

# A toast to dynamic NMR spectroscopy: towards a better comprehension of palatable emulsions

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## Introduction

Memories of summer vacations on the Mediterranean would not be complete without recollections of the refreshing anise-flavoured alcoholic beverages served during the hot afternoons. Though varying in name and composition across cultures (Raki in Turkey, Arak in Lebanon, Ouzo in Greece, Sambuca in Italy and Pastis in France), extracts from star anis (*Illicium floridanum*) are a common principal ingredient of the plant extracts used in their production. Besides their pungent anis aroma, another well-known trademark of these drinks is their transition from a clear solution to an opalescent milky-white substance when they are diluted with water. This sudden change in physical appearance will not go unnoticed by a scientist, who may summarily assign it to the precipitation or separation of the hydrophobic organic fragrances due to the addition of water. We know this not to be entirely correct,<sup>1</sup> however, as it should lead to a rather unappetising biphasic mixture in which the essential oils would float on top of a large volume of water and alcohol. The truth behind this apparently simple phenomenon is actually much more complex—and very interesting indeed!

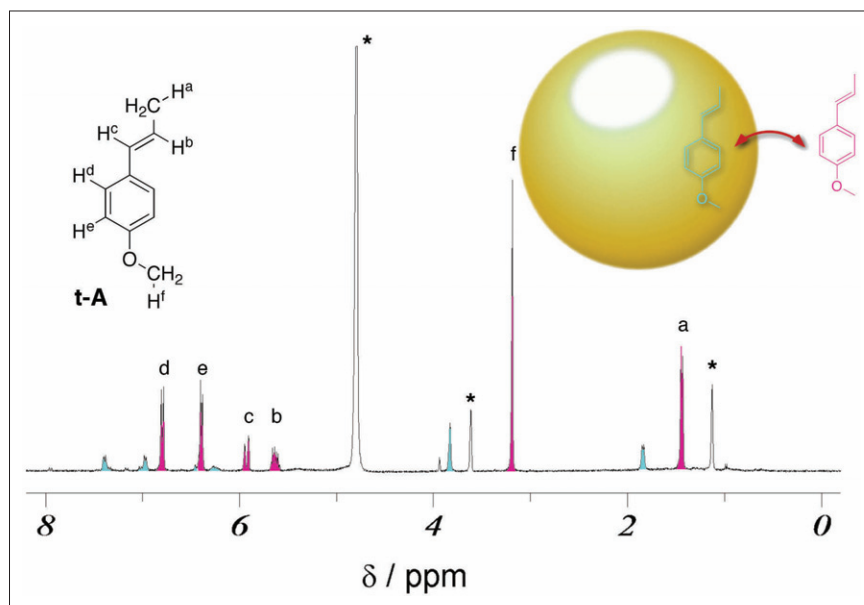
The principal component extracted from star anis is *trans*-anethole (*E*-4-methoxyphenylpropene, *t*-A), which is a naturally-occurring flavouring compound present in numerous essential oils, including fennel, star anis

and aniseed. One of the most intriguing properties of *t*-A, however, is its capability to undergo spontaneous emulsification upon rapid dilution of a concentrated solution with a miscible but non-solubilising solvent. This phenomenon, for which the term “ouzo effect” has been coined by some authors,<sup>2</sup> is responsible for the cloudy aspect of the above-mentioned beverages as the alcohol content of the hydro-alcoholic solution is dropped from above 40% to ca 5% v/v and affects the photochemistry of *t*-A.<sup>3</sup> Self-emulsification phenomena are the subject of continuing investigation due to their importance in the chemical and pharmaceutical industries, and yet more applications, such as the formation of nanoparticles and nanocapsules, are emerging.

In the case of *t*-A, dynamic light scattering (DLS) and small-angle neutron scattering (SANS) techniques have been employed to elucidate the origins of the self-emulsification process.<sup>4</sup> However, to obtain information on the origin of the droplets formed at the initial stages of emulsification, it is necessary to employ techniques that are capable of directly monitoring the exchange between the free (dissolved) and the aggregated fractions of the immiscible component. For this, nuclear magnetic resonance (NMR) spectroscopy is particularly well adapted as it is capable, in favourable cases when the individual components possess different spectral signatures, to directly monitor the exchange between mole-

cules that are in different environments.<sup>5</sup> In many occurrences, however, the NMR spectra of emulsions are not very informative due to inhomogeneous line-broadening of the signals. Surprisingly, this is not the case for emulsions of *t*-A in water, though, as convincingly shown in Figure 1. The sharp and clearly distinguishable signals are attributed to *t*-A located in different environments: a moderately polar aromatic environment within the aggregates, and a hydrophilic aqueous phase. Because the exchange between these two environments is much slower than the frequency difference of the chemical shift of the protons, the resultant spectrum shows a sharp signal for each proton in either of the environments.

The possibility of following spectroscopically the occurrence of *t*-A in the aggregated and aqueous phase simultaneously opens exciting possibilities for further elucidating the emulsification process. For example, the quantification of the component in each phase—a sometimes inextricable problem—becomes trivial and can be performed in real-time, limited only by the rate of data acquisition of the spectrometer. But NMR spectroscopy can do so much more than take simple <sup>1</sup>H spectra! By using specific pulse sequences, for example, it is possible to generate spin populations whose exchange and decay can be followed. In this particular study, the use of exchange spectroscopy (EXSY) and diffusion-



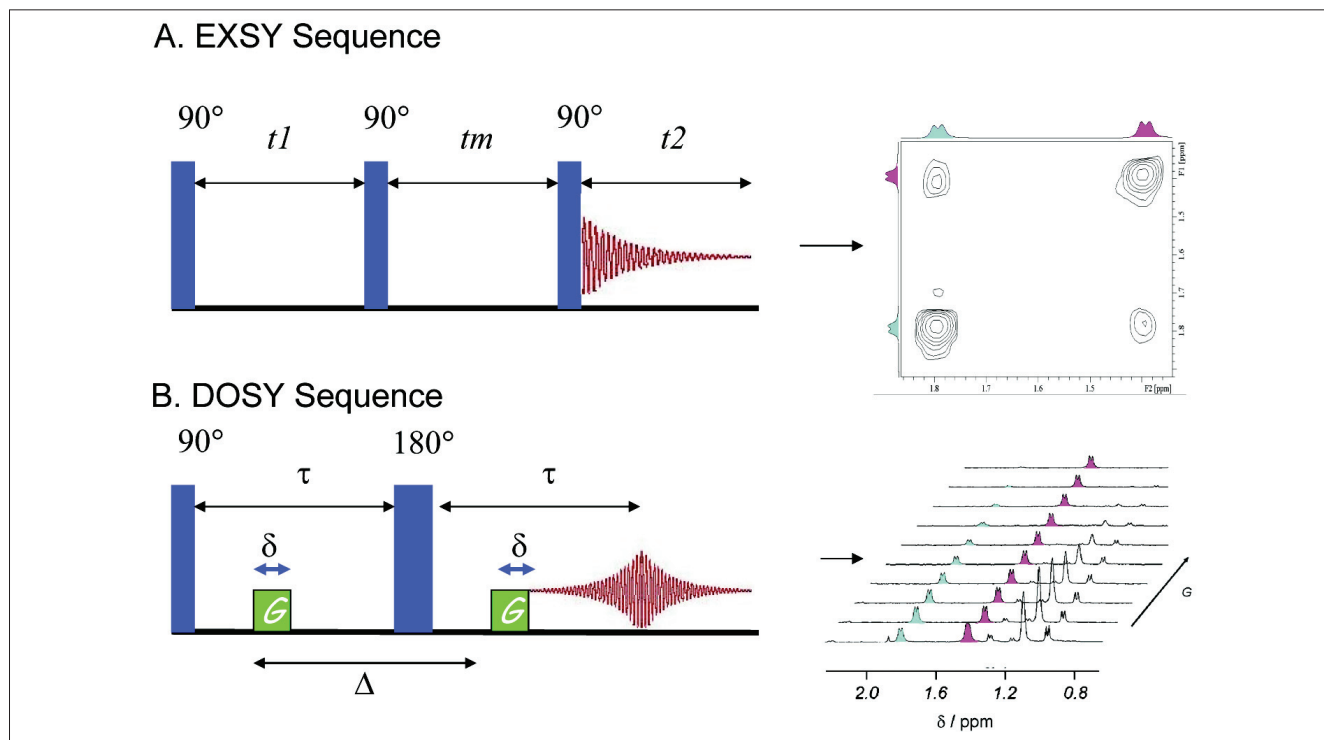
**Figure 1.**  $^1\text{H}$  NMR (400.13 MHz) of a 5 mM *t*-A solution in 5% EtOD/95%  $\text{D}_2\text{O}$  10 min after the mixture preparation. The signals assigned to the free (cyan) and aggregated (magenta) *t*-A are sharp and well separated. Residual solvent peaks are marked by an asterisk (\*).

ordered spectroscopy (DOSY) proved particularly useful to characterise the kinetics of the exchange of *t*-A between each sub-phase, and the growth of the aggregates, over time.

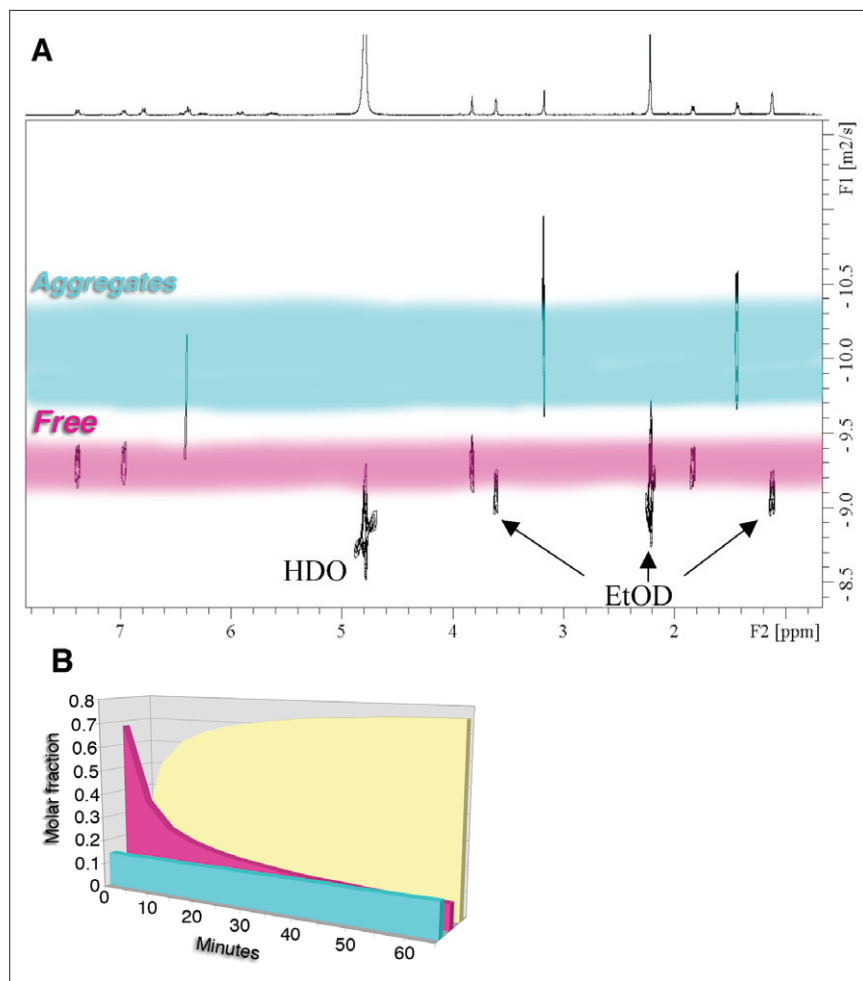
### Methods

The EXSY (Exchange Spectroscopy) NMR experiment is a powerful methodology to investigate exchange processes in molecular systems under a

dynamic equilibrium.<sup>6</sup> The principle is relatively simple: radiofrequency pulses are applied to create a magnetic non-equilibrium between exchanging species in chemical equilibrium. It is the subsequent return to the magnetic equilibrium, which is under the influence of the chemical exchange, that 2D EXSY NMR permits one to observe in a particularly vivid representation. Let us take the case of a system in which two compounds are in exchange: free *t*-A resonating at  $\omega_1$  and aggregated *t*-A resonating at  $\omega_2$ . The NMR sequence (Figure 2A) is composed of a first  $90^\circ$  pulse that creates the transverse spin magnetisation. In the course of the following evolution period  $t_1$ , the transverse magnetisation vectors evolve at their characteristic resonance frequencies (in our case  $\omega_1$  for free *t*-A and  $\omega_2$  for aggregated *t*-A) and become "frequency labelled". The exchange process predominantly takes place after the second  $90^\circ$  pulse, which replaces the magnetisation along the z axis (longitudinal magnetisation) during the mixing time period  $t_m$ . The last  $90^\circ$  pulse is named the "reading pulse", and rotates the magnetisation



**Figure 2.** (A) EXSY sequence and spectrum of a hydroalcoholic solution of *t*-A 5 mM recorded with a 60 ms mixing time showing the interconversion between the free (cyan) and aggregated (magenta) forms of *t*-A. (B) DOSY sequence and spectra at different applied gradients ( $G$ ), showing the decay of the free and aggregated forms.



**Figure 3.** (A) 2-dimensional DOSY spectrum of a hydroalcoholic solution of *t*-A 5 mM, showing two distinct diffusion coefficient values allowing the assignment of the free (cyan) and aggregated (magenta) forms. (B) Evolution of the molar fraction of free (magenta) and aggregated (cyan) *t*-A over time as determined from solution  $^1\text{H}$  NMR. The proportion of *t*-A that is not detected (calculated from the conservation of mass) is shown in yellow and is attributed to *t*-A located in small droplets.

in the  $x$ - $y$  detection plane. The signal is recorded all along the  $t_2$  period during which the magnetisation components precess at their new resonance frequencies owing to the exchange process. The experiment is then repeated for a number of equally spaced values of  $t_1$ , and the result is a data matrix  $s(t_1, t_2)$  for which a double Fourier transform gives a 2D spectrum  $S(\omega_1, \omega_2)$ . The appearance of an off-diagonal peak at frequencies  $\omega_1, \omega_2$  in the 2D spectrum indicates that exchange occurs between free *t*-A and aggregated *t*-A.

The DOSY (Diffusion Ordered Spectroscopy) NMR experiment is a method that gives rise to the measure-

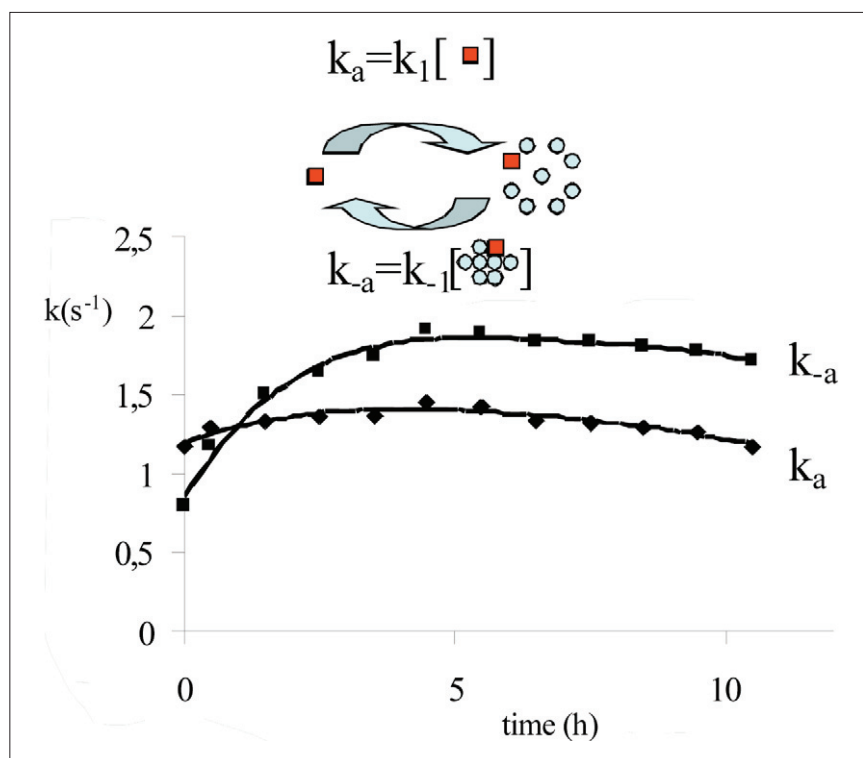
ment of a translational diffusion coefficient ( $D$ ), and thus allows differentiation of species based on their diffusion in solution. The NMR pulse sequence, that was proposed by Stejskal and Tanner in 1965,<sup>7</sup> is based on a spin echo sequence in which two magnetic gradient pulses  $G$ , of  $\delta$  duration and separated by a delay  $\Delta$ , are inserted during the echo periods  $\tau$  (Figure 2B). At the beginning of the experiment, the net magnetisation is oriented along the  $z$  axis and the  $90^\circ$  pulse rotates the magnetisation in the "reading plane"  $x$ - $y$ . At a time point  $t$ , a pulse gradient of  $\delta$  duration and  $G$  magnitude is applied, and at this time each spin experiences its particular char-

acteristic phase. Then two scenarios are possible. If no diffusion occurs, the  $180^\circ$  pulse cancels the effect of the two gradient pulses and all the spins will refocus leading to a maximum signal at the echo (i.e. at the end of the second  $\tau$  period, see Figure 2B). But if the spins have moved during the  $\Delta$  delay, the degree of dephasing will be proportional to the displacement of the spin during this delay, inducing an attenuation of the signal. The greater the diffusion, the larger the attenuation of the echo signal. This is illustrated in Figure 2B (right) in which the signal of free *t*-A is more attenuated than the aggregated *t*-A.<sup>8</sup>

## Results

Figure 1 shows a proton spectrum of *t*-A obtained approximately 10 min after emulsification. According to expected pattern for the proton spectrum, two distinct sets of readily assigned resonances can be distinguished, suggesting that *t*-A can exist in at least two forms. Three types of NMR experiments confirm that these two forms correspond to free and aggregated *t*-A. First, EXSY experiments acquired on the mixture clearly demonstrate a slow exchange between the two species, confirming that the same compound is undergoing exchange between two states. Second, DOSY experiments performed on the mixture show that the two species have very different diffusion coefficient values: about  $6 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$  and  $1 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$  allowing the unambiguous assignment of the free and the aggregated forms, respectively, as shown in the DOSY spectrum displayed in Figure 2. Third, proton spectra recorded at different ethanol concentrations (up to 60% ethanol) show that only the free form of *t*-A is present in solutions containing >40% ethanol.

When water is added to an alcoholic solution of *t*-A, the mixture instantaneously takes a cloudy aspect that appears visually stable for at least six to eight hours, after which creaming can be observed. This stability, however, is only apparent: Figure 3 displays the 9- $\text{CH}_3$  signals acquired over the course of time. It shows that while the intensity of the free form ( $\delta$  1.78) remains constant,



**Figure 4.** Time-dependence of the rates of exchange of *t*-A between the isotropic aqueous phase,  $k_{-a}$  (filled circles), and the aggregates,  $k_a$  (filled diamonds), for a 4 mM *t*-A solution in 5% EtOD/95% D<sub>2</sub>O as determined from EXSY experiments.

the signal assigned to the aggregated form ( $\delta$  1.39) decreases with time. This decrease can be quantified thanks to the presence of a small amount of methanol (5 mM) added to the mixture as an internal standard. From these results, it is possible to deduce the proportion of *t*-A which is invisible by NMR (Figure 3B). After 40 min, the latter plateaus at 3.5 mM, which represents *ca* 70% of the total *t*-A concentration. At the same time, the proportion of small aggregates decreases to a concentration close to 0.7 mM whereas the proportion of free *t*-A (which is determined by its solubility in the hydroalcoholic solution) remains constant (0.8 mM).

The fraction of *t*-A not visible in the NMR proton spectrum recorded using classical liquid techniques is assigned to *t*-A present as small droplets that are several micrometres in size and that are responsible for the cloudy aspect of the solution. In agreement with this assignment, their concentration can be fit to a monoexponential function that is consistent with an Ostwald ripening process.

This behaviour is typical of droplet growth and agrees with previous investigations of *t*-A droplets by Grillo *et al.*<sup>4</sup> using small-angle neutron scattering.

In the case of *t*-A emulsions, EXSY NMR experiments allow one to observe the exchange of magnetisation between the free and the aggregated forms: such an exchange is adequately described by the bimolecular process illustrated in Figure 4, which assumes that the observed rate constants  $k_a$  and  $k_{-a}$  are pseudo-first order rate constants that depend on the concentration of free *t*-A and *t*-A present in small aggregates, respectively. Following the evolution of  $k_a$  and  $k_{-a}$  over time (Figure 4) shows that two growth regimes are present. At early times,  $k_a$  is greater than  $k_{-a}$  and the population of small aggregates is growing. After *ca* 1.5 h,  $k_{-a}$  becomes greater than  $k_a$ , implying that the small aggregates are now dissolving. This can be understood by taking into consideration the formation of the larger droplets, which are now growing by Ostwald ripening at the expense of free *t*-A in solution. This, in

turn induces the preferential dissolution of the smaller aggregates due to their higher ratio of surface area to mass.

## Conclusion

Taken together, our findings indicate that the initial steps of the spontaneous emulsification of *t*-A are, in fact, somewhat more complex than previously thought. A model emerges in which small, nanometre-sized aggregates are initially formed upon rapid dilution of more concentrated hydroalcoholic solutions. These then slowly disappear in favour of the larger droplets previously observed by light scattering experiments. While using dynamic NMR spectroscopy to follow emulsification phenomena may appear counter-intuitive, we hope this study serves to demonstrate the contrary: it is a wonderful technique that offers enormous potential to obtain high-quality information not otherwise available. The principal conditions for its applicability are that the exchange between species be slow on the NMR timescale, and, of course, that the experimentally-accessible concentration range be suitable for NMR spectroscopy. Cheers!

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