Importance of the Reference Spectrum on Generalized Two-Dimensional Correlation Spectroscopy: Relation between Intensity Variations and Synchronism

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Generalized two-dimensional correlation spectroscopy (2D-COS) establishes correlations between intensity variations within a series of ordered spectra generated by an external perturbation. The influence of the reference spectrum on the synchronicity has been investigated by using spectral simulations and mathematical analysis. With a two-state model, it is found that for two synchronous bands, 2D asynchronous peaks appear when no reference is used, whereas when the mean or the first spectrum of the series is chosen, no asynchronous peak occurs, as expected. In the latter cases, the intensity ratio of the dynamic spectra is constant throughout the experiment, which is not the case if a reference is not subtracted. The proportionality constant is equal to the ratio of the amplitudes of the intensity variations. This result is mathematically demonstrated and generalized to any form of intensity variation: if the intensity ratio of two bands is constant throughout the experiment, the elements of the 2D asynchronous matrix are zero at any wavenumber. In addition, it is established that any spectrum of the series can be used as a reference to evidence the occurrence of synchronisms. In the case of linear intensity variations, the correlations between two bands are always synchronous as long as a spectrum of the series or the mean spectrum is chosen as the reference. Thus, it is very difficult to determine whether the intensity variations have different variation rates. All the conclusions drawn from the mathematical analysis are confirmed with spectral simulations. These mathematical considerations are applied to absorbance spectra.

Introduction

Generalized two-dimensional correlation spectroscopy (2D-COS) establishes correlations (in-phase and out-of-phase) between intensity variations occurring at independent frequencies.[1] This method of statistical analysis can be used for any series of ordered spectra that represents the response of a system to an applied external perturbation. This perturbation can be of any nature and can follow any type of variation.[1] 2D-COS reveals spectral intensity variations that can be synchronous or asynchronous. This allows the establishment of band assignments and sequences of events for samples subject to an external stress. This is an interesting advantage that lead to numerous studies in order to understand different physical processes such as dynamic polymer stretching,[2,3] phase transitions,[4–6] time-dependent hydrogen–deuterium exchange in proteins,[7,8] or heat-induced denaturation of proteins.[9]

In 2D-COS, the correlation intensities are given by two matrixes that correspond to the covariance and the out-of-phase correlation of the spectral intensity variations. The calculation is in fact made with the so-called dynamic spectra that are obtained by the subtraction of a reference spectrum to all original spectra. This reference can be the first spectrum of the series, the mean spectrum of the series, or zero (no reference).[1,10] Little attention has been paid so far to the importance of the choice of the reference. Schlutz et al.[9] have found that with a reference spectrum, smaller out-of-phase variations can be detected than if no reference is used. Šašić and colleagues[11] have shown that the results of 2D-COS strongly depend on the pretreatment

(mean-centering and normalization) made on the spectra. This in turn is related to the reference problem. Šašić and colleagues have found that the normalization led to the disappearance of the asynchronous spectra in a two-component system. It was concluded that the normalization is important for systems with large intensity variations. Finally, Czarnecki[12] observed that a band with no intensity change can generate 2D peaks if no reference is used, whereas these peaks disappear with a reference.

In this study, simulations have been used to investigate the influence of different references on the spectral correlations and on the synchronism. A simple two-state model is first presented. It is shown that the choice of the reference is crucial to detect in-phase intensity correlations, whereas normalization is unimportant. The simulated results are mathematically rationalized and generalized to different types of intensity variations. The notion of synchronicity and the criterion for a reference to be valid are described mathematically. Special attention is paid to linear intensity variations and to absorption spectra.

Experimental Section

Simulations. Spectra were simulated by using two Lorentzian bands located at 950 and 1050 cm^{-1} that decrease in intensity without wavenumber shift or bandwidth change. Except for the two-state model presented below, one of the two bands arbitrary decreases from 10 to 7 arbitrary units, whereas the other one decreases from 2 to 1. The rates and delays of the intensity variations were controlled, and different types of variations were used (see below). The spacing between the data points was 1 cm^{-1}. For each simulation, the series was composed of 13 spectra.

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Two-Dimensional Correlation Analysis. The synchronous ($\mathbf{\Phi}$) and asynchronous ($\mathbf{\Psi}$) matrixes were calculated and the corresponding maps plotted with a homemade program written with the Mathlab 5.3 software for Windows (MathSoft Inc., Cambridge, MA). The calculation was carried out in the matrix form[11] using the Hilbert transform:[10]

$$\mathbf{\Phi} = \frac{1}{N-1}\mathbf{M}\mathbf{M}^{\mathrm{T}} \quad \text{and} \quad \mathbf{\Psi} = \frac{1}{N-1}\mathbf{M}(\mathbf{H}\mathbf{M}^{\mathrm{T}})$$

where N is the number of spectra of the series, $\mathbf{M}$ is the matrix of the spectra of the series (spectra are put in columns), $\mathbf{M}^{\mathrm{T}}$ its transposed matrix, and $\mathbf{H}$ is the Hilbert−Noda matrix whose elements $H_{mn} = 0$ if $m = n$ and $H_{mn} = 1/(n - m)$ otherwise.

A filter was used to eliminate very small correlation intensities. It sets to zero the elements $\mathbf{\Phi}_{mn}$ of $\mathbf{\Phi}$ and the elements $\mathbf{\Psi}_{mn}$ of $\mathbf{\Psi}$ that obey the conditions

$$|\mathbf{\Phi}_{mn}| < (\mathbf{\Phi}_{\max} - \mathbf{\Phi}_{\min})/100$$

and

$$|\mathbf{\Psi}_{mn}| < (\mathbf{\Psi}_{\max} - \mathbf{\Psi}_{\min})/100$$

respectively.

Results and Discussion

I. Impact of the Reference. To demonstrate the impact of the reference spectrum on 2D-COS, a simple two-state model is first presented. Consider two bands, centered at wavenumbers $\bar{\nu}_1$ and $\bar{\nu}_2$, that are associated to two species A and B such that

$$A \rightleftharpoons B \tag{1}$$

This equilibium depends on a variable t that represents an external perturbation that is applied to the system under study. The two bands at $\bar{\nu}_1$ and $\bar{\nu}_2$ evolve as a result of this perturbation and are represented by their intensities $y_1(t)$ and $y_2(t)$. The potential presence of band shifts and changes in bandwidth is neglected here. Before the perturbation is applied, the initial intensities y_1^i and y_2^i are constant and have different values. For example, for infrared absorption spectra, y_1^i and y_2^i may have different values because the absorption coefficients of the vibrations at $\bar{\nu}_1$ and $\bar{\nu}_2$ are different and because the initial concentrations of the two species are different. When the perturbation is applied, the intensities change as a function of t, with $t \in [-T/2, T/2]$, where T is the total time of the experiment. The intensities are described by two functions $g_1(t)$ and $g_2(t)$ that represent the time-dependent response of the system at $\bar{\nu}_1$ and $\bar{\nu}_2$ and by the amplitudes of the intensity variations δy_1 and δy_2. The intensities can then be written as

$$\begin{cases} y_1(t) = y_1^i + \delta y_1 g_1(t) \\ y_2(t) = y_2^i + \delta y_2 g_2(t) \end{cases} \tag{2}$$

Figure 1 shows the simulated spectra of two bands representative of a two-state model. In this example, the species A is characterized by a band located at 950 cm^{-1} and the species B by a band at 1050 cm^{-1}. The concentration of A is initially 100% of the total and that of B is zero. During the simulated experiment, 75% of A is converted into B. To describe the intensity variations, a hyperbolic tangent function has been chosen as illustrated in Figure 2. This function is a good
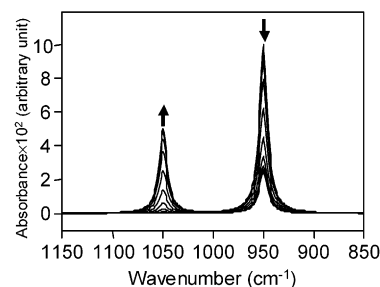

Figure 1. Simulated absorbance spectra corresponding to a two-state model. One band is located at $\bar{\nu}_1 = 950$ cm^{-1} and decreases in intensity as a function of the external variable t. The second band, located at $\bar{\nu}_2 = 1050$ cm^{-1}, increases in intensity. The two bands are synchronous with $y_1^i = 0.1$, $y_2^i = 0$, $\delta y_1 = 0.075$, and $\delta y_2 = \delta y_1/1.5 = 0.05$.
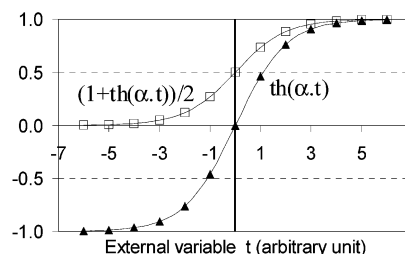

Figure 2. Plot of the function th(αt) (eq 3) and the function $(1 + \text{th}(\alpha t))/2$ in eq 4 for $t \in [-6, +6]$ (step of unity) and $\alpha = 0.5$.

representation of phenomena such as phase transitions or titration curves, etc., and its expression is given by

$$\text{th}(\alpha t) = \frac{\exp(\alpha t) - \exp(-\alpha t)}{\exp(\alpha t) + \exp(-\alpha t)} \tag{3}$$

where the constant α is the slope of the curve at the origin. Hence, it characterizes the speed (or the rate) of the intensity variations. The intensity variations at $\bar{\nu}_1$ and $\bar{\nu}_2$ are characterized by their speed α_1 and α_2, respectively. Due to the conversion of the species A into B for a two-state model (or reciprocally B into A), the variations must be synchronous. Therefore, the type of variation must be identical at $\bar{\nu}_1$ and $\bar{\nu}_2$ ($g_1(t) \equiv g_2(t) \equiv g(t)$), and the variation rates must be equal ($\alpha_1 = \alpha_2 = \alpha$). The amplitudes δy_1 and δy_2 can be a priori different. For example, for infrared absorption spectra, $\delta y_1 \neq \delta y_2$ because each absorption coefficient at $\bar{\nu}_1$ and $\bar{\nu}_2$ is specific to the corresponding vibration mode. Hence, $y_1(t)$ and $y_2(t)$ can be written

$$\begin{cases} y_1(t) = y_1^i - \delta y_1 g(t) = y_1^i - \delta y_1 \dfrac{1 + \text{th}(\alpha t)}{2} \\ y_2(t) = y_2^i + \delta y_2 g(t) = y_2^i + \delta y_2 \dfrac{1 + \text{th}(\alpha t)}{2} \end{cases} \tag{4}$$

The function $g(t) = (1 + \text{th}(\alpha t))/2$ has been more specifically chosen because it varies between 0 and 1, whereas the function th(αt) varies between -1 and $+1$, as seen in Figure 2. Thus, $y_1^i - \delta y_1 \leq y_1(t) \leq y_1^i$ and $y_2^i \leq y_2(t) \leq y_2^i + \delta y_2$. The opposite sign in the expressions of $y_1(t)$ and $y_2(t)$ in eq 4 stems from the fact that one band decreases while the other increases for a two-state model. A potential delay, noted β, can be introduced by using a translation of the intensity variation along the t axis (replacing t by $t - \beta$). Of course, for a two state model, the values of β must be equal for $y_1(t)$ and $y_2(t)$. Therefore, by definition, two synchronous (in-phase) bands must follow the same type of variation (the same function), have the same speed
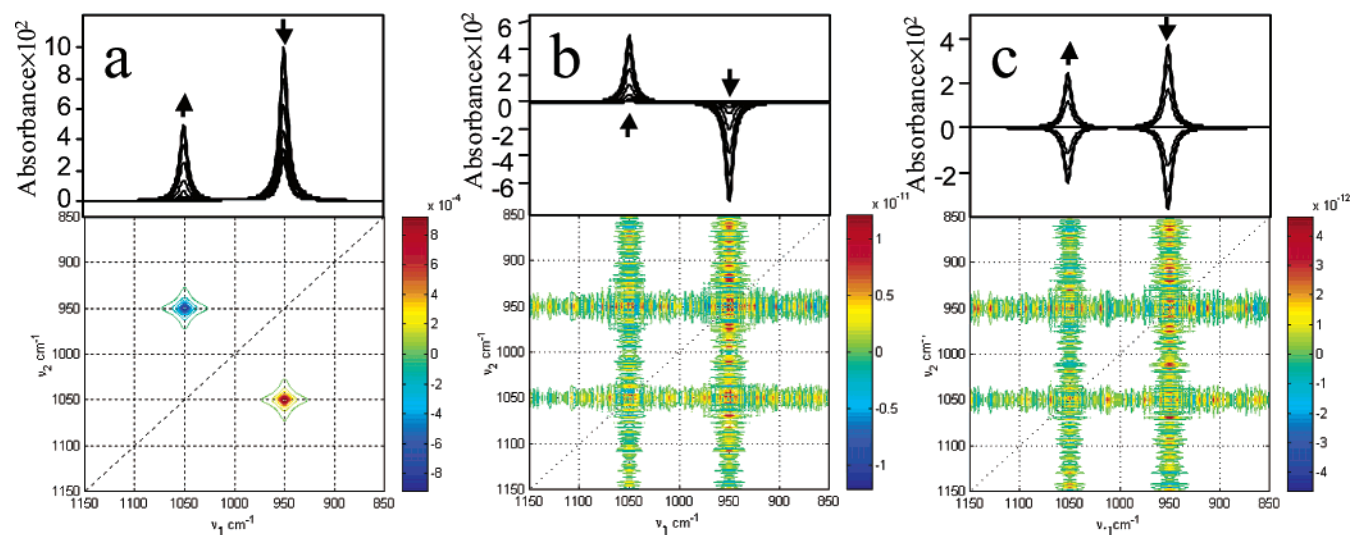
Figure 3. Asynchronous maps Ψ generated by the spectra of Figure 1. The maps were obtained for different references: (a) no reference; (b) first spectrum; (c) mean spectrum. The simulated dynamic spectra are shown in the outer frames. The minimum and maximum values of the correlation intensities are indicated at the bottom of the maps.
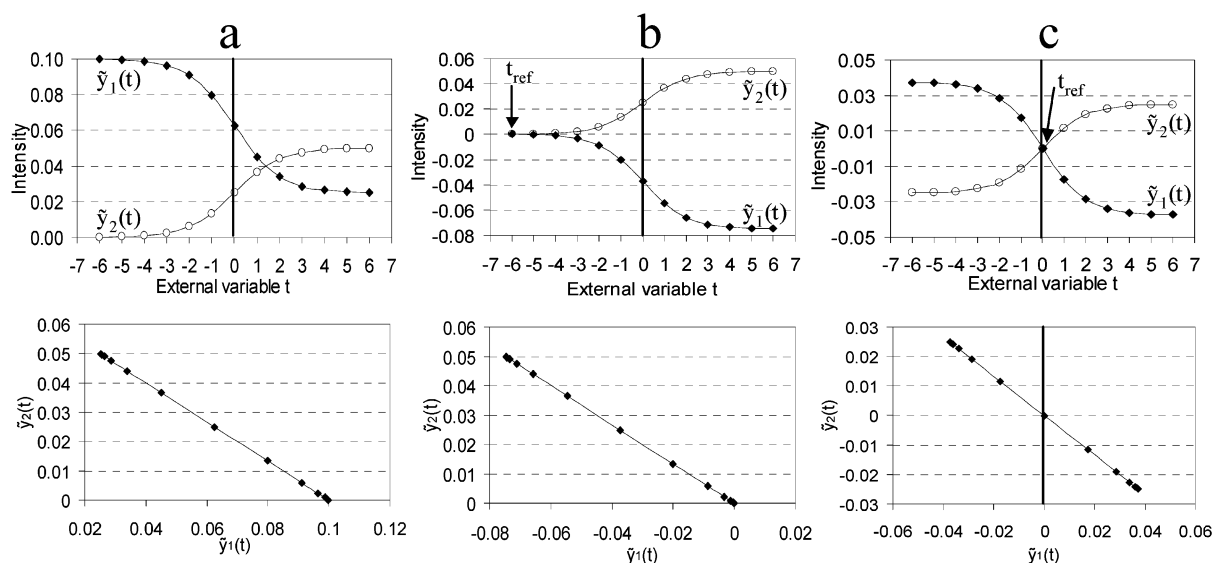

Figure 4. Intensities $\tilde{y}_1(t)$ and $\tilde{y}_2(t)$ as a function of the external variable t (above) and intensity $\tilde{y}_1(t)$ as a function of $\tilde{y}_2(t)$ (below), corresponding to the simulated spectra of Figure 1 and calculated for different references: (a) no reference; (b) first spectrum; (c) mean spectrum.

(the same value of α), and be characterized by the same delay (the same value of β). In Figure 1, α is 0.5, β is 0, and $t \in [-6, +6]$ in arbitrary time units.

Figure 3 presents the asynchronous maps generated by the spectra of Figure 1 and calculated with different references (i.e., with different dynamic spectra). The usual references suggested by Noda[1] are used, i.e., the first spectrum, the mean (average) spectrum, and no reference. Without reference, the asynchronous map exhibits a peak at 950/1050 cm^{-1}, indicating that the band at 950 cm^{-1} is delayed or varies at a different rate than that at 1050 cm^{-1},[12] although the spectral variations are synchronous as defined above. When the mean spectrum or the first spectrum is used as a reference, the maps only exhibit noise, which is characteristic of two bands that are in-phase. Thus, different results are obtained depending on the reference.

Figure 4, in its upper part, presents the dynamic intensities $\tilde{y}_1(t)$ and $\tilde{y}_2(t)$ of the two bands shown in Figure 1 as a function of t, obtained after subtraction of different references. Without reference, $\tilde{y}_1(t) = y_1(t)$ and $\tilde{y}_2(t) = y_2(t)$. The use of a reference only results in an offset of the intensity variations, the two dynamic intensities at $\bar{\nu}_1$ and $\bar{\nu}_2$ being zero at the same time.

This time, noted t_{ref} in the following, corresponds to the time of the chosen reference and is defined by

$$\tilde{y}_1(t_{ref}) = \tilde{y}_2(t_{ref}) = 0 \qquad (5)$$

Then, $y_1^{ref} = y_1(t_{ref})$ and $y_2^{ref} = y_2(t_{ref})$ are the reference intensities of the reference spectrum corresponding to the time t_{ref}. In the time domain $[-6, +6]$ of this two-state model, t_{ref} is -6 if the first spectrum is the reference (Figure 4b) and 0 if the mean spectrum is chosen (Figure 4c). In its lower part, Figure 4 shows the plots of $\tilde{y}_1(t)$ as a function of $\tilde{y}_2(t)$. It can be seen that, as a result of the offset, the relation between $\tilde{y}_1(t)$ and $\tilde{y}_2(t)$ is a straight line that is linear (that passes through the origin of the axis), i.e.,

$$\tilde{y}_1(t) = c\tilde{y}_2(t) \qquad (6)$$

where c is a constant with respect to t. Therefore, the dynamic intensities at $\bar{\nu}_1$ and $\bar{\nu}_2$ increase in a proportional manner; i.e., the intensity ratio of the two synchronous bands is constant throughout the experiment if a reference is used. The cor-

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respondence between synchronicity and the linear relationship between the intensity variations has been emphasized very recently.[13] Without a reference, the straight line is affine (does not passes through the origin), i.e.,

$$\tilde{y}_1(t) = c\tilde{y}_2(t) + d, \quad \text{with } d \neq 0 \tag{7}$$

and the intensity ratio is not constant with respect to t.

These conclusions can be easily demonstrated mathematically. If the first spectrum is the reference, the dynamic intensities are

$$\begin{cases} \tilde{y}_1(t) = y_1(t) - y_1^{\text{i}} = -\delta y_1 \dfrac{1 + \text{th}(\alpha t)}{2} \\ \tilde{y}_2(t) = y_2(t) - y_2^{\text{i}} = \delta y_2 \dfrac{1 + \text{th}(\alpha t)}{2} \end{cases} \tag{8}$$

Therefore, the intensity ratio r of the dynamic intensities as a function of t is

$$r = \frac{\tilde{y}_1(\alpha_1, t)}{\tilde{y}_2(\alpha_2, t)} = -\frac{\delta y_1}{\delta y_2} \tag{9}$$

which is identical to eq 6. Then, the intensity ratio r of the two bands is constant for any t and is given by the ratio of the amplitudes of the intensity variations. The negative sign only reflects that the two bands change in opposite directions.

For $t \in [-T/2, +T/2]$, the mean value of $\text{th}(\alpha t)$ is zero because this function is odd. Then, the mean (average) intensities $\bar{y}_1$ and $\bar{y}_2$ are

$$\begin{cases} \bar{y}_1 = \dfrac{1}{T} \int_{-T/2}^{T/2} y_1(t) \, dt = y_1^{\text{i}} - \dfrac{\delta y_1}{2} \\ \bar{y}_2 = \dfrac{1}{T} \int_{-T/2}^{T/2} y_2(t) \, dt = y_2^{\text{i}} + \dfrac{\delta y_2}{2} \end{cases} \tag{10}$$

If the reference is the mean spectrum, the dynamic intensities are given by

$$\begin{cases} \tilde{y}_1(t) = y_1(t) - \bar{y}_1 = -\dfrac{\delta y_1}{2}\text{th}(\alpha t) \\ \tilde{y}_2(t) = y_2(t) - \bar{y}_2 = \dfrac{\delta y_2}{2}\text{th}(\alpha t) \end{cases} \tag{11}$$

and their ratios also follow eq 9. One can notice that the dynamic intensities plotted in parts b and c of Figure 4 as a function of t are well represented by eqs 8 and 11, respectively. In the example shown in Figure 1, $\delta y_1 = 0.075$ and $\delta y_2 = 0.05$, so that r should be equal to -1.5, which is actually observed in Figure 4b,c.

If no reference is used, the intensity ratio is

$$r = \frac{\tilde{y}_1(t)}{\tilde{y}_2(t)} = \frac{y_1(t)}{y_2(t)} = \frac{y_1^{\text{i}} - \delta y_1[1 + \text{th}(\alpha t)]/2}{y_2^{\text{i}} + \delta y_2[1 + \text{th}(\alpha t)]/2} \tag{12}$$

and it can be demonstrated that the relation between $\tilde{y}_1(t)$ and $\tilde{y}_2(t)$ follows eq 7. Then, r is not constant with respect to t even if $\alpha_1 = \alpha_2$. Therefore, the constancy of r (i.e., the linearity between $\tilde{y}_1(t)$ and $\tilde{y}_2(t)$) is a criterion for two bands to be synchronous. Using a nonzero reference, the synchronicity between two bands is revealed even if the magnitude of their intensity changes is different of if their initial intensities are different. The choice of the reference is thus very important to detect synchronicities in 2D-COS. However, the normalization of the intensity is unimportant to demonstrate the occurrence

of synchronicities, even though the correlation intensities of the 2D maps do depend on the amplitudes of the intensity variations of the dynamic spectra.

The above mathematical results have been obtained considering two intensities located at fixed wavenumbers. Of course, the results can be extended to two wavenumber domains that represent the (more realistic) absorption of two vibration modes (see Figure 3). As long as the bands do not experience wavenumber shift of change in bandwidth, the 2D correlations between intensities at any wavenumber of the first band and intensities at any wavenumber of the second band will follow the same rules given above.

II. General Case: Criterion for Two Intensity Variations To Be Synchronous (In-phase). Equations 6 and 7 have been deduced from synchronous intensity variations that follow a hyperbolic tangent function, but are these equations valid for any synchronous variations? Consider two dynamic intensities at $\bar{v}_1$ and $\bar{v}_2$ whose intensity ratio is constant for any t such as

$$r = \frac{\tilde{y}_1(t)}{\tilde{y}_2(t)} = \frac{y_1(t) - y_1^{\text{ref}}}{y_2(t) - y_2^{\text{ref}}} = c \tag{13}$$

where c is a constant and y_1^{ref} and y_2^{ref} are the reference intensities at $\bar{v}_1$ and $\bar{v}_2$ of the reference spectrum. From the Fourier transform formalism of 2D-COS, the elements $\Phi(\bar{v}_1, \bar{v}_2)$ and $\Psi(\bar{v}_1, \bar{v}_2)$ of the synchronous map Φ and asynchronous map Ψ can be written as follows:[1]

$$\Phi(\bar{v}_1, \bar{v}_2) = \frac{1}{\pi T}\int_0^\infty [\tilde{Y}_1^{\text{Re}}(\omega)\tilde{Y}_2^{\text{Re}}(\omega) + \tilde{Y}_1^{\text{Im}}(\omega)\tilde{Y}_2^{\text{Im}}(\omega)] \, d\omega \tag{14}$$

$$\Psi(\bar{v}_1, \bar{v}_2) = \frac{1}{T}\int_0 [\tilde{Y}_1^{\text{Im}}(\omega)\tilde{Y}_2^{\text{Re}}(\omega) - \tilde{Y}_1^{\text{Re}}(\omega)\tilde{Y}_2^{\text{Im}}(\omega)] \, d\omega \tag{15}$$

where $\tilde{Y}_j^{\text{Re}}(\omega)$ and $\tilde{Y}_j^{\text{Im}}(\omega)$ ($j = 1$ or 2) are the real and imaginary parts of the Fourier transform $\tilde{Y}_j(\omega)$ of $y_j(t)$, respectively. If $\tilde{y}_1(t) = c\tilde{y}_2(t)$, the Fourier transforms are related by

$$\tilde{Y}_1(\omega) = c\tilde{Y}_2(\omega) \tag{16}$$

Therefore, for two complex numbers that are proportional, we have

$$\begin{cases} \tilde{Y}_1^{\text{Re}}(\omega) = c\tilde{Y}_2^{\text{Re}}(\omega) \\ \tilde{Y}_1^{\text{Im}}(\omega) = c\tilde{Y}_2^{\text{Im}}(\omega) \end{cases} \tag{17}$$

From eqs 14, 15, and 17, we obtain

$$\Phi(\bar{v}_1, \bar{v}_2) = \frac{c}{\pi T}\int_0^\infty |\tilde{Y}_2(\omega)|^2 \, d\omega \tag{18}$$

$$\Psi(\bar{v}_1, \bar{v}_2) =$$
$$\frac{c}{\pi T}\int_0^\infty [\tilde{Y}_2^{\text{Im}}(\omega)\tilde{Y}_2^{\text{Re}}(\omega) - \tilde{Y}_2^{\text{Re}}(\omega)\tilde{Y}_2^{\text{Im}}(\omega)] \, d\omega = 0 \tag{19}$$

It is demonstrated that when the ratio of two intensities is constant for any t (i.e., if eq 13 is satisfied), the asynchronous correlation is zero and the two intensity variations are synchronous. A similar demonstration can be done using the Hilbert–Noda formalism.

III. General Case: General Functions That Satisfy the Criterion of Synchronicity. The above two-state model, for which a hyperbolic function $\text{th}(\alpha t)$ represents the intensity variations, is a particular case that illustrates the synchronism

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between two bands. One can wonder what the forms of the dynamic intensities are that satisfy the condition given in eq 13. Consider the intensity $y_j(t)$ (for $j = 1$ or 2) at the wavenumber $\bar{v}_j$ such that

$$y_j(t) = A_j g(\alpha_j, t) + B_j \qquad (20)$$

for $t \in [0,T]$ (but identical results can be obtained for t ranging between two arbitrary values T_1 and T_2). A_j and B_j are two constants with respect to t that depend on the studied system and on $\bar{v}_j$. The function $g(\alpha_j, t)$ is arbitrary and is characterized by a single parameter α_j which is related to the speed (the rate) of the intensity variation and which is a constant with respect to t. The values of α_1 and α_2 can be a priori different. A potential delay can be added to $y_1(t)$ or $y_2(t)$ by replacing t by $t - \beta_j$, where β_j is constant and characterizes the band at $\bar{v}_j$. In the following, β_1 and β_2 are set to zero. Examples of $g(\alpha,t)$ functions are th(αt), exp$(-\alpha t)$, ln(αt), sin(αt), polynomials, etc. Some boundary conditions can be imposed on $y_i(t)$ and we note δy_j the amplitude of the intensity variations such as

$$\begin{cases} y_j(0) = y_j^i \\ y_j(T) = y_j^f \\ \delta y_j = y_j^f - y_j^i \end{cases} \qquad (21)$$

Given a and b are the boundary values of $g(\alpha_j,t)$ and δg the difference between b and a

$$\begin{cases} g(\alpha_j, t = 0) = a \\ g(\alpha_j, t = T) = b \\ \delta g = b - a \end{cases} \qquad (22)$$

the boundary conditions (21) become

$$\begin{cases} y_j(0) = y_j^i = A_j a + B_j \\ y_j(T) = y_j^f = A_j b + B_j \\ \delta y_j = y_j^f - y_j^i = A_j(b - a) \end{cases} \qquad (23)$$

This leads to another form for eq 20:

$$y_j(t) = \frac{1}{\delta g}[\delta y_j g(\alpha_j,t) - (a y_j^f - b y_j^i)]$$

$$= \frac{\delta y_j}{\delta g}[g(\alpha_j,t) - a] + y_j^i \qquad (24)$$

A representative scheme of the general function $g(\alpha,t)$ and the intensities $y_j(t)$ of two bands located at different wavenumbers are given in Figure 5. For two bands that have different variation amplitudes and different initial and final intensities, $g(\alpha,t)$ represents a common function that describes their (synchronous) intensity variation. It can also be viewed as the representation of the external perturbation. Indeed, for small amplitudes of the perturbation, the response of the system will be, in a first approximation, of the same type as the perturbation, i.e., will follow the same function. Thus, the intensity variations of the bands will be of the same type, with potential delays β_j with respect to the perturbation. Each parameter that describes the bands depends on the applied perturbation, on the system under study, and on the wavenumber. Therefore, y^i_j, y^f_j, and δy_j depend on the perturbation and on the intrinsic properties of the system. This is reflected by eq 23 where a and b characterize the perturbation, whereas $A_j(\bar{v}_j)$ and $B_j(\bar{v}_j)$ characterize the system at the wavenumber $\bar{v}_j$.
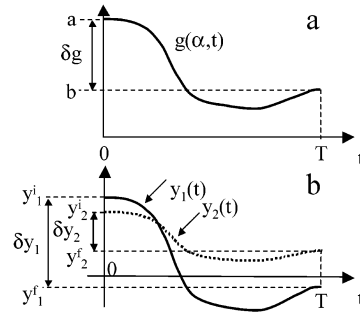

Figure 5. Symbolic representation of (a) the general function $g(\alpha,t)$ that can describe intensity variations and (b) two synchronous intensity variations $y_1(t)$ and $y_2(t)$ that follow this general function. The amplitudes and the initial and final values of the intensities are also represented.

If no reference is used, the intensity ratio of the dynamic intensities represented by eq 24 is not constant with respect to t even if $\alpha_1 = \alpha_2$ and $\beta_1 = \beta_2$. This is also true even if $y_1^i = y_2^i$, if $y_2^f = y_1^f$, or if $\delta y_1 = \delta y_2$. It can be demonstrated that the relation between $y_1(t)$ and $y_2(t)$ can be rearranged in the form given by eq 7. Consequently, the two intensity variations will generate 2D asynchronous peaks even if the bands are actually synchronous.

If the first spectrum is chosen as the reference, the dynamic intensities are given by

$$\tilde{y}_j(t) = y_j(t) - y_j^i = \frac{\delta y_j}{\delta g}[g(\alpha_j,t) - a] \qquad (25)$$

Then, the ratio of the dynamic intensities is given by

$$r = \frac{\tilde{y}_1(t)}{\tilde{y}_2(t)} = \frac{\dfrac{\delta y_1}{\delta g}[g(\alpha_1,t) - a]}{\dfrac{\delta y_2}{\delta g}[g(\alpha_2,t) - a]} = \frac{\delta y_1}{\delta y_2} \qquad \text{if } \alpha_1 = \alpha_2 \quad (26)$$

which satisfies eq 13.

The mean intensities are given by

$$\bar{y}_j = \frac{1}{T}\int_0^T y_j(t)\, dt = \frac{1}{\delta g}\left[\frac{\delta y_j}{T}\left(\int_0^T g(\alpha_j,t)\, dt\right) - (a y^f_j - b y^i_j)\right]$$

$$= \frac{\delta y_j}{\delta g}\left[\frac{1}{T}\int_0^T g(\alpha_j,t)\, dt - a\right] + y^i_j \qquad (27)$$

If the mean spectrum is chosen as a reference, the dynamic intensities are given by

$$\tilde{y}_j(t) = y_j(t) - \bar{y}_j = \frac{y_j}{g}\left[g(\alpha_j,t) - \frac{1}{T}\int_0^T g(\alpha_j,t)\, dt\right] \qquad (28)$$

Again, it can be seen that the expressions between the brackets are equal to each other for the intensities at $\bar{v}_1$ and $\bar{v}_2$ if $\alpha_1 = \alpha_2$ and if there is no delay between the variations. The ratio r is again equal to the ratio of the amplitudes. Thus, using a nonzero reference leads to the expected absence of asynchronisms in Ψ for synchronous bands. But, is it the case for any nonzero reference?

Given y_j^γ the intensity at wavenumber $\bar{v}_j$ of a spectrum situated between the first and the last spectrum of the series such that

$$y_j^\gamma = y_j(t = \gamma T), \quad \text{with } 0 \leq \gamma \leq 1 \qquad (29)$$

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If this spectrum is used as a reference, it is found that

$$\tilde{y}_j(t) = y_j(t) - y_j^{\gamma} = \frac{\delta y_j}{\delta g}[g(\alpha_j, t) - g(\alpha_j, t = \gamma T)] \quad (30)$$

It can be seen that eq 25 is a particular case of eq 30 when $\gamma = 0$ (initial spectrum). From eq 30, r satisfies eq 13 if $\alpha_1 = \alpha_2$ and is equal to the ratio of the variation amplitudes. Therefore, as soon as any spectrum of the series is used as a reference, two synchronous bands will not generate asynchronous peaks. It can be noticed that the two dynamic intensities $\tilde{y}_1(t)$ and $\tilde{y}_2(t)$ both follow eq 5 for $t_{\text{ref}} = \gamma T$ (which corresponds to the time of the chosen reference y_j^{γ}).

The fact that the choice of the reference may be arbitrary has been previously mentioned.[1] It is demonstrated here that it should not be zero and that it may be any spectrum of the series or the mean spectrum. With such references, the results of 2D-COS will be qualitatively equivalent (2D asynchronous peaks located at the same positions with the same signs), but the 2D correlation intensity values may be different depending on the chosen reference. Without reference, the correlations will be asynchronous, although the two bands can be actually synchronous. It has to be noted that correct results will be obtained if any spectrum of the series or if the mean spectrum is the reference but, of course, the reference cannot be completely arbitrary. First of all, the reference should have a physical meaning. Second, the convenient references (i.e., those that will provide correct results for synchronous bands) must be restricted to those that obey eq 13.

The function $g(\alpha,t)$ can be monotonic or nonmonotonic. As long as the two variations follow the same function $g(\alpha,t)$, they will be in-phase if a spectrum is used as a reference, if $\alpha_1 = \alpha_2$, and if $\beta_1 = \beta_2$. This has been confirmed, for example, with simulations for which the intensity variations follow a sinusoidal or a polynomial function of degree 3 such as $\alpha t^3 - t$ (data not shown). But, as pointed out by Šašić and colleagues, the interpretation of synchronous maps generated by nonmonotonic intensity variations may be complex.[11] However, most of the conclusions drawn from the synchronous map can be directly obtained from a conventional analysis of the dynamic spectra. Such an analysis is highly recommended before any 2D-COS investigation, especially for nonmonotonic variations.

The above mathematical considerations can be extended to noncontinuous functions (discrete series of spectra), which is the practical case. The conclusion is the same; i.e., for two bands, the intensity variations will be experimentally synchronous using 2D-COS if the intensity ratio is constant throughout the experiment and if a nonzero reference is used. It has to be stressed that the conclusions relative to the choice of the reference and to the type of intensity variation that have been drawn from this mathematical analysis have all been confirmed by spectral simulations as described in the Experimental Section.

It has to be noted that the problem of the reference also occurs for asynchronous bands (results not presented). It can be shown that the sign of the cross-peaks in the synchronous and asynchronous maps can be incorrect if no reference is used. In fact, it appears that the sign of the 2D peaks depends on the direction of the band intensity variations. This clearly can lead to misinterpretation of the data. The problem does not arise with a reference, which emphasizes the importance of the reference.

As mentioned in the Introduction, additional 2D peak may be observed by using a nonzero reference spectrum. This stems from the fact that the correlation intensities in 2D-COS depends on both the degree of correlation and the band intensity. Therefore, 2D peaks due to weak bands can be masked by stronger ones if no reference is used. Using a reference spectrum, the initial subtraction diminishes the band intensities, highlighting the small intensity variations.

IV. Special Case: Linear Variations. The relationships between intensity variations of two synchronous bands represented by arbitrary functions have been seen above. A special case that should deserve consideration is linear intensity variation. Suppose that the intensity variations are linear and satisfy eq 21. The intensity can be written as

$$y_j(t) = \frac{\delta y_j}{T}t + y_j^{\text{i}} \quad (31)$$

The coefficient $\delta y_j/T$ is the slope of the straight line. It also represents the speed α_j of the intensity variations with

$$\alpha_j = \frac{\delta y_j}{T} \quad (32)$$

As a consequence, the speed and the amplitude of the intensity variations are directly related, which is not the case in general.

When the first spectrum is chosen as the reference, the dynamic intensities are given by (the same demonstrations can be made and the same conclusions are reached for other references)

$$\tilde{y}_j(t) = y_j(t) - y^{\text{i}}_j = \frac{\delta y_j}{T} \quad (33)$$

and then

$$r = \frac{\tilde{y}_1(t)}{\tilde{y}_2(t)} = \frac{\delta y_1}{\delta y_2} = \frac{\alpha_1}{\alpha_2} \quad (34)$$

Thus, the above general rule is also valid for linear variations; i.e., for two synchronous bands, the ratio of their dynamic intensity is constant and equal to the ratio of the variation amplitudes when a convenient nonzero reference is used. But in this case, the variation rates α_1 and α_2 do not have to be identical, because the amplitude and the rate are not independent. A difference in variation amplitude is the same as a difference in variation rate. The ratio r is then also equal to the intensity ratio of the rate of changes. Therefore, for two bands that linearly vary in intensity, their correlations will always be found as synchronous as long as a convenient spectrum reference is used. This has been verified with spectral simulations (data not shown).

V. Application to Absorption Spectra. The above mathematical considerations can be applied to spectroscopic signals represented by their intensity $y_j(t)$. As a particular case, we now consider absorption spectra that follow the Beer−Lambert's law. Suppose that under static conditions ($t \leq 0$) the absorbance $A_j(t)$ at wavenumbers $\bar{v}_j$ is given by

$$A_j(t \leq 0) = A^{\text{i}}_j = \epsilon_j^{\circ}c_j^{\circ} \quad (35)$$

where the constants ϵ_j° and c_j° are the initial molar absorptivity and initial concentration, respectively (we assume that the optical path length of the sample is constant and equal to unity during the experiment).

For $t > 0$, the absorbance $A_j(t)$ can be written

$$A_j(t) = \epsilon_j^{\circ}c_j^{\circ} + \tilde{\epsilon}_j(t)\tilde{c}_j(t) \quad (36)$$

The functions $\tilde{\epsilon}_j(t)$ and $\tilde{c}_j(t)$ are the time-dependent responses at wavenumbers $\bar{v}_j$ of the molar absorption coefficient and concentration, respectively. They represent the dynamic part of the spectra.

Two cases can be encountered:

1. The changes in absorbance are associated to variations in concentration of the species ($\tilde{\epsilon}_j(t) = \epsilon_j^o$).

This corresponds, for example, to a dilution or to any two-state systems including isomerization, phase transitions, etc. In this case,

$$A_j(t) = \epsilon_j^o[c_j^o + \tilde{c}_j(t)] = \epsilon_j^o[c_j^o + \delta c_j(t)g(\alpha_j,t)] \quad (37)$$

where δc_j is the amplitude of the concentration changes of the species that absorb at $\bar{v}_j$.

With respect to eq 24, eq 37 corresponds to $a = 0$ and $b = 1$. Thus, $A_j(t)$ is exactly represented by the intensity $y_j(t)$ that has been discussed above and $A_j(t)$ can be treated similarly. The above conclusions drawn for $y_j(t)$ can be applied to $A_j(t)$. Therefore, for two bands that vary as a consequence of concentration changes only, the existence of in-phase variations will be detected by a nonzero reference spectrum. For a two-state model such as that described above, $\delta c_2 = -\delta c_1 = \delta c$ and $\alpha_1 = \alpha_2 = \alpha$. Thus eq 37 becomes for $j = 1$ and $j = 2$:

$$\begin{cases} A_1(t) = \epsilon_1^o c_1^o - \epsilon_1^o \delta c g(\alpha,t) \\ A_2(t) = \epsilon_2^o c_2^o + \epsilon_2^o \cdot \delta c g(\alpha,t) \end{cases} \quad (38)$$

It can be seen that the amplitudes of the intensity variations $\delta y_1 = \epsilon_1^o \delta c$ and $\delta y_2 = \epsilon_2^o \delta c$ are different as a result of different values of the absorption coefficients. Thus, a nonzero reference has to be used to reveal the synchronism of the two bands.

2. The changes in absorbance are associated to changes in the molar absorptivity ($\tilde{c}_j(t) = c_j^o$).

This can arise if the absorption coefficient directly depends on the perturbation (temperature, for example) or as a result of a change in the molecule orientation (due to an electric field, a magnetic field, a stretching, etc.). Then, the absorbance takes the form

$$A_j(t) = c_j^o[\epsilon_j^o + \tilde{\epsilon}_j(t)] = c_j^o[\epsilon_j^o + \delta\epsilon_j(t)g(\alpha_j,t)] \quad (39)$$

where $\delta\epsilon_j$ is the amplitude of the molar absorptivity changes of the species absorbing at $\bar{v}_j$. The absorbance variations of two synchronous bands can be again well described by the above analysis.

A more complicated case arises for linear variations of the absorbance. As seen above, for linear variations, a difference in amplitude between intensity variations at two different wavenumbers is related to a difference in the rate of the variations (eq 32). But one can wonder after all how to determine the linear absorbance variations that are synchronous (with different variation amplitudes) and those that are asynchronous (with similar variation amplitudes) using 2D-COS. In fact, this is impossible because a difference in amplitude is directly associated to a difference in rate of the variations (eq 32). If a linear external perturbation is applied, it can be anticipated that the response will (often) also be linear. Since the two bands initially have different absorbances, the observed amplitude of the spectral variations will (in general) also be different. The amplitude of the absorbance changes will then be different, but it cannot be ascribed to a different amplitude or to a different rate of the variations. In the case where a change in concentration is at the origin of the spectral variations (case

1 above), the slopes of the straight lines are different and the spectral changes cannot be ascribed to a different amplitude of concentration changes or to a different rate of the variations. One needs additional data, i.e., the final concentrations of the two species. If the initial and final concentrations are equal, and if an asynchronous peak is observed, it can be assessed that the two bands are asynchronous. However, even if the magnitudes of the concentration changes of the two species are nonequal, it cannot be excluded that the rate of the concentration changes are also different. The same argument can be used if the spectral changes are due to variations of the absorption coefficient (case 2 above).

Conclusion

In conclusion, the results shown above demonstrate that it is highly recommended to use a nonzero reference for 2D-COS analysis. Otherwise, peak artifacts in the asynchronous maps can be obtained for synchronous intensity variations. The reference can be any spectrum of the series or the mean spectrum. It could also be demonstrated (data not shown) that, without a reference, error in the signs of the synchronous as well as asynchronous peaks can arise for nonsynchronous bands, whereas the signs are correct if the reference is one of the above spectra. Therefore, not only is the reference spectrum crucial for synchronous bands as shown in the present analysis, but it is so for any band variation. Moreover, even using a nonzero reference, the application of a linear perturbation in view of 2D-COS analysis should be avoided at least for small amplitudes, because the response of the system may also be linear. As a consequence, the determination of synchronisms and asynchronisms may be very complex. Even for large-amplitude variations of the perturbation, one should verify that the responses of the bands are not linear as a function of the external variable. The conclusions reached in this study for generalized 2D-COS are valid for the different forms of hybrid 2D-COS[14] and for the global phase angle description of 2D-COS[15] as well.

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