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Optimization of homonuclear 2D NMR for fast quantitative analysis: Application to tropine–nortropine mixtures

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Abstract

Quantitative analysis by ^{1}H NMR is often hampered by heavily overlapping signals that may occur for complex mixtures, especially those containing similar compounds. Bidimensional homonuclear NMR spectroscopy can overcome this difficulty. A thorough review of acquisition and post-processing parameters was carried out to obtain accurate and precise, quantitative 2D *J*-resolved and DQF-COSY spectra in a much reduced time, thus limiting the spectrometer instabilities in the course of time. The number of t_1 increments was reduced as much as possible, and standard deviation was improved by optimization of spectral width, number of transients, phase cycling and apodization function. Localized polynomial baseline corrections were applied to the relevant chemical shift areas. Our method was applied to tropine–nortropine mixtures. Quantitative *J*-resolved spectra were obtained in less than 3 min and quantitative DQF-COSY spectra in 12 min, with an accuracy of 3% for *J*-spectroscopy and 2% for DQF-COSY, and a standard deviation smaller than 1%.

Keywords: 2D NMR; Quantitative analysis; Tropine; Nortropine

1. Introduction

^{1}H NMR spectroscopy is a powerful tool for quantitative analysis in various domains, such as drug analysis [1], natural product authentication or metabolome analysis [2]. However, precise quantification is often made difficult by the presence of homonuclear scalar couplings and large overlaps between peaks.

Various methods have been proposed to overcome these difficulties. Many of them rely on 2D *J*-resolved spectroscopy, first described by Aue et al. [3]. This method was improved by Bax et al. [4] to obtain a better resolution, by developing the 'constant-time experiment' where the signal was detected at a constant time after the 90° pulse. Unfortunately, the areas of the peaks were then no longer proportional to the equilibrium magnetization. Woodley and Freeman [5] developed a data-processing algorithm based on pattern recognition, to convert 2D *J*-spectra into decoupled ^{1}H spectra. However, this technique fails in the presence of important overlaps. Nuzillard [6] showed that *J*-resolved spectra could be improved by extrapolating the 2D signal towards the negative t_1 values, using linear prediction. This makes possible the suppression of all dispersion components in the spectrum. Mutzenhardt et al. [7] developed a close procedure to obtain pure absorption 2D *J*-spectra by means of linear prediction for the negative t_2 values. This method was supposed to lead to quantitative fully *J*-decoupled homonuclear spectra, however it seemed rather complicated to implement in routine procedures. Two-dimensional correlated spectroscopy [8] with double quantum filtering [9] (DQF-COSY) was also used as a quantitative tool for metabolite quantitation [10], however to our knowledge, this method was never studied in terms of accuracy and standard deviation.

This study aims at showing that 2D NMR can be used to perform precise and accurate quantitative analysis of complex mixtures. For this, no new acquisition method is required, but existing basic methods were carefully optimized to make them suitable for quantitative analysis with maximum accuracy and precision. In this paper, a thorough review of acquisition and post-processing parameters was carried out for *J*-resolved spectroscopy and DQF-COSY. Both techniques were studied in terms of accuracy and standard deviation by plotting calibration curves

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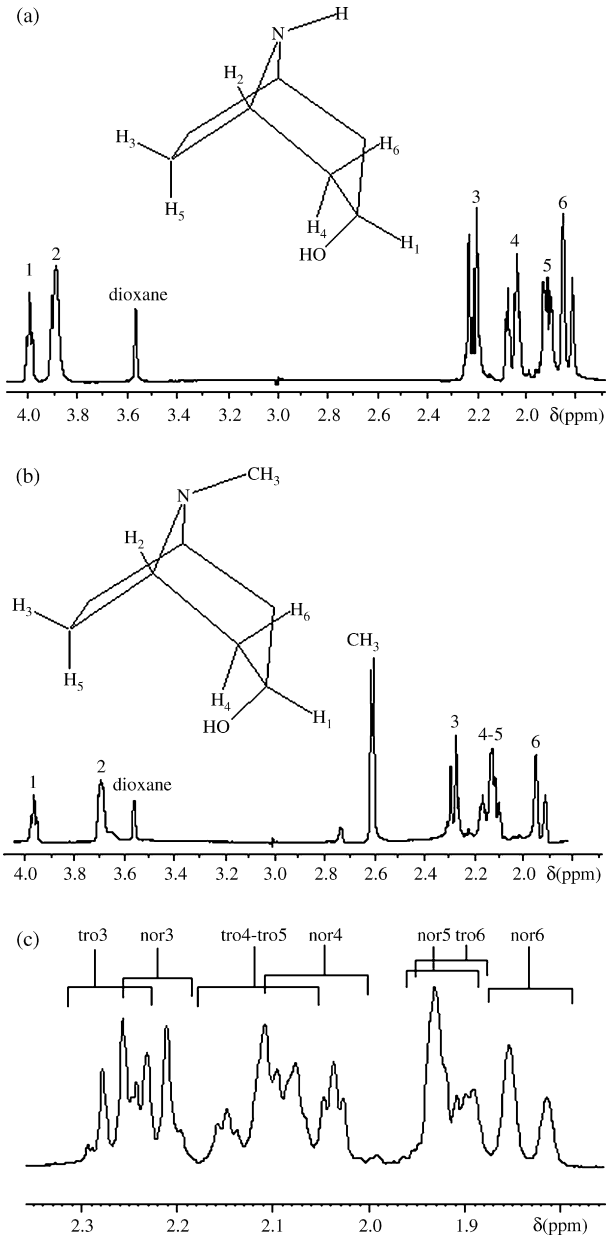

Fig. 1. Nortropine (a) and tropine (b) molecules and corresponding 400 MHz ^{1}H NMR spectra, and spectra of an equimolar mixture of tropine and nortropine (c). ^{1}H sites are numbered in decreasing chemical shift. Spectra were recorded at 298 K with two transients, applying water signal presaturation.

with repeated experiments. Moreover, particular attention was paid to decreasing the experiment duration, which highly contributes towards reducing the standard deviation.

These methods were applied to mixtures of tropine and nortropine (Fig. 1a and b), which constitute a typical example where 1D NMR is inadequate for quantitative analysis. These tropane alkaloids are members of a group of compounds that have important pharmaceutical activity [11]. Better means of determining their metabolism helps improve the development of therapeutic drugs based on these compounds. They are usually found in complex aqueous media such as biological fluids or fermentation supernatants, in which the compounds to be studied are mixed with many others. Consequently, their respective amounts are to be measured without any preliminary extraction. Contrary to other common techniques such as gas chromatography, NMR makes this measurement possible.

2. Experimental

Tropine, purchased from Acros organics and nortropine, purchased from Boehringer Ingelheim, were dissolved in water to obtain 50 mmol L^{-1} solutions. Six mixtures of 1 mL were prepared from these solutions, with the following nortropine/tropine concentration ratios: 0.25; 0.67; 1.00; 1.50; 2.33; 3.00. For each sample, 70 μL of phosphate buffer solution (pH 2; 2 M) were added to avoid peak shifting due to pH variations. Fifty microliters of dioxane-d_8 were added as a lock compound. After homogenization, each sample was filtered and analyzed in a 5 mm tube.

All ^{1}H NMR spectra were recorded at 298 K, on a Bruker Avance 400 DPX spectrometer, at a frequency of 400.13 MHz with a 5 mm probe and $p_w(90°) = 12.6$ μs. ^{1}H sites were numbered in decreasing chemical shift. Spectra were analyzed using the Bruker program X-WinNMR 2.6, including 2D integration tool for peak volume determination. For all spectra, water signal suppression was performed by applying an attenuated RF field of 0.8 mW during recovery delays.

Longitudinal relaxation times (T_1) were determined for the equimolar mixture by using an inversion-recovery sequence with water signal presaturation. There, the baseline was corrected automatically and the areas of the peaks were analyzed directly within the Bruker software. For both molecules, H-3 T_1 were equal to 1 s. Consequently, for each 1D and 2D spectra, a $5T_1 = 5$ s repetition time was applied to obtain accurate quantitative data.

1D spectra were acquired with two transients preceded by two dummy scans. Free induction decays (FIDs) were accumulated in 4 K channels and zero-filled to 32 K, with a spectral width of 2395 Hz and an acquisition time of 1 s. An exponential apodization function of 0.3 Hz was applied to the FID prior to Fourier transform.

For *J*-resolved spectra, FIDs in F_2 dimension were accumulated in 2 K channels, with a spectral width of 1008 Hz and an acquisition time of 1 s. An exponential apodization function of 0.3 Hz was applied in each dimension before Fourier transform. Data matrix were zero-filled to 4 K in F_2 dimension and 256 in F_1 dimension. A polynomial baseline correction ($n = 2$) was applied in F_2 dimension between 2.18 and 2.33 ppm. All other parameters were optimized as described in the main section.

For DQF-COSY spectra, FIDs in F_2 dimension were accumulated in 0.5 K channels, with a spectral width of 504 Hz and an acquisition time of 1 s. A $\pi/24$ shifted square sine-bell function and a $\pi/8$ shifted sine-bell function were applied in F_2 and F_1 dimensions, respectively. Data matrix were zero-filled to 512 and 64 points in F_2 and F_1 dimensions, respectively. Polynomial baseline corrections were applied in F_2 ($n = 3$, 2.18–2.33 ppm) and F_1 ($n = 1$, 1.84–2.17 ppm) dimensions. All other parameters are detailed in the main section.

All integration results are the average of five experiments.

3. Results and discussion

Tropine and nortropine (Fig. 1a and b) are two similar molecules consisting of an alicyclic nitrogen-containing alcohol structure. At the temperature and pH considered, their spectra are composed of six different ^{1}H groups (plus a CH_3 group for tropine), as both molecules present a plane of symmetry. Note that the low resolution observed in Fig. 1 is a consequence of the presence of water, which leads to line-broadening. Tropine and nortropine have very similar spectra due to their close structures. Consequently, when both molecules are present in the same sample, large overlaps between peaks occur on 1D ^{1}H spectra (Fig. 1c), which makes impossible the determination of their relative amounts. Therefore, 2D NMR should be useful to obtain the relative concentrations of these compounds in a mixture, as it allows a better separation between peaks.

A major drawback of 2D NMR is that it is often time-consuming. Numerous t_1 increments and the collection of several independent transients are required to obtain a spectrum with a good resolution, which leads to very long acquisition times, up to several hours when following the standard protocol given by the manufacturer. The reduction of the experiment duration is very important, for timetable constraints of course, but also to limit the spectrometer instabilities in the course of time, which strongly influence accuracy and standard deviation. It was then decided to optimize successively acquisition and post-processing parameters so as to obtain accurate and repeatable results in a reduced time. In the following study, all the optimization procedure will be done on an equimolar mixture of tropine and nortropine as described in the experimental section. Even if this case is not very likely, a calibration will allow an extension of the method to a range of concentration ratios, as discussed further in the text.

First, the classical *J*-resolved experiment [3] was evaluated. The corresponding spectrum is presented in Fig. 2. The choice of the peaks used for quantitative analysis was made as follow: H-1 and H-2 peaks were not considered for quantitative analysis because their volume was strongly influenced by water signal presaturation. Nortropine and tropine H-4–H-6 peaks were not separated enough on the 2D spectrum and their signal-to-noise ratio was lower than for other peaks. Tropine CH_3 peak would have been interesting for quantitative analysis, as its doublet (due to the nitrogen protonation at a low pH) was well separated from other peaks, and because of its high signal-to-noise ratio. However, this study aimed at developing a method which may be used for any complex mixture, where such a well-separated peak often lacks. Consequently, for both compounds, H-3 groups were quantified, as they give rise to four well-separated peaks, contrary to the corresponding peaks on the 1D spectrum. Four distinct rectangular integration zones were then defined as described in Fig. 2. *J*-resolved spectra are usually presented in the absolute-value mode, however partial cancellation between peaks may occur. A preliminary study on a model compound showed that phase-sensitive display [12] was then more suitable for quantitative analysis. Phase correction was performed after Fourier transform in F_2 dimension. Moreover, the common 45° tilt procedure was not applied here, as it leads to

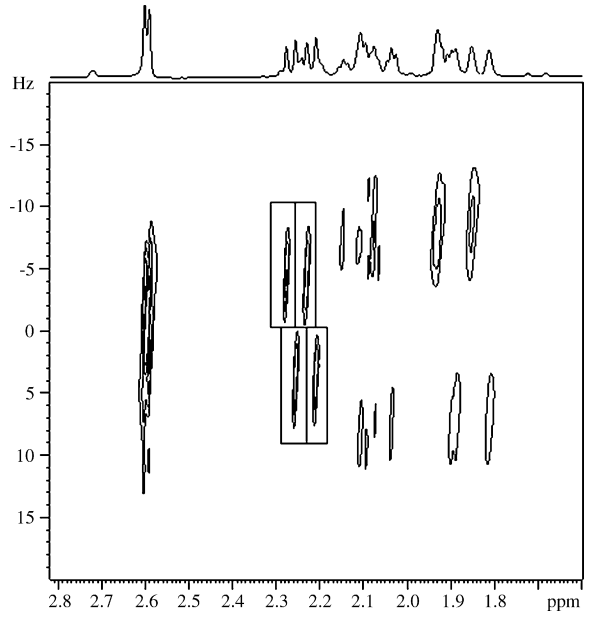

Fig. 2. Four hundred megahertz 2D *J*-resolved spectrum of an equimolar mixture of tropine and nortropine, recorded after parameter optimization in 2.7 min at 298 K, with water signal presaturation. Framed areas correspond to integration zones.

partial overlap between 2D peaks, which makes the integration procedure more difficult.

Table 1 summarizes the influence of acquisition and post-processing parameters on the nortropine/tropine peaks volume ratio, which should theoretically be equal to 1. First, the influence of the spectral width (SW) in the F_1 dimension was studied. A 40 Hz value was sufficient to observe all peaks, however it leaded to bad standard deviation and accuracy values, whereas the standard deviation was divided by 3 when using a SW of 80 Hz. Actually, the SW is the reciprocal of the dwell time (digital sampling interval), thus increasing the SW leads to a shorter dwell time, which allows a better digitalization of the FID. It can be noted that increasing SW above 80 Hz did not improve standard deviation any more.

The number of points on the pseudo-FID in the F_1 dimension (TD_1) is of the utmost importance for the total duration of the experiment. It should be as short as possible, as it determines the number of increments in the F_1 dimension. To study the effect of decreasing TD_1, a 128 data points FID was cut off at various lengths to simulate different TD_1 values. Fig. 3 shows

Table 1
Influence of acquisition and processing parameters on integration results for a 2D *J*-resolved spectrum of tropine and nortropine equimolar mixture

Spectral width (F_1)	40 Hz	80 Hz	80 Hz	80 Hz	80 Hz
Data points (F_1)	128	128	12	12	12
No. of transients	2	2	2	8	2
Phase cycling	2 steps	2 steps	2 steps	8 steps	2 steps
Baseline correction	No	No	No	No	Yes
Peak volume ratio	1.25	1.21	1.19	1.18	1.03
S.D.	15.2%	5.8%	2.2%	3.2%	0.7%

Measurements were performed at 298 K and 400 MHz, with water signal suppression. Each peak volume ratio is the average of five experiments.

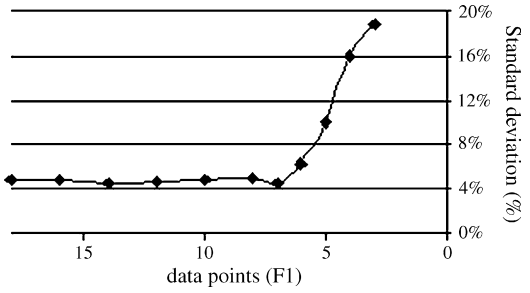

Fig. 3. Influence of the number of increments in F_1 dimension (TD_1) on the standard deviation, for five *J*-resolved spectra recorded with 128 F_1 data points.

the effect on standard deviation, for the nortropine/tropine peak volume ratio. One observes that $TD_1 = 7$ is sufficient to keep a good standard deviation value, as it remains constant at about 5 % when TD_1 is higher than 7. A TD_1 value of 12 was finally chosen to keep a safety margin. New experiments performed with this value enabled us to decrease the standard deviation to 2.2 % (Table 1). In this way, the experiment duration was divided by 10, thus minimizing the spectrometer instabilities in time. To avoid truncation of the FID which would occur with a short TD_1 value, the apodization function was optimized together with TD_1 value. For a weakly coupled IS spin system, the FID envelope in both dimensions after a spin echo possesses a cosinusoidal modulation in *J*. As a consequence, the FID in F_1 dimension has a maximum value for $t_1 = 0$, thus the appropriate apodization function should start from a maximum value at $t_1 = 0$, and decrease to zero for the last points of the FID. For this reason, an exponential window function was chosen, which emphasizes the resonances due to the sample in the earlier part of the FID.

The experiment duration also depends on the number of transients, which is related to phase cycling. The usual phase cycling for *J*-resolved spectroscopy consists of 16 steps, so at least 16 transients are required in this case. Our results showed that a two-step phase cycling with two transients was sufficient for quantitative analysis. It was chosen to compensate for the 180° pulse imperfections. The first pulse and the receiver phases were kept constant, whereas the second pulse phase was alternated. It can be seen in Table 1 that further phase cycling amelioration does not improve standard deviation, as the spectrometer instabilities increase with the experiment duration. Moreover, the imperfection compensation was improved by using a 180° composite pulse formed by three rectangular pulses: $(75^\circ)_0$ $(285^\circ)_{110}$ $(75^\circ)_0$, as described by Simbrunner and Zieger [13].

Finally, it was shown (Table 1) that it was necessary to apply a polynomial baseline correction in both dimensions, as described in the experimental section, to obtain a good accuracy. To be efficient, this correction must be restricted to the relevant chemical shift area.

The use of a gradient-selected experiment to optimize the *J*-resolved experiment for quantitative analysis was also evaluated. However, this did not improve our results, probably because phase cycling and number of transients were already reduced as much as possible.

This optimization enabled us to obtain accurate quantitative data, with a standard deviation better than 1%. The experiment duration was reduced to 2.7 min, which is very short for a conventional 2D experiment. Our method was applied to six different mixtures of tropine and nortropine to obtain a calibration curve between nortropine/tropine peak ratios and relative concentrations. The results are shown in Table 2, column a. The correlation coefficient shows that our method is reliable for accurate quantitative analysis, as it presents a good linearity for nortropine/tropine ratios lower than 3. One could object that the results could be affected by the 'phase-twist' shape which characterizes peaks in a 2D *J*-resolved spectrum and can increase overlap between peaks. However, this problem is bypassed by the calibration curve. Note that it could explain that the slope is not exactly 1.000.

The method was tested for higher ratio values, however the adequacy between experimental and expected values is not so good, since one of the peaks is close to noise level. For example, accuracy is approximately 10% when the concentration ratio reaches a value of 4. However, the method, even if less precise, may still be used to obtain an estimation of relative amounts of nortropine and tropine.

In order to check the robustness of our method, it was applied to tropine CH_3 peak. Indeed, this peak presents coupling constants and relaxation properties different from those of the H-3 proton. Baseline correction area was extended to take it into account. A calibration was performed by plotting the ratio between nortropine H-3 peak and tropine CH_3 peak as a function of the concentration ratio, for the same range as described above. The results are shown in Table 2(column b). The correlation coefficient is almost as good as the one obtained when using similar peaks, which indicates that the

Table 2
Calibration curve parameters obtained when plotting the dependence of nortropine/tropine cross-peak ratio on the concentration ratio, for optimized *J*-resolved experiments (a and b) and DQF-COSY experiments (c) performed at 298 K and 400 MHz

	J-resolved spectroscopy		DQF-COSY (c)
	(a)	(b)	
Cross-peak intensity ratio	H-3 (nortropine)/H-3 (tropine)	H-3 (nortropine)/CH_3 (tropine)	H-3 (nortropine)/H-3 (tropine)
Slope	0.980	1.017	1.025
y-Intercept	−0.030	−0.022	0.078
r^2	0.999	0.991	0.995

Each calibration curve was obtained using six different mixtures with concentration ratios ranging from 0.25 to 3.00. Every point in the curve is the average of five experiments.

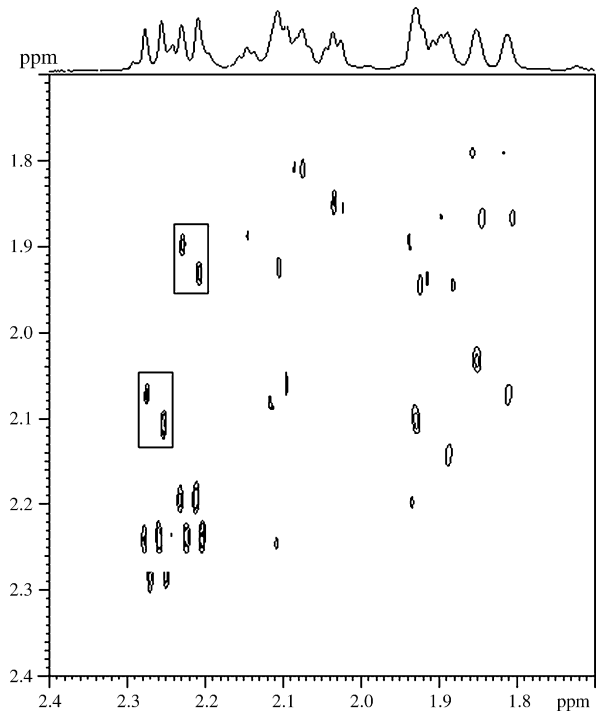

Fig. 4. 400 MHz 2D DQF-COSY spectrum of an equimolar mixture of tropine and nortropine, recorded after parameter optimization in 12 min at 298 K, with water signal presaturation. Framed areas correspond to integration zones. Only the positive components are plotted.

method is still reliable when comparing peaks with different properties. However, such a well-separated peak may lack in complex mixtures and could increase the experiment duration due to higher longitudinal relaxation times.

A major drawback of the *J*-resolved experiment is that it may become less precise or even unusable when overlap between peaks is too large. Thus, it would be interesting to develop a method allowing a better separation between peaks. The standard COSY experiment is inappropriate for quantitative analysis, because of partial cancellations due to the absolute-value mode. Therefore, it was chosen to optimize the DQF-COSY experiment [9] which is more suitable for quantitative analysis. This technique involves a third 90° pulse immediately following the second 90° pulse of a conventional COSY experiment: $90°–t_1–90°–\tau–90°–\mathrm{Acq}(t_2)$, where τ is a very short delay (3 μs).

Phase-sensitive display was obtained using time-proportional phase incrementation method [14]. The corresponding spectrum is shown in Fig. 4. The separation between nortropine and tropine cross-peaks is far better than for the *J*-resolved spectrum. In order to compare the efficiency of both methods, H-3 cross-peaks were chosen to quantify relative amounts of nortropine and tropine. Two integration regions were defined as described in Fig. 4.

Baseline polynomial correction was restricted to the relevant area containing H-3 cross-peaks. Spectral width in both dimensions was set to 1.26 ppm. With a shorter value, aliasing would occur on the spectrum, whereas using a bigger SW would lead to baseline distorsion due to water signal presaturation.

For the DQF-COSY cross-peaks, the FID envelope in both dimensions possesses a sinusoidal modulation in *J*. In F_1 dimension, the signal reaches a maximum value for $t_1 = 1/(2J)$. Contrary to *J*-resolved spectroscopy, relevant information is not located at the beginning of the FID. As a consequence, the number of points in F_1 dimension, TD_1, could not be reduced as much as for *J*-resolved spectroscopy. A TD_1 value of 64 increments was found the minimum value to preserve essential information. A shifted sine-bell window function was applied to emphasize this information and to avoid truncation of the FID.

As for *J*-resolved spectroscopy, the experiment duration was reduced as much as possible by using a two-step phase cycle, consisting of an alternation of the last pulse and receiver phases, whereas other phases were kept constant. Increasing the phase cycling or the number of transients did not improve accuracy or stability in time.

This optimization enabled us to obtain accuracy better than 2% with a 0.4% standard deviation, for the equimolar mixture of tropine and nortropine. The experiment lasted for 12 min, which is still a reasonable value for a 2D experiment. The use of symmetrization, which is quite a common procedure, increased the difference between measured and true value to 13%, with a 5.25% standard deviation, so it was not applied for quantitative measurements.

In order to compare this method to the precedent one, it was applied to 6 different mixtures to obtain a calibration curve. The results are shown in Table 2(column c). As for *J*-resolved spectroscopy, a very good linearity was obtained, which indicates that DQF-COSY is suitable for accurate quantitative analysis. For nortropine/tropine ratios higher than 3, a slight linearity decrease was observed, however the method would still give a good estimation of relative amounts, with accuracy better than 10%.

In the case of DQF-COSY, multiplet components are in antiphase, which can lead to partial cancellations when a negative component of a multiplet overlaps with a positive component of another one (which is not the case here). However, the calibration curve would help to circumvent this difficulty.

For pharmaceutical purposes, it is interesting to study the sensitivity of our optimized methods. The signal-to-noise ratios measured in both cases are of the same order of magnitude (260 for *J*-resolved spectroscopy and 310 for DQF-COSY, for the equimolar mixture). According to the ICH guidance [15], a signal-to-noise ratio of 3 is generally considered acceptable for estimating the limit of detection (LOD), and a value of 10 is typical for the limit of quantification (LOQ). Following these recommendations, the values obtained from both methods are approximately $\mathrm{LOD} = 0.5\ \mathrm{mmol\ L^{-1}}$ and $\mathrm{LOQ} = 1.5\ \mathrm{mmol\ L^{-1}}$. In the mixtures employed to perform the calibration curves, the minimum concentration for tropine and nortropine is $10\ \mathrm{mmol\ L^{-1}}$, which is much higher than the limits calculated above. However, it would be interesting to perform calibration curves with smaller concentrations to check these sensitivity limits.

4. Conclusion

It is demonstrated that *J*-resolved spectroscopy and DQF-COSY are efficient techniques for precise and accurate quantitative analysis of complex mixtures in a short time, when acquisition and post-processing parameters are carefully opti-

mized. Reducing the experiment duration is essential to obtain a low standard deviation. In this study, *J*-resolved quantitative spectra were obtained in less than 3 min. However, it may be inadequate when overlap between peaks is too important, then DQF-COSY should be a more efficient tool to obtain accurate quantitative data.

Both optimized experiments allow a very important time-saving, and may be applied each time precise quantitative analysis is required and cannot be performed on 1D spectra. There is no need for advanced data processing methods such as linear prediction [16], maximum entropy [17] or filter diagonalization method [18]. However, the use of these techniques to improve our quantitative experiments could be considered in later works. Moreover, an optimization of heteronuclear 2D NMR spectra for quantitative analysis is currently under investigation.

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