Metabolic response to exogenous ethanol in yeast: An *in vivo* statistical total correlation NMR spectroscopy approach

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In vivo NMR spectroscopy, together with selectively 13C-labelled substrates and 'statistical total correlation spectroscopy' analysis (STOCSY), are valuable tools to collect and interpret the metabolic responses of a living organism to external stimuli. In this study, we applied this approach to evaluate the effects of increasing concentration of exogenous ethanol on the *Saccharomyces cerevisiae* fermentative metabolism. We show that the STOCSY analysis correctly identifies the different types of correlations among the enriched metabolites involved in the fermentation, and that these correlations are quite stable even in presence of a stressing factor such as the exogenous ethanol.

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1. Introduction

The responses of living organisms to exogenous stresses and their interpretation in terms of biochemical consequences are primary objectives for the metabolic approaches (Fiehn 2002; Nicholson *et al*. 2002).

Nuclear magnetic resonance (NMR), due to its ability to simultaneously identify the concentration of many metabolites (Shulman 1983), has emerged as a valuable tool to observe the consequences of external stimuli on the metabolic profile of many organisms (Nicholls *et al* 2003; Griffin and Shockcor 2004).

However, the high amount of information stored in an NMR spectrum can turn the analysis of a biological sample into a challenging task. For this reason a statistical approach is required to identify the metabolic network structure and quantify the metabolic consequences of exogenous stimuli (Dumas *et al.* 2006).

In this study, we adopted a 'statistical total correlation spectroscopy' (STOCSY) approach (Cloarec *et al*. 2005; Bohus *et al*. 2009; Noda 2010) to examine a set of *in vivo* 13C-NMR experiments of *Saccharomyces cerevisiae* fermentation (den Hollander *et al.* 1979).

The experiments were performed in the presence of increasing concentration of exogenous ethanol (0, 20 and 50 g/L respectively) to investigate the effects of stress conditions on the yeast fermentative processes. Although *Saccharomyces cerevisiae* is a highly ethanol-tolerant yeast, past studies have demonstrated that exogenous ethanol leads to inhibition of cell growth and viability, resulting in a reduced fermentation productivity and ethanol yield (Martini *et al.* 2006).

These phenomena were further investigated by using the *in vivo* 13C NMR data as input for the STOCSY analysis.

STOCSY delivers information about the structural and time course (i.e. metabolic-related) relationship between peaks across a set of NMR spectra (Cloarec *et al.* 2005; Eads and Noda 2002; Sasic 2008; Alves *et al* 2009; Robinette *et al*. 2009).

It should be pointed out that in our study the term 'time course' refers to spectra acquired from one sample at different times (Maher *et al*. 2009).

As results of our statistical analysis of the *in vivo* 13C NMR experiments of yeast metabolism, we constructed three

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STOCSY matrices (one matrix for each exogenous ethanol concentration).

The STOCSY matrices, in addition to the structural information, highlighted the correlations between the metabolic pathways involved in the yeast fermentation and how these are affected by the exogenous ethanol.

2. Materials

All high-purity reagents were purchased from Sigma-Aldrich (Milano, Italy), Merck Eurolab (Milano, Italy), Carlo Erba (Rodano, MI, Italy) and Serva (Heidelberg, Germany). All the water used was Milli-Q quality (Millipore, Bedford, USA). (1-13C)-D-glucose was from Cambridge Isotope Laboratories (Andover, MA, USA).

2.1 *Yeast strain and culture conditions and sample preparation*

This study uses previously published data sets containing three 13C *in vivo* NMR spectra acquired during yeast fermentation (Ricci *et al*. 2004).

The yeast used in the study was *Saccharomyces cerevisiae* (strain K310) isolated from naturally fermenting must during the vinification process (Trabalzini *et al.* 2003). The yeast was grown in YPD at 30°C with rotary shacking until it reached a cell concentration of 1×10^4 cells/mL. An aliquot of the yeast culture was inoculated in YPD adjusting the pH to 4.5 by adding citrate-phosphate buffer and containing 100 g/L unlabelled glucose. The cell suspension was incubated at 28°C without shaking, and samples were collected at different times during the growth. At each sampling the pH of the cell suspension was checked and the cell density was monitored by measuring the absorbance of the culture at 660 nm. Once the cell suspension reached the concentration of 1×10^6 cells/mL, it was centrifuged and the pellet was re-suspended in the a 13C-enriched medium containing 100 g/L of (1-13C)- D-glucose as yeast substrate and 20% (v/v) of D_2O as lock reference. Three experiments were performed with exogenous ethanol concentrations of 0, 20 and 50 g/L.

2.2 *NMR experiments*

The NMR sample consisted in a 2 mL of yeast suspension placed in a 10 mm outside diameter NMR-tube. 13C-NMR spectra were collected with a Bruker Avance DRX 600 spectrometer operating at 600 and 150 MHz for proton and carbon nuclei respectively. Carbon spectra were recorded using the inverse-gated-decoupling sequence (Freeman *et al*. 1972). The recycle delay was set to 20 s, the acquisition time was 0.270 s and the number of scans was 8.

FIDs (free induction decays) were acquired into 16 K complex data points with a spectral width of 201.1562 ppm and the NMR probe was thermostated at 30°C.

During the experiments all the spectra were sequentially arrayed (Shulman *et al*. 1979; Johnson 1999) in a pseudo-2D spectrum by an in-house developed sequence. The time interval between two consecutive spectra was about 3 min.

The arrayed experiments were processed as standard pseudo-2D spectra, like those used to measure the relaxation times (Vold *et al.* 1968) using Bruker XwinNMR software (ver 3.5). Spectra were multiplied by an exponential window function with a line broadening of 1 Hz, Fourier transformed, phase and baseline corrected. Peak areas were determined by line integration using the software XwinNMR.

The starting concentration of glucose was established during the experimental design, while the total concentration of ethanol was estimated at the end of the experiment by an enzymatic assay by Sigma-Aldrich (kit code 332-C).

The substrate and the end-products concentration were also calculated from the area of the NMR peaks by building a calibration curve with standard ethanol and glucose solution in YPD using TMS as external standard.

The area of the peaks of α and β-D-1-glucose were used to evaluate the progression of the experiment. A ratio of 0.01 between the current area of these peaks and their values at the beginning of the experiment were adopted for determining the end of the experiment (figure 1).

2.3 *STOCSY analysis*

The STOCSY analysis of the three sets of experiments was performed by a suite of in-house developed scripts running in Scilab 5.3 (Consortium Scilab - Digiteo (2011)).

Before to import the experimental data into SciLab, we extracted each 1D spectrum from the pseudo-2D spectra. The extraction was performed by a Cshell script that saved the 1D spectra in an ASCII table of ppm and intensities.

3. Discussion

In this study we analysed NMR-based data of yeast fermentation by a statistical approach NMR experiments were carried out using 1-13C glucose as limiting substrate and ethanol as exogenous stressing factor.

During the yeast fermentation – as described in previous studies (Ricci *et al.* 2004; Martini *et al.* 2004) – the kinetics of glucose degradation and of the endogenous ethanol production (e.g. the fermentation end-product) were strongly affected by the presence of exogenous ethanol.

In fact, in the absence of exogenous ethanol, fermentation ended after 38 h and the endogenous ethanol reached a

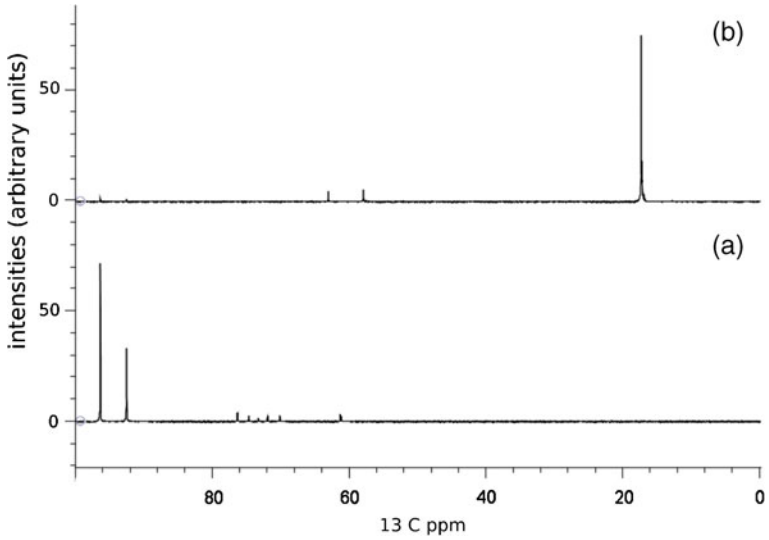

Figure 1. Proton decoupled 13C-NMR spectra of yeast medium during fermentation: (**a**) representative spectra at the beginning and (**b**) at the end of the fermentation. 13C chemical shift in parts per million (13C ppm) were as follow: panel (**a**), β-D-1-glucose, 96.5; α−D -1-glucose, 92.7; β- D-3-glucose and α- D-3-glucose, 76.4; β- D-2-glucose, 74.8; β- D-2-glucose and α- D-2-glucose, 72; α- D-3-glucose C-3, 73.4; α- D-5-glucose, 72.1; β-D-4-glucose and α-D-4-glucose, 70.2; β-D-6-glucose, 61.4; α-D-6-glucose, 61.2; panel (**b**): 2-glycerol, 63.1; 1-glycerol and 3-glycerol, 58.1; 1-ethanol, 58.0; 2-ethanol, 17.4. Chemical shifts are reported in ppm relative to TMS.

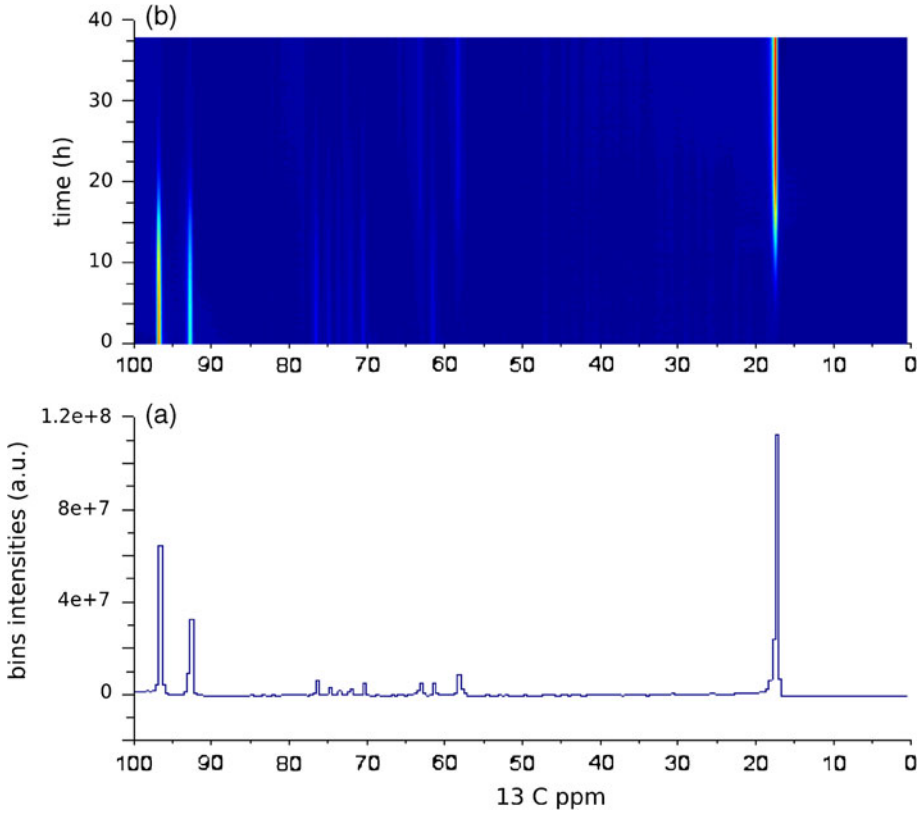

Figure 2. (**a**) A representative binned spectrum and (**b**) the data matrix M (see text) from the experiment conducted in absence of exogenous ethanol. The representative binned spectrum the mean of the bins contained in the spectrum.

concentration of 45 g/L. In the presence of 20 and 50 g/L of exogenous ethanol the fermentation ended after 43 and 55 h with a concentration of endogenous ethanol of 41 and 28 g/L respectively.

To further investigate the effects of the exogenous ethanol on fermentative metabolism of the yeast, we performed a STOCSY analysis using the pseudo-2D-spectra as input data (Fukuda *et al*. 2009).

The STOCSY approach is based on the computation of a correlation matrix from a set of NMR spectra. This approach, applied to a set of NMR spectra, generates a matrix that displays the temporal and structural correlations among the

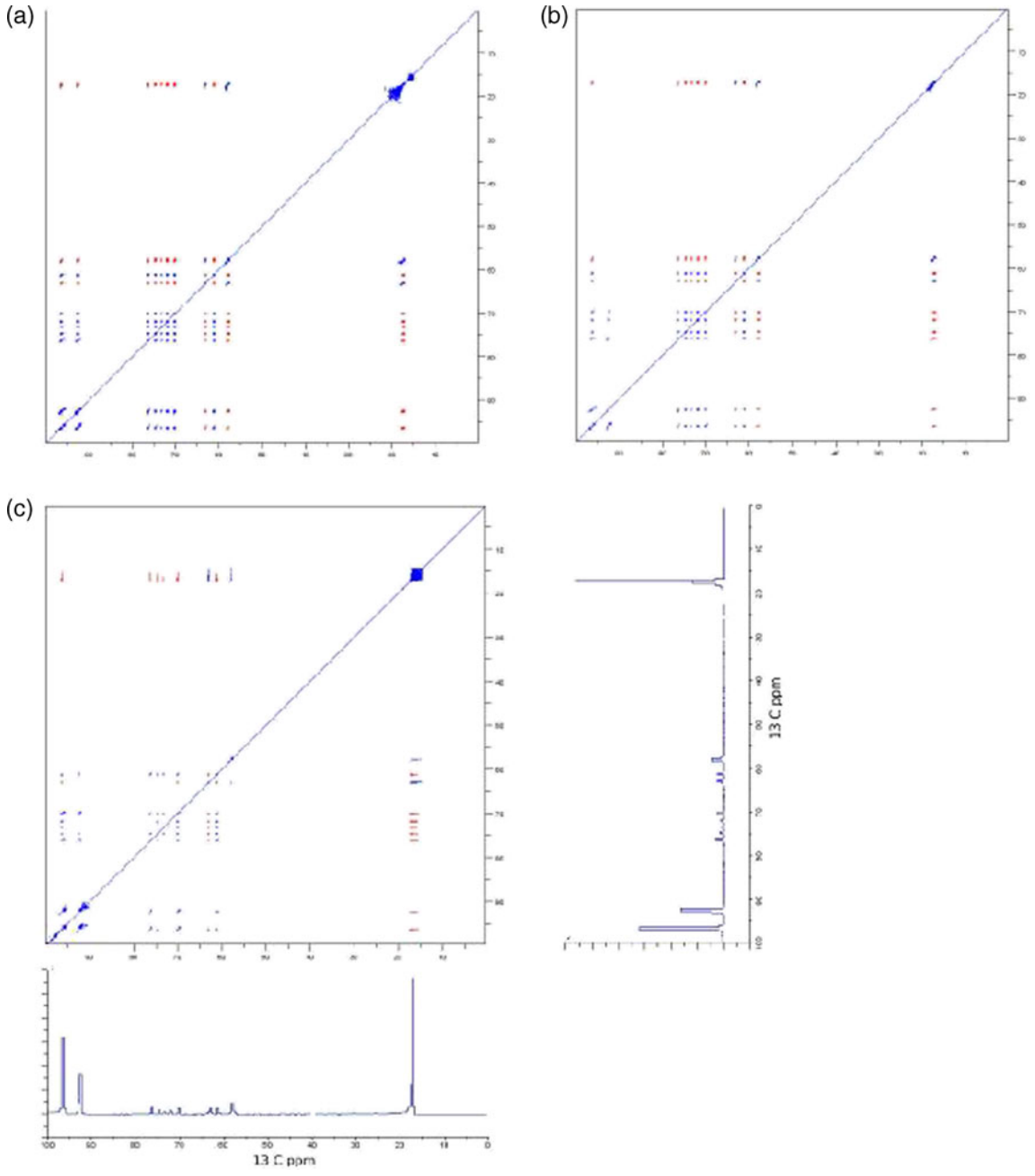

Figure 3. The correlation matrices obtained from the three sets of 13C *in vivo* NMR experiments: (**a**) in the absence of exogenous ethanol, (**b**–**c**) in the presence of 20 g/L and 50 g/L of exogenous ethanol, respectively. The pattern showed by the three correlation matrices reveals which metabolites have positive (blue) or negative (red) Pearson correlation coefficient (*r*). The analysis correctly identifies the negative correlation between glucose and ethanol; glucose and glycerol; and the positive correlation between glycerol and ethanol.

intensities of the various peaks across the whole sample (Cloarec *et al*. 2005).

Thus, the STOCSY correlation peaks were generated by calculating a time vector of correlations from a given chemical shift (variable) to all the other variables in the set of spectra.

A high value of the correlation coefficients between two chemical shifts implies either that the signals originate from the same molecule or that their concentrations are related by a common metabolic process.

Data were imported into the SciLab 5.3.3 environment (*www.scilab.org*) and only region between 0 and 100 ppm were retained.

Each NMR spectrum was reduced to bins of 0.1964416 ppm in order to prevent the effect of chemical shift and to allow a more manageable set of variables for the subsequent statistical analysis (Craig *et al*. 2006) (figure 2a).

After the binning, the pseudo-2D-spectra were transformed in data matrices (M) containing the binned spectra represented in rows and the vector time series (v) in columns (figure 2b).

The different length of the experiments, due to the increasing concentrations of exogenous ethanol and it inhibition effects, resulted in three matrices having dimension of 758×509, 843×509 and 1100×509.

To mitigate the effects of the peaks arising form the exogeneous ethanol the SciLab script checked the values of the first (v_i=1) and last (v_i=m) element in the time vector; if the value of v_i=m was higher than v_i=1 then the script subtracts v_i=1 from the vector v.

A STOCSY spectrum was calculated as a symmetrical matrix in which an element – at position i, j – is defined as the Pearson's coefficient (r) between ith and jth vector time series (equation 1).

$$r_{ij} = corr(v_i, v_j); \quad (1)$$

The autocorrelation value of each peak on the NMR spectra will appear on the diagonal of the correlation matrix, while other peaks having a significant correlation with the diagonal peaks, will appear off the diagonal at their chemical shift.

A positively (negatively) higher coefficient means the existence of a positive (negative) correlation between ith and jth peaks throughout the spectra.

Peaks arising from nuclei involved in structural correlations show high positive values of r; on the other hand nuclei involved in time course correlation (delineating metabolism related connectivity) show either positive or negative value of r. In the latter case r-values would be high but not as high as for resonance from the same molecules.

Figure 4 reports the STOCSY matrices obtained from the analysis of the *in vivo* 13C experiments of the yeast fermentation. The three STOCSY matrices refer to the experiments performed at increasing exogenous ethanol concentrations (panel a: 0 g/L, panel b: 20 g/L and panel c: 50 g/L) and display correlations above an absolute threshold coefficient of 0.9.

According to literature (Zhang *et al.* 2008; Sands *et al*. 2011), absolute values of r larger than 0.9 represent a statistically significant relationship between two NMR peaks. The STOCSY matrices allow to correctly identify the cross peaks arising from the structural correlations (delineating intramolecular connectivity) and non-structural correlations (delineating metabolism-related connectivity).

For a further analysis of the correlations taking place between the observed metabolites we focused on the STOCSY spectra originated by the experiment without exogeneous ethanol.

Considering the horizontal slice of the STOCSY corresponding to the resonance of α and β C1-glucose (92.7 ppm and 96.50 ppm, respectively), it is possible to highlight the following correlations: (a) the structural

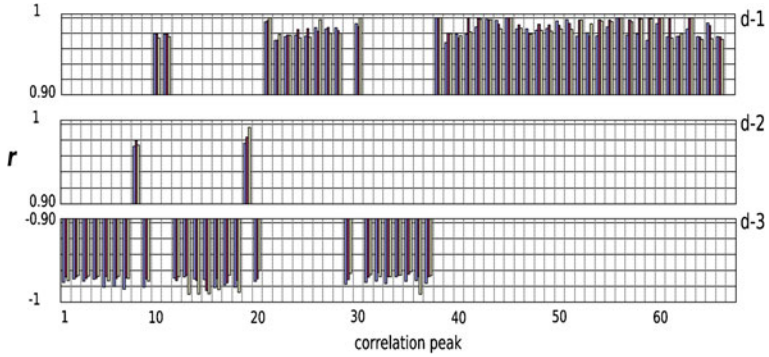

Figure 4. The correlation peaks intensities (r) have been reported for the structural correlations (panel d1), and for the negative and positive time course correlations (panel d2 and d3 respectively). The three sets of the experiments are reported in different colours: purple for the experiment in absence of exogenous ethanol and red and yellow for the experiments at 20 and 50 g/L respectively. The values of the intensities of the STOCSY peaks are very close, highlighting a weak or negligible effect of the exogenous ethanol on the time course and structural correlation between the observed metabolite.

correlations arising from the resonance of the α β C2–C5 glucose peaks (ranging from 67 to 77 ppm) and α−β glucose C6 peaks (ranging from 61 to 62 ppm); (b) the time course correlation arising from the resonance of glycerol C1; C3 (63.1 ppm), the ethanol C1 (58 ppm) and ethanol C2 (17.4 ppm). The latter three correlation peaks correctly show negative values because of the opposite kinetics of the glucose vs ethanol and glycerol.

As expected, the slice corresponding to the ethanol C2 (17.4 ppm), shows negative correlations with the glucose α−β C1–C5 (ranging from 96.50 to 70.20 ppm) and α−β glucose C6 peaks, and two positive correlations arising respectively from a structural correlation with the C1 ethanol and from a time course correlation with the glycerol (63 ppm).

The other two STOCSY matrices obtained from experiments conducted in the presence of exogenous ethanol (figure 3a) showed an equivalent pattern in the displacement of the correlation peaks. The intensities of the correlation peaks are reported in figure 4 (panel d–d3); time course correlation peaks and structural correlation peaks – for the three experiments performed at increasing exogenous ethanol concentration – showed comparable values: the positive time course correlation peaks (panel d2) have mean values of: 0.974 (sd=0.003), 0.982 (sd=0.002) and 0.985 (sd=0.015); the negative time course correlation peaks (panel d3) have mean values of: −0.980 (sd=0.005), −0.975 (sd=0.004), −0.978 (sd=0.01) and finally the structural correlation peaks (panel d1) have mean values of 0.984 (sd=0.009), 0.990 (sd=0.009), 0.987 (sd=0.010).

4. Conclusion

In this study we adopted a STOCSY analysis in combination with *in vivo* 13C NMR to investigate the effects induced by the exogenous ethanol on the *Saccharomyces cerevisiae* fermentative process. In previously published works, authors have demonstrated that the presence of exogenous ethanol reduced the yeast metabolic activity leading to a lowering in the total fermentation rate.

STOCSY analysis was carried out on three sets of *in vivo* 13C NMR experiments of yeast fermentation performed at increasing concentrations of exogenous ethanol (0, 20 and 50 g/L respectively).

According to the 13C-enriched substrates used in this study, the correlation matrices we obtained from the STOCSY analysis correctly identify the pattern of the structural and temporal correlation peaks arising from the NMR signals of the molecules involved in the fermentation.

The comparison of the STOCSY matrices obtained from experiments at different exogenous ethanol concentrations highlights the same intensities and displacement pattern of the correlation peaks.

Thus, our study demonstrated that, in contrast to the effects on the fermentation rate, the exogenous ethanol leaves the pattern of the correlations between the enriched metabolites involved in the yeast fermentation unchanged.

The SciLab and CShell scripts used in this study, as well as the arrayed NMR sequence, are available up on request from Dr M Ricci.

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