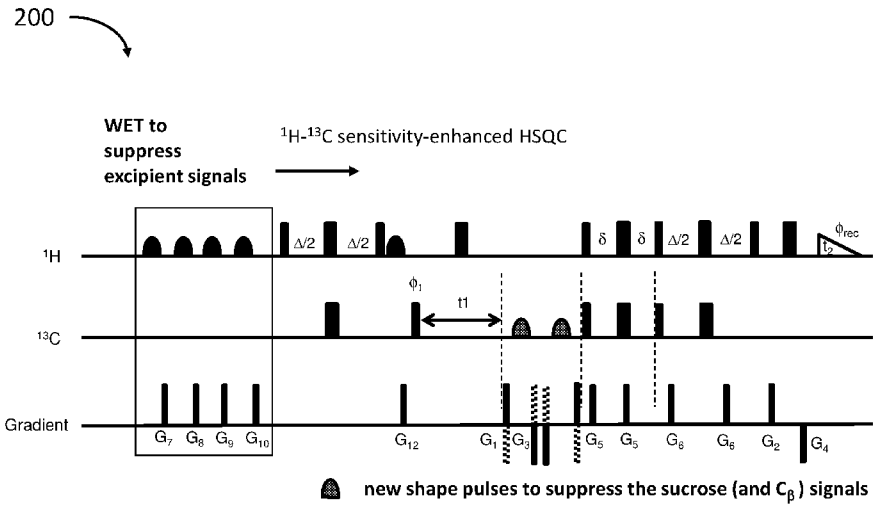

FIGURE 2

(57) Abstract: Methods of fingerprinting a specific molecule in a composition using nuclear magnetic resonance (NMR) is disclosed. The disclosed NMR methods provide several modifications and improvements over existing NMR techniques. In some embodiments, the methods include applying a cycle of signal processing steps, including applying a radio frequency (RF) pulse, applying a gradient pulse having a pulse length less than or equal to 1000 μs, and applying a water suppression technique (WET). In some embodiments, the methods further include repeating the cycle for at least 3 times to acquire an enhanced signal of the composition. In some embodiments, the methods further include fingerprinting the specific molecule based on the enhanced signal of the composition.

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METHODS OF FINGERPRINTING THERAPEUTIC PROTEINS VIA A TWO-DIMENSIONAL (2D) NUCLEAR MAGNETIC RESONANCE TECHNIQUE AT NATURAL ABUNDANCE FOR FORMULATED BIOPHARMACEUTICAL PRODUCTS

SEQUENCE LISTING

[0001] The present application is being filed with a sequence listing in electronic format. The sequence listing provided as a file titled, "041925-0924_SL.txt," created January 6, 2020, and is 265 KB in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Pharmaceutically active proteins, such as antibodies and recombinant therapeutic proteins (as a class, "therapeutic proteins"), are frequently formulated in liquid solutions, such as for parenteral injection. Pharmaceutical compositions can comprise agents for modifying, maintaining or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition.

[0003] In general, excipients can be classified on the basis of the mechanisms by which they stabilize proteins against various chemical and physical stresses. Some excipients alleviate the effects of a specific stress or regulate a particular susceptibility of a specific polypeptide. Other excipients more generally affect the physical and covalent stabilities of proteins. Common excipients of pharmaceutical liquid protein formulations are described, for example, by Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB. 2011, Protein-excipient interactions: Mechanisms and biophysical characterization applied to protein formulation development, *Adv Drug Deliv Rev* 63: 1118-59.

[0004] During the development, manufacture, and formulation of pharmaceutical formulations/compositions, the higher order structure (*e.g.,* secondary, tertiary, and quaternary structures; HOS) of therapeutic proteins is assessed to ensure therapeutic protein effectiveness and safety since HOS is a critical quality attribute (CQA) that can impact quality, stability, safety and efficacy (with an increase potential for immunogenicity of loss of function if HOS changes overtime). CQAs are chemical, physical, or biological properties that are present

within a specific value or range of values. For large polypeptide therapeutic molecules, physical attributes and modifications of amino acids (the building blocks of polypeptides) are important CQAs that are monitored during and after manufacturing (as well as during drug development). Likewise, HOS is a CQA, but detecting the HOS of a formulated therapeutic protein can be challenging because of the strong interference of excipients in formulations (for example, sucrose and acetate) with the methyl peaks of the therapeutic protein (such as an antibody, or fragments thereof, or derivatives and analogues thereof) using, for example nuclear magnetic resonance (NMR).

[0005] Methods and techniques based on NMR are useful to detect the HOS of proteins but can be challenging to implement when directed to fingerprinting target proteins in a multi-component solution. A challenge remains to improve NMR techniques to detect target signals from a target molecule (such as a therapeutic protein) over signals from other molecules in solution, especially those that produce signals in the same detection regions of the generated NMR spectra, especially those generated by a therapeutic protein. Therefore, an innovative approach to solving this challenge is needed.

SUMMARY

[0006] An exemplary method of fingerprinting a specific molecule in a composition using nuclear magnetic resonance (NMR) is described herein. The method includes providing the composition having at least a first molecule having a first NMR signal, a second molecule having a second NMR signal, and a third molecule having a third NMR signal. In the method, each of the signals arises from each of the respective molecules having a nuclear spin differing from zero. The method includes applying a cycle of signal processing steps. The cycle includes applying a radio frequency (RF) pulse, applying a gradient pulse having a pulse length less than or equal to 1000 μs, and applying a water suppression technique (WET). In the method, the first NMR signal, the second NMR signal, and the third NMR signal are located in the defined regions of NMR spectra. The method also includes repeating the cycle for at least 3 times to acquire an enhanced signal of the composition. The method further includes fingerprinting the specific molecule based on the enhanced signal of the composition.

[0007] Another exemplary method of fingerprinting a specific molecule in a composition using NMR is described herein. The method includes providing the composition having at least a first molecule having a first NMR signal, a second molecule having a second NMR signal, and a third molecule having a third NMR signal. In the method, each of the signals arises from each of

the respective molecules having a nuclear spin differing from zero. The method includes applying a cycle of signal processing steps. The cycle includes applying a RF pulse and applying a gradient pulse. In the method, the first NMR signal, the second NMR signal, and the third NMR signal are located in a region of NMR spectral window from about 5 ppm to about 150 ppm. The method also includes repeating the cycle for at least 3 times to acquire an enhanced signal of the composition. The method further includes fingerprinting the specific molecule based on the enhanced signal of the composition.

[0008] Yet another exemplary method of fingerprinting a specific molecule in a composition using NMR is described herein. The method includes providing the composition having at least a first molecule having a first NMR signal, a second molecule having a second NMR signal, and a third molecule having a third NMR signal. In the method, each of the signals arises from each of the respective molecules having a nuclear spin differing from zero. The method includes applying a RF pulse to the composition to excite the first NMR signal while suppressing the second NMR signal. The RF pulse includes at least one of a Refocusing Band-Selective Pulse with Uniform Response and Phase (Reburp) pulse, a combination of a broadband inversion pulse (BIP) and a Gaussian (G3) inversion pulse, and an asymmetric adiabatic pulse. The method also includes applying a gradient pulse having a pulse length less than or equal to 1000 μs and applying a WET sequence to suppress the third NMR signal. The method also includes repeating the cycle for at least 3 times to acquire an enhanced signal of the composition. The method further includes fingerprinting the specific molecule based on the enhanced signal of the composition.

[0009] These and other aspects and implementations are discussed in detail below. The foregoing information and the following detailed description include illustrative examples of various aspects and implementations and provide an overview or framework for understanding the nature and character of the disclosed aspects and implementations. The drawings provide illustration and a further understanding of the various aspects and implementations and are incorporated in and constitute a part of this specification.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] The accompanying drawings are not intended to be drawn to scale. Like reference numbers and designations in the various drawings indicate like elements. For purposes of clarity, not every component may be labeled in every drawing. In the drawings:

[0011] **Figure 1** shows an exemplary NMR signal enhancement technique using a combination of the conventional proton-carbon (^{1}H-^{13}C) sensitivity-enhanced Heteronuclear Single Quantum Coherence (HSQC) experiment and additional signal processing steps based on an experimental scheme disclosed herein.

[0012] **Figure 2** shows another example of a NMR signal enhancement technique based on an ^{1}H-^{13}C sensitivity-enhanced HSQC experimental scheme as disclosed herein.

[0013] **Figures 3A-3F** show exemplary excitation profiles of pulses with different shapes to suppress the ^{13}C sucrose signals.

[0014] **Figure 4** shows a graphical comparison of signal intensities for sucrose, acetate and methyl peaks based on an ^{1}H-^{13}C sensitivity-enhanced HSQC experimental scheme.

[0015] **Figure 5** shows a graphical comparison of signal intensities for sucrose and methyl peaks based on an ^{1}H-^{13}C sensitivity-enhanced HSQC experimental scheme disclosed herein using different RF pulses in exemplary HSQC experiments.

[0016] **Figures 6A-6C** show different ^{13}C 2D methyl fingerprinting plots for comparing the effectiveness of particular NMR enhancement methods.

[0017] **Figure 7** shows another example of a NMR signal enhancement technique based on an ^{1}H-^{13}C sensitivity-enhanced HSQC experimental scheme, in accordance with various embodiments.

[0018] **Figure 8** shows the spectra from the first increment of HSQC data without (802) and with (804) for the suppression of signals from 10 mM glutamate and 10 mM acetate in sample 1 of Example 2.

[0019] **Figure 9A** displays the 2D methyl region of HSQC spectra without the suppression of signals from 10 mM glutamate and 10 mM acetate in sample 1 of Example 2.

[0020] **Figure 9B** displays the 2D methyl region of HSQC spectra with the suppression of signals from 10 mM glutamate and 10 mM acetate in sample 1 of Example 2.

[0021] **Figure 10** shows the spectra from the first increment of HSQC data without (1002) and with (1004) for the suppression of signals from 15 mM glutamate in sample 3 of Example 2.

[0022] **Figure 11A** displays the 2D methyl region of HSQC spectra without the suppression of signals from 15 mM glutamate in sample 3 of Example 2.

[0023] **Figure 11B** displays the 2D methyl region of HSQC spectra with the suppression of signals from 15 mM glutamate in sample 3 of Example 2.

[0024] **Figure 12** shows the spectra from the first increment of HSQC data without (1202) and with (1204) for the suppression of signals from 200 mM proline and 10 mM acetate in sample 2 of Example 2.

[0025] **Figure 13** shows another example of a NMR signal enhancement technique based on double WET scheme, in accordance with various embodiments.

[0026] **Figure 14A** displays the 2D methyl region of HSQC spectra without the suppression of signals from 200 mM proline and 10 mM acetate in sample 2 of Example 2.

[0027] **Figure 14B** displays the 2D methyl region of HSQC spectra with the suppression of signals from 200 mM proline and 10 mM acetate in sample 2 of Example 2.

[0028] **Figures 15A-15E** show exemplary excitation profiles of pulses with different shapes to suppress the ^{13}C sucrose signals.

[0029] **Figure 16A** displays the 2D methyl region of HSQC spectra using the [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 50, 0.1 Tp] for pulse length 375 μs with transmitter offset at 16 ppm as the refocusing element, and the WET sequence to suppress the 1H acetate signal.

[0030] **Figure 16B** displays the 2D methyl region of HSQC spectra using the [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 70, 0.1 Tp] for pulse length 750 μs with transmitter offset at 18 ppm.

[0031] **Figure 17** shows a graphical comparison of signal intensities for methyl peaks based on an ^{1}H-^{13}C sensitivity-enhanced HSQC experimental scheme using different RF pulses in exemplary HSQC experiments obtained using a 800 MHz NMR system.

DETAILED DESCRIPTION

[0032] The disclosure generally relates to methods of fingerprinting a complex therapeutic protein, via a two-dimensional (2D) nuclear magnetic resonance technique for mapping the structure of the chemical composition.

[0033] The current state of the art NMR techniques or methods have not been applied for the assessment of HOS for formulated proteins containing high concentrations of aliphatic excipients, such as sucrose and acetate, even though 2D ^{13}C NMR methyl fingerprinting methods have been recently introduced for mapping the structure of protein molecules, such as monoclonal antibodies (mAbs). Applications of these techniques are hampered by spectral interference from these excipients. This excipient interreference can be especially problematic for applications where excipient signals are often orders of magnitude larger than that of the target chemical composition, such as a protein, negatively influencing chemometric analysis

through introduction of baseline distortions or impacting the fidelity of picked peak parameters in the vicinity of the excipient signal.

[0034] The disclosed NMR methods provide modifications and improvements over existing NMR techniques to overcome strong interference in sucrose and acetate signals with regards to the methyl peaks. Applicants have discovered, upon various experiments on several samples and sample types to evaluate the effectiveness of using the described modified NMR techniques, that the above-described problems of interference have been overcome.

[0035] Thus, what has been surprisingly found is that changing the pulse profile can drastically influence the signal-to-noise ratio of various NMR regions. For example, a particular pulse profile can be used to excite the ^{13}C methyl signals from a therapeutic molecule while suppressing a ^{13}C excipient signal, such as that coming from a sucrose. The signals can be further enhanced by applying shorter gradient pulses less than 1 millisecond (ms) to increase the intensities of the ^{13}C methyl signals.

[0036] What follows is discussion of the evaluation and validation of the effectiveness of the various specific factors in the improved NMR methods, as well as related embodiments utilizing various combinations of these specifically described factors.

[0037] In accordance with related embodiments of the disclosed NMR methods, a method can include application of at least one of a Refocusing Band-Selective Pulse with Uniform Response and Phase (Reburp) pulse, a broad band inversion pulse (BIP) and a Gaussian (G3) inversion pulse, and an asymmetric adiabatic pulse. The application of at least one of the three different types of pulse excites the ^{13}C methyl signals of a therapeutic molecule while suppressing the ^{13}C excipient signal, such as those coming from sucrose. The method can also apply a water suppression technique (WET) sequence to suppress the signal of ^{1}H acetate (and/or signals from other excipients) which ^{13}C signal falls into the methyl region, that cannot be suppressed by the at least one of the three different types of pulses (Reburp, BIP, G3, adiabatic). The method can further include applying shorter gradient pulses to increase the intensities of ^{13}C methyl signals of a therapeutic molecule. The application of the aforementioned pulses culminates in the disclosed NMR methods that can be used for performing 2D ^{13}C NMR methyl fingerprinting to detect specific compositions, including peptides and proteins in pharmaceutical formulations, *etc.*

[0038] Now referring to the figures, Figure 1 shows an example NMR signal enhancing pulse profile 100 that uses a combination of an ^{1}H-^{13}C sensitivity-enhanced HSQC experiment and

additional signal processing steps according to some embodiments. Figure 2 shows another example of a NMR signal enhancing pulse profile 200 based on an ^{1}H-^{13}C sensitivity-enhanced HSQC experimental scheme, according to some embodiments. Figures 3A-3F show example excitation profiles 300a, 300b, and 300c, respectively, of pulses with different shapes to suppress the ^{13}C-sucrose signals, according to some embodiments. The example NMR signal enhancement techniques shown in Figures 1, 2, and 3A-3F are for illustrative purposes only.

[0039] Figure 1 shows an implementation of additional signal processing steps to the current state of the art ^{1}H-^{13}C sensitivity-enhanced HSQC experiment with a particular set of signal processing steps that has been applied to 2D ^{13}C NMR methyl fingerprinting for mAbs. As illustrated, the pulse profile 100 of Figure 1, a RF pulse with a specific signal profile is applied to induce proton (^{1}H) magnetization, which is subsequently transferred to the directly attached carbon (^{13}C) magnetization by Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) processing step. In Figure 1, Δ=1/2*J, δ=1/8*J, where J was set to 145 Hz, φ_1=0, 2; and φ_{rec}=0, 2. G1=80% with 1 ms and G2=20.1% with 1ms (or G1=80% with 250 μs and G2=20.1% with 246 μs). G7=-80% with 1 ms, G8=-40% with 1 ms, G9=-20% with 1 ms, G10=-10% with 1 ms, G11=50% with 1 ms, G5=5% with 600 μs, G6=-2% with 1 ms. The maximum gradient strength at 100% was about 53.5 G/cm (t1 and t2 are periods to acquire time domain data in F1 (frequency 1 after Fourier transform of t1 data points) and F2 (frequency 2 after Fourier transform of t2 data points) dimensions, respectively).

[0040] Upon application of the INEPT processing step, the carbon frequency is encoded in the carbon magnetization after the T_1 evolution period. The carbon magnetization is subsequently transferred back to the proton magnetization for detection through application of the sensitivity-enhanced reverse INEPT processing step. In various implementations, the coherence selection of ^{1}H-^{13}C magnetization, suppression of proton magnetization attached to ^{12}C (not NMR active), and absorption line shape in 2D data are accomplished by accompanying gradient pulses and the echo/anti-echo scheme, such as described by Davis, A.L.; Keeler, J.; Laue, E. D.; Moskau, D.; Experiments for recording pure-absorption heteronuclear correlation spectra using pulsed field gradients, *J. Magn. Reson*. 1992, 98, 207-216; Kay, L.; Keifer, P.; Saarinen, T.; Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity, *J. Am. Chem. Soc*. 1992, 114, 10663-10665; and J. Schleucher, J.; Schwendinger, M.; Sattler, M.; Schmidt, P.; Schedletzky, O.; Glaser, S. J.; Sorensen, O. W.; and Griesinger, O.W.; A general enhancement scheme in heteronuclear

multidimensional NMR employing pulsed field gradients, *J. Biomol. NMR* 1994, 4, 301-306). In the current NIST protocol for 2D ^{13}C NMR methyl fingerprinting, the carbon bandwidth is set between 7 to 35 ppm with the transmitter frequency at 21 ppm. Since the carbon signals of sucrose range from 60 to 103 ppm (as shown in Figure 3A), the signals result in aliasing in the 7 to 35 ppm range in the HSQC spectrum. In some instances, the aliased sucrose signals cannot be properly phased and result in dispersion of the signal in the tail regions of the F_2 domain. In some instances, these aliased signals interfere with the methyl peak analysis as further explained in detail with respect to Figure 6A.

[0041] To resolve the alias issue of sucrose signals in Figure 1, the disclosed NMR method includes improving the pulse design with a modified pulse profile to excite the ^{13}C methyl signals while suppressing the ^{13}C sucrose signals in the encoding period of echo/anti-echo scheme. In related embodiments, the pulse profile can be designed to suppress the ^{13}C sucrose signals. In related embodiments, the pulse profile can be designed to suppress the ^{1}H sucrose signals. In related embodiments, suppressing the ^{13}C sucrose signals can be straighter forward than suppressing the ^{1}H sucrose signals because carbon signals are more dispersed than the proton signals. Since the excitation band shown in Figure 1 covers 7 ppm to 35 ppm and the suppression band is 60 ppm and beyond, the transition band can be set, for example, to between 60 and 35 ppm. Therefore, for an NMR system operating at 600 MHz, 25 ppm bandwidth is 3772.5 Hz (150.9 Hz/ppm). However, the proton transition can only be about 1.5 ppm (900 Hz, 600 Hz/ppm) between 3.5 and 2 ppm, or less. The bandwidth can change according to the NMR operating frequency, which can be from 100 MHz to 2000 MHz. In accordance with various embodiments, the NMR operating frequency can range from about 100 MHz to about 2000 MHz, about 500 MHz to about 2000 MHz, about 500 MHz to about 1000 MHz, about 500 MHz to about 900 MHz, about 600 MHz to about 800 MHz, inclusive of any frequency ranges therebetween. In accordance with various embodiments, the NMR system can operate at a frequency of about 100 MHz, about 200 MHz, about 300 MHz, about 400 MHz, about 500 MHz, about 600 MHz, about 700 MHz, about 800 MHz, about 900 MHz, about 1000 MHz, about 1100 MHz, about 1200 MHz, about 1300 MHz, about 1400 MHz, about 1500 MHz, about 1600 MHz, about 1700 MHz, about 1800 MHz, about 1900 MHz, about 2000 MHz, inclusive of any frequency therebetween. For illustrative purposes, the experiments of examples 1 and 2 described herein use a 600 MHz NMR system, and the experiment of example 3 uses an 800 MHz NMR system. For other field strengths, certain parameters for various pulses

discussed below can be adjusted, such as lengths of Reburp and G3, and the position of transmitter offset at the ppm scale for asymmetric adiabatic pulses. Moreover, depending on the operating frequency, certain parameters for various pulses can be adjusted, such as lengths of G2 or G4. For example, at 800 MHz NMR, the pulse length of gradient can be 248 μs, G2 could be 40.00% to 40.50%, and G4 can be -40.00% to -40.50%. However, the performance of asymmetric adiabatic pulses is independent of field strength.

[0042] In the example shown in Figure 2, a disclosed NMR method includes using the CLUB sandwich approach, such as described by for example, Mandelshtam, V. A.; Hu, H.; Shaka, A. J., Two-dimensional HSQC NMR spectra obtained using a self-compensating double pulsed field gradient and processed using the filter diagonalization method, *Magn. Reson. Chem.* 1998, 36, S17-S28; and Hu, H.; Shaka, A. J., Composite pulsed field gradients with refocused chemical shifts and short recovery time. *J. Magn. Reson.* 1999, 136, 54-62, during the encoding period of echo/anti-echo scheme. When using the double-echo approach to design a refocusing pulse, the design process is simplified to investigate the inversion profile of the element used in the double-echo sequence, where the phase at the end of double-echo sequence is the same as that at the start of the sequence. With this approach, the refocusing profile is then probability of spin flip using an inversion element squared as described, for example, by Hwang, T.-L.; Shaka, A. J., Water suppression that works. Excitation sculpting using arbitrary waveforms and pulsed field gradients. *J. Magn. Reson. A* 1995, 112, 275-279. This is unlike the design of Reburp or similar refocusing pulses, where both amplitude and phase responses of magnetization under the influence of RF pulses and offsets need to be considered.

[0043] As explained above, Figures 3A-3F show example excitation profiles of pulses with different shapes to suppress the ^{13}C sucrose signals, according to some embodiments. The sample used in the measurement is 1% water with 0.1 mg/ml gadolinium chloride ($GdCl_3$) in deuterated water (D_2O). As stated above, Figure 3A shows a pulse profile 300a of ^{13}C signal for sucrose and acetate signal regions. In the figure, the relative intensities of both the sucrose and acetate signals can be observed.

[0044] Figure 3B shows a pulse profile 300b of a Reburp profile, according to related embodiments. In various implementations, the disclosed NMR method includes a Reburp refocusing pulse 300b as shown in Figure 3B to remove the sucrose signals by replacing a conventional hard pulse with a 750 μs Reburp refocusing pulse with transmitter offset at 21 ppm, which covers the excitation bandwidth for the methyl ^{13}C region. Although there are

excited side lobes in the transition period, the intensities of excited peaks are small around the 60 ppm area, as shown in Figure 3B.

[0045] Figure 3C shows a combination of BIP and G3 pulse profile 300c, according to related embodiments. The excitation profile of this pulse combination shown in Figure 3C leads to good suppression of the sucrose signals. As illustrated in Figure 2, the first CLUB sandwich element uses the combination of a broadband BIP pulse with 120 μs duration positioned at 55 ppm to excite a wide range of magnetization and a G3 inversion pulse with 500 μs duration positioned at 81.5 ppm to suppress the sucrose signals.

[0046] Some experiments using NMR measurement techniques require inversion or excitation for magnetization in one side of bandwidth. In various implementations, an asymmetric adiabatic full passage containing two half passages from HS1/2 and tanh/tan modulation functions, such as described, for example, by Hwang, T.-L.; van Zijl, P. C. M.; Garwood, M., Asymmetric adiabatic pulses for NH selection. *J. Magn. Reson.* 1999, *138*, 173-177, with different R values (R = pulse length in second * bandwidth in Hz) and pulse lengths (Tp) can narrow the transition bandwidth while achieving the broadband inversion or excitation on one side of spectrum.

[0047] Figures 3D, 3E, and 3F show three example asymmetric adiabatic pulses 300d, 300e, and 300f, respectively, which are optimized with different pulse lengths for inversion of ^{13}C methyl signals while suppression of ^{13}C sucrose signals. In each of the Figures 3D, 3E, and 3F, T_x is the transmitter offset and the profiles were generated by incrementing the offset with 1 ppm interval.

[0048] Figure 3D shows a pulse profile 300d, shown as (1) [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 140, 0.1 Tp] for pulse length 1500 μs with transmitter offset at 43 ppm as described, for example, by Hwang, T.-L.; van Zijl, P. C. M.; Garwood, M., Asymmetric adiabatic pulses for NH selection. *J. Magn. Reson.* 1999, 138, 173-177. As a result, the excitation band can cover the methyl region, while sucrose carbon signals are suppressed. The transition bandwidth of [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 140, 0.1 Tp] for pulse length 1500 μs is about 700 Hz (Figure 3D). Note that the entire pulse profile can be moved around according to the position of transmitter offset for the pulse. In other words, if the transmitter offset of the pulse is positioned at 21 ppm, the excitation band moves to a lower ppm range accordingly, which still covers the methyl region while C_β carbon signals are suppressed.

[0049] Figure 3E shows a pulse profile 300e, shown as (2) [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 70, 0.1 Tp] for pulse length 750 μs with transmitter offset at 30 ppm. The excitation band covers the methyl region of a therapeutic molecule, while sucrose carbon signals are suppressed.

[0050] Figure 3F shows a pulse profile 300f, shown as (3) [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 50, 0.1 Tp] for pulse length 375 μs with transmitter offset at 2 ppm. Similarly, the excitation band can cover the methyl region of a therapeutic molecule, while sucrose carbon signals are suppressed. In Figure 3F, although the transition bandwidth of [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 50, 0.1 Tp] for pulse length 375 μs is much wider, the shorter pulse length reduces the intensity loss of methyl peaks due to the very short T_2 and $T_{1\rho}$ relaxation of mAbs' magnetization.

[0051] Figure 4 is a graph 400 of a spectrum that is the result of Fourier transformation of time-domain free-induction decay data into frequency domain data, thus visualizing NMR peaks appearing at different ppm. The X-axis is expressed as ppm and is independent of spectrometer frequency, which allows for the comparison of spectra at different field strength. As shown in Figure 4, graph 400 shows the comparison of signal intensities for sucrose, acetate and methyl peaks based on an ^{1}H-^{13}C sensitivity-enhanced HSQC experimental scheme, according to related embodiments. The intensities of different components in the ^{1}H-^{13}C HSQC experiments are measured using a hard refocusing pulse in the encoding period of echo/anti-echo. As shown in Figure 4, the intensities of sucrose signals are much greater than those of the methyl peaks, causing the signal interference issue in the 2D spectrum.

[0052] Figure 5 is a graph 500 showing a spectrum that is Fourier transformed of time domain-free induction decay data into frequency domain data, enabling visualization of NMR peaks appearing at different ppm. The X-axis is expressed as ppm and is independent of spectrometer frequency, which allows for the comparison of spectra at different field strength. As shown in Figure 5, graph 500 shows the comparison of signal intensities for sucrose and methyl peaks based on the inventive ^{1}H-^{13}C sensitivity-enhanced HSQC experimental scheme using different proposed RF pulses in the encoding period of echo/anti-echo scheme, according to some embodiments. In particular, the signal profiles shown in Figure 5 are from the signal intensities of different components measured via the ^{1}H-^{13}C HSQC experiments using the newly proposed refocusing pulses (*i.e.*, Reburp, BIP+G3, and asymmetric adiabatic pulses) in the encoding period of echo/anti-echo scheme. In various implementations, the water suppression

technique (WET) scheme is applied to suppress the acetate signal. In various implementations, a digital filter is applied to further remove the water signal.

[0053] Figure 5 also shows that the intensities of sucrose signals are about the same order of magnitude as those of the methyl peaks. In the 2D spectrum, these sucrose signals behave like T_1 noises, and do not interfere with the methyl peak analysis (as shown in Figures 6B and 6C). These spectra also show that the intensities of methyl peaks vary slightly for pulses with different pulse lengths. For example, the pulse profile of [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 140, 0.1 Tp] with a pulse length 1500 μs positioned at 21 ppm does not excite the C_β signals, and the corresponding H_β peaks around 3 ppm disappears as shown in Figure 5.

[0054] In various implementations, the T_2 and $T_{1\rho}$ relaxations of signals for small peptides are much slower than those of large mAbs. Conversely, the intensity loss due to the T_2 and $T_{1\rho}$ relaxation of mAbs and/or diffusion effect can be significant at slight differences in the pulse lengths. As a result, any slight differences in the pulse lengths can have significant effects on the intensities of methyl peaks for mAbs. In accordance with related embodiments of the disclosed NMR methods, the pulse sequences can be improved by shortening the gradient pulses from 1000 μs to 250 μs for the echo/anti-echo period. This approach is experimented using Sample 3. Because different polarity of gradients in the CLUB sandwich can cancel the eddy currents, the gradient recovery can be further reduced from the conventional 200 μs to 50 μs. Upon applying these optimized values to current and new ^{1}H-^{13}C HSQC experiments by integrating the methyl peak area between -0.5 to 2 ppm, the relative integral values from different experiments are compared in Table 1 below.

Table 1

Comparison of relative methyl intensities from different experiments

Experimental conditions for the echo/anti-echo schemes	Relative methyl intensity
[1]Hard pulse, G1=80% with 250 μs, G2=20.1% with 246 μs	1
[1]Reburp for pulse length 750 μs with transmitter offset at 21 ppm, G1=80% with 250 μs, G2=20.1% with 246 μs	0.88
[2][$HS\frac{1}{2}$, R = 10, 0.9 T_p; tanh/tan, R = 50, 0.1 T_p] for pulse length 375 μs with transmitter offset at 2 ppm	0.88

[2]BIP pulse with 120 μs duration positioned at 55 ppm and a G3 inversion pulse with 500 μs duration positioned at 81.5 ppm	0.84
[2][HS$\frac{1}{2}$, R = 10, 0.9 T_p; tanh/tan, R = 70, 0.1 T_p] for pulse length 750 μs with transmitter offset at 30 ppm	0.84
[2][HS$\frac{1}{2}$, R = 10, 0.9 T_p; tanh/tan, R = 140, 0.1 T_p] for pulse length 1500 μs with transmitter offset at 43 ppm	0.76
[2][HS$\frac{1}{2}$, R = 10, 0.9 T_p; tanh/tan, R = 140, 0.1 T_p] for pulse length 1500 μs with transmitter offset at 21 ppm	0.76
[1]Hard pulse, G1=80% with 1000 μs, G2=20.1% with 1000 μs	0.73

[1] Pulse sequence in Figure 1. The maximum gradient strength is about 53.5 G/cm at 100%. Gradient recovery = 200 μs.
[2] Pulse sequence in Figure 2. For these experiments, G1=80% with 250 μs, G2=40.11% with 246 μs, G3= -80% with 250 μs, G4= -40.08% with 246 μs, gradient recovery = 50 μs.

[0055] The data in Table 1 show the original hard refocusing experiment with gradients at 1 ms (1000 μs) lengths has the lowest relative intensity at 0.73. After shorting the gradient pulse lengths to about 250 μs, the relative methyl intensities increase significantly to 1.

[0056] Figures 6A-6C show different ^{13}C 2D methyl fingerprinting plots 600a, 600b, and 600c, respectively, for comparing effectiveness of particular NMR enhancement methods. Figure 6A shows the experimental result using the conventional NMR method (*i.e.*, the NIST protocol) on a sample containing mAb1, 50 mg/ml, 9% sucrose, 10 mM acetate, 0.01% polysorbate (PS) 80 at pH = 5.2 with 3% D_2O. The sucrose signals aliased to the methyl region and strip of acetate signals showed up around 2 ppm. These artifacts interfered with the methyl peak analysis. In contrast, Figure 6B displays a clean methyl region without the interference from sucrose and acetate signals. The result is obtained by using the [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 50, 0.1 Tp] for pulse length 375 μs with transmitter offset at 2 ppm as the refocusing element, and the WET sequence to suppress the ^1H acetate signal. Figure 6C presents that C_β region can be further suppressed by using the [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 140, 0.1 Tp] for pulse length 1500 μs with transmitter offset at 21 ppm.

Therapeutic Proteins

[0057] "Therapeutic protein" refers to any protein molecule which exhibits therapeutic biological activity. The therapeutic protein molecule can be, for example, a full-length protein.

In other embodiments, the therapeutic protein is an active fragment of a full-length protein. The therapeutic protein may be produced and purified from its natural source. Alternatively, the term "recombinant therapeutic protein" includes any therapeutic protein obtained via recombinant DNA technology.

[0058] Proteins, including those that bind to one or more of the following, can be used in the disclosed methods. These include CD proteins, including CD3, CD4, CD8, CD19, CD20, CD22, CD30, and CD34; including those that interfere with receptor binding. HER receptor family proteins, including HER2, HER3, HER4, and the EGF receptor. Cell adhesion molecules, for example, LFA-I, MoI, pl50, 95, VLA-4, ICAM-I, VCAM, and alpha v/beta 3 integrin. Growth factors, such as vascular endothelial growth factor ("VEGF"), growth hormone, thyroid stimulating hormone, follicle stimulating hormone, luteinizing hormone, growth hormone releasing factor, parathyroid hormone, Mullerian-inhibiting substance, human macrophage inflammatory protein (MIP-I -alpha), erythropoietin (EPO), nerve growth factor, such as NGF-beta, platelet-derived growth factor (PDGF), fibroblast growth factors, including, for instance, aFGF and bFGF, epidermal growth factor (EGF), transforming growth factors (TGF), including, among others, TGF- α and TGF-β, including TGF-βl, TGF-β2, TGF-β3, TGF- β4, or TGF- β 5, insulin-like growth factors-I and -II (IGF-I and IGF-II), des(l-3)-IGF-I (brain IGF-I), and osteoinductive factors. Insulins and insulin-related proteins, including insulin, insulin A-chain, insulin B-chain, proinsulin, and insulin-like growth factor binding proteins. Coagulation and coagulation-related proteins, such as, among others, factor VIII, tissue factor, von Willebrands factor, protein C, alpha-1-antitrypsin, plasminogen activators, such as urokinase and tissue plasminogen activator ("t-PA"), bombazine, thrombin, and thrombopoietin; other blood and serum proteins, including but not limited to albumin, IgE, and blood group antigens. Colony stimulating factors and receptors thereof, including the following, among others, M-CSF, GM-CSF, and G-CSF, and receptors thereof, such as CSF-1 receptor (c-fms). Receptors and receptor-associated proteins, including, for example, flk2/flt3 receptor, obesity (OB) receptor, LDL receptor, growth hormone receptors, thrombopoietin receptors ("TPO-R," "c-mpl"), glucagon receptors, interleukin receptors, interferon receptors, T-cell receptors, stem cell factor receptors, such as c-Kit, and other receptors. Receptor ligands, including, for example, OX40L, the ligand for the OX40 receptor. Neurotrophic factors, including bone-derived neurotrophic factor (BDNF) and neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6). Relaxin A-chain, relaxin B-chain, and prorelaxin; interferons and interferon receptors, including for example,

interferon-α, -β, and -γ, and their receptors. Interleukins and interleukin receptors, including IL-I to IL-33 and IL-I to IL-33 receptors, such as the IL-8 receptor, among others. Viral antigens, including an AIDS envelope viral antigen. Lipoproteins, calcitonin, glucagon, atrial natriuretic factor, lung surfactant, tumor necrosis factor-alpha and -beta, enkephalinase, RANTES (regulated on activation normally T-cell expressed and secreted), mouse gonadotropin-associated peptide, DNAse, inhibin, and activin. Integrin, protein A or D, rheumatoid factors, immunotoxins, bone morphogenetic protein (BMP), superoxide dismutase, surface membrane proteins, decay accelerating factor (DAF), AIDS envelope, transport proteins, homing receptors, addressins, regulatory proteins, immunoadhesins, antibodies. Myostatins, TALL proteins, including TALL-I, amyloid proteins, including but not limited to amyloid-beta proteins, thymic stromal lymphopoietins ("TSLP"), RANK ligand ("OPGL"), c-kit, TNF receptors, including TNF Receptor Type 1, TRAIL-R2, angiopoietins, and biologically active fragments or analogs or variants of any of the foregoing.

[0059] Other therapeutic proteins include Activase® (Alteplase); alirocumab, Aranesp® (Darbepoetin-alfa), Epogen® (Epoetin alfa, or erythropoietin); Avonex® (Interferon β-Ia); Bexxar® (Tositumomab); Betaseron® (Interferon-β); bococizumab (anti-PCSK9 monoclonal antibody designated as L1L3, see U.S.P.N. 8,080,243); Campath® (Alemtuzumab); Dynepo® (Epoetin delta); Velcade® (bortezomib); MLN0002 (anti-α4β7 Ab); MLN1202 (anti-CCR2 chemokine receptor Ab); Enbrel® (etanercept); Eprex® (Epoetin alfa); Erbitux® (Cetuximab); evolocumab; Genotropin® (Somatropin); Herceptin® (Trastuzumab); Humatrope® (somatropin [rDNA origin] for injection); Humira® (Adalimumab); Infergen® (Interferon Alfacon-1); Natrecor® (nesiritide); Kineret® (Anakinra), Leukine® (Sargamostim); LymphoCide® (Epratuzumab); BenlystaTM (Belimumab); Metalyse® (Tenecteplase); Mircera® (methoxy polyethylene glycol-epoetin beta); Mylotarg® (Gemtuzumab ozogamicin); Raptiva® (efalizumab); Cimzia® (certolizumab pegol); SolirisTM (Eculizumab); Pexelizumab (Anti-C5 Complement); MEDI-524 (Numax®); Lucentis® (Ranibizumab); Edrecolomab (Panorex®); Trabio® (lerdelimumab); TheraCim hR3 (Nimotuzumab); Omnitarg (Pertuzumab, 2C4); Osidem® (IDM-I); OvaRex® (B43.13); Nuvion® (visilizumab); Cantuzumab mertansine (huC242-DMl); NeoRecormon® (Epoetin beta); Neumega® (Oprelvekin); Neulasta® (Pegylated filgastrim, pegylated G-CSF, pegylated hu-Met-G-CSF); Neupogen® (Filgrastim); Orthoclone OKT3® (Muromonab-CD3), Procrit® (Epoetin alfa); Remicade® (Infliximab), Reopro® (Abciximab), Actemra® (anti-IL6 Receptor Ab), Avastin® (Bevacizumab), HuMax-CD4 (zanolimumab), Rituxan® (Rituximab);

Tarceva® (Erlotinib); Roferon-A®-(Interferon alfa-2a); Simulect® (Basiliximab); StelaraTM (Ustekinumab); Prexige® (lumiracoxib); Synagis® (Palivizumab); 146B7-CHO (anti-IL15 antibody, see U.S.P.N. 7.153,507), Tysabri® (Natalizumab); Valortim® (MDX-1303, anti-B. anthracis Protective Antigen Ab); ABthraxTM; Vectibix® (Panitumumab); Xolair® (Omalizumab), ETI211 (anti-MRSA Ab), IL-I Trap (the Fc portion of human IgGl and the extracellular domains of both IL-I receptor components (the Type I receptor and receptor accessory protein), VEGF Trap (Ig domains of VEGFRl fused to IgGl Fc), Zenapax® (Daclizumab); Zenapax® (Daclizumab), Zevalin® (Ibritumomab tiuxetan), Atacicept (TACI-Ig), anti-α4β7 Ab (vedolizumab); galiximab (anti-CD80 monoclonal antibody), anti-CD23 Ab (lumiliximab); BR2-Fc (huBR3 / huFc fusion protein, soluble BAFF antagonist); SimponiTM (Golimumab); Mapatumumab (human anti-TRAIL Receptor-1 Ab); Ocrelizumab (anti-CD20 human Ab); HuMax-EGFR (zalutumumab); M200 (Volociximab, anti-α5β1 integrin Ab); MDX-010 (Ipilimumab, anti-CTLA-4 Ab and VEGFR-I (IMC-18F1); anti-BR3 Ab; anti-C. difficile Toxin A and Toxin B C Abs MDX-066 (CDA-I) and MDX-1388); anti-CD22 dsFv-PE38 conjugates (CAT-3888 and CAT-8015); anti-CD25 Ab (HuMax-TAC); anti-TSLP antibodies; anti-TSLP receptor antibody (see U.S.P.N. 8,101,182); anti-TSLP antibody designated as A5 (see U.S.P.N. 7,982,016); (see anti-CD3 Ab (NI-0401); Adecatumumab (MT201, anti-EpCAM-CD326 Ab); MDX-060, SGN-30, SGN-35 (anti-CD30 Abs); MDX-1333 (anti- IFNAR); HuMax CD38 (anti-CD38 Ab); anti-CD40L Ab; anti-Cripto Ab; anti-CTGF Idiopathic Pulmonary Fibrosis Phase I Fibrogen (FG-3019); anti-CTLA4 Ab; anti-eotaxinl Ab (CAT-213); anti-FGF8 Ab; anti-ganglioside GD2 Ab; anti-sclerostin antibodies (see, U.S.P.N. 8,715,663 or U.S.P.N.7,592,429) anti-sclerostin antibody designated as Ab-5 (see U.S.P.N. 8,715,663 or U.S.P.N. 7,592,429); anti-ganglioside GM2 Ab; anti-GDF-8 human Ab (MYO-029); anti-GM-CSF Receptor Ab (CAM-3001); anti-HepC Ab (HuMax HepC); MEDI-545, MDX-1103 (anti-IFNα Ab); anti-IGFIR Ab; anti-IGF-IR Ab (HuMax-Inflam); anti-IL12/IL23p40 Ab (Briakinumab); anti-IL-23p19 Ab (LY2525623); anti-IL13 Ab (CAT-354); anti-IL-17 Ab (AIN457); anti-IL2Ra Ab (HuMax-TAC); anti-IL5 Receptor Ab; anti-integrin receptors Ab (MDX-Ol8, CNTO 95); anti-IPIO Ulcerative Colitis Ab (MDX- 1100); anti-LLY antibody; BMS-66513; anti-Mannose Receptor/hCGβ Ab (MDX-1307); anti-mesothelin dsFv-PE38 conjugate (CAT-5001); anti-PDlAb (MDX-1 106 (ONO- 4538)); anti-PDGFRα antibody (IMC-3G3); anti-TGFβ Ab (GC-1008); anti-TRAIL Receptor-2 human Ab (HGS-ETR2); anti-TWEAK Ab; anti-VEGFR/Flt-1 Ab; anti- ZP3 Ab (HuMax-ZP3); NVS Antibody #1; NVS Antibody #2; and an amyloid-beta monoclonal antibody comprising sequences, SEQ ID NO:8 and SEQ ID NO:6 (see U.S.P.N. 7,906,625).

[0060] Examples of antibodies that can be used in the disclosed methods include the antibodies shown in Table A. Other examples of suitable antibodies include infliximab, bevacizumab, ranibizumab, cetuximab, ranibizumab, palivizumab, abagovomab, abciximab, actoxumab, adalimumab, afelimomab, afutuzumab, alacizumab, alacizumab pegol, ald518, alemtuzumab, alirocumab, alemtuzumab, altumomab, amatuximab, anatumomab mafenatox, anrukinzumab, apolizumab, arcitumomab, aselizumab, altinumab, atlizumab, atorolimiumab, tocilizumab, bapineuzumab, basiliximab, bavituximab, bectumomab, belimumab, benralizumab, bertilimumab, besilesomab, bevacizumab, bezlotoxumab, biciromab, bivatuzumab, bivatuzumab mertansine, blinatumomab, blosozumab, brentuximab vedotin, briakinumab, brodalumab, canakinumab, cantuzumab mertansine, cantuzumab mertansine, caplacizumab, capromab pendetide, carlumab, catumaxomab, cc49, cedelizumab, certolizumab pegol, cetuximab, citatuzumab bogatox, cixutumumab, clazakizumab, clenoliximab, clivatuzumab tetraxetan, conatumumab, crenezumab, cr6261, dacetuzumab, daclizumab, dalotuzumab, daratumumab, demcizumab, denosumab, detumomab, dorlimomab aritox, drozitumab, duligotumab, dupilumab, ecromeximab, eculizumab, edobacomab, edrecolomab, efalizumab, efungumab, elotuzumab, elsilimomab, enavatuzumab, enlimomab pegol, enokizumab, enokizumab, enoticumab, enoticumab, ensituximab, epitumomab cituxetan, epratuzumab, erlizumab, ertumaxomab, etaracizumab, etrolizumab, exbivirumab, exbivirumab, fanolesomab, faralimomab, farletuzumab, fasinumab, fbta05, felvizumab, fezakinumab, ficlatuzumab, figitumumab, flanvotumab, fontolizumab, foralumab, foravirumab, fresolimumab, fulranumab, futuximab, galiximab, ganitumab, gantenerumab, gavilimomab, gemtuzumab ozogamicin, gevokizumab, girentuximab, glembatumumab vedotin, golimumab, gomiliximab, gs6624, ibalizumab, ibritumomab tiuxetan, icrucumab, igovomab, imciromab, imgatuzumab, inclacumab, indatuximab ravtansine, infliximab, intetumumab, inolimomab, inotuzumab ozogamicin, ipilimumab, iratumumab, itolizumab, ixekizumab, keliximab, labetuzumab, lebrikizumab, lemalesomab, lerdelimumab, lexatumumab, libivirumab, ligelizumab, lintuzumab, lirilumab, lorvotuzumab mertansine, lucatumumab, lumiliximab, mapatumumab, maslimomab, mavrilimumab, matuzumab, mepolizumab, metelimumab, milatuzumab, minretumomab, mitumomab, mogamulizumab, morolimumab, motavizumab, moxetumomab pasudotox, muromonab-cd3, nacolomab tafenatox, namilumab, naptumomab estafenatox, narnatumab, natalizumab, nebacumab, necitumumab, nerelimomab, nesvacumab, nimotuzumab, nivolumab, nofetumomab merpentan, ocaratuzumab, ocrelizumab,

odulimomab, ofatumumab, olaratumab, olokizumab, omalizumab, onartuzumab, oportuzumab monatox, oregovomab, orticumab, otelixizumab, oxelumab, ozanezumab, ozoralizumab, pagibaximab, palivizumab, panitumumab, panobacumab, parsatuzumab, pascolizumab, pateclizumab, patritumab, pemtumomab, perakizumab, pertuzumab, pexelizumab, pidilizumab, pintumomab, placulumab, ponezumab, priliximab, pritumumab, PRO 140, quilizumab, racotumomab, radretumab, rafivirumab, ramucirumab, ranibizumab, raxibacumab, regavirumab, reslizumab, rilotumumab, rituximab, robatumumab, roledumab, romosozumab, rontalizumab, rovelizumab, ruplizumab, samalizumab, sarilumab, satumomab pendetide, secukinumab, sevirumab, sibrotuzumab, sifalimumab, siltuximab, simtuzumab, siplizumab, sirukumab, solanezumab, solitomab, sonepcizumab, sontuzumab, stamulumab, sulesomab, suvizumab, tabalumab, tacatuzumab tetraxetan, tadocizumab, talizumab, tanezumab, taplitumomab paptox, tefibazumab, telimomab aritox, tenatumomab, tefibazumab, telimomab aritox, tenatumomab, teneliximab, teplizumab, teprotumumab, TGN1412, tremelimumab, ticilimumab, tildrakizumab, tigatuzumab, TNX-650, tocilizumab, toralizumab, tositumomab, tralokinumab, trastuzumab, TRBS07, tregalizumab, tremelimumab, tucotuzumab celmoleukin, tuvirumab, ublituximab, urelumab, urtoxazumab, ustekinumab, vapaliximab, vatelizumab, vedolizumab, veltuzumab, vepalimomab, vesencumab, visilizumab, volociximab, vorsetuzumab mafodotin, votumumab, zalutumumab, zanolimumab, zatuximab, ziralimumab and zolimomab aritox.

[0061] Most preferred antibodies for use in the disclosed methods are adalimumab, bevacizumab, blinatumomab, cetuximab, conatumumab, denosumab, eculizumab, erenumab, evolocumab, infliximab, natalizumab, panitumumab, rilotumumab, rituximab, romosozumab, and trastuzumab, and antibodies selected from Table A.

TABLE A

Examples of therapeutic antibodies

Target (informal name)	Conc. (mg/ml)	Viscosity (cP)	HC Type (including allotypes)	LC Type	pI	LC SEQ ID NO	HC SEQ ID NO
anti-amyloid	142.2	5.0	IgG1 (f) (R;EM)	Kappa	9.0	1	2
GMCSF (247)	139.7	5.6	IgG2	Kappa	8.7	3	4
CGRPR	136.6	6.3	IgG2	Lambda	8.6	5	6

Target (informal name)	Conc. (mg/ml)	Viscosity (cP)	HC Type (including allotypes)	LC Type	pI	LC SEQ ID NO	HC SEQ ID NO
RANKL	152.7	6.6	IgG2	Kappa	8.6	7	8
Sclerostin (27H6)	145.0	6.7	IgG2	Kappa	6.6	9	10
IL-1R1	153.9	6.7	IgG2	Kappa	7.4	11	12
Myostatin	141.0	6.8	IgG1 (z) (K;EM)	Kappa	8.7	13	14
B7RP1	137.5	7.7	IgG2	Kappa	7.7	15	16
Amyloid	140.6	8.2	IgG1 (za) (K;DL)	Kappa	8.7	17	18
GMCSF (3.112)	156.0	8.2	IgG2	Kappa	8.8	19	20
CGRP (32H7)	159.5	8.3	IgG2	Kappa	8.7	21	22
CGRP (3B6.2)	161.1	8.4	IgG2	Lambda	8.6	23	24
PCSK9 (8A3.1)	150.0	9.1	IgG2	Kappa	6.7	25	26
PCSK9 (492)	150.0	9.2	IgG2	Kappa	6.9	27	28
CGRP	155.2	9.6	IgG2	Lambda	8.8	29	30
Hepcidin	147.1	9.9	IgG2	Lambda	7.3	31	32
TNFR p55)	157.0	10.0	IgG2	Kappa	8.2	33	34
OX40L	144.5	10.0	IgG2	Kappa	8.7	35	36
HGF	155.8	10.6	IgG2	Kappa	8.1	37	38
GMCSF	162.5	11.0	IgG2	Kappa	8.1	39	40
Glucagon R	146.0	12.1	IgG2	Kappa	8.4	41	42
GMCSF (4.381)	144.5	12.1	IgG2	Kappa	8.4	43	44
Sclerostin (13F3)	155.0	12.1	IgG2	Kappa	7.8	45	46
CD-22	143.7	12.2	IgG1 (f) (R;EM)	Kappa	8.8	47	48
INFgR	154.2	12.2	IgG1 (za) (K;DL)	Kappa	8.8	49	50
Ang2	151.5	12.4	IgG2	Kappa	7.4	51	52
TRAILR2	158.3	12.5	IgG1 (f) (R;EM)	Kappa	8.7	53	54
EGFR	141.7	14.0	IgG2	Kappa	6.8	55	56
IL-4R	145.8	15.2	IgG2	Kappa	8.6	57	58
IL-15	149.0	16.3	IgG1 (f) (R;EM)	Kappa	8.8	59	60

Target (informal name)	Conc. (mg/ml)	Viscosity (cP)	HC Type (including allotypes)	LC Type	pI	LC SEQ ID NO	HC SEQ ID NO
IGF1R	159.2	17.3	IgG1 (za) (K;DL)	Kappa	8.6	61	62
IL-17R	150.9	19.1	IgG2	Kappa	8.6	63	64
Dkk1 (6.37.5)	159.4	19.6	IgG2	Kappa	8.2	65	66
Sclerostin	134.8	20.9	IgG2	Kappa	7.4	67	68
TSLP	134.2	21.4	IgG2	Lambda	7.2	69	70
Dkk1 (11H10)	145.3	22.5	IgG2	Kappa	8.2	71	72
PCSK9	145.2	22.8	IgG2	Lambda	8.1	73	74
GIPR (2G10.006)	150.0	23.0	IgG1 (z) (K;EM)	Kappa	8.1	75	76
Activin	133.9	29.4	IgG2	Lambda	7.0	77	78
Sclerostin (2B8)	150.0	30.0	IgG2	Lambda	6.7	79	80
Sclerostin	141.4	30.4	IgG2	Kappa	6.8	81	82
c-fms	146.9	32.1	IgG2	Kappa	6.6	83	84
α4β7	154.9	32.7	IgG2	Kappa	6.5	85	86
PD-1	-	-	IgG2	Kappa	-	87	88

* An exemplary concentration suitable for patient administration; ^HC – antibody heavy chain; LC – antibody light chain.

Mutein

[0062] Mutein is a protein having at least amino acid change due to a mutation in the nucleic acid sequence, such as a substitution, deletion or insertion. Exemplary muteins comprise amino acid sequences having at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, or has greater than about 90% (*e.g.*, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%) sequence identity to the wild type amino acid sequence. In addition, the mutein may be a fusion protein as described above. In exemplary embodiments, the mutein comprises an amino acid sequence comprising at least one amino acid substitution relative to the wild-type amino acid sequence, and the amino acid substitution(s) is/are conservative amino acid substitution(s). As used herein, the term

"conservative amino acid substitution" refers to the substitution of one amino acid with another amino acid having similar properties, *e.g.*, size, charge, hydrophobicity, hydrophilicity, and/or aromaticity, and includes exchanges within one of the following five groups:

I. **Small aliphatic, nonpolar or slightly polar residues**: Ala, Ser, Thr, Pro, Gly;

II. **Polar, negatively charged residues and their amides and esters**: Asp, Asn, Glu, Gln, cysteic acid and homocysteic acid;

III. **Polar, positively charged residues**: His, Arg, Lys; Ornithine (Orn)

IV. **Large, aliphatic, nonpolar residues**: Met, Leu, Ile, Val, Cys, Norleucine (Nle), homocysteine

V. **Large, aromatic residues**: Phe, Tyr, Trp, acetyl phenylalanine.

[0063] In exemplary embodiments, the mutein comprises an amino acid sequence comprising at least one amino acid substitution relative to the wild-type amino acid sequence, and the amino acid substitution(s) is/are non-conservative amino acid substitution(s). As used herein, the term "non-conservative amino acid substitution" is defined herein as the substitution of one amino acid with another amino acid having different properties, *e.g.*, size, charge, hydrophobicity, hydrophilicity, and/or aromaticity, and includes exchanges outside the above five groups.

[0064] In exemplary aspects, the mutein comprises an amino acid sequence comprising at least one amino acid substitution relative to the wild-type amino acid sequence, and the substitute amino acid is a naturally-occurring amino acid. By “naturally-occurring amino acid” or “standard amino acid” or “canonical amino acid” is meant one of the 20 alpha amino acids found in eukaryotes encoded directly by the codons of the universal genetic code (Ala, Val, Ile, Leu, Met, Phe, Tyr, Trp, Ser, Thr, Asn, Gln, Cys, Gly, Pro, Arg, His, Lys, Asp, Glu). In exemplary aspects, the mutein comprises an amino acid sequence comprising at least one amino acid substitution relative to the wild-type amino acid sequence, and the substitute amino acid is a non-standard amino acid, or an amino acid which is not incorporated into proteins during translation. Non-standard amino acids include, but are not limited to: selenocysteine, pyrrolysine, ornithine, norleucine, β-amino acids (*e.g.*, β-alanine, β-aminoisobutyric acid, β-phenlyalanine, β-homophenylalanine, β-glutamic acid, β-glutamine, β-homotryptophan, β-leucine, β-lysine), homo-amino acids (*e.g.*, homophenylalanine, homoserine, homoarginine, monocysteine, homocystine), *N*-methyl amino acids (*e.g.*, L-abrine, *N*-methyl-alanine, *N*-

methyl-isoleucine, *N*-methyl-leucine), 2-aminocaprylic acid, 7-aminocephalosporanic acid, 4-aminocinnamic acid, alpha-aminocyclohexanepropionic acid, amino-(4-hydroxyphenyl)acetic acid, 4-amino-nicotinic acid, 3-aminophenylacetic acid, and the like.

BiTE® Molecules

[0065] Bispecific T cell engager (BiTE) molecules are a bispecific antibody construct or bispecific fusion protein comprising two antibody binding domains (or targeting regions) linked together. One arm of the molecule is engineered to bind with a protein found on the surface of cytotoxic T cells, and the other arm is designed to bind to a specific protein found primarily on tumor cell. When both targets are engaged, the BiTE molecule forms a bridge between the cytotoxic T cell and the tumor cell, which enables the T cell to recognize the tumor cell and fight it through an infusion of toxic molecules. For example, the tumor-binding arm of the molecule can be altered to create different BiTE antibody constructs that target different types of cancer

[0066] The term "binding domain" in regard to a BiTE molecule refers to a domain which (specifically) binds to / interacts with / recognizes a given target epitope or a given target site on the target molecules (antigens). The structure and function of the first binding domain (recognizing the tumor cell antigen), and preferably also the structure and/or function of the second binding domain (cytotoxic T cell antigen), is/are based on the structure and/or function of an antibody, *e.g.* of a full-length or whole immunoglobulin molecule.

[0067] The "epitope" refers to a site on an antigen to which a binding domain, such as an antibody or immunoglobulin or derivative or fragment of an antibody or of an immunoglobulin, specifically binds. An “epitope” is antigenic and thus the term epitope is sometimes also referred to herein as “antigenic structure” or “antigenic determinant”. Thus, the binding domain is an “antigen interaction site”. Said binding/interaction is also understood to define a “specific recognition”.

[0068] For example, the BiTE molecule comprises a first binding domain characterized by the presence of three light chain "complementarity determining regions" (CDRs) (*i.e.* CDR1, CDR2 and CDR3 of the VL region) and three heavy chain CDRs (*i.e.* CDR1, CDR2 and CDR3 of the VH region). The second binding domain preferably also comprises the minimum structural requirements of an antibody which allow for the target binding. More preferably, the second binding domain comprises at least three light chain CDRs (*i.e.* CDR1, CDR2 and CDR3 of the VL region) and/or three heavy chain CDRs (*i.e.* CDR1, CDR2 and CDR3 of the VH region). It is

envisaged that the first and/or second binding domain is produced by or obtainable by phage-display or library screening methods rather than by grafting CDR sequences from a pre-existing (monoclonal) antibody into a scaffold.

[0069] A binding domain may typically comprise an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH); however, it does not have to comprise both. Fd fragments, for example, have two VH regions and often retain some antigen-binding function of the intact antigen-binding domain. Examples of (modified) antigen-binding antibody fragments include (1) a Fab fragment, a monovalent fragment having the VL, VH, CL and CH1 domains; (2) a F(ab')2 fragment, a bivalent fragment having two Fab fragments linked by a disulfide bridge at the hinge region; (3) an Fd fragment having the two VH and CH1 domains; (4) an Fv fragment having the VL and VH domains of a single arm of an antibody, (5) a dAb fragment (Ward *et al.*, (1989) Nature 341 :544-546), which has a VH domain; (6) an isolated complementarity determining region (CDR), and (7) a single chain Fv (scFv) , the latter being preferred (for example, derived from an scFV-library).

[0070] The terms “(specifically) binds to”, (specifically) recognizes", “is (specifically) directed to”, and “(specifically) reacts with” regarding a BiTE molecule refers to a binding domain that interacts or specifically interacts with one or more, preferably at least two, more preferably at least three and most preferably at least four amino acids of an epitope located on the target protein or antigen.

[0071] The term "variable" refers to the portions of the antibody or immunoglobulin domains that exhibit variability in their sequence and that are involved in determining the specificity and binding affinity of a particular antibody (*i.e.*, the "variable domain(s)"). The pairing of a variable heavy chain (VH) and a variable light chain (VL) together forms a single antigen-binding site. The CH domain most proximal to VH is designated as CH1. Each light (L) chain is linked to a heavy (H) chain by one covalent disulfide bond, while the two H chains are linked to each other by one or more disulfide bonds depending on the H chain isotype.

[0072] Variability is not evenly distributed throughout the variable domains of antibodies; it is concentrated in sub-domains of each of the heavy and light chain variable regions. These sub-domains are called “hypervariable regions” or “complementarity determining regions” (CDRs). The more conserved (*i.e.*, non-hypervariable) portions of the variable domains are called the "framework" regions (FRM) and provide a scaffold for the six CDRs in three-dimensional space to form an antigen-binding surface. The variable domains of naturally occurring heavy and light

chains each comprise four FRM regions (FR1, FR2, FR3, and FR4), largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRM and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site (see Kabat et al., 1991, Sequences of Proteins of Immunological Interest, Public Health Service N.I.H., Bethesda, MD). The constant domains are not directly involved in antigen binding, but exhibit various effector functions, such as, for example, antibody-dependent, cell-mediated cytotoxicity and complement activation.

[0073] The CDR3 of the light chain and, particularly, the CDR3 of the heavy chain may constitute the most important determinants in antigen binding within the light and heavy chain variable regions. In some antibody constructs, the heavy chain CDR3 appears to constitute the major area of contact between the antigen and the antibody. In vitro selection schemes in which CDR3 alone is varied can be used to vary the binding properties of an antibody or determine which residues contribute to the binding of an antigen. Hence, CDR3 is typically the greatest source of molecular diversity within the antibody-binding site. H3, for example, can be as short as two amino acid residues or greater than 26 amino acids.

[0074] The sequence of antibody genes after assembly and somatic mutation is highly varied, and these varied genes are estimated to encode 1010 different antibody molecules (Immunoglobulin Genes, 2nd ed., eds. Jonio *et al.*, Academic Press, San Diego, CA, 1995). Accordingly, the immune system provides a repertoire of immunoglobulins. The term "repertoire" refers to at least one nucleotide sequence derived wholly or partially from at least one sequence encoding at least one immunoglobulin. The sequence(s) may be generated by rearrangement in vivo of the V, D, and J segments of heavy chains, and the V and J segments of light chains. Alternatively, the sequence(s) can be generated from a cell in response to which rearrangement occurs, *e.g.*, in vitro stimulation. Alternatively, part or all of the sequence(s) may be obtained by DNA splicing, nucleotide synthesis, mutagenesis, and other methods, see, *e.g.*, U.S. Patent 5,565,332. A repertoire may include only one sequence or may include a plurality of sequences, including ones in a genetically diverse collection.

[0075] The term “bispecific” as used herein refers to an antibody construct which is “at least bispecific”, *i.e.*, it comprises at least a first binding domain and a second binding domain, wherein the first binding domain binds to one antigen or target, and the second binding domain binds to another antigen or target. Accordingly, antibody constructs within a BiTE molecule

comprise specificities for at least two different antigens or targets. The term "bispecific antibody construct" of the invention also encompasses multispecific antibody constructs such as trispecific antibody constructs, the latter ones including three binding domains, or constructs having more than three (*e.g.* four, five...) specificities.

[0076] The at least two binding domains and the variable domains of the antibody construct within a BiTE molecule may or may not comprise peptide linkers (spacer peptides). The term "peptide linker" defines in accordance with the present invention an amino acid sequence by which the amino acid sequences of one (variable and/or binding) domain and another (variable and/or binding) domain of the antibody construct of the invention are linked with each other. An essential technical feature of such peptide linker is that said peptide linker does not comprise any polymerization activity. Among the suitable peptide linkers are those described in U.S. Patents 4,751,180 and 4,935,233 or WO 88/09344.

[0077] In the event that a linker is used, this linker is preferably of a length and sequence sufficient to ensure that each of the first and second domains can, independently from one another, retain their differential binding specificities. For peptide linkers which connect the at least two binding domains in the antibody construct within a BiTE molecule (or two variable domains), those peptide linkers are preferred which comprise only a few number of amino acid residues, *e.g.* 12 amino acid residues or less. Thus, peptide linker of 12, 11, 10, 9, 8, 7, 6 or 5 amino acid residues are preferred. An envisaged peptide linker with less than 5 amino acids comprises 4, 3, 2 or one amino acid(s) wherein Gly-rich linkers are preferred. A particularly preferred "single" amino acid in context of said "peptide linker" is Gly. Accordingly, said peptide linker may consist of the single amino acid Gly. Another preferred embodiment of a peptide linker is characterized by the amino acid sequence Gly-Gly-Gly-Gly-Ser, *i.e.* Gly4Ser, or polymers thereof, *i.e.* (Gly4Ser)x, where x is an integer of 1 or greater. The characteristics of said peptide linker, which comprise the absence of the promotion of secondary structures are known in the art and are described *e.g.* in Dall'Acqua *et al.* (Biochem. (1998) 37, 9266-9273), Cheadle *et al.* (Mol Immunol (1992) 29, 21-30) and Raag and Whitlow (FASEB (1995) 9(1), 73-80). Peptide linkers which also do not promote any secondary structures are preferred. The linkage of said domains to each other can be provided by, *e.g.* genetic engineering, as described in the examples. Methods for preparing fused and operatively linked bispecific single chain constructs and expressing them in mammalian cells or bacteria are well-known in the art (*e.g.* WO

99/54440 or Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001).

[0078] The BiTE molecules of the disclosure may comprise an antibody construct in a format selected from the group consisting of (scFv)2, scFv-single domain mAb, diabodies and oligomers of any of the aforementioned formats.

[0079] According to a particularly preferred embodiment, and as documented in the appended examples, the antibody construct within a BiTE molecule is a "bispecific single chain antibody construct", more preferably a bispecific "single chain Fv" (scFv). Although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form a monovalent molecule; see *e.g.*, Huston *et al.* (1988) Proc. Natl. Acad. Sci USA 85:5879-5883). These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are evaluated for function in the same manner as are whole or full-length antibodies. A single-chain variable fragment (scFv) is hence a fusion protein of the variable region of the heavy chain (VH) and of the light chain (VL) of immunoglobulins, usually connected with a short linker peptide of about ten to about 25 amino acids, preferably about 15 to 20 amino acids. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. This protein retains the specificity of the original immunoglobulin, despite removal of the constant regions and introduction of the linker.

[0080] Bispecific single chain molecules are known in the art and are described in WO 99/54440, Mack, J. Immunol. (1997), 158, 3965-3970, Mack, PNAS, (1995), 92, 7021-7025, Kufer, Cancer Immunol. Immunother., (1997), 45, 193-197, Löffler, Blood, (2000), 95, 6, 2098-2103, Brühl, Immunol., (2001), 166, 2420-2426, Kipriyanov, J. Mol. Biol., (1999), 293, 41-56. Techniques described for the production of single chain antibodies (see, inter alia, US Patent 4,946,778, Kontermann and Dübel (2010), loc. cit. and Little (2009), loc. cit.) can be adapted to produce single chain antibody constructs specifically recognizing (an) elected target(s).

[0081] Bivalent (also called divalent) or bispecific single-chain variable fragments (bi-scFvs or di-scFvs having the format (scFv)2) can be engineered by linking two scFv molecules. If these two scFv molecules have the same binding specificity, the resulting (scFv)2 molecule will preferably be called bivalent (*i.e.* it has two valences for the same target epitope). If the two

scFv molecules have different binding specificities, the resulting (scFv)2 molecule will preferably be called bispecific. The linking can be done by producing a single peptide chain with two VH regions and two VL regions, yielding tandem scFvs (see *e.g.* Kufer P. *et al.*, (2004) Trends in Biotechnology 22(5):238-244). Another possibility is the creation of scFv molecules with linker peptides that are too short for the two variable regions to fold together (*e.g.* about five amino acids), forcing the scFvs to dimerize. This type is known as diabodies (see *e.g.* Hollinger, Philipp *et al.*, (July 1993) Proceedings of the National Academy of Sciences of the United States of America 90 (14): 6444-8.).

[0082] Single domain antibodies comprise merely one (monomeric) antibody variable domain which is able to bind selectively to a specific antigen, independently of other V regions or domains. The first single domain antibodies were engineered from heavy chain antibodies found in camelids, and these are called VHH fragments. Cartilaginous fishes also have heavy chain antibodies (IgNAR) from which single domain antibodies called VNAR fragments can be obtained. An alternative approach is to split the dimeric variable domains from common immunoglobulins *e.g.* from humans or rodents into monomers, hence obtaining VH or VL as a single domain Ab. Although most research into single domain antibodies is currently based on heavy chain variable domains, nanobodies derived from light chains have also been shown to bind specifically to target epitopes. Examples of single domain antibodies are called sdAb, nanobodies or single variable domain antibodies.

[0083] A (single domain mAb)2 is hence a monoclonal antibody construct composed of (at least) two single domain monoclonal antibodies, which are individually selected from the group comprising VH, VL, VHH and VNAR. The linker is preferably in the form of a peptide linker. Similarly, an "scFv-single domain mAb" is a monoclonal antibody construct composed of at least one single domain antibody as described above and one scFv molecule as described above. Again, the linker is preferably in the form of a peptide linker.

[0084] Exemplary BiTE molecules include anti-CD33 and anti-CD3 BiTE molecule, anti-BCMA and anti-CD3 BiTE molecule, anti-FLT3 and anti-CD3 BiTE, anti-CD19 and anti-CD3 BiTE, anti-EGFRvIII and anti-CD3 BiTE molecule, anti-DLL3 and anti-CD3 BiTE, BLINCYTO (blinatumomab) and Solitomab.

Pharmaceutical composition formulation and components

[0085] Acceptable pharmaceutical components preferably are nontoxic to patients at the dosages and concentrations used. Pharmaceutical compositions can comprise agents for modifying, maintaining or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition.

[0086] In general, excipients can be classified on the basis of the mechanisms by which they stabilize proteins against various chemical and physical stresses. Some excipients alleviate the effects of a specific stress or regulate a particular susceptibility of a specific polypeptide. Other excipients more generally affect the physical and covalent stabilities of proteins.
Common excipients of liquid and lyophilized protein formulations are shown in Table B (see also Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR, Volkin DB. 2011. Protein-excipient interactions: mechanisms and biophysical characterization applied to protein formulation development. *Adv Drug Deliv Rev* 63: 1118-59.

TABLE B

Examples of excipient components for polypeptides formulations

Component	**Function**	**Examples**
Buffers	Maintaining solution pH Mediating buffer-ion specific interactions with polypeptides	Citrate, Succinate, Acetate, Glutamate, Aspartate, Histidine, Phosphate, Tris, Glycine
Sugars and carbohydrates	Stabilizing polypeptides Tonicifying agents Acting as carriers for inhaled drugs (*e.g.*, lactose) Providing dextrose solutions during IV administration	Sucrose, Trehalose, Sorbitol, Mannitol, Glucose, Lactose, Cyclodextrin derivatives
Stabilizers and bulking agents	Enhancing product elegance and preventing blowout Providing structural strength to a lyo cake	Mannitol, Glycine

Component	Function	Examples
Osmolytes	Stabilizing against environmental stress (temperature, dehydration)	Sucrose, Trehalose, Sorbitol, Glycine, Proline, Glutamate, Glycerol, Urea
Amino acids	Mediating specific interactions with polypeptides Providing antioxidant activity (*e.g.*, His, Met) Buffering, tonicifying	Histidine, Arginine, Glycine, Proline, Lysine, Methionine, Amino acid mixtures (*e.g.*, Glu/Arg)
Polypeptides and polymers	Acting as competitive inhibitors of polypeptide adsorption Providing bulking agents for lyophilization Acting as drug delivery vehicles	HSA, PVA, PVP, PLGA, PEG, Gelatin, Dextran, Hydroxyethyl starch, HEC, CMC
Anti-oxidants	Preventing oxidative polypeptides damage Metal ion binders (if a metal is included as a cofactor or is required for protease activity) Free radical scavengers	Reducing agents, Oxygen scavengers, Free radical scavengers, Chelating agents (*e.g.*, EDTA, EGTA, DTPA), Ethanol
Metal ions	Polypeptides cofactors Coordination complexes (suspensions)	Magnesium, Zinc
Specific ligands	Stabilizers of native conformation against stress-induced unfolding Providing conformation flexibility	Metals, Ligands, Amino acids, Polyanions
Surfactants	Acting as competitive inhibitors of polypeptides adsorption Acting as competitive inhibitor of polypeptides surface denaturation Providing liposomes as drug delivery vehicles	Polysorbate 20, Polysorbate 80, Poloxamer 188, Anionic surfactants (*e.g.*, sulfonates and sulfosuccinates), Cationic surfactants, Zwitterionic surfactants

Component	Function	Examples
	Inhibiting aggregation during lyophilization Acting as reducer of reconstitution times of lyophilized products	
Salts	Tonicifying agents Stabilizing or destabilizing agents for polypeptides, especially anions	NaCl, KCl, $NaSO_4$
Preservatives	Protecting against microbial growth	Benzyl alcohol, M-cresol, Phenol

[0087] As discussed above, that has been surprisingly found is that changing the pulse profile can drastically influence the signal-to-noise ratio of various NMR regions. For example, a particular pulse profile with inverted pulses can be used to excite the ^{13}C methyl signals from a therapeutic molecule while suppressing the ^{13}C excipient signal, such as that coming from a sucrose; these signals can be enhanced with shorter gradient pulses. These various factors that affect the signal enhancement and noise suppression are further emphasized in the embodiments below.

[0088] An exemplary method of fingerprinting a specific molecule in a composition using NMRis described herein, in accordance with related embodiments. The method includes providing the composition having at least a first molecule having a first NMR signal, a second molecule having a second NMR signal, and a third molecule having a third NMR signal. In the method, each of the signals arises from each of the respective molecules having a nuclear spin differing from zero. The method includes applying a cycle of signal processing steps. The cycle includes applying a radio frequency (RF) pulse, applying a gradient pulse having a pulse length less than or equal to 1000 μs, and applying a water suppression technique (WET). In the method, the first NMR signal, the second NMR signal, and the third NMR signal are located in a region of NMR spectra in vicinity defined ppm range of ^{13}C methyl signal. The method also includes repeating the cycle for at least 3 times to acquire an enhanced signal of the composition. The method further includes fingerprinting the specific molecule based on the enhanced signal of the composition.

[0089] In this and related embodiments, the region of NMR spectra includes a NMR ^{13}C spectral window from about 5 ppm to about 150 ppm. The region of NMR spectra includes a NMR spectral window from about 5 ppm to about 100 ppm, from about 5 ppm to about 50 ppm, or from about 7 ppm to about 35 ppm. Moreover, for example, when using oxidized met, the NMR spectral window can be from about 7 ppm to about 40 ppm.

[0090] The RF pulse includes at least one of a Reburp pulse, a combination of a broadband inversion pulse (BIP) and a Gaussian (G3) inversion pulse, and an asymmetric adiabatic pulse. In the case of the Reburp pulse, this pulse excites the first NMR signal. In the case of the BIP, the BIP excites a wide range of NMR signals and the G3 inversion pulse suppresses the second NMR signal. In the case of the asymmetric adiabatic pulse, this pulse excites the first NMR signal while suppressing the second NMR signal.

[0091] The first NMR signal is a NMR signal related to ^{13}C methyl of a therapeutic molecule, the second NMR signal is a signal related to ^{13}C sucrose, and the third NMR signal is a signal related to at least ^{1}H acetate or other $^{1}H/^{13}C$ NMR signals.

[0092] The exemplary method for using NMR can be conducted at a frequency range from about 100 MHz to about 2000 MHz, such as 1200 MHz, as is currently customarily available.

[0093] The Reburp pulse has a pulse length from about 500 μs to about 1000 μs. the Reburp pulse has a pulse length from about 600 μs to about 900 μs, or from about 600 μs to about 800 μs.

[0094] The combination of the BIP and the G3 inversion pulses has a total pulse length from about 200 μs to about 2500 μs. The combination of the BIP and the G3 inversion pulse has a pulse length from about 200 μs to about 2000 μs, from about 200 μs to about 1500 μs, from about 250 μs to about 1000 μs, or from about 250 μs to about 750 μs. The combination of the BIP and the G3 inversion pulse has a pulse length of about 620 μs. The BIP has a pulse length of about 120 μs and the G3 inversion pulse has a pulse length of about 500 μs.

[0095] The asymmetric adiabatic pulse has a pulse length from about 50 μs to about 2500 μs, from about 50 μs to about 2000 μs, from about 50 μs to about 1500 μs, from about 50 μs to about 1000 μs, or from about 100 μs to about 800 μs.

[0096] The gradient pulse has a pulse length less than equal to about 1500 μs or less than or equal to about 1000 μs. The gradient pulse has a pulse length from about 50 μs to about 1500 μs, from about 50 μs to about 1200 μs, from about 50 μs to about 1000 μs, from about 50 μs to about 800 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from

about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about 250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

[0097] The gradient pulse is followed by at least one inverted gradient pulse having a pulse length from about 50 μs to about 990 μs, from about 50 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about 250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

[0098] The at least one inverted gradient pulse is followed by another gradient pulse having a pulse length from about 50 μs to about 990 μs, from about 50 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about 250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

[0099] Another exemplary method of fingerprinting a specific molecule in a composition using NMR is described herein. The method includes providing the composition having at least a first molecule having a first NMR signal, a second molecule having a second NMR signal, and a third molecule having a third NMR signal. Each of the signals arises from each of the respective molecules having a nuclear spin differing from zero. The method includes applying a cycle of signal processing steps. The cycle includes applying a radio frequency (RF) pulse and applying a gradient pulse. In the method, the first NMR signal, the second NMR signal, and the third NMR signal are located in a region of NMR spectral window from about 5 ppm to about 150 ppm. The method also includes repeating the cycle for at least 3 times to acquire an enhanced signal of the composition. The method further includes fingerprinting the specific molecule based on the enhanced signal of the composition.

[0100] The cycle further includes applying a water suppression technique (WET) sequence.

[0101] The region of NMR spectra includes a NMR spectral window from about 5 ppm to about 100 ppm, from about 5 ppm to about 50 ppm, or from about 7 ppm to about 35 ppm.

[0102] The RF pulse include at least one of a Reburp pulse, a combination of a broadband inversion pulse (BIP) and a Gaussian (G3) inversion pulse, or an asymmetric adiabatic pulse.

[0103] In the case of a Reburp pulse, this pulse excites the first NMR signal. The broadband inversion pulse excites a wide range of NMR signals and the G3 inversion pulse suppresses the second NMR signal. The asymmetric adiabatic pulse excites the first NMR signal while suppressing the second NMR signal.

[0104] The first NMR signal is a NMR signal related to ^{13}C methyl of a therapeutic molecule, the second NMR signal is a signal related to ^{13}C sucrose, and the third NMR signal is a signal related to at least ^{1}H acetate or other $^{1}H/^{13}C$ NMR signals.

[0105] The exemplary method for using NMR can be conducted at a frequency range from about 100 MHz to about 2000 MHz, including 1200 MHz.

[0106] The Reburp pulse has a pulse length from about 300 μs to about 1000 μs, from about 600 μs to about 900 μs, or from about 600 μs to about 800 μs.

[0107] The combination of the BIP and the G3 inversion pulses has a total pulse length from about 200 μs to about 2500 μs, from about 200 μs to about 2000 μs, from about 200 μs to about 1500 μs, from about 250 μs to about 1000 μs, or from about 250 μs to about 750 μs. The combination of the BIP and the G3 inversion pulse has a pulse length of about 620 μs to 660 μs. The BIP has a pulse length of about 120 μs to 160 μs and the G3 inversion pulse has a pulse length of about 500 μs.

[0108] The asymmetric adiabatic pulse has a pulse length from about 50 μs to about 2500 μs, from about 50 μs to about 2000 μs, from about 50 μs to about 1500 μs, from about 50 μs to about 1000 μs, or from about 100 μs to about 800 μs.

[0109] The gradient pulse has a pulse length less than or equal to 1000 μs. In some implementations, the gradient pulse has a pulse length from about 50 μs to about 990 μs, from about 50 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about 250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

[0110] In some implementations, the gradient pulse is followed by at least one inverted gradient pulse having a pulse length less than or equal to 1000 μs. The gradient pulse is followed by at least one inverted gradient pulse having a pulse length from about 50 μs to about 990 μs, from about 50 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about

250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

[0111] The at least one inverted gradient pulse is followed by another gradient pulse having a pulse length less than or equal to 1000 μs. The at least one inverted gradient pulse is followed by another gradient pulse having a pulse length from about 50 μs to about 990 μs, from about 50 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about 250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

[0112] Another exemplary method of fingerprinting a specific molecule in a composition using NMR is described herein. The method includes providing the composition having at least a first molecule having a first NMR signal, a second molecule having a second NMR signal, and a third molecule having a third NMR signal. In the method, each of the signals arises from each of the respective molecules having a nuclear spin differing from zero. The method includes applying a radio frequency (RF) pulse to the composition to excite the first NMR signal while suppressing the second NMR signal. The RF pulse includes at least one of a Reburp pulse, a combination of a broadband inversion pulse and a Gaussian inversion pulse, or an asymmetric adiabatic pulse. The method also includes applying a gradient pulse having a pulse length less than or equal to 1000 μs and applying a water suppression technique (WET) sequence to suppress the third NMR signal. The method also includes repeating the cycle for at least 3 times to acquire an enhanced signal of the composition. The method further includes fingerprinting the specific molecule based on the enhanced signal of the composition.

[0113] The first NMR signal, the second NMR signal, and the third NMR signal are located in a region of NMR spectra in the vicinity of 13C methyl signal.

[0114] The first NMR signal, the second NMR signal, and the third NMR signal are located in an NMR spectral window from about 5 ppm to about 150 ppm. In various implementations, the first NMR signal, the second NMR signal, and the third NMR signal are located in an NMR spectral window from about 5 ppm to about 100 ppm, from about 5 ppm to about 50 ppm, or from about 7 ppm to about 35 ppm.

[0115] The exemplary method for using NMR can be conducted at a frequency range from about 100 MHz to about 2000 MHz, such as 1200 MHz, as is currently customarily available.

[0116] The Reburp pulse has a pulse length from about 300 μs to about 1000 μs, from about 600 μs to about 900 μs, or from about 600 μs to about 800 μs.

[0117] The combination of the BIP and the G3 inversion pulses has a total pulse length from about 200 μs to about 2500 μs, from about 200 μs to about 2000 μs, from about 200 μs to about 1500 μs, from about 250 μs to about 1000 μs, or from about 250 μs to about 750 μs.

[0118] The combination of the BIP and the G3 inversion pulses has a pulse length of about 620 μs to 660 μs. The BIP has a pulse length of about 120 μs to 160 μs and the G3 inversion pulse has a pulse length of about 500 μs.

[0119] The asymmetric adiabatic pulse has a pulse length from about 50 μs to about 2500 μs, from about 50 μs to about 2000 μs, from about 50 μs to about 1500 μs, from about 50 μs to about 1000 μs, or from about 100 μs to about 800 μs.

[0120] The gradient pulse has a pulse length from about 50 μs to about 1500 μs, from about 50 μs to about 1200 μs, from about 50 μs to about 1000 μs, from about 50 μs to about 800 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about 250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

[0121] The gradient pulse is followed by at least one inverted gradient pulse having a pulse length from about 50 μs to about 990 μs, from about 50 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about 250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

[0122] The at least one inverted gradient pulse is followed by another gradient pulse having a pulse length from about 50 μs to about 990 μs, from about 50 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about 250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

[0123] In various implementations, applying the RF pulse, the gradient pulse, and the WET sequence constitutes a cycle of signal processing steps, and the method further includes repeating the cycle for at least 3 times.

[0124] The method includes repeating the cycle for less than 1024 times, less than 512 times, less than 500 times, less than 400 times, less than 300 times, less than 256 times, less than 250 times, less than 200 times, less than 150 times, less than 128 times, less than 100 times, less than 96 times, less than 80 times, less than 70 times, less than 64 times, less than 60 times, less than 50 times, less than 48 times, less than 40 times, less than 36 times, less than 30 times, less than 25 times, less than 20 times, or less than 16 times.

[0125] Other excipients are known in the art (*e.g.*, see Powell MF, Nguyen T, Baloian L. 1998. Compendium of excipients for parenteral formulations. *PDA J Pharm Sci Technol* 52: 238-311). Those skilled in the art can determine what amount or range of excipient can be included in any particular formulation to achieve a biopharmaceutical composition that promotes retention in stability of the biopharmaceutical. For example, the amount and type of a salt to be included in a biopharmaceutical composition can be selected based on to the desired osmolality (*i.e.*, isotonic, hypotonic or hypertonic) of the final solution as well as the amounts and osmolality of other components to be included in the formulation.

TABLE OF ABBREVIATIONS

Abbreviation	Definition
2D	Two-Dimensional
BIP	Broadband Inversion Pulse
CQA	Critical Quality Attribute
G3	Gaussian
HOS	Higher Order Structure
HSQC	Heteronuclear Single Quantum Coherence
INEPT	Insensitive Nuclei Enhanced by Polarization Transfer
NIST	National Institute of Standards and Technology
PS	Polysorbate
Reburp	Refocusing Band-Selective Pulse with Uniform Response and Phase
RF	Radio Frequency
WET	Water Suppression Technique

EXPERIMENTAL RESULTS, MATERIALS AND METHODS

Example 1

[0126] To conduct measurements in Example 1, a Bruker Avance III 600 MHz NMR spectrometer (10040043) equipped with a 5 mm CPTCI cryoprobe $^{1}H\{^{19}F\}$-$^{13}C/^{15}N$/D-ZGRD z-gradient was used to acquire NMR data at 310 K (37 °C). The data processing was carried out using the spectrometer software (TopSpin, Bruker BioSpin North America; Billerica, MA), and MNova software (Mestrelab Research S.L. (USA); Escondido, CA).

[0127] The following samples were used for evaluation of the disclosed NMR methods.

[0128] Sample 1: A peptide with 42 amino acids and M.W. 4651.38 Da, 30 mg/ml, 6 mM with 50 mM acetate, 5% sucrose, 0.01% PS80, pH=5 with 5 % D2O. About 200 µl of solution was placed into a 4 mm Shigemi tube for NMR analysis.

[0129] Sample 2: mAb1, 50 mg/ml, 9% sucrose, 10 mM acetate, 0.01% PS80, pH=5.2 with 3% D_2O. About 600 µl of solution was placed into a 5 mm Wilmad tube for NMR analysis.

[0130] Sample 3: Proline, 32.22 mg (~ 280 mM) (Sigma-Aldrich; St. Louis, MO), Sucrose, 87.92 mg (Sigma-Aldrich), dissolved in ~ 1 mL D_2O, 99.9% D, (Sigma-Aldrich). About 600 µl of solution was placed into a 5 mm Wilmad tube for NMR analysis.

[0131] Sample 4: 1% water with 0.1 mg/ml $GdCl_3$ in D_2O.

Example 2

[0132] To conduct measurements in Example 2, a Bruker Avance III 600 MHz NMR spectrometer (S/N 10040043) equipped with a 5 mm CPTCI cryoprobe $^{1}H\{^{19}F\}$-$^{13}C/^{15}N$/D-ZGRD z-gradient (S/N Z128744/0001) was used to acquire NMR data for samples 1 and 2 at 310 K (37 °C) and sample 3 at 300 K (27 °C).

[0133] In this example, a 2D methyl fingerprinting pulse sequence is applied to suppress excipient signals in mAb1 samples in the A52Su buffer (10 mM acetate, 9% sucrose, pH:5.2) spiking with (1) 10 mM glutamate, or (2) 200 mM proline, and "Protein 1" (an antigen binding protein having a canonical BiTE molecule structure) in the G42Su buffer (15 mM glutamate, 9% sucrose, pH: 4.2).

[0134] The following three samples were made to test the capability of NMR pulse sequence to suppress the signals from glutamate and proline, in addition to the suppression of signals from sucrose and acetate:

[0135] Sample 1: mAb1 , 50 mg/ml, 9% sucrose, 10 mM acetate, spiking with 10 mM glutamate and 5% D_2O.

[0136] Sample 2: mAb1, 50 mg/ml, 9% sucrose, 10 mM acetate, spiking with 200 mM proline and 5% D_2O.

[0137] Sample 3: Protein 1, 10 mg/ml, 9 % sucrose, 15 mM glutamate and 5% D_2O.

[0138] Now referring to the Figure 7, which shows an example NMR signal enhancement pulse sequence 700 based on an ^{1}H-^{13}C sensitivity-enhanced HSQC experimental scheme to suppress the excipient signals from sucrose. As shown in Figure7, the WET portion of the pulse sequence is used to suppress the proton signal of acetate, whereas the new shaped pulses in the middle of HSQC experiment are used to excite the carbon signals from the methyl region of therapeutic proteins while suppressing the carbon signals from sucrose. In this example, the pulses used in the WET portion of the sequence is re-designed to suppress the signals from other excipients, exemplified with glutamate and proline. Depending on which signals from excipients need to be suppressed, the pulses in the WET portion of the sequence can be generated using the Bruker Topspin software.

[0139] Figure 8 shows spectra 800 from the first increment of HSQC data without (802) and with (804) for the suppression of signals from 10 mM glutamate and 10 mM acetate in sample 1 in example 2. The WET pulse was specifically designed to suppress the signals from glutamate and acetate. The peak intensity at 2.418 ppm is reduced to the baseline level. Although the peak intensities at 2.144 and 2.080 ppm were reduced by about 50%, these peaks have roughly the same intensities as peaks in the methyl region.

[0140] Figure 9A displays the 2D methyl region of HSQC spectra 900a without the suppression of signals from 10 mM glutamate and 10 mM acetate in sample 1 of Example 2. Figure 9B displays the 2D methyl region of HSQC spectra 900b with the suppression of signals from 10 mM glutamate and 10 mM acetate in sample 1 of Example 2. These spectra demonstrate that if the signal intensities from excipients are comparable to those from the methyl peaks as shown in Figure 8, these signals may not produce strips along the carbon dimension or cause phasing issues in the 2D spectra. Artifacts from strips and the phasing issue can interfere with the data analysis of the methyl peaks near the artifacts.

[0141] Figure 10 shows spectra 1000 from the first increment of HSQC data without (1002) and with (1004) for the suppression of signals from 15 mM glutamate in sample 3 of example 2. The peaks from glutamate are efficiently suppressed by using the WET sequence.

[0142] Figure 11A displays the 2D methyl region of HSQC spectra 1100a without the suppression of signals from 15 mM glutamate in sample 3 of Example 2. Figure 11B displays the

2D methyl region of HSQC spectra 1100b with the suppression of signals from 15 mM glutamate in sample 3 of Example 2. These spectra reveal that if the signal intensities from excipients are much higher than those from the methyl peaks, these signals produce strips in the carbon dimension, which could interfere with the analysis of peaks near the strips in the methyl region.

[0143] Figure 12 shows spectra 1200 from the first increment of HSQC data without (1202) and with (1204) for the suppression of signals from 200 mM proline and 10 mM acetate in sample 2 of example 2. The intensities from 200 mM of proline are much larger than those from peaks in the methyl region.

[0144] Figure 13 shows another example NMR signal enhancement pulse sequence 1300 based on double WET scheme, in accordance with various embodiments. The double WET scheme shown in Figure 13 was used to suppress the proline signals down to the baseline level. Double WET scheme was shown to be more efficient than the single WET scheme to effectively suppress the peaks from proline, resulting in no strips in the carbon dimension, as shown in Figures 14A and 14B. Nonetheless, the intensities of peaks in the methyl region was dropped by approximately 15% when using the double WET scheme as compared to those obtained from the single WET scheme.

[0145] Figure 14A displays the 2D methyl region of HSQC spectra 1400a without the suppression of signals from 200 mM proline and 10 mM acetate in sample 2 of Example 2. Figure 14B displays the 2D methyl region of HSQC spectra 1400b with the suppression of signals from 200 mM proline and 10 mM acetate in sample 2 of Example 2. Without suppression of the peaks from proline, there are strips along the carbon and proton dimensions, as shown in Figure 14A. When using the double WET sequence to suppress the proline signals, the 2D spectrum in Figure 14B is suitable for the analysis of peaks in the methyl region.

Example 3

[0146] As described herein, when applying these pulses in an NMR spectrometer with a different magnetic field strength, the pulses can be scaled in pulse length or the transmitter offset can be positioned differently. The results in this example demonstrate such application at 800 MHz. In particular, example 3 was conducted using the following parameters: 800 MHz NMR data on mAb1, 50 mg/ml, 9% sucrose, 10 mM acetate, 0.01% polysorbate (PS) 80 at pH = 5.2 with 3% D_2O.

[0147] When using the same kind of probes for the experiments, a 800 MHz NMR system has higher sensitivity and better resolution of spectra compared to a 600 MHz NMR system; that is, for example, 1 ppm in the carbon dimension is 200 Hz and 150 Hz at the 800 and 600 MHz NMR systems, respectively. Therefore, peaks can further spread out in the spectra from the 800 MHz NMR system.

[0148] Figures 15A-15E show exemplary excitation profiles of pulses with different shapes that can be applied at 800 MHz to suppress the ^{13}C sucrose signals. Figure 15A shows a pulse profile 1500a of ^{13}C signal for sucrose signal regions. Figure 15B shows a pulse profile 1500b of a Reburp profile that is scaled to 575 μs to keep the same excitation profile as that of a 750 μs Reburp pulse at 600 MHz. Figure 15C shows a pulse profile 1500c. Since the carbon spectral width in Hz is larger at 800 MHz, the transmitter offset is positioned at 16 ppm for [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 140, 0.1 Tp] with pulse length 375 μs at 800 MHz, instead of transmitter offset at 2 ppm at 600 MHz, to keep similar excitation profiles, as shown Figure 15C. Figure 15D shows a pulse profile 1500d having the parameters [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 70, 0.1 Tp] with pulse length 750 μs with a transmitter offset at 18 ppm. Figure 15E shows a pulse profile 1500e having the parameters [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 1400, 0.1 Tp] with pulse length 1500 μs with a transmitter offset at 27 ppm. The profiles 1500d and 1500e are used to suppress the C_β carbon signals above 40 ppm.

[0149] Figures 16A and 16B show different ^{13}C 2D methyl fingerprinting plots 1600a and 1600b for comparing effectiveness of particular NMR enhancement methods obtained on a 800 MHz NMR spectrometer**.** Figure 16A shows a clean methyl region obtained by using the [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 50, 0.1 Tp] for pulse length 375 μs with transmitter offset at 16 ppm as the refocusing element, and the WET sequence to suppress the ^{1}H acetate signal. Figure 16B presents that the C_β region can be suppressed by using the [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 70, 0.1 Tp] for pulse length 750 μs with transmitter offset at 18 ppm.

[0150] Figure 17 shows a graphical comparison of signal intensities 1700 for methyl peaks based on an ^{1}H-^{13}C sensitivity-enhanced HSQC experimental scheme using different RF pulses in exemplary HSQC experiments obtained using a 800 MHz NMR system. Note that the H_β signals around 3 ppm disappear when using shape pulses [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 70, 0.1 Tp] with pulse length 750 μs and transmitter offset at 18 ppm and for [HS1/2, R = 10, 0.9 Tp; tanh/tan, R = 1400, 0.1 Tp] with pulse length 1500 μs and transmitter offset at 27 ppm. The relative methyl intensities by integrating the peak area between -0.5 to 2 ppm in Figure 17 are

shown in Table 2. The intensity of methyl peak area by using the Reburp pulse was normalized to 0.88, in order to compare the values in Table 2 to those in Table 1. The relative methyl intensities obtained at 600 MHz and 800 MHz are similar.

Table 2

Comparison of relative methyl intensities from different experiments obtained at 800 MHz

Experimental conditions for the echo/anti-echo schemes	Relative methyl intensity
[1]Reburp for pulse length 575 μs with transmitter offset at 21 ppm, G1=80% with 250 μs, G2=20.1% with 246 μs	0.88
[2][HS$\frac{1}{2}$, R = 10, 0.9 T_p; tanh/tan, R = 50, 0.1 T_p] for pulse length 375 μs with transmitter offset at 16 ppm	0.92
[2][HS$\frac{1}{2}$, R = 10, 0.9 T_p; tanh/tan, R = 70, 0.1 T_p] for pulse length 750 μs with transmitter offset at 18 ppm	0.85
[2][HS$\frac{1}{2}$, R = 10, 0.9 T_p; tanh/tan, R = 140, 0.1 T_p] for pulse length 1500 μs with transmitter offset at 27 ppm	0.76

[1] Pulse sequence in Figure 1. The maximum gradient strength is about 53.5 G/cm at 100%. Gradient recovery = 200 μs.

[2] Pulse sequence in Figure 2. For these experiments, G1=80% with 250 μs, G2=40.11% with 248 μs, G3= -80% with 250 μs, G4= -40.08% with 248 μs, gradient recovery = 50 μs.

[0151] While this specification contains many specific implementation details, these should not be construed as limitations on the scope of any inventions or of what may be claimed, but rather as descriptions of features specific to particular implementations of particular inventions. Certain features that are described in this specification in the context of separate implementations can also be implemented in combination in a single implementation. Conversely, various features that are described in the context of a single implementation can also be implemented in multiple implementations separately or in any suitable sub-combination. Moreover, although features may be described above as acting in certain combinations and even initially claimed as such, one or more features from a claimed combination can in some cases be excised from the combination, and the claimed combination may be directed to a sub-combination or variation of a sub-combination.

[0152] Similarly, while operations are depicted in the drawings in a particular order, this should not be understood as requiring that such operations be performed in the particular

order shown or in sequential order, or that all illustrated operations be performed, to achieve desirable results.

[0153] References to "or" may be construed as inclusive so that any terms described using "or" may indicate any of a single, more than one, and all of the described terms. The labels "first," "second," "third," and so forth are not necessarily meant to indicate an ordering and are generally used merely to distinguish between like or similar items or elements.

[0154] Various modifications to the implementations described in this disclosure may be readily apparent to those skilled in the art, and the generic principles defined herein may be applied to other implementations without departing from the spirit or scope of this disclosure. Thus, the claims are not intended to be limited to the implementations shown herein, but are to be accorded the widest scope consistent with this disclosure, the principles and the novel features disclosed herein.

[0155] All cited references, in permitted jurisdictions, are incorporated herein by reference.

CLAIMS

1. A method of fingerprinting a specific molecule in a composition using nuclear magnetic resonance (NMR), the method comprising:

providing the composition comprising at least a first molecule having a first NMR signal, a second molecule having a second NMR signal, and a third molecule having a third NMR signal, wherein each of the signals arises from each of the respective molecules having a nuclear spin differing from zero; and

applying a cycle of signal processing steps, the cycle comprising:

applying a radio frequency (RF) pulse;

applying a gradient pulse having a pulse length less than or equal to 1000 μs; and

applying a water suppression technique (WET),

wherein the first NMR signal, the second NMR signal, and the third NMR signal are located in a region of NMR spectra in a defined ppm range of ^{13}C methyl signal;

repeating the cycle for at least 3 times to acquire an enhanced signal of the composition; and

fingerprinting the specific molecule based on the enhanced signal of the composition.

2. The method of claim 1, wherein the region of NMR spectra includes a NMR spectral window from about 5 ppm to about 150 ppm.

3. The method of claim 1, wherein the region of NMR spectra includes a NMR spectral window from about 5 ppm to about 100 ppm.

4. The method of claim 1, wherein the region of NMR spectra includes a NMR spectral window from about 5 ppm to about 50 ppm.

5. The method of claim 1, wherein the region of NMR spectra includes a NMR spectral window from about 7 ppm to about 35 ppm.

6. The method of claim 1, wherein the RF pulse includes at least one of a Reburp pulse; a combination of a broadband inversion pulse (BIP) and a Gaussian (G3) inversion pulse; or an asymmetric adiabatic pulse.

7. The method of claim 6, wherein the Reburp pulse excites the first NMR signal.

8. The method of claim 6, wherein the broadband inversion pulse excites each of the NMR signals and the G3 inversion pulse suppresses the second NMR signal.

9. The method of claim 6, wherein the asymmetric adiabatic pulse excites the first NMR signal while suppressing the second NMR signal.

10. The method of claim 1, wherein the first NMR signal is a NMR signal related to ^{13}C methyl, the second NMR signal is a signal related to ^{13}C sucrose, and the third NMR signal is a signal related to at least ^{1}H acetate or $^{1}H/^{13}C$ NMR signals from other excipients from one of Glutamate, Proline, Arginine, or Mannitol.

11. The method of claim 1, wherein the method for using NMR is conducted at a frequency range from about 100 MHz to about 2000 MHz.

12. The method of claim 6, wherein the Reburp pulse has a pulse length from about 500 μs to about 1000 μs.

13. The method of claim 6, wherein the Reburp pulse has a pulse length from about 600 μs to about 900 μs.

14. The method of claim 6, wherein the Reburp pulse has a pulse length from about 600 μs to about 800 μs.

15. The method of claim 6, wherein the combination of the BIP and the G3 inversion pulse has a pulse length from about 200 μs to about 2500 μs.

16. The method of claim 6, wherein the combination of the BIP and the G3 inversion pulse has a pulse length from about 200 μs to about 2000 μs.

17. The method of claim 6, wherein the combination of the BIP and the G3 inversion pulse has a pulse length from about 200 μs to about 1500 μs.

18. The method of claim 6, wherein the combination of the BIP and the G3 inversion pulse has a pulse length from about 250 μs to about 1000 μs.

19. The method of claim 6, wherein the combination of the BIP and the G3 inversion pulse has a pulse length from about 250 μs to about 750 μs.

20. The method of claim 6, wherein the combination of the BIP and the G3 inversion pulse has a pulse length of about 620 μs to 660 μs.

21. The method of claim 20, wherein the BIP has a pulse length of about 120 μs to 160 μs and the G3 inversion pulse has a pulse length of about 500 μs.

22. The method of claim 6, wherein the asymmetric adiabatic pulse has a pulse length from about 50 μs to about 2500 μs.

23. The method of claim 6, wherein the asymmetric adiabatic pulse has a pulse length from about 50 μs to about 2000 μs.

24. The method of claim 6, wherein the asymmetric adiabatic pulse has a pulse length from about 50 μs to about 1500 μs.

25. The method of claim 6, wherein the asymmetric adiabatic pulse has a pulse length from about 50 μs to about 1000 μs.

26. The method of claim 6, wherein the asymmetric adiabatic pulse has a pulse length from about 100 μs to about 800 μs.

27. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 990 μs.

28. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 900 μs.

29. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 800 μs.

30. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 700 μs.

31. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 600 μs.

32. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 500 μs.

33. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 400 μs.

34. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 300 μs.

35. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 250 μs.

36. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 200 μs.

37. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 150 μs.

38. The method of claim 1, wherein the gradient pulse has a pulse length range from about 50 μs to about 100 μs.

39. The method of any of claims 27-38, wherein the gradient pulse is followed by at least one inverted gradient pulse having the same pulse length range.

40. The method of claim 39, wherein the at least one inverted gradient pulse is followed by another gradient pulse having the same pulse length range.

41. The method of claim 1, wherein repeating the cycle for at least 3 times includes a delay in the repeating ranging from about 10 μs to about 990 μs.

42. The method of claim 41, wherein the delay is from about 30 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 100 μs to about 600 μs, from about 150 μs to about 500 μs, or from about 200 μs to about 300 μs.

43. A method of fingerprinting a specific molecule in a composition using nuclear magnetic resonance (NMR), the method comprising:

providing the composition comprising at least a first molecule having a first NMR signal, a second molecule having a second NMR signal, and a third molecule having a third NMR signal, wherein each of the signals arises from each of the respective molecules having a nuclear spin differing from zero; and

applying a cycle of signal processing steps, the cycle comprising:

applying a radio frequency (RF) pulse; and

applying a gradient pulse;

wherein the first NMR signal, the second NMR signal, and the third NMR signal are located in a region of NMR spectral window from about 5 ppm to about 150 ppm;

repeating the cycle for at least 3 times to acquire an enhanced signal of the composition; and

fingerprinting the specific molecule based on the enhanced signal of the composition.

44. The method of claim 43, wherein the cycle further comprises:

applying a water suppression technique (WET) sequence to suppress the third NMR signal.

45. The method of claim 43, wherein the region of NMR spectra includes a NMR spectral window from about 5 ppm to about 100 ppm, from about 5 ppm to about 50 ppm, or from about 7 ppm to about 35 ppm.

46. The method of claim 43, wherein the RF pulse includes at least one of a Reburp pulse, a combination of a broadband inversion pulse (BIP) and a Gaussian (G3) inversion pulse, and an asymmetric adiabatic pulse.

47. The method of claim 46, wherein the Reburp pulse excites the first NMR signal.

48. The method of claim 46, wherein the broadband inversion pulse excites a wide range of NMR signals and the G3 inversion pulse suppresses the second NMR signal.

49. The method of claim 46, wherein the asymmetric adiabatic pulse excites the first NMR signal while suppressing the second NMR signal.

50. The method of claim 43, wherein the first NMR signal is a NMR signal related to ^{13}C methyl, the second NMR signal is a signal related to a NMR signal related to ^{13}C sucrose, and the third NMR signal is a signal related to at least ^{1}H acetate or ^{13}C NMR signals from one of Glutamate, Proline, Arginine, or Mannitol.

51. The method of claim 43, wherein the method for using NMR is conducted at a frequency range from about 100 MHz to about 2000 MHz.

52. The method of claim 46, wherein the Reburp pulse has a pulse length from about 500 μs to about 1000 μs, from about 600 μs to about 900 μs, or from about 600 μs to about 800 μs.

53. The method of claim 46, wherein the combination of the BIP and the G3 inversion pulse has a pulse length from about 200 μs to about 2500 μs, from about 200 μs to about 2000 μs, from about 200 μs to about 1500 μs, from about 250 μs to about 1000 μs, or from about 250 μs to about 750 μs.

54. The method of claim 46, wherein the combination of the BIP and the G3 inversion pulse has a pulse length of about 620 μs to 660 μs.

55. The method of claim 54, wherein the BIP has a pulse length of about 120 μs to 160 μs and the G3 inversion pulse has a pulse length of about 500 μs.

56. The method of claim 46, wherein the asymmetric adiabatic pulse has a pulse length from about 50 μs to about 2500 μs, from about 50 μs to about 2000 μs, from about 50 μs to about 1500 μs, from about 50 μs to about 1000 μs, or from about 100 μs to about 800 μs.

57. The method of claim 43, wherein the gradient pulse has a pulse length less than or equal to 1000 μs.

58. The method of claim 43, wherein the gradient pulse has a pulse length range from about 50 μs to about 1000 μs, from about 50 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about 250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

59. The method of any of claims 57 or 58, wherein the gradient pulse is followed by at least one inverted gradient pulse having the same pulse length or the same pulse length range.

60. The method of claim 59, wherein the at least one inverted gradient pulse is followed by another gradient pulse having the same pulse length range.

61. The method of claim 43, wherein repeating the cycle for at least 3 times includes a delay in the repeating ranging from about 10 μs to about 990 μs, from about 30 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 100 μs to about 600 μs, from about 150 μs to about 500 μs, or from about 200 μs to about 300 μs.

62. A method of fingerprinting a specific molecule in a composition using nuclear magnetic resonance (NMR), the method comprising:

providing the composition comprising at least a first molecule having a first NMR signal, a second molecule having a second NMR signal, and a third molecule having a third NMR signal, wherein each of the signals arises from each of the respective molecules having a nuclear spin differing from zero;

applying a radio frequency (RF) pulse to the composition to excite the first NMR signal while suppressing the second NMR signal, the RF pulse comprising at least one of a Reburp pulse, a combination of a broadband inversion pulse and a Gaussian inversion pulse, and an asymmetric adiabatic pulse,

applying a gradient pulse having a pulse length less than or equal to 1000 μs;

applying a water suppression technique (WET) sequence to suppress the third NMR signal;

acquiring an enhanced signal of the composition; and

fingerprinting the specific molecule based on the enhanced signal of the composition.

63. The method of claim 62, wherein the first NMR signal, the second NMR signal, and the third NMR signal are located in a region of NMR spectra in the vicinity of ^{13}C methyl signal.

64. The method of claim 62, wherein the first NMR signal, the second NMR signal, and the third NMR signal are located in a NMR spectral window from about 5 ppm to about 150 ppm.

65. The method of claim 62, wherein the first NMR signal, the second NMR signal, and the third NMR signal are located in a NMR spectral window from about 5 ppm to about 100 ppm, from about 5 ppm to about 50 ppm, or from about 7 ppm to about 35 ppm.

66. The method of claim 62, wherein the method for using NMR is conducted at a frequency range from about 100 MHz to about 2000 MHz.

67. The method of claim 62, wherein the Reburp pulse has a pulse length from about 500 μs to about 1000 μs, from about 600 μs to about 900 μs, or from about 600 μs to about 800 μs.

68. The method of claim 62, wherein the combination of the BIP and the G3 inversion pulse has a pulse length from about 200 μs to about 2500 μs, from about 200 μs to about 2000 μs, from about 200 μs to about 1500 μs, from about 250 μs to about 1000 μs, or from about 250 μs to about 750 μs.

69. The method of claim 62, wherein the combination of the BIP and the G3 inversion pulse has a pulse length of about 620 μs to 660 μs.

70. The method of claim 69, wherein the BIP has a pulse length of about 120 μs to 160 μs and the G3 inversion pulse has a pulse length of about 500 μs.

71. The method of claim 62, wherein the asymmetric adiabatic pulse has a pulse length from about 50 μs to about 2500 μs, from about 50 μs to about 2000 μs, from about 50 μs to about 1500 μs, from about 50 μs to about 1000 μs, or from about 100 μs to about 800 μs.

72. The method of claim 62, wherein the gradient pulse has a pulse length range from about 50 μs to about 990 μs, from about 50 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 50 μs to about 600 μs, from about 50 μs to about 500 μs, from about 50 μs to about 400 μs, from about 50 μs to about 300 μs, from about 50 μs to about 250 μs, from about 50 μs to about 200 μs, from about 50 μs to about 150 μs, or from about 50 μs to about 100 μs.

73. The method of claim 72, wherein the gradient pulse is followed by at least one inverted gradient pulse having the same pulse length range.

74. The method of claim 73, wherein the at least one inverted gradient pulse is followed by another gradient pulse having the same pulse length range.

75. The method of claim 62, wherein the applying the RF pulse, the gradient pulse, and the WET sequence constitutes a cycle of signal processing steps, the method further comprising:

repeating the cycle for at least 3 times to acquire the enhanced signal of the composition.

76. The method of claim 75, wherein repeating the cycle for at least 3 times includes a delay in the repeating ranging from about 10 μs to about 990 μs, from about 30 μs to about 900 μs, from about 50 μs to about 800 μs, from about 50 μs to about 700 μs, from about 100 μs to about 600 μs, from about 150 μs to about 500 μs, or from about 200 μs to about 300 μs.

77. The method of any of claims 10 or 50, wherein the first NMR signal related to ^{13}C methyl is contributed by a protein selected from the group consisting of a BiTE® molecule selected from the group consisting of anti-CD33 and anti-CD3 BiTE molecule, anti-BCMA and anti-CD3 BiTE molecule, anti-FLT3 and anti-CD3 BiTE, anti-CD19 and anti-CD3 BiTE, anti-EGFRvIII and anti-CD3 BiTE molecule, anti-DLL3 and anti-CD3 BiTE, BLINCYTO (blinatumomab) and Solitomab; an antibody selected from the group consisting of adalimumab, bevacizumab, blinatumomab, cetuximab, conatumumab, denosumab, eculizumab, erenumab, evolocumab, infliximab, natalizumab, panitumumab, rilotumumab, rituximab, romosozumab, and trastuzumab, and antibodies selected from Table A; and combinations thereof.

78. The method of claim 62, wherein the first NMR signal is a NMR signal related to ^{13}C methyl, the second NMR signal is a signal related to a NMR signal related to ^{13}C sucrose, and the third NMR signal is a signal related to at least ^{1}H acetate or $^{1}H/^{13}C$ NMR signals from one of Glutamate, Proline, Arginine, or Mannitol.

79. The method of any of claims 11, 51, or 66, wherein the method for using NMR is conducted at a frequency range from about 500 MHz to about 2000 MHz.

80. The method of any of claims 11, 51, or 66, wherein the method for using NMR is conducted at a frequency range from about 500 MHz to about 1000 MHz.

81. The method of any of claims 11, 51, or 66, wherein the method for using NMR is conducted at a frequency range of about 900 MHz.

82. The method of any of claims 11, 51, or 66, wherein the method for using NMR is conducted at a frequency range of about 800 MHz.

83. The method of any of claims 11, 51, or 66, wherein the method for using NMR is conducted at a frequency range of about 700 MHz.

84. The method of any of claims 11, 51, or 66, wherein the method for using NMR is conducted at a frequency range of about 600 MHz.

85. The method of any of claims 11, 51, or 66, wherein the method for using NMR is conducted at a frequency range of about 500 MHz.

86. The method of claim 10, 50, or 78, wherein the third NMR signal is related to glutamate or proline.

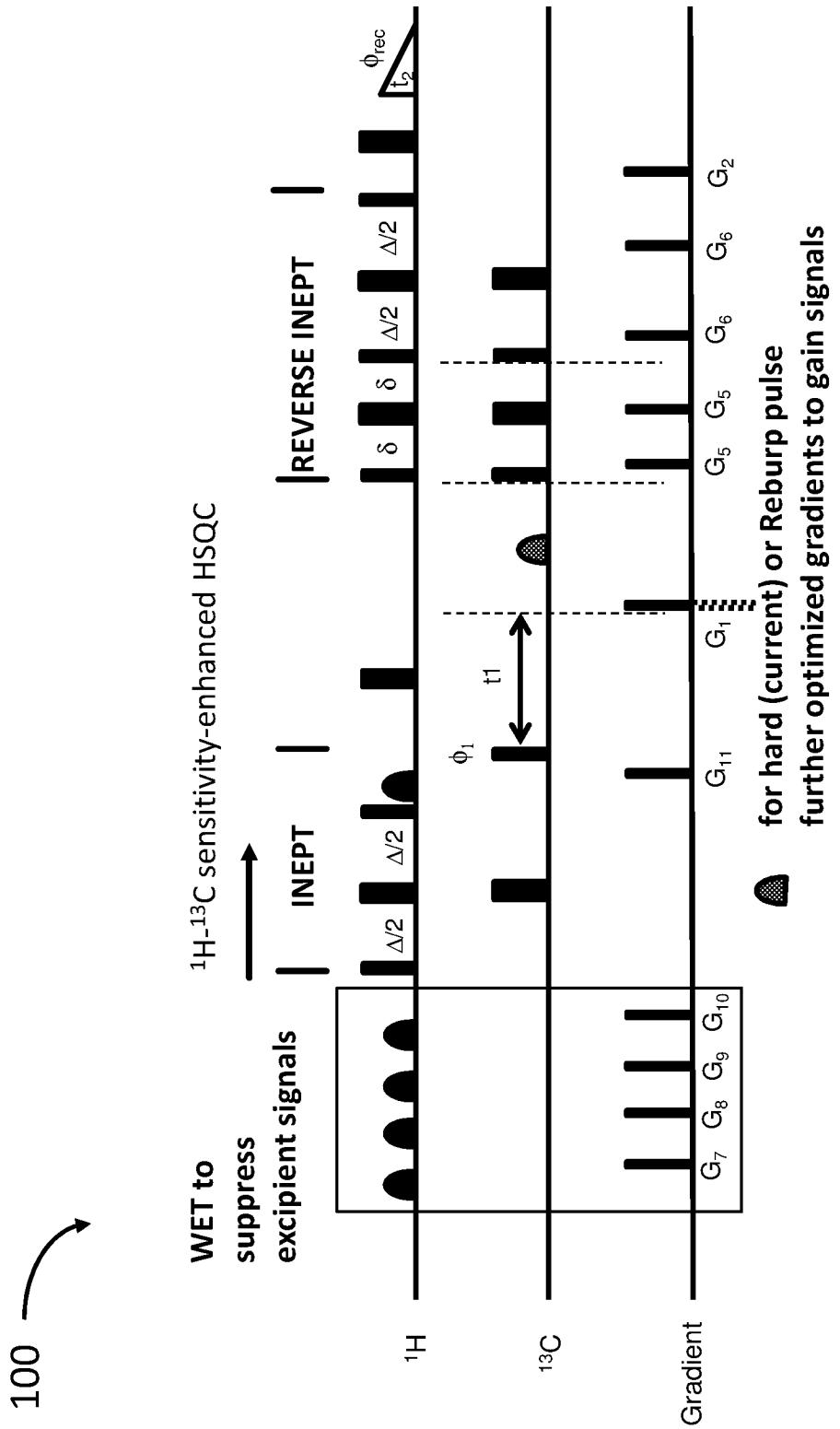
100
WET to suppress excipient signals
^{1}H-^{13}C sensitivity-enhanced HSQC
INEPT
REVERSE INEPT
Δ/2
δ
ϕ_1
t1
ϕ_{rec}
t_2
G_1 G_2 G_5 G_6 G_7 G_8 G_9 G_{10} G_{11}
^{1}H
^{13}C
Gradient
for hard (current) or Reburp pulse
further optimized gradients to gain signals

FIGURE 1

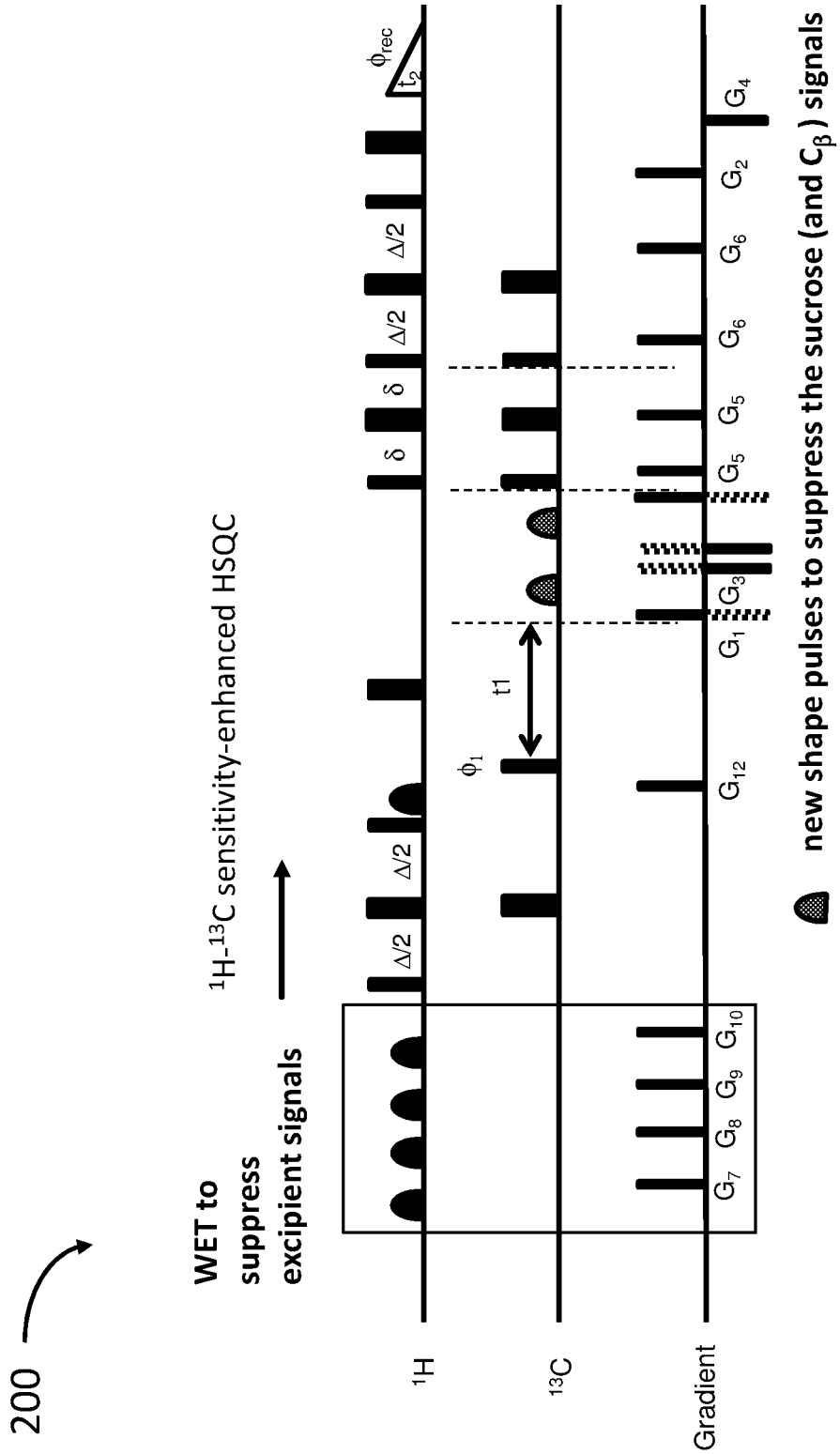
200
WET to suppress excipient signals
^{1}H-^{13}C sensitivity-enhanced HSQC
^{1}H
^{13}C
Gradient
Δ/2
Δ/2
Δ/2
δ
δ
Δ/2
Δ/2
ϕ_{rec}
t_2
ϕ_1
t1
G_{12}
G_1
G_3
G_5
G_5
G_6
G_6
G_2
G_4
G_7
G_8
G_9
G_{10}
new shape pulses to suppress the sucrose (and C_β) signals

FIGURE 2

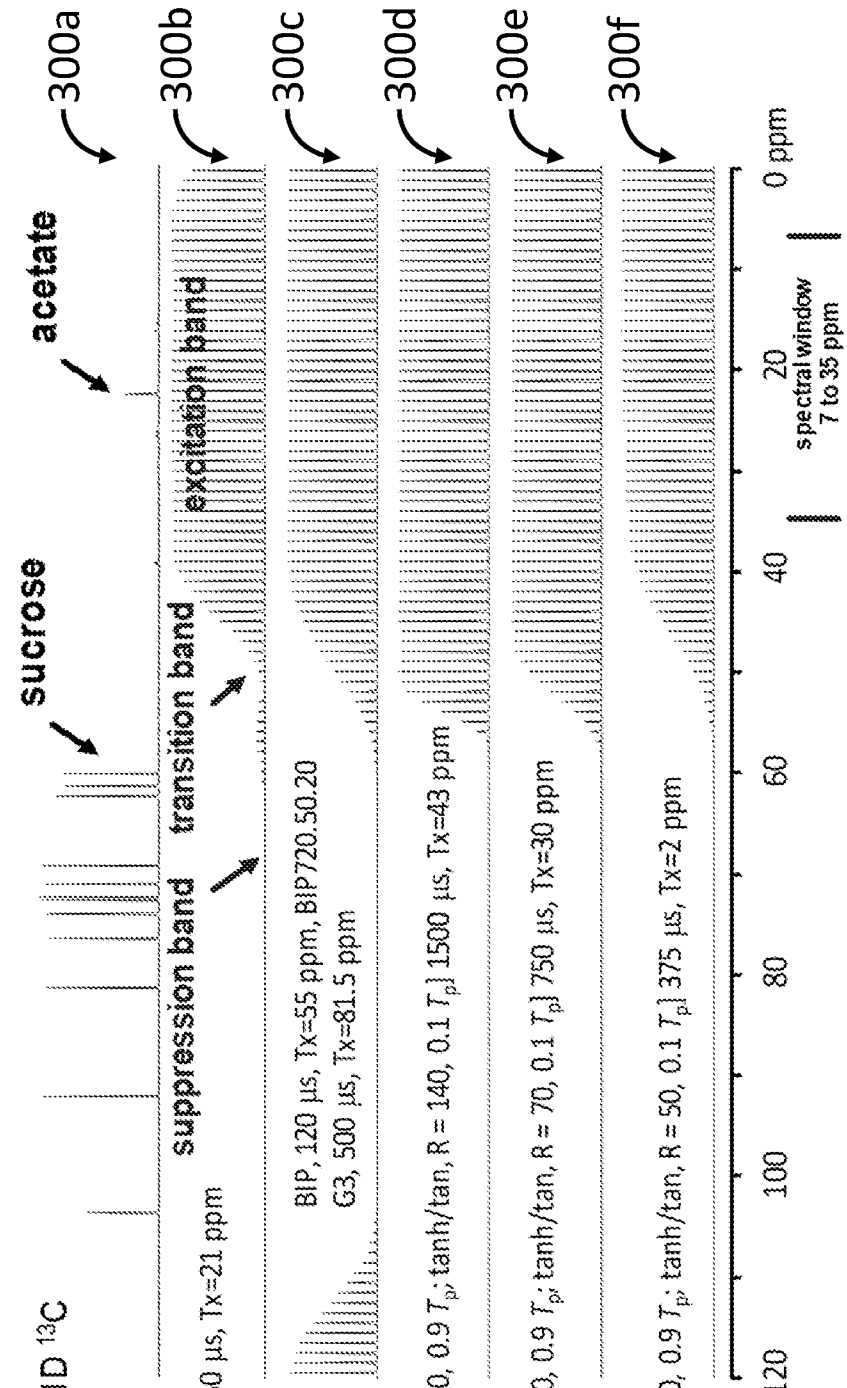

FIGURE 3A

FIGURE 3B

FIGURE 3C

FIGURE 3D

FIGURE 3E

FIGURE 3F

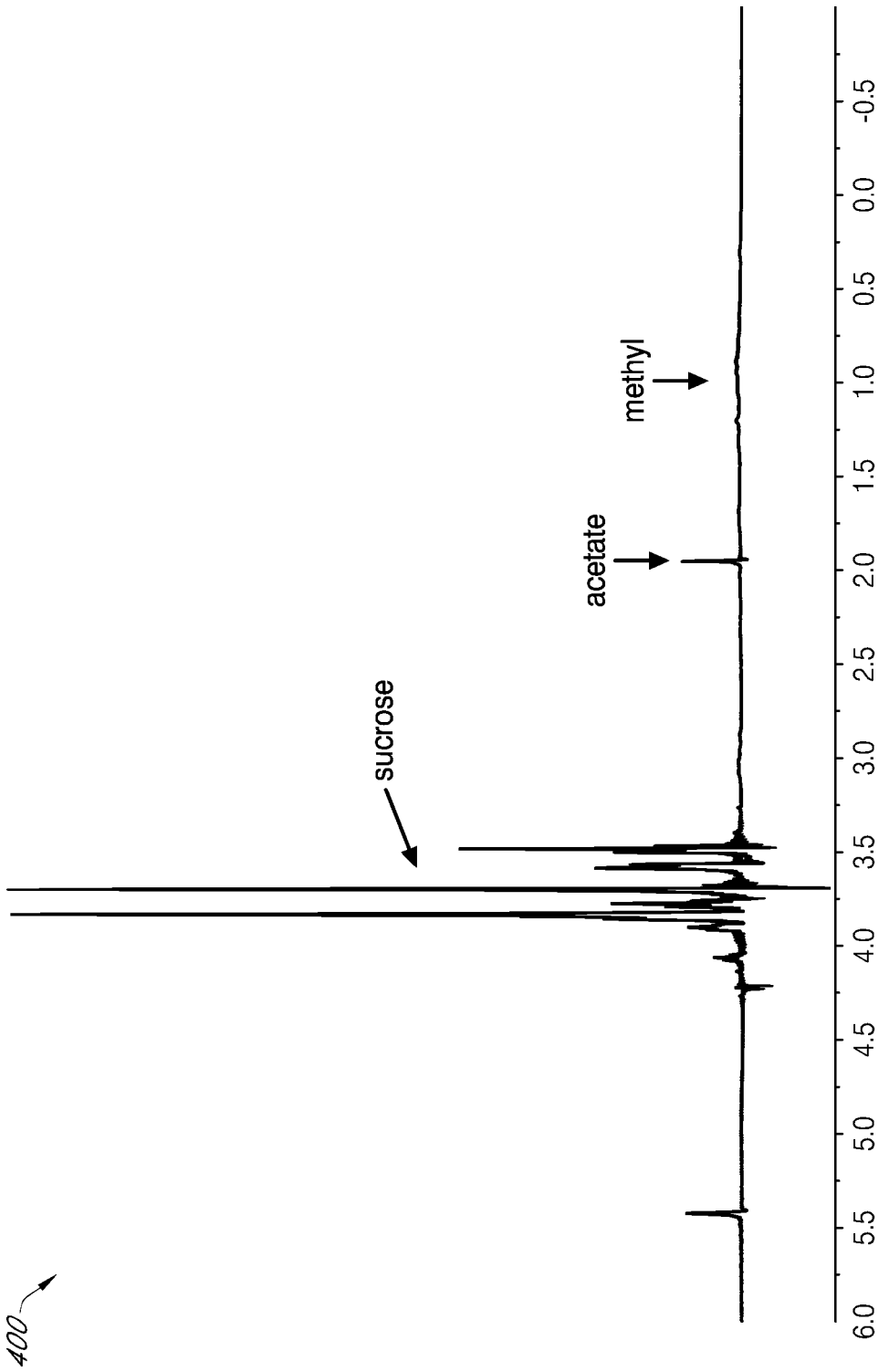
400
sucrose
acetate
methyl
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5

FIGURE 4

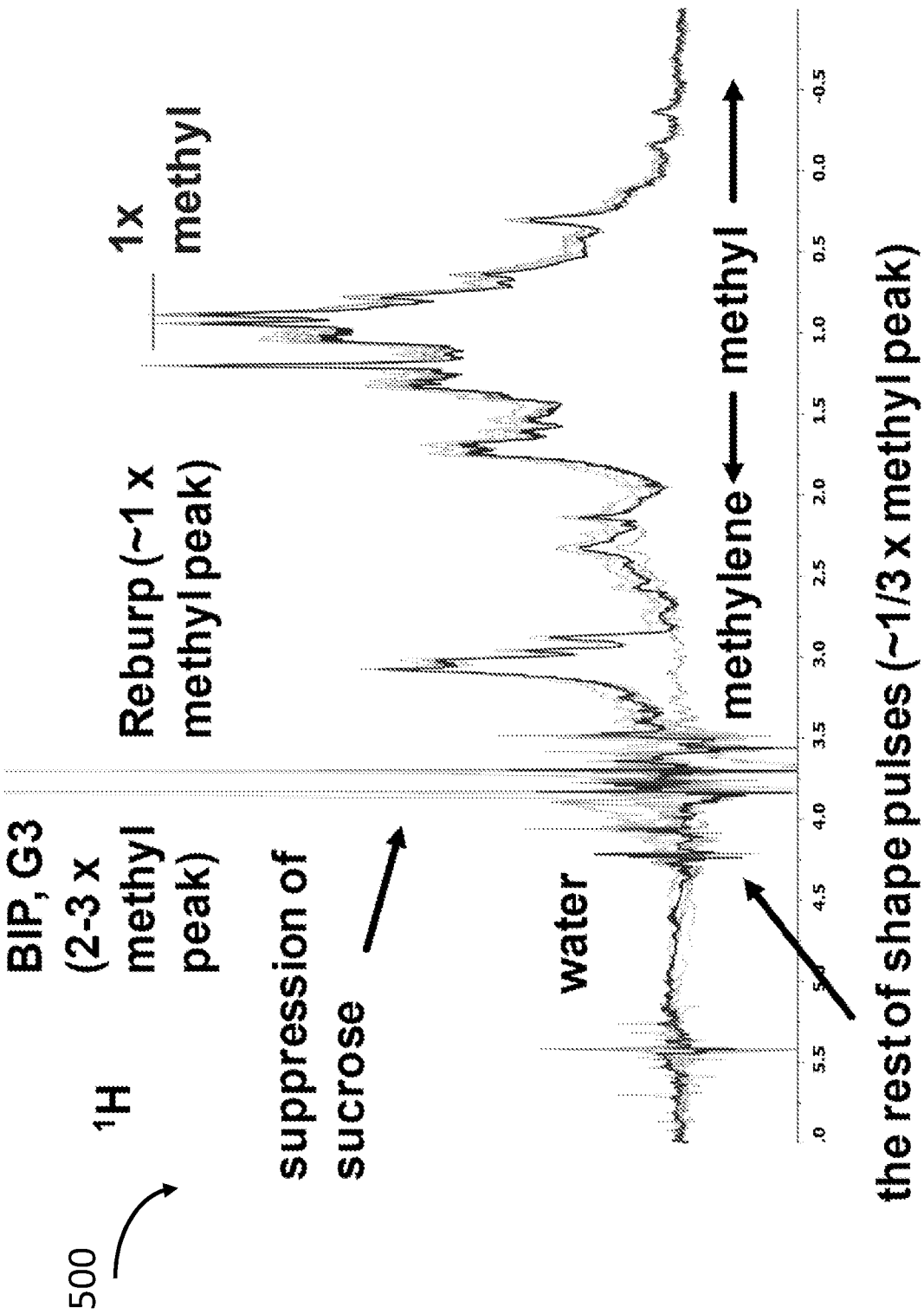
500
^{1}H
BIP, G3
(2-3 x
methyl
peak)
suppression of
sucrose
Reburp (~1 x
methyl peak)
1x
methyl
water
methylene
methyl
the rest of shape pulses (~1/3 x methyl peak)
.0
5.5
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5

FIGURE 5

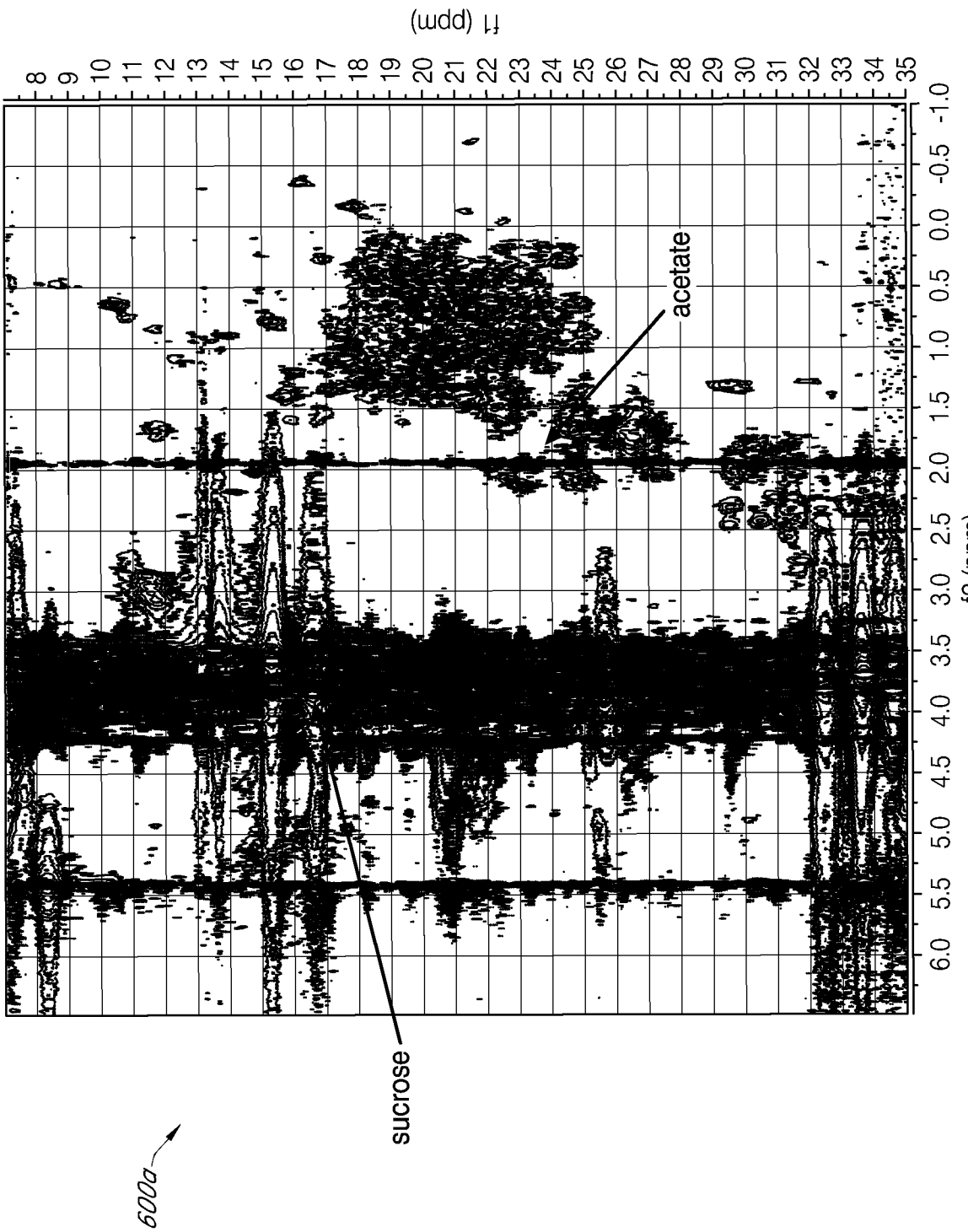
f1 (ppm)
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
f2 (ppm)
6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
acetate
sucrose
600a

FIGURE 6A

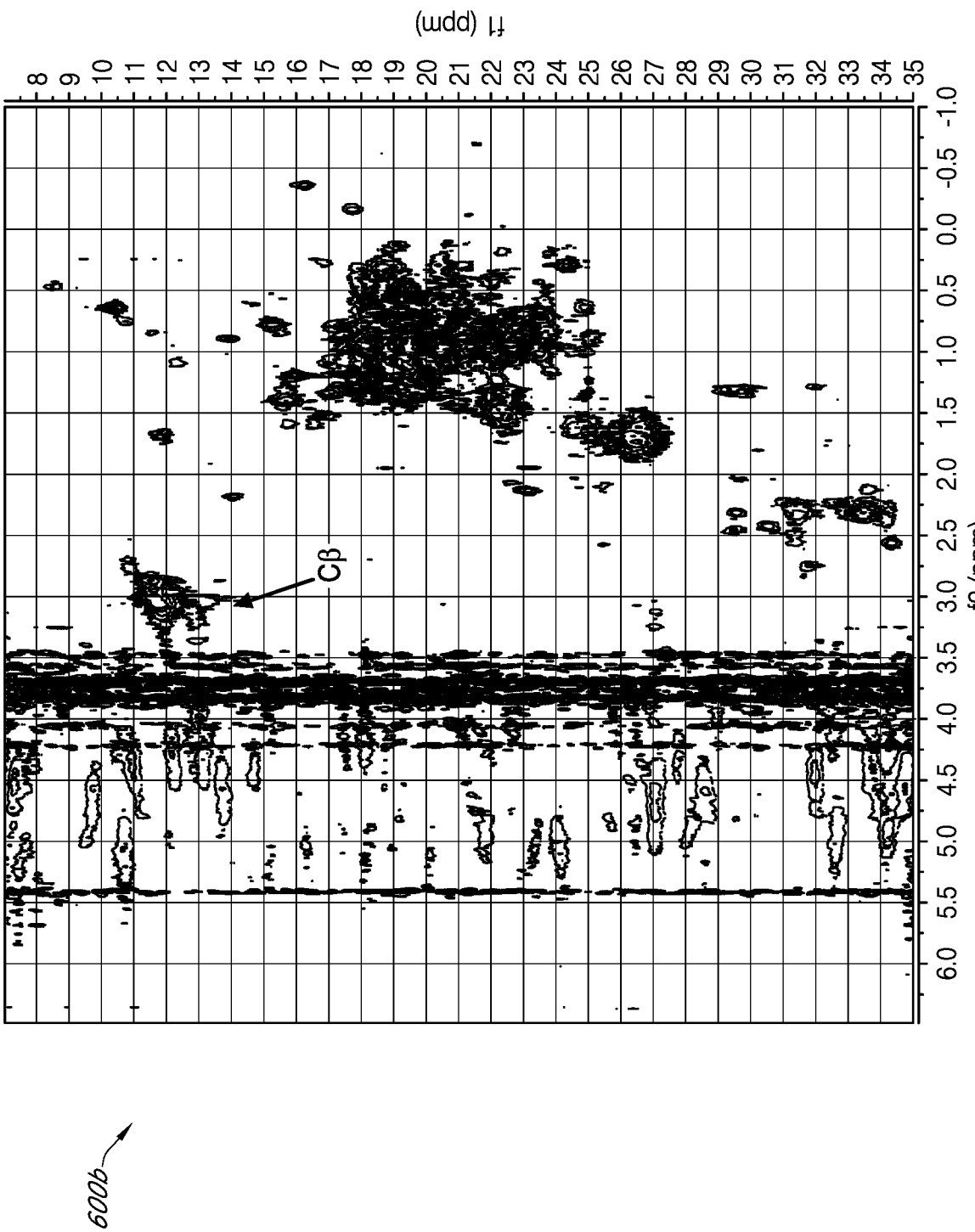
f1 (ppm)
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
Cβ
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
f2 (ppm)
600b

FIGURE 6B

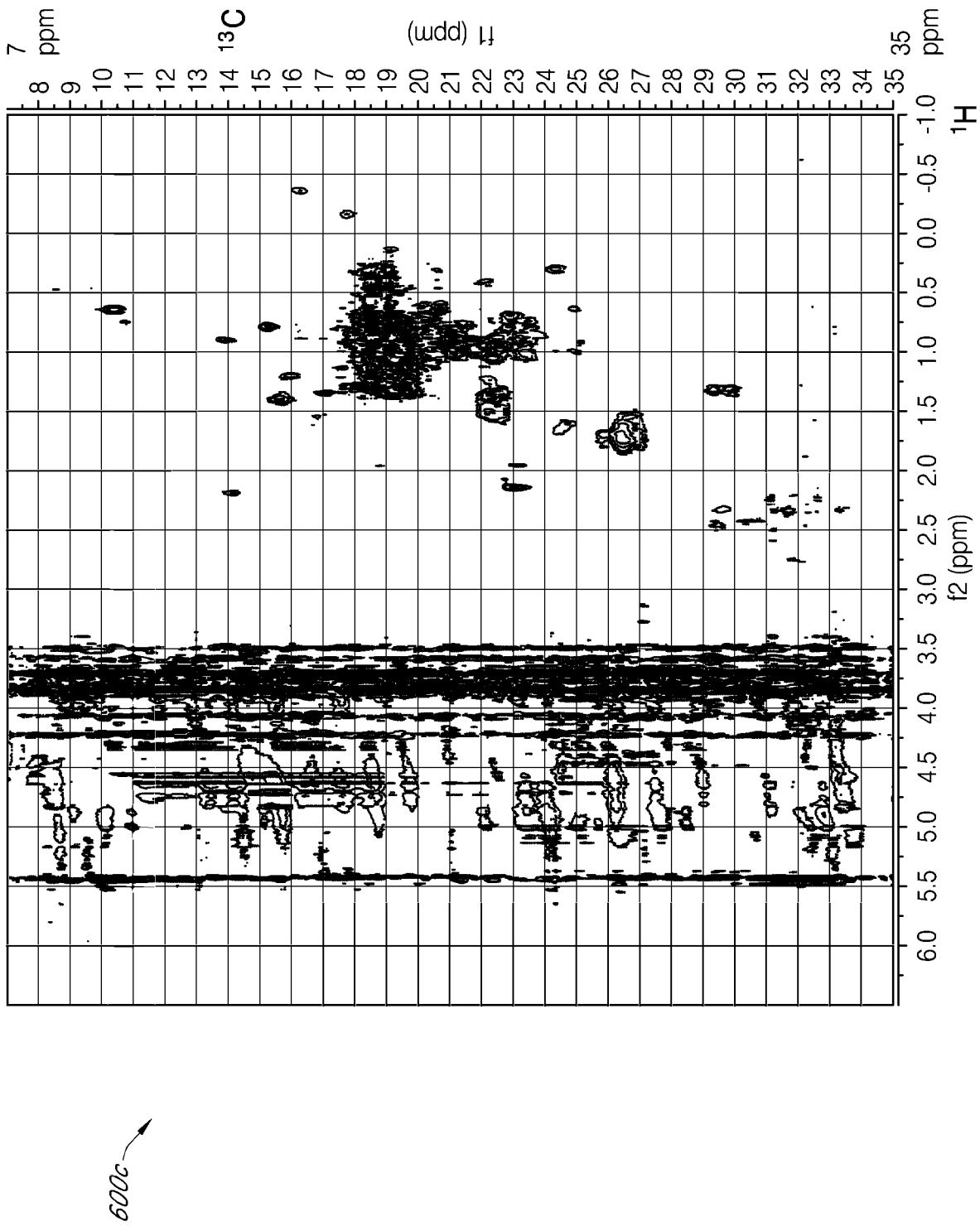

FIGURE 6C

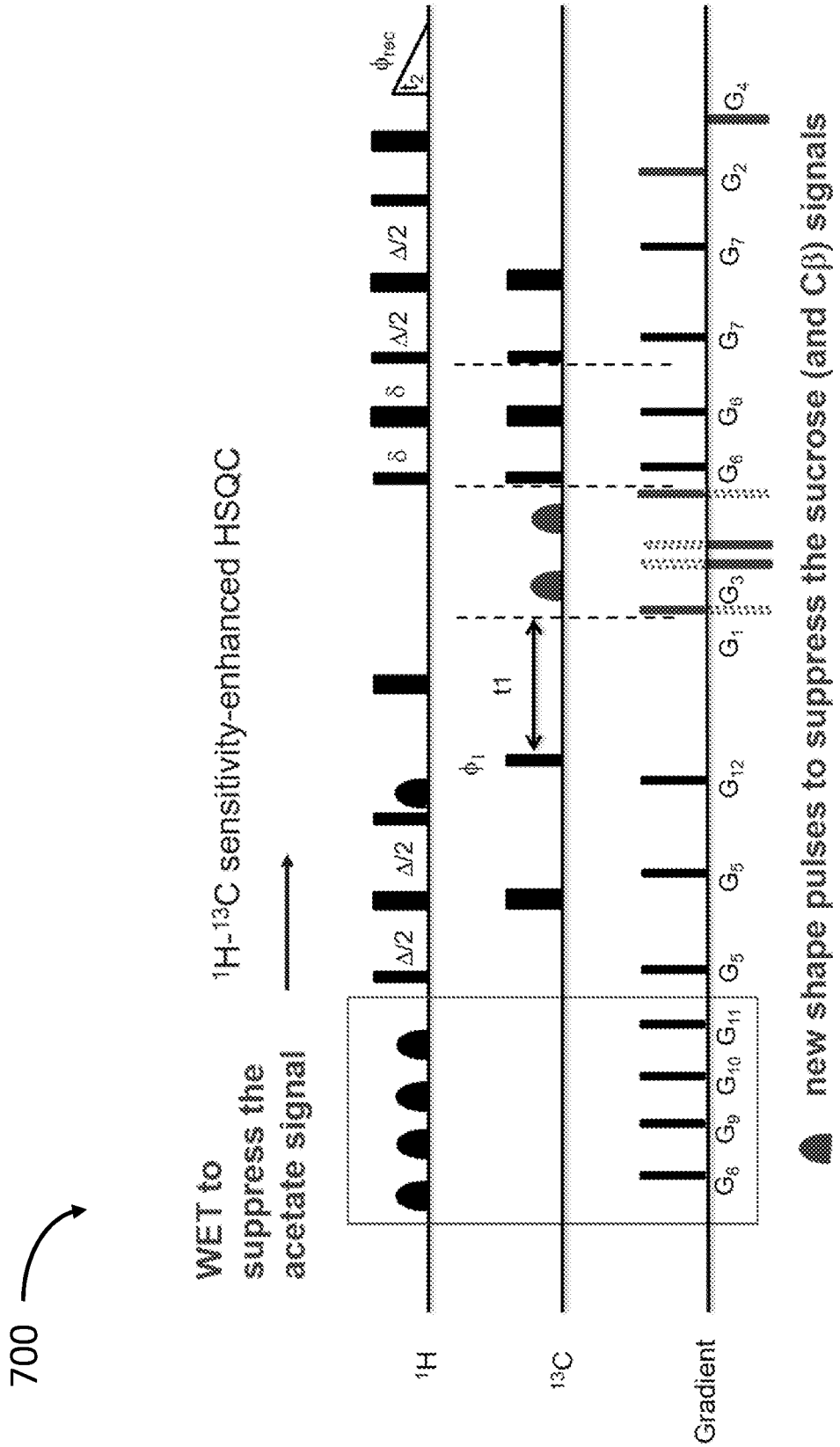
700
WET to suppress the acetate signal
^{1}H-^{13}C sensitivity-enhanced HSQC
new shape pulses to suppress the sucrose (and Cβ) signals
^{1}H
^{13}C
Gradient
Δ/2
δ
t1
ϕ_1
ϕ_{rec}
G_1 G_2 G_3 G_4 G_5 G_6 G_7 G_8 G_9 G_{10} G_{11} G_{12}

FIGURE 7

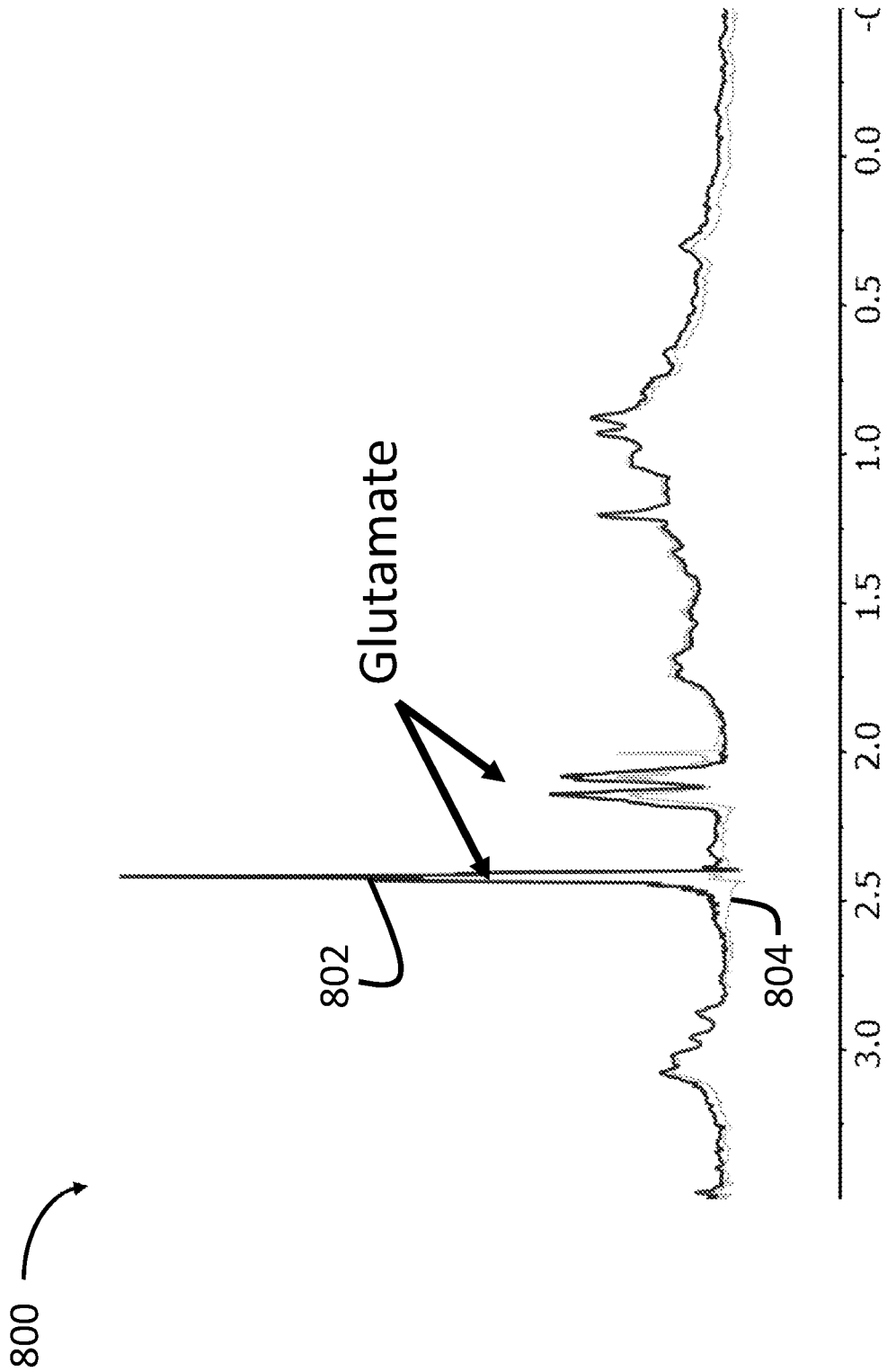

FIGURE 8

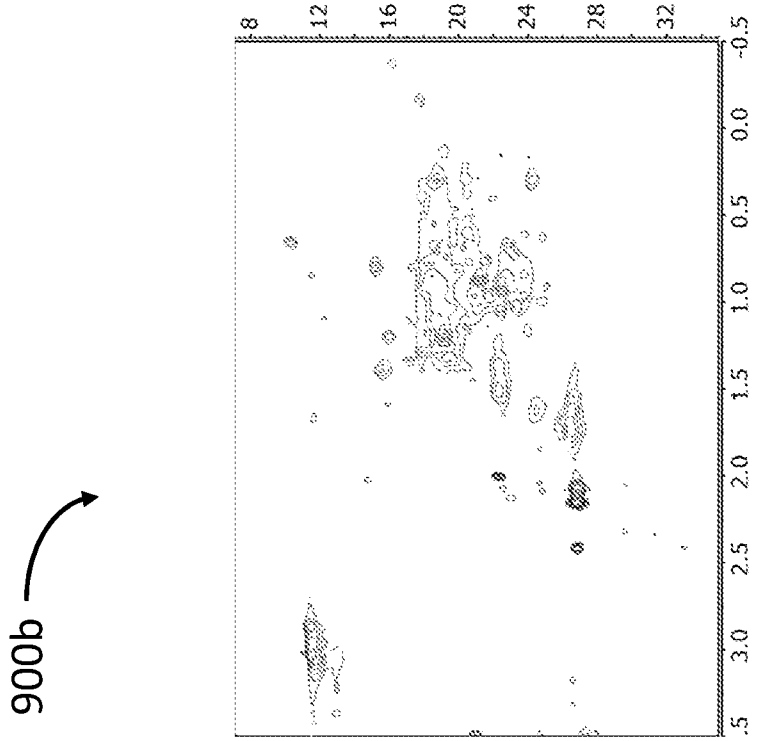

FIGURE 9B

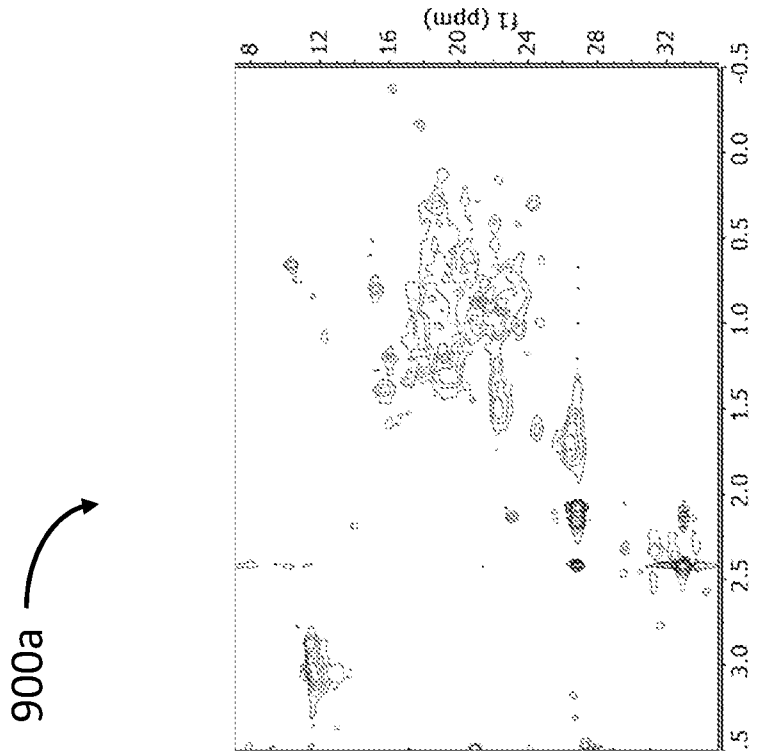

FIGURE 9A

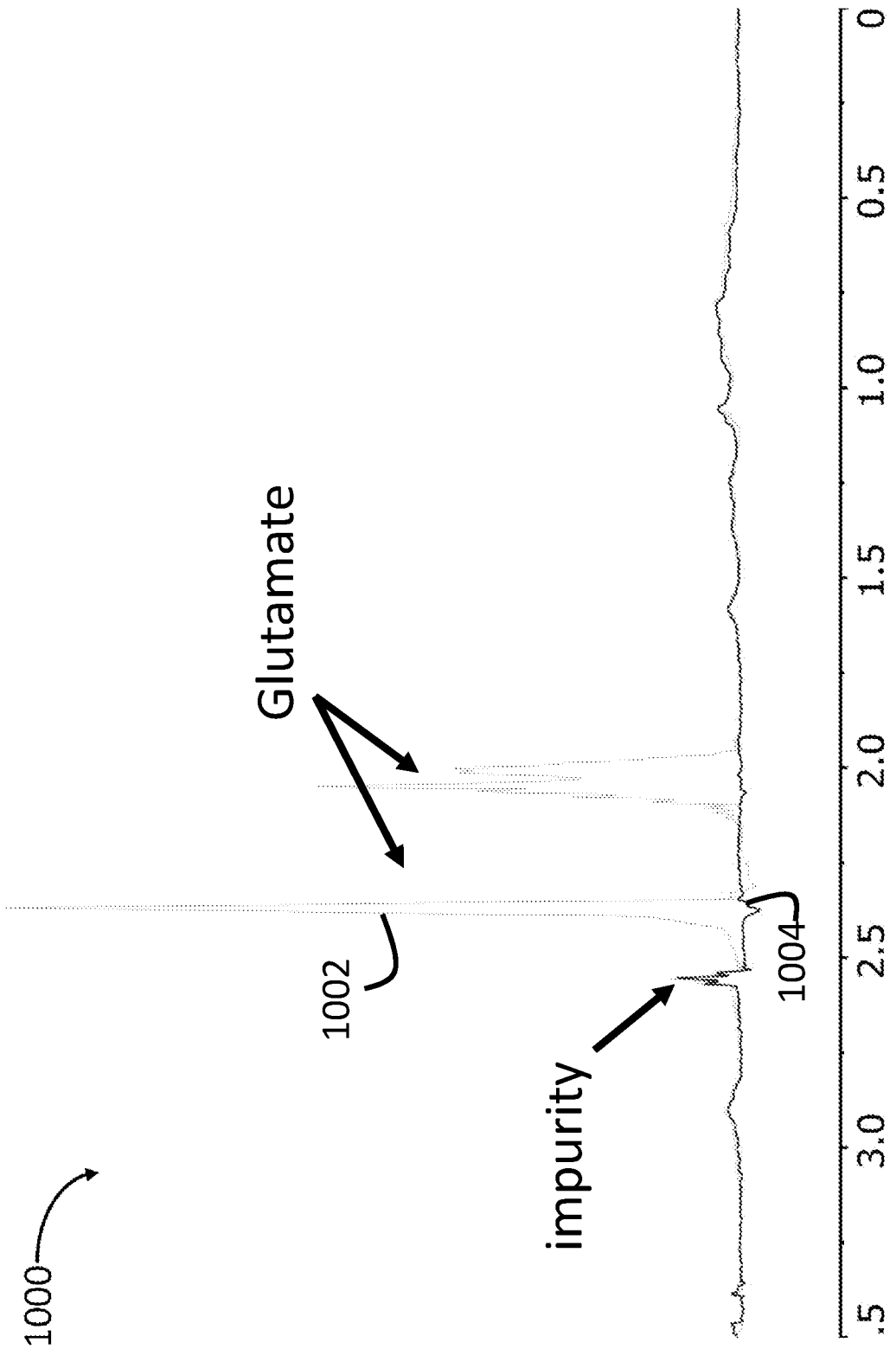

FIGURE 10

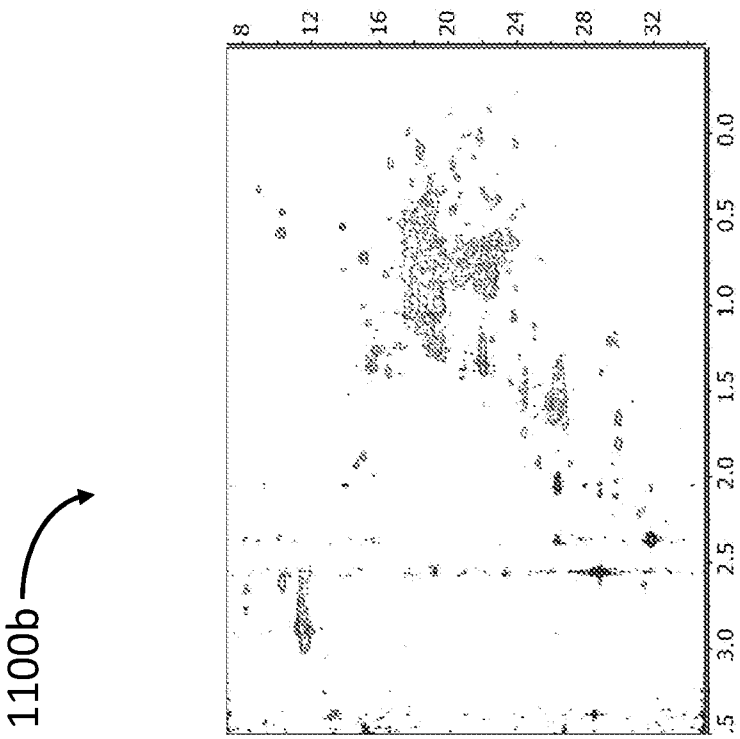

FIGURE 11B

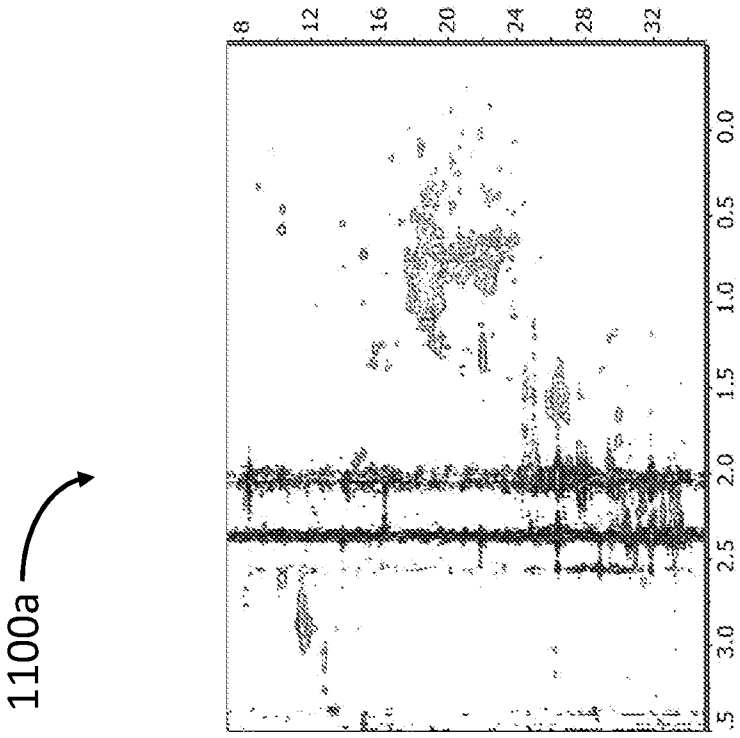

FIGURE 11A

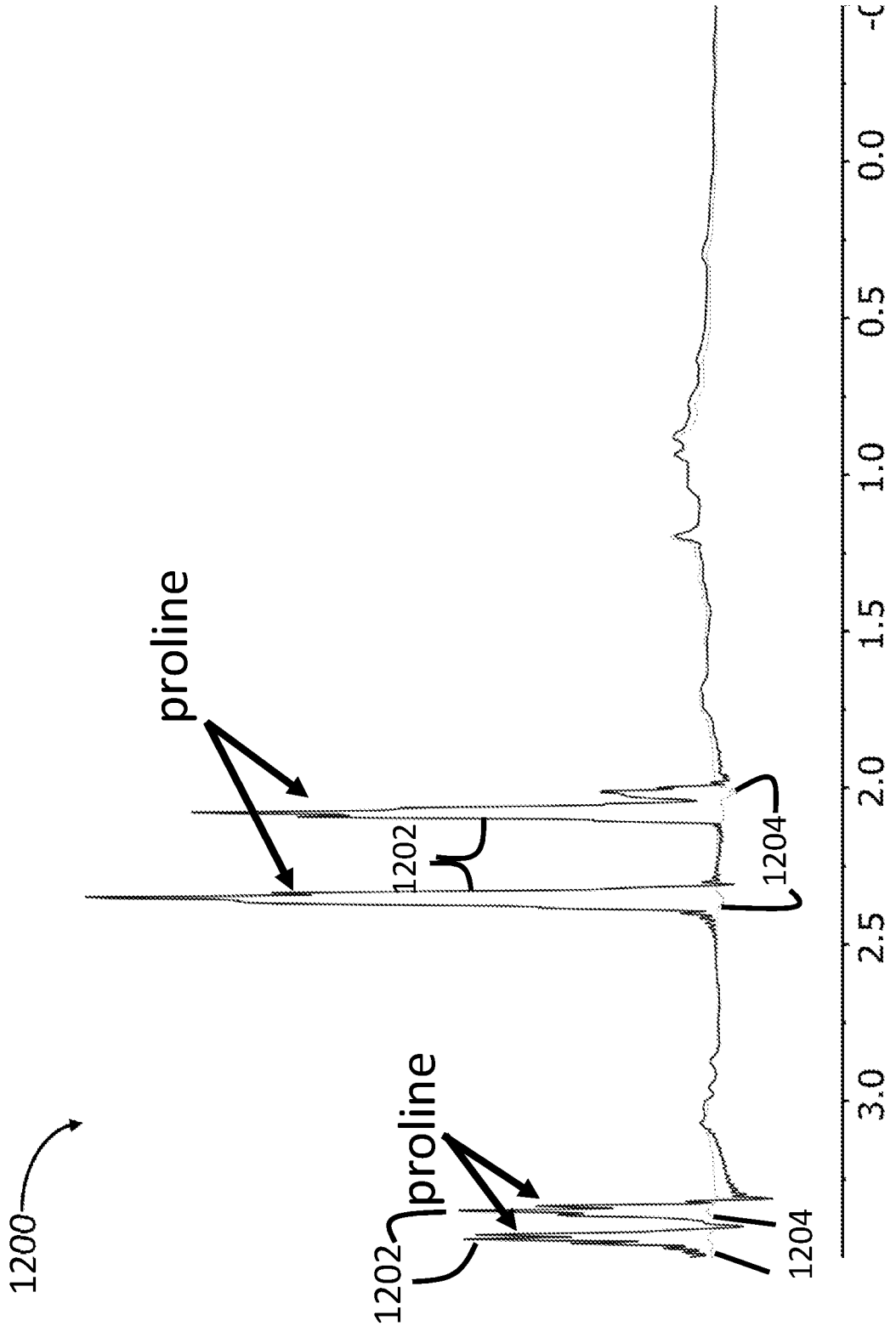

FIGURE 12

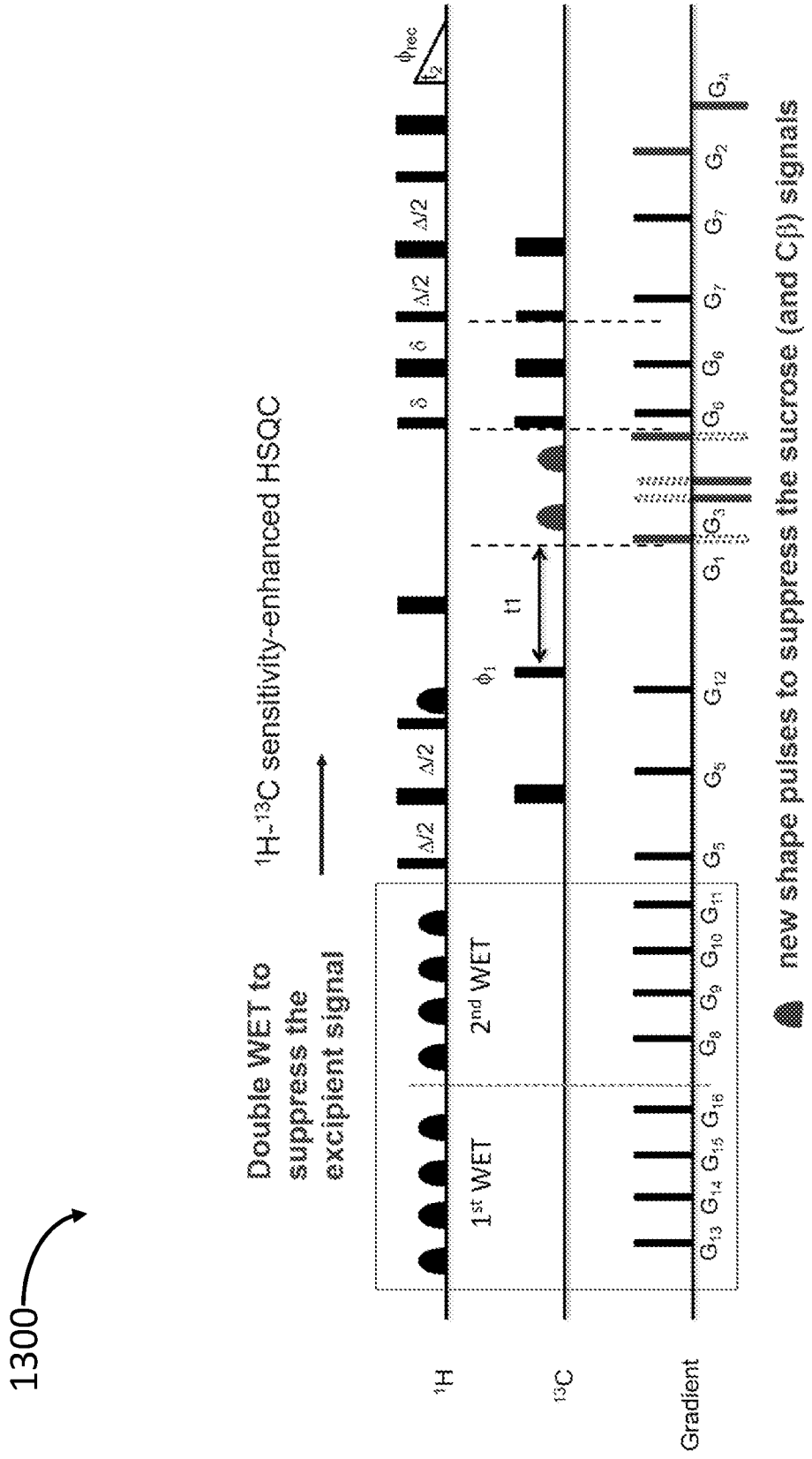
1300
Double WET to suppress the excipient signal
^1H-^{13}C sensitivity-enhanced HSQC
1st WET
2nd WET
^1H
^{13}C
Gradient
new shape pulses to suppress the sucrose (and Cβ) signals

FIGURE 13

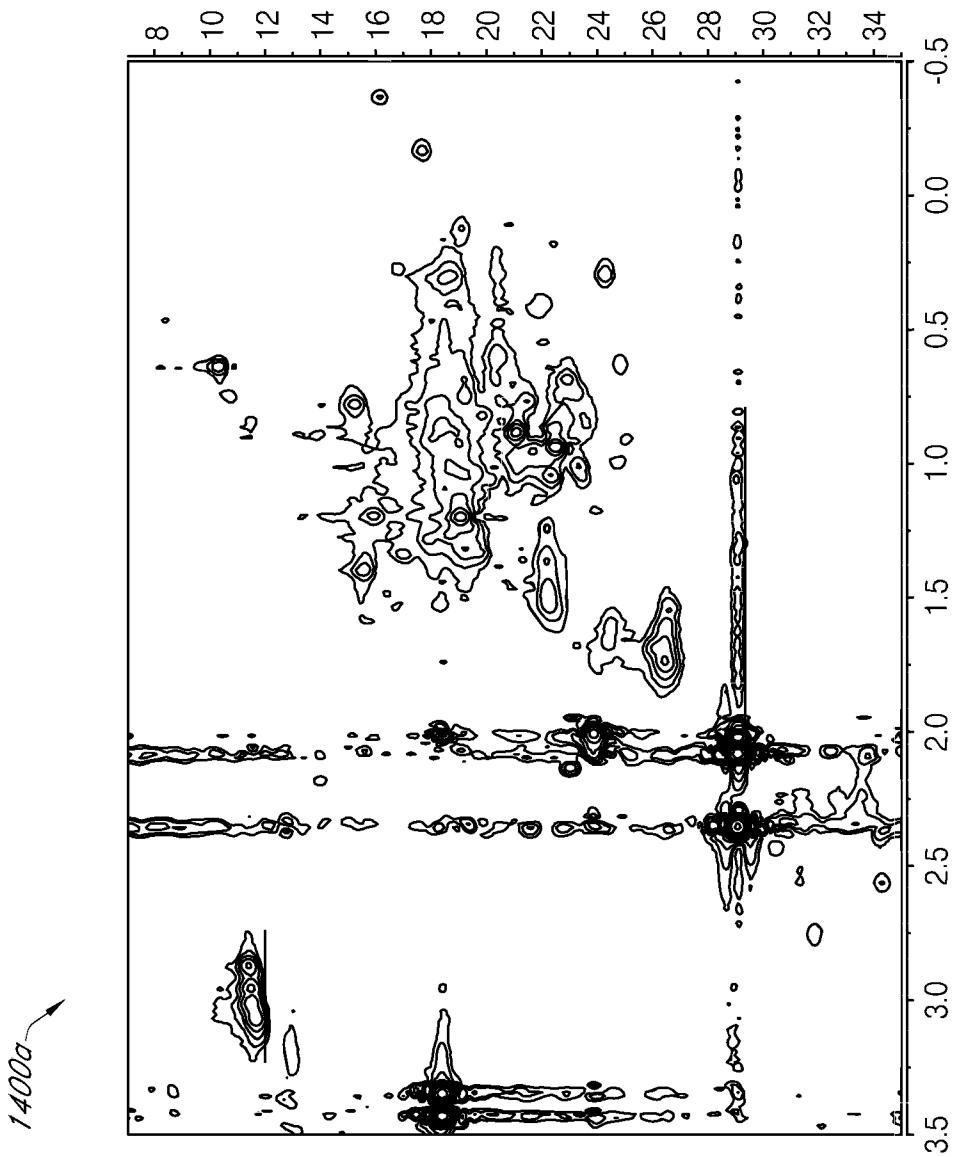
8
10
12
14
16
18
20
22
24
26
28
30
32
34
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
1400a

FIGURE 14A

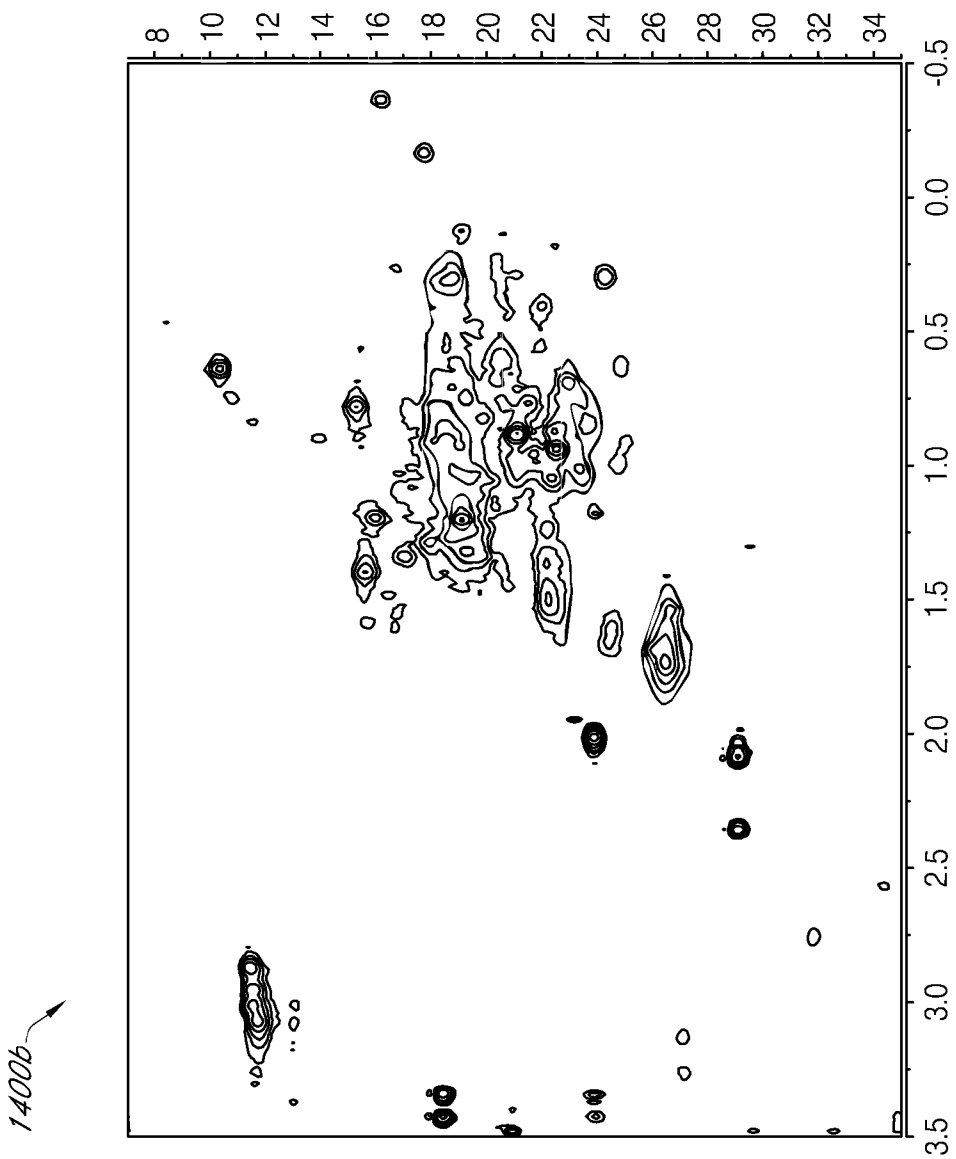
8
10
12
14
16
18
20
22
24
26
28
30
32
34
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
1400b

FIGURE 14B

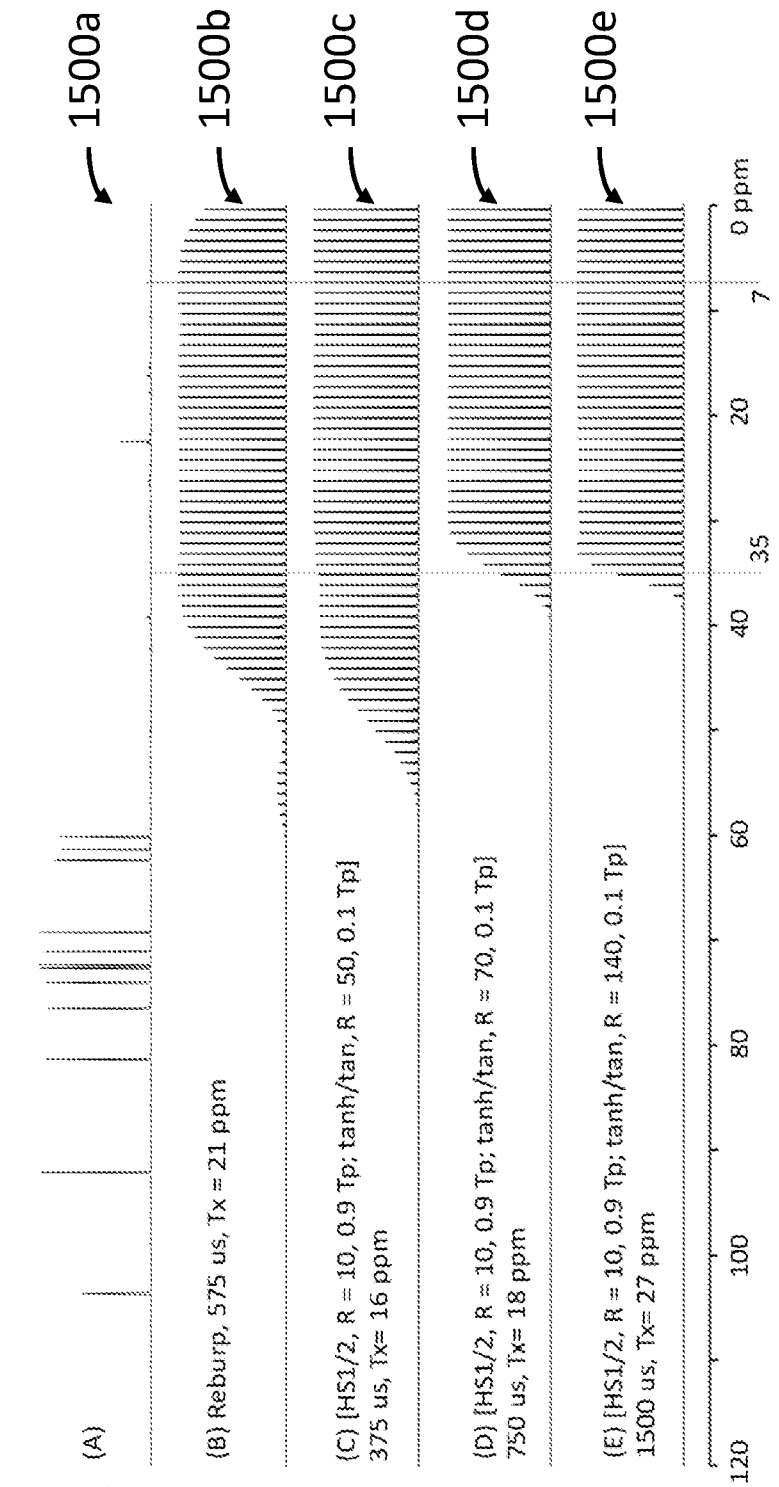

FIGURE 15A

FIGURE 15B

FIGURE 15C

FIGURE 15D

FIGURE 15E

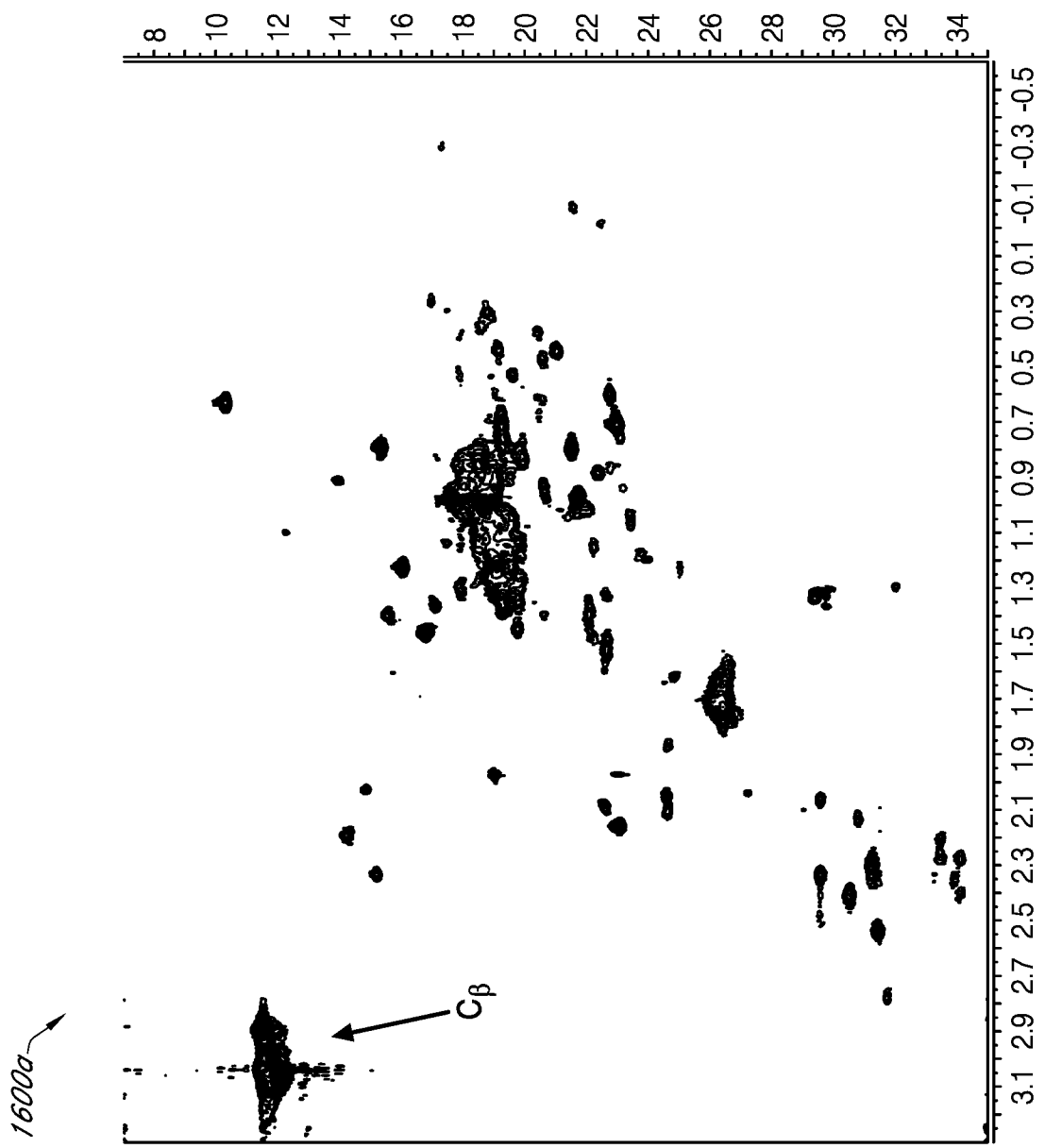
1600a
C_β
8 10 12 14 16 18 20 22 24 26 28 30 32 34
3.1 2.9 2.7 2.5 2.3 2.1 1.9 1.7 1.5 1.3 1.1 0.9 0.7 0.5 0.3 0.1 -0.1 -0.3 -0.5

FIGURE 16A

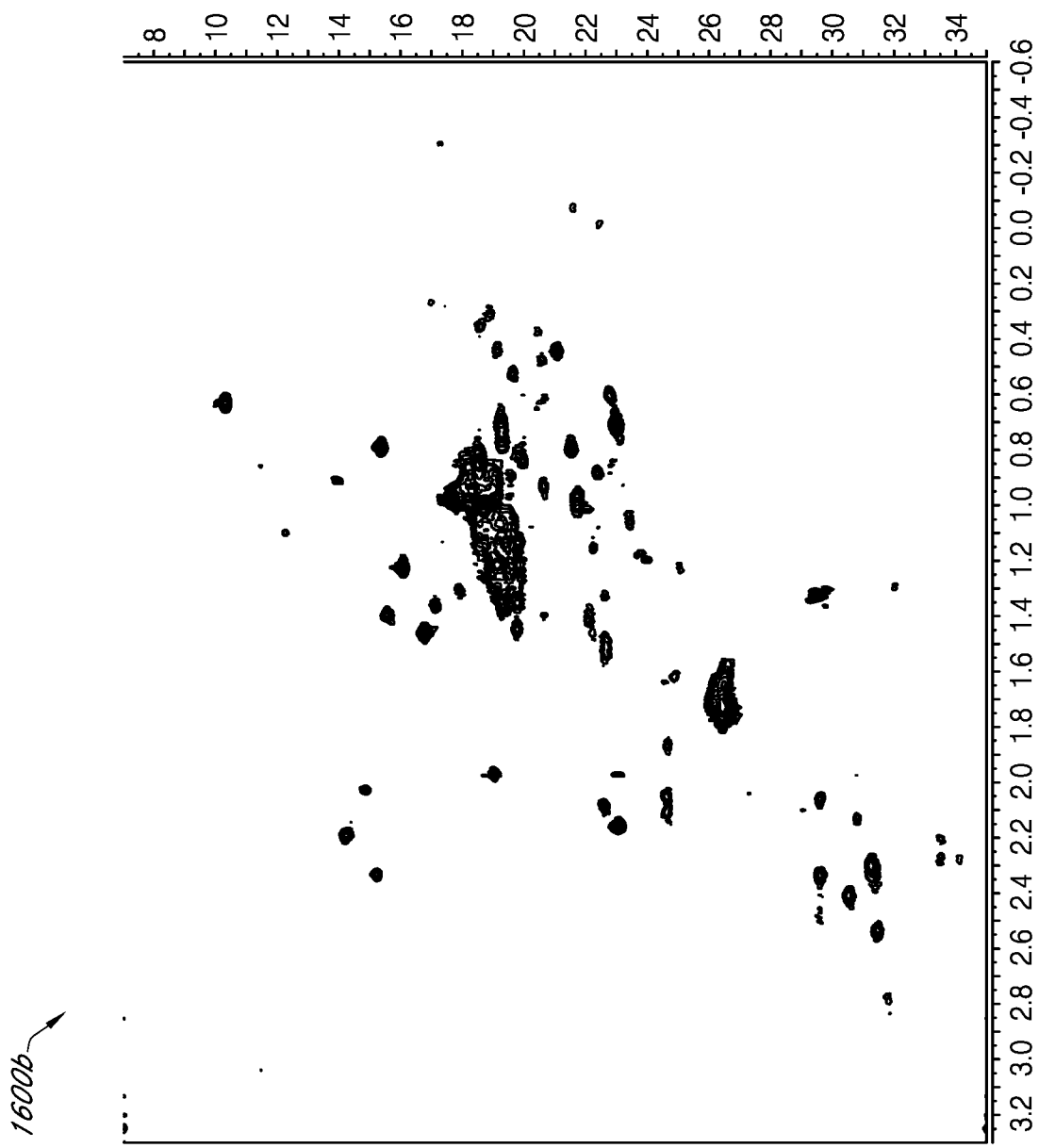
1600b
8 10 12 14 16 18 20 22 24 26 28 30 32 34
3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4 0.2 0.0 -0.2 -0.4 -0.6

FIGURE 16B

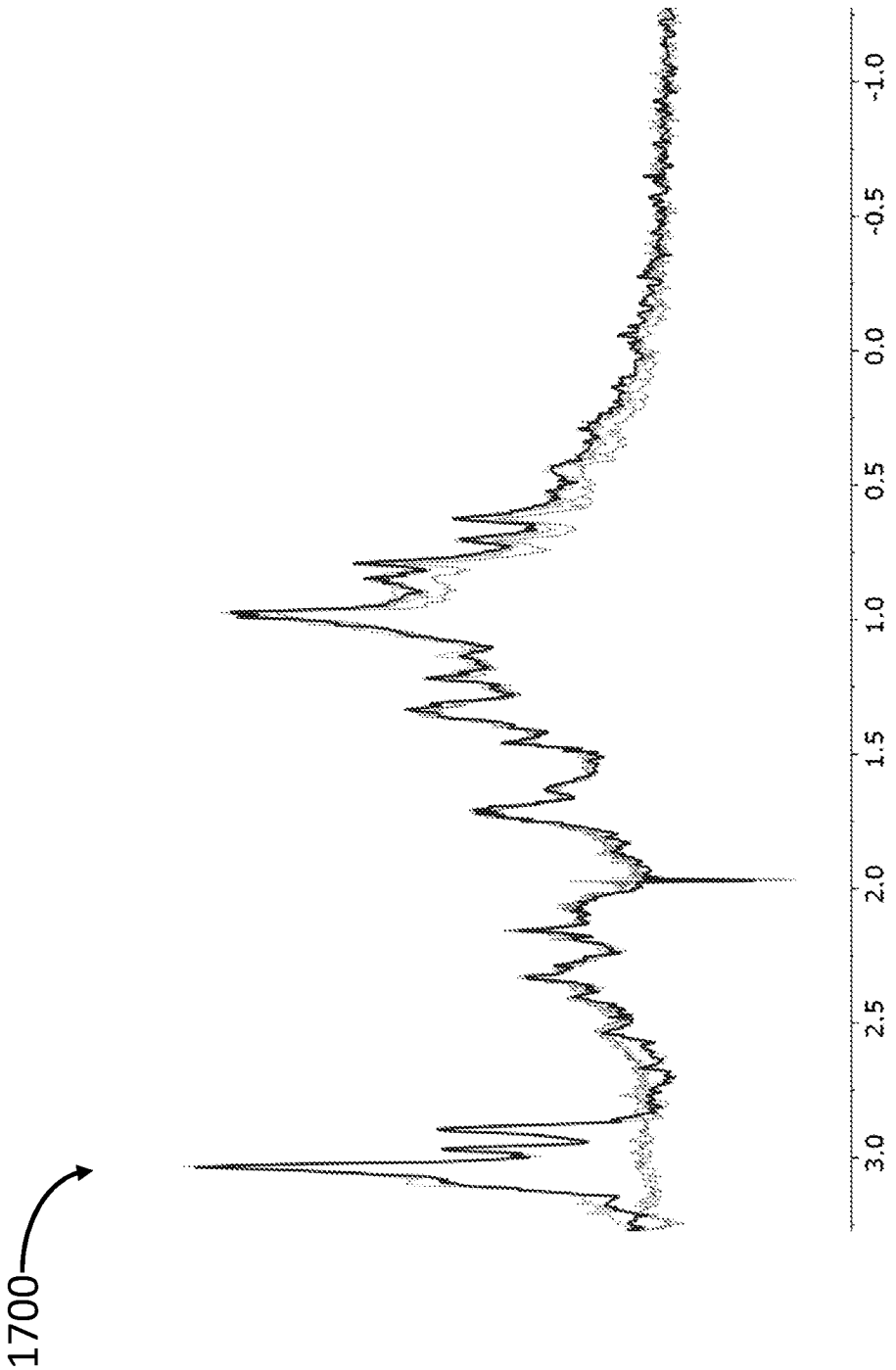

FIGURE 17

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/025078

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N24/08 G01R33/46 G01R33/465 G01R33/485
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N G01R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARBOGAST LUKE W ET AL: "Selective suppression of excipient signals in 2D1H-13C methyl spectra of biopharmaceutical products", JOURNAL OF BIOMOLECULAR NMR, ESCOM, LEIDEN, NL, vol. 72, no. 3, 27 November 2018 (2018-11-27), pages 149-161, XP036659000, ISSN: 0925-2738, DOI: 10.1007/S10858-018-0214-1 [retrieved on 2018-11-27] the whole document ----- -/--	1-86

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 June 2020	21/07/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vanhaecke, Nicolas

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/025078

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HUAWEI LIU ET AL: "Selective Excitation with Asymmetric Adiabatic Pulses for NMR Spectroscopy", CHEMPHYSCHEM - A EUROPEAN JOURNAL OF CHEMICAL PHYSICS & PHYSICALCHEMISTRY., vol. 16, no. 3, 16 December 2014 (2014-12-16), pages 621-627, XP055701866, DE ISSN: 1439-4235, DOI: 10.1002/cphc.201402705 the whole document -----	6,7,9, 12-14, 22-26, 46,47, 49,52, 56,62, 67,71
A	OGG R J ET AL: "WET, A T1- AND B1-INSENSITIVE WATER-SUPPRESSION METHOD FOR IN VIVO LOCALIZED 1H NMR SPECTROSCOPY", JOURNAL OF MAGNETIC RESONANCE. SERIES B, ACADEMIC PRESS, ORLANDO, FL, US, vol. 104, no. 1, 1 May 1994 (1994-05-01), pages 1-10, XP000538758, ISSN: 1064-1866, DOI: 10.1006/JMRB.1994.1048 the whole document -----	75,76
A	Lyndon Emsley ET AL: "GAUSSIAN PULSE CASCADES: NEW ANALYTICAL FUNCTIONS FOR RECTANGULAR SELECTIVE INVERSION AND IN-PHASE EXCITATION IN NMR", CHEMICAL PHYSICS LETTERS Volume 165, number 6, 2 February 1990 (1990-02-02), pages 469-476, XP055702409, DOI: https://doi.org/10.1016/0009-2614(90)87025-M Retrieved from the Internet: URL:https://doi.org/10.1016/0009-2614(90)87025-M [retrieved on 2020-06-08] the whole document -----	6,8, 15-21, 46,48, 53-55, 62-76, 78-86
A	PETER C. M. VAN ZIJL ET AL: "Optimized Excitation and Automation for High-Resolution NMR Using B 1 -Insensitive Rotation Pulses", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 118, no. 23, 12 June 1996 (1996-06-12), pages 5510-5511, XP055702329, US ISSN: 0002-7863, DOI: 10.1021/ja9602612 the whole document -----	6,8, 15-21, 46,48, 53-55, 62-76, 78-86
	-/--	

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/025078

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP 2003 194750 A (SUMITOMO CHEMICAL CO) 9 July 2003 (2003-07-09) paragraphs [0014], [0020] - [0021] -----	75,76

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2020/025078

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2003194750 A	09-07-2003	NONE	