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RESEARCH ARTICLE

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J-filter: An experiment to simplify and isolate specific signals in ^{1}H NMR spectra of complex mixtures based on scalar coupling constants

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Abstract

One-dimensional selective NMR experiments relying on a J-filter element are proposed to isolate specific signals in crowded ^{1}H spectral regions. The J-filter allows the edition or filtering of signals in a region of interest of the spectrum by exploiting the specific values of their ^{1}H-^{1}H coupling constants and certain parameters of protons coupled to them that appear in less congested parts of the spectrum (chemical shifts and coupling constants). The new experiments permitted the isolation of specific peaks of phytosterol components in a sample obtained from a liquid nutraceutical recommended for lowering blood cholesterol levels in regions with complete overlap in the ^{1}H spectrum.

KEYWORDS

NMR of mixtures, NMR scalar coupling filter, selective experiment, signal edition and filtering

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1 | INTRODUCTION

Solution NMR spectroscopy is a versatile tool for the study of a variety of samples of pure compounds and mixtures such as food, cosmetics, and biofluids, among many others. To this end, the one-dimensional (1D) proton spectrum offers high sensitivity per measurement time, but its relative short chemical shift range compounded with signal splitting due to ^{1}H-^{1}H scalar couplings (J) often results in severe signal overlapping, which in samples of some complexity can even impede structure assignment or quantitative analysis.

Some NMR solutions to deal with crowded spectra are aimed at simplifying the spectrum, for instance by using ^{1}H pure shift methods[1] or by relying on multidimensional NMR (nD) to spread the resonances into a second or higher dimension at the cost of longer measurement times. Although a variety of methods have been developed to accelerate these measures,[2,3] there remain, however, difficult cases of strong overlap that may not be resolved by using these methods and/or the required number of scans to detect compounds at very low concentration reduces their applicability.

For cases where only partial spectral information is strictly necessary, for instance, to identify or quantify some relevant compound/s, 1D frequency selective or 2D band-selective experiments are the most efficient and sensitive. Many selective experiments of these types are based on the excitation-sculpting experiment proposed by Hwang and Shaka.[4] The recently introduced ultra-selective GEMSTONE (Gradient-Enhanced Multiplet-Selective Targeted-Observation NMR Experiment) proposed by Kiraly et al.[5] affords a drastic reduction of the selected band width by only a few Hz, without the pernicious effects of J-modulation associated with the use of extremely long selective pulses. Of course, strong overlap of signals limits the use of any frequency selective experiment, because at least a certain number of Hz of separation between the signal(s) of interest and the others is required.

Herein, we build upon the ideas of GEMSTONE[5] and focus on expanding selective NMR experiments to cases of severe signal spectral overlap as it frequently occurs in mixtures of substances.[6] We propose a J-filter pulse sequence for the edition/filtering (selection/rejection) of signals attending to the magnitude of their homonuclear scalar J-coupling constants. To distinguish between overlapping signals with similar values of their coupling constants, the J-filter can also discriminate signals by exploiting the presence of specific coupling partner/s in another part/s of the proton spectrum. A J-filter can easily be incorporated as a building block with other NMR pulse sequences to enhance the signal selection. Thus, in this work, we combined a J-filter with the ultra-selective GEMSTONE experiment. In what follows, we describe the pulse sequences and test them with a sample of phytosterols isolated from a liquid nutraceutical recommended for lowering blood cholesterol levels. It is shown that some key signals of these phytosterols that severely overlap in the proton spectrum can be efficiently isolated and assigned with the experiments proposed.

2 | MATERIALS AND METHODS

2.1 | Sample preparation

A commercial nutraceutical drink to lower cholesterol with added plant stanol esters (Danacol®) was lyophilized and the white powder obtained was hydrolyzed following the procedure established by the Official Journal of the European Union.[7] A complex mixture of phytosterols was obtained as a colorless oil. The sample used for the NMR studies was prepared in a standard 5-mm tube by mixing 35 mg of the mixture of phytosterols obtained from the hydrolysis reaction in 0.75 mL of $CDCl_3$.

2.2 | Nuclear magnetic resonance measurements

NMR experiments were conducted at 25°C on a Bruker NEO 17.6 T spectrometer (proton resonance 750 MHz), equipped with a ^{1}H-^{19}F/^{13}C/^{15}N triple resonance PA-TXI probe with deuterium lock channel and shielded PFG *z*-gradient. The spectrometer control software was TopSpin 4.0. Spectra were processed and analyzed with Mestrenova software v.14.3.2 (Mestrelab Inc.).

2.3 | Theoretical background

The pulse sequences proposed in this work are shown in Figure 1. They begin with the proposed J-filter element aimed to simplify the spectrum by exploiting the specific values of the ^{1}H-^{1}H coupling constants and coupling constant partners of the signals of interest. The basic 1D J-filter experiment is shown in Figure 1a and consists of a spin-echo followed by the adiabatic *z*-filtering of zero-quantum (ZQ) coherences[8] and the remanent transversal single-quantum coherences (SQ) that are not completely refocused depending on the duration Δ of the spin-echo. The sequence is reminiscent of the 2D adiabatic *z*-filtered J-resolved experiment of Luy et al.[9] Here, the variable delay for the t_1 evolution of the J-resolved dimension is replaced by a fixed constant echo delay, Δ, that is adjusted to select or reject signals with specific

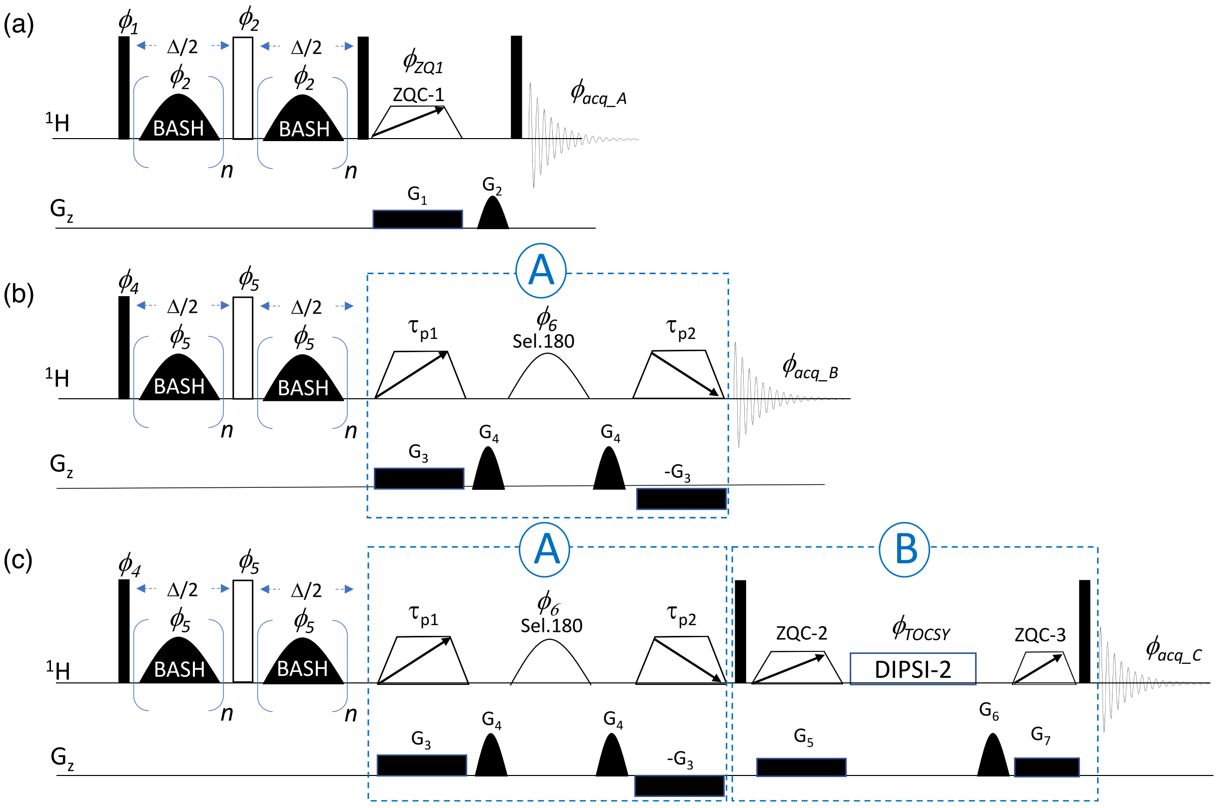

FIGURE 1 Pulse sequence of the experiments proposed in this work. (a) 1D J-filter, (b) 1D ultra-selective J-filter-GEMSTONE experiment, and (c) 1D ultra-selective J-filter-GEMSTONE-TOCSY. The zero and single quantum-coherence filters (ZQC) are indicated for the trapezoidal pulses ZQC-1, ZQC-2, and ZQC-3. It uses adiabatic inversion CHIRP pulse of duration 20, 20, and 15 ms, respectively simultaneous with a gradient.[7] The delay Δ is adjusted depending on the desired value of J to select/suppress. The BASH pulses are optional and consist of a train of r-SNOB shaped pulses with a duration, τ_{BASH}, of 5 ms. They covered a band width (BW) 466 Hz equivalent to 0.6 ppm at 750 MHz. In (b) and (c), the part of the sequence marked as A is the GEMSTONE ultra-selective experiment of Kiraly et al.[5] and the pulses and gradients use similar conditions to those described therein. Pulses t_{p1} and t_{p2} are of type WURST-80 with frequency sweep in opposite directions, with a duration of 60 ms (BW 3750 Hz, 5 ppm at 750 MHz). In (c), the part of the sequence marked as B corresponds to the TOCSY part that uses the DIPSI-2 scheme with the phase of the pulses applied along the *y*-axis. Unless indicated otherwise, the phase of the pulses is applied along the *x*-axis. The phases are $\phi_1 = x, -x$, $\phi_2 = [y]_4,[-y]_4$, $\phi_{\mathrm{ZQ1}} = -y$, $\phi_{\mathrm{acq\_A}} = x,\text{-}x,\text{-}x, x$, $\phi_4 = x, x,\text{-}x,\text{-}x$, $\phi_5 = y$, $\phi_6 = [x]_4,[y]_4,[-x]_4,[-y]_4$, and $\phi_{\mathrm{acq\_B}} = \phi_{\mathrm{acq\_C}} = x, x,\text{-}x,\text{-}x,\text{-}x,\text{-}x, x, x$. The pulse field gradients indicated as filled black Gaussian envelopes are 1 ms in length with an amplitude according to a smoothed rectangle (10% smoothing on either side). The gradients indicated with rectangles are used for spatial phase encoding. They are applied simultaneously with the corresponding RF pulse on ^{1}H and have a rectangular shape. Gradients G_2 and G_6 are used for purging transversal magnetization. Gradients G_4 at both sides of the selective 180-degree pulse are used for the removal of transversal magnetization not affected by the selective pulse. The constant *n* is the number of cycles for the repetition of the BASH pulse with a total duration of Δ/2. The gradient amplitudes expressed in *G*/cm are $G_1 = 2.71$, $G_2 = 3.58$, $G_3 = 0.26$, $G_4 = 7.68$, $G_5 = 2.05$, $G_6 = 15.87$ and $G_7 = 2.71$.

values of J-couplings. After the spin-echo, the ZQ filtering rejects out-of-phase coherences that would introduce unwanted antiphase dispersive components in the spectrum, leaving only the absorptive part. The observed intensity in J-filter for a multiplet is modulated by the matching/mismatching of its coupling constants, typically $^2J_{\mathrm{HH}}$ and $^3J_{\mathrm{HH}}$, with the adjustable echo delay Δ. Previously, the work of Zijl et al. had derived equations for the intensity modulation of the double spin-echo experiment for different types of spin systems.[10] The same equations hold here for the J-filter, except that only the absorptive part should be considered. In the absence of strong coupling, the absorptive component of a signal A of an A_nX_m spin system where the subscripts *n* and *m* refer to the number of equivalent protons is modulated by the J-couplings (J) according to Equation (1).

$$\text{Intensity } \alpha \ \cos^m(\pi J \Delta) \tag{1}$$

Therefore, according to Equation (1), by adjusting the delay Δ, the J-filter experiment can maximize the intensity or eliminate certain multiplets from the spectrum. The J-filter (see Figure 1a) includes, as an option, the possibility of decoupling specific protons to simplify a specific spin system. This is accomplished by a train of band-selective refocusing pulses of relative short duration (approximately ~3 to 5 ms), which are denoted as BASH (BASH: BAnd-Selective-Homodecoupling)[11] in Figure 1. The objective of this train is to deal with cases of overlapping when there are signals with the same or similar couplings in a region of interest of the spectrum (ROI). The BASH train is applied at a chemical shift (δ_{BASH}) where a coupling partner is expected for a given signal in the ROI. Thus, in favorable situations, the simplification of the spin system introduced by BASH, by eliminating the particular $^{n}J_{HH}$ evolution during the Δ period, can lead to differences in the intensity calculated by Equation (1) for ROI signals. An illustrative example of the effect of BASH pulses in the J-filter experiment is given in Figure S1.

The J-filter sequence of Figure 1a can be easily incorporated as a building block in some of the available 1D or nD homonuclear or heteronuclear experiments to solve cases of signal overlap. With this purpose, we combined the J-filter with the ultra-selective GEMSTONE[5] and GEMSTONE-TOCSY[12] experiments, resulting in the J-filter-GEMSTONE and J-filter-GEMSTONE-TOCSY sequences of Figure 1b,c, respectively.

2.4 | Practical optimization of J-filter parameters for the study of complex mixtures

The two adjustable parameters of the J-filter are δ_{BASH}, the chemical shift of a coupling partner of the signal of interest at which the BASH selective pulse is applied, and the other is the evolution delay Δ. For relatively simple compounds, δ_{BASH} can be obtained from NMR databases (e.g., MMCD,[13] HMDB,[14] and BMRB[15]) or empirically predicted from the known chemical structure.[16] Nevertheless, other more complicated situations can occur if the signals to suppress contain additional couplings that are not covered by the cases supported by Equation (1), or if the ROI contains many overlapping signals with large variations of intensity because it may happen that no setting of the J-filter parameters can achieve the required outcome.

For a practical use of the J-filter in cases of ROIs with complex overlapping, the parameter Δ and δ_{BASH} can be optimized experimentally by exploring a number of values with a grid search and then selecting the spectrum that produces the most favorable outcome. Such optimization is preferably made by a pseudo-2D version of the J-filter of Figure 1a that allows to rapidly explore an array of values of the adjustable parameter. Two examples of pseudo-2D J-filter spectra are given in Figure S1.

2.5 | Measurement of 1D J-filter spectrum

J-filter spectra of phytosterols sample were measured with adjusted parameters to isolate the signal **H-29** of β-sitosterol at 0.85 ppm, **H-26** of β-sitosterol at 0.84 ppm, or to isolate the **H-28** signal of campesterol and dihydrobrassicasterol, 0.76 and 0.77 ppm, respectively. The optimization of the J-filter parameters, Δ and δ_{BASH}, was carried out with a pseudo-2D version of the sequence of Figure 1a in which parameter Δ is arrayed to explore a series of values for a given fixed value of δ_{BASH}. Eighteen values of the delay Δ were explored between 10 and 180 ms in steps of 10 ms and with each value providing a 1D J-filter sub-spectrum. A total of six pseudo-2D spectra were repeated to explore values of δ_{BASH} at positions at which coupling partners can be expected between 2.0 and 4.5 ppm. The minimum step size for the exploration of Δ values is twice the duration of the BASH pulse, τ_{BASH}, which must be short enough to nullify the evolution of coupling constants during Δ (Equation 2):

$$\tau_{BASH} \ll \frac{1}{2 \cdot J_{HH}} = \frac{\Delta}{2} \tag{2}$$

where τ_{BASH} is at least four times smaller than Δ/2. Because the duration τ_{BASH} is inversely related to the BW covered by BASH, a compromise must be reached for the duration of τ_{BASH} between satisfying Equation (2) and having a suitable BW. In our case, τ_{BASH} was set to 0.5 ms, which corresponds to a BW of approximately 0.6 ppm at 750 MHz. The step size to explore values of δ_{BASH} was also set in concordance with the BW covered by the BASH pulses. The values of δ_{BASH} explored were 4.5, 4.0, 3.5, 3.0, 2.5, and 2.0 ppm. Other experimental parameters are described in the caption of Figure 1. After processing of the six pseudo-2D, the 1D trace with the cleaner selection of the signal of interest by the J-filter was found. The best parameters found for the selection of signal **H-29** of β-sitosterol in the mixture of phytosterols were Δ of 130 ms and δ_{BASH} of 3.5 ppm, for signal **H-26** of β-sitosterol were Δ of 20 ms and δ_{BASH} of 4.5 ppm and for signal **H-28** of campesterol and dihydrobrassicasterol were Δ of 110 ms and δ_{BASH} of 3.5 ppm. Each pseudo-2D spectrum was acquired with eight scans per Δ value in approximately 8 min.

2.6 | 1D J-filter-GEMSTONE and 1D J-filter-GEMSTONE-TOCSY spectra

These experiments were measured for the sample of phytosterols obtained from a nutraceutical drink with the sequences of Figure 1b,c, respectively. These two types of

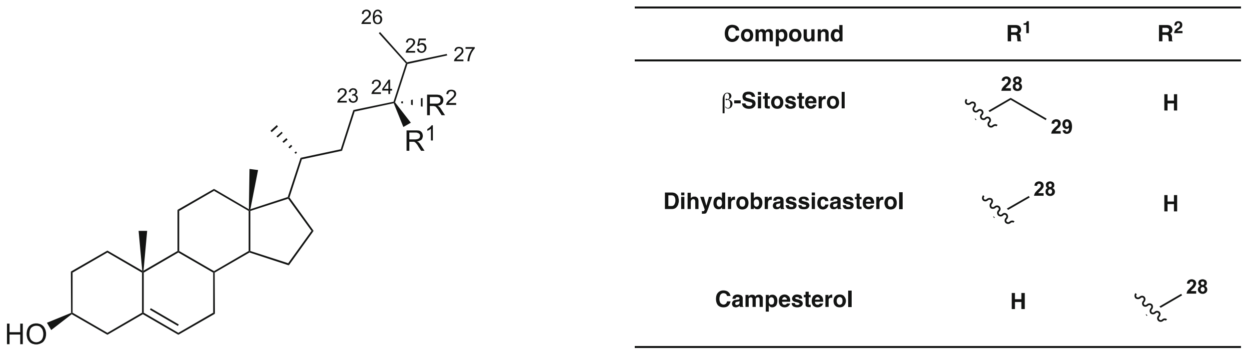

FIGURE 2 Chemical structures of saturated side chain phytosterols, β-sitosterol, campesterol, and dihydrobrassicasterol, showing the numbering scheme used to refer to certain protons in the text.

spectra were calibrated for the selection of signal **H-29** of β-sitosterol at 0.85 ppm using the pre-optimized parameters of Δ and δ_{BASH} obtained above. Analogously, the two types of spectra were also measured with the selection of signal **H-26** of β-sitosterol at 0.84 ppm and **H-28** of campesterol and dihydrobrassicasterol at 0.76–0.77 ppm. Other parameters are described in the caption of Figure 1. The TOCSY mixing time was 80 ms, and the number of scans to measure each spectrum was 128 with a measurement time of approximately 8 min.

3 | RESULTS AND DISCUSSION

Phytosterols are a subtype of sterols naturally present in plants and marine materials, the chemical structures of which are similar to cholesterol, differing mostly in the substituents and the degree of unsaturation of their side chains.[17] Plant sterols are mainly obtained from vegetable oils and are widely used in the food and cosmetic industries. These vegetable oils present large differences in composition and total sterol content, with saturated side chain phytosterols being the major sterol components[18,19] (Figure 2). The determination of the sterol profile of oils and other products allows the assessment of their quality, safety, authenticity, and the detection of fraud by adulteration with lower value oils and, conversely, to establish the origin of the phytosterols.[20] Therefore, it is desirable to develop a discriminating and reliable method for the analysis of phytosterols in vegetable oils and other food products that permits their rapid identification with minimal sample preparation.

The J-filter experiments of Figure 1 were applied to a sterol mixture isolated from a nutraceutical drink. This type of sample may contain β-sitosterol, campesterol, and/or dihydrobrassicasterol as main components.[21] These sterols differ structurally only at position **28** (Figure 2): A methyl group is present in campesterol and dihydrobrassicasterol, whereas in β-sitosterol, a CH_2 group attached to a methyl group is present at that position. In principle, it should be straightforward to discern the presence of the above sterols in a 1D proton spectrum, because a triplet signal is expected for the methyl **H-29** of β-sitosterol and doublets for the methyls at **H-28** of campesterol and dihydrobrassicasterol. However, the 1D proton spectrum shows a complex mixture of sterols, and there is signal crowding in the relevant region of the methyl groups from 0.7 to 0.9 ppm (see Figure S2 and Table S1).[22–24]

The 1D J-filter experiments of Figure 3 allowed us to discern and confidently assign the methyl signal **H-29** of β-sitosterol. The J-filter-GEMSTONE of Figure 3b was calibrated by the pseudo-2D spectrum of Figure S3 to select the triplet of **H-29** and simultaneously null the signals of other overlapping peaks with the same magnitude of their scalar couplings. The J-filter-GEMSTONE-TOCSY of Figure 3c confirmed its assignment by showing the expected TOCSY correlations with **H-24** and **H-28**. The large intensity of the signal **H-29** indicates that β-sitosterol is the major sterol in this mixture. It is illustrative of the effect of the J-filter to compare the spectra in Figure 3b,c with their respective ultra-selective GEMSTONE and GEMSTONE-TOCSY spectra in Figure S4 measured under analog conditions. The number of artifact responses caused by signals that overlap with triplet **H-29** is drastically reduced in the experiments that incorporate the J-filter. Apart from this difference of intensity, they are easily identifiable in the case of the spectra with J-filter because the calibration made them appear with opposite phase. The presence of these small artifacts is due to additional couplings for the suppressed signals and also due to the calibration of the J-filter, which has to be optimized in time steps of 10 ms due to the duration of the BASH pulses employed. Despite the limitation, one of the advantages of the J-filter is that once calibrated for a given application, it does not require extremely precise calibration of the parameters and in particular for the selectivity for the signal of interest. This makes it suitable for the high-throughput study of

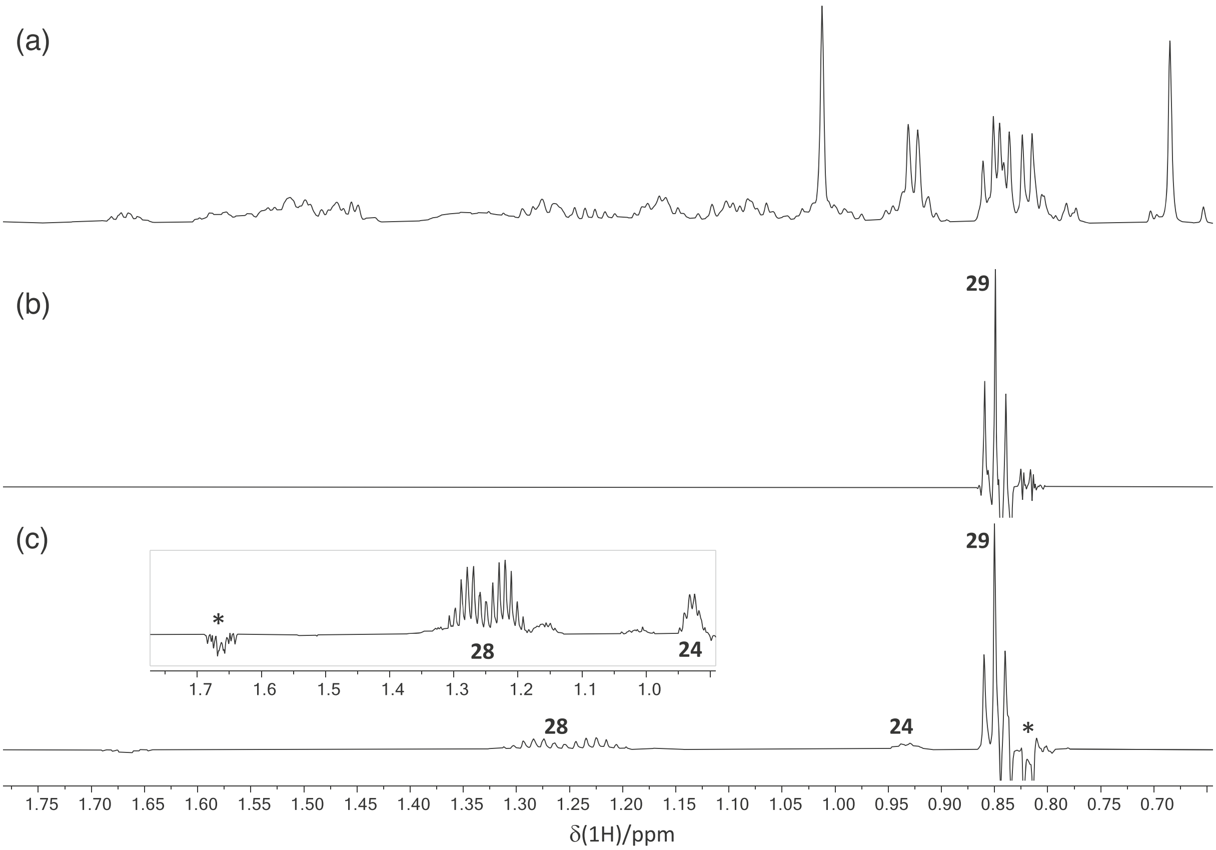

FIGURE 3 J-filter experiments applied to the selection of the triplet of methyl **H-29** of β-sitosterol at δ 0.85 ppm in the sample of sterols isolated from a nutraceutical drink. (a) 1D ^{1}H spectrum, (b) 1D J-filter-GEMSTONE, and (c) 1D J-filter-GEMSTONE-TOCSY. The delay Δ of the J-filter was set to 130 ms. BASH pulses were applied centered at δ_{BASH} of 3.5 ppm to filter couplings that overlap with signal **H-29**. The center of the spectrum and the ultra-selective pulse of the GEMSTONE part were set at the frequency of **H-29**. The asterisks denote small artefact signals with opposite phase to **H-29** generated by other signals that overlap with this peak and are strongly attenuated by the experiment.

complex mixtures such as food or biofluids in automated mode, where subtle variations in chemical shifts can occur (e.g., variations of pH and salinity).

A further confirmation of the presence of β-sitosterol in this sample was obtained by the selection of the doublet of the methyl protons **H-26** and the detection of the expected TOCSY correlations with protons **H-27** and **H-25** using the J-filter experiments explained in the previous case (see Figure S5).

On the other hand, two doublets at 0.76 and 0.77 ppm were detected in the 1D proton spectrum of the sample (see Figure S6). The chemical shift and the low intensity of these signals are consistent with the presence of campesterol and dihydrobrassicasterol as minor saturated chain sterols in the mixture. The doublet at 0.76 ppm is tentatively assigned to the methyl group **H-28** of campesterol, and the doublet at 0.77 ppm to the methyl **H-28** of dihydrobrassicasterol.[25,26] The 1D J-filter experiments of Figure 4 allowed us to assign the methyl signal **H-28** of campesterol and dihydrobrassicasterol. The J-filter-GEMSTONE of Figure 4b was calibrated to select the doublets of **H-28**, and the J-filter-GEMSTONE-TOCSY of Figure 4c confirmed its assignment by showing the TOCSY correlations with **H-23** and **H-24**.

Therefore, in this work, a J-filter is proposed that provides a convenient filtering/editing of signals of the ^{1}H spectrum according to scalar coupling values and the chemical shifts of their coupling partners.

The parameters controlling the selectivity of the J-filter are simple to adjust and only require some a priori knowledge of the chemical structure or substructure whose signals are to be filtered/edited. The selectivity of the J-filter is controlled by an adjustable delay (Δ) during which transversal relaxation operates; therefore, it is more adequate to select spin systems with sufficiently long transversal relaxation times and/or to the selection of signals with large H-H couplings for which the required delay is shorter. These considerations indicate that the J-filter is more appropriate to study molecules of moderate size or their mixtures.

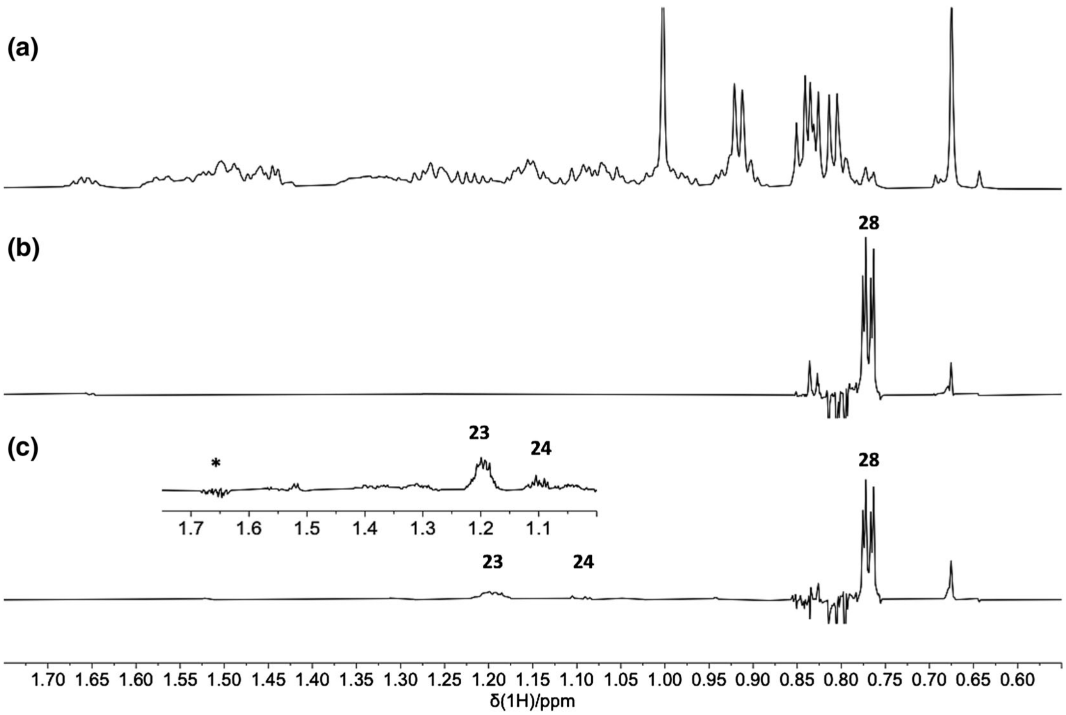

FIGURE 4 J-filter experiments applied to the selection of the doublets of the methyl groups **H-28** of dihydrobrassicasterol and campesterol at δ 0.77 and 0.76 ppm respectively in the sample of sterols isolated from a nutraceutical drink. (a) 1D ^{1}H spectrum, (b) 1D J-filter-GEMSTONE, and (c) 1D J-filter-GEMSTONE-TOCSY. The delay Δ of the J-filter was set to 110 ms. BASH pulses were applied centered at δ_{BASH} of 3.5 ppm to filter couplings that overlaps with signals **H-28**. The center of the spectrum and the ultra-selective pulse of the GEMSTONE part were set at the frequency of **H-28**. The asterisks denote small artefact signals with opposite phase to **H-28** generated by other signals that overlap with this peak and are strongly attenuated by the experiment.

The J-filter is highly versatile and can easily be incorporated as a building block into other pulse sequences. A variety of these hybrid experiments can be proposed in 1D or nD to solve cases of signal overlapping exploiting several properties of the NMR signals such as relaxation, diffusivity, chemical shifts, and isotope filters. In this work, we found a convenient synergy in the combination of the J-filter with the ultra-selective GEMSTONE experiments.[5,12] The J-filter-GEMSTONE hybrid sequences were very efficient for the isolation and assignment of specific signals in crowded ^{1}H spectral regions without the need to recur to experiments with additional dimensions that would be impractical for sensitivity compromised samples, as it was demonstrated for the sample of phytosterols obtained from a nutraceutical drink.

Future applications of the J-filter experiments could extend to the study of other types of samples with a complex ^{1}H spectrum such as biofluids (urine, serum) or food. These samples contain a mixture of metabolites, and the proposed experiments can aid to discern and quantify specific ones in a time-efficient manner.

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DATA AVAILABILITY STATEMENT

The pulse sequences and the experimental data are available upon request.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/mrc.5396.

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SUPPORTING INFORMATION

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