Monitoring Alcoholic Fermentation by Low-Resolution Pulsed Nuclear Magnetic Resonance

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Low-resolution NMR is used to determine residual sugars and alcohol content in the course of an oenological fermentation process. The measurement requires the use of a relaxation reagent ($MnCl_2$, 1 mM) to attenuate the water proton signal. Acquisition of the signal after a spin-echo sequence permits observation of the contributions of the soluble substances, sugars or alcohol, with good precision. The linear correlations observed between the NMR signal and the total sugars content, in the range 0–200 g/L, or the ethanol content, in the range 0–12% (v/v), can be used to predict the composition of a fermentation medium (standard deviations of 4–8 g/L for total sugars and 0.05–0.1% (v/v) for ethanol). The calibration curves can be used to follow the kinetics of the oenological fermentation process. The rapidity of the measurement (3.5 min) and the simple sample preparation make this technique suitable for on-line control of fermentation processes.

1. INTRODUCTION

Numerous fermentations are carried out in industry for the production of biomass or substrates. However, they are often difficult to monitor because of a lack of suitable sensors for carrying out rapid, or more particularly, on-line analyses.

In the food industry, for alcoholic fermentation for example, only certain physical parameters, such as temperature, can be effectively monitored.

Extensive research has been undertaken to develop methods for monitoring alcoholic fermentations. These methods can be divided into two groups according to whether information on the stage of fermentation is obtained directly or indirectly. The former uses physical parameters such as density, refraction index, weight loss, or volume of gas produced, which can be correlated to the concentration of sugar consumed or of ethanol produced (El Haloui et al., 1987). The latter analyzes the substrate and fermentation products directly by several techniques: mass spectrometry (Lloyd et al., 1985), gas chromatography (Comberbach and Bu'lock, 1983), near-infrared spectrometry (Dumoulin et al., 1987), liquid chromatography (Dinwoodie and Mehnert, 1985; Fischer Ross and Chapital, 1987), or Raman spectroscopy (Shope et al., 1987). Nevertheless, all these methods require a lengthy preparation of the samples, which is impractical for use in industry at present.

We therefore became interested in using the technique of low-resolution nuclear magnetic resonance to measure sugars and alcohol concentrations during a fermentation process. Compared to high-resolution NMR, this technique has the advantage of using a less costly instrument, making it more suitable for use in industry. We showed recently (Guillou and Tellier, 1988) that it is possible, using this technique, to measure specifically the alcohol content in solution in water or in alcoholic beverages, in a concentration range of 0–70% (v/v), without prior preparation or denaturation of the sample.

In the present work, we have developed a method, based on low-resolution NMR, which enables the determination, simultaneously, of the ethanol and sugar content in an oenological fermentation medium.

2. EXPERIMENTAL SECTION

2.1. Fermentations. Fermentations were carried out in a 1.5-hL cylindrical fermenter, which was fitted with sensors and a temperature control system. The concentrated grape musts from the Carignan or Macabeu grape varieties were diluted to approximately 1080 g/L at the start of fermentation (Table I). The musts were inoculated with a *Saccharomyces cerevisiae* KI strain, obtained in dehydrated form from the INRA (Institut National de la Recherche Agronomique). A silicon-based foam inhibitor (Rhodorsyl 426) was added before the start of fermentation. Samples (30 mL) were taken throughout the fermentation process.

In preparation for subsequent NMR analysis, the samples were filtered on a 0.45-μm (Sartorius-type) membrane, stabilized with mustard oil, and the samples were stored at +4 °C. Before the NMR determinations were carried out, the samples were centrifuged at low speed for 5 min to eliminate any particles that may have appeared during storage.

2.2. Chemical Analysis. An analytical control of the fermentation process was achieved by measuring the sugar, ethanol, and glycerol contents of the samples. The total sugar content was calculated by Bertrand's method, whereas glucose and fructose concentrations were measured enzymatically (Boehringer). Ethanol and glycerol concentrations were also determined by an enzymatic method, using, respectively, alcohol dehydrogenase (Boehringer) and the method described by Eggtein and Kuhlmann (1974) (Table I). Accuracies of these reference analyses were estimated from 0.1 to 0.2% (v/v) for ethanol, 0.1 g/L for glycerol, and 0.2 g/L in the 0–20 g/L range and around 2 g/L over 20 g/L for the sugars.

2.3. Parameters for NMR Measurements. NMR measurements were obtained on a low-field (0.47-T), low-resolution spectrometer operating by radio-frequency pulses (20 MHz) (Bruker Minispec PC 120). The spectrometer was fitted with a thermostatically controlled, 10-mm-diameter, quantitative probe. The 90° and 180° pulse widths were determined on the first fermentation sample (starting medium), and the signal was recorded with a diode detector. Recordings were carried out at 20 °C.

The principle on which the alcohol content determination by low-resolution NMR is based has been treated in full in a previous paper (Guillou and Tellier, 1988) and will only be outlined briefly here. During a spin-echo sequence ($90°x-\tau/2-180°y-\tau/2$ acquisition), the presence in the sample of molecules with a J_{H-H} coupling system leads to incomplete refocusing of the magnetization at time τ (Freeman and Hill, 1971). For ethanol, the amplitude modulation is maximum for $\tau = 1/2J \simeq 68$ ms, and

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Table I. Characteristics of the Fermentations[a]

fermentation	grape variety	grape must vol mass, g/L	starting medium, g/L: overall sugar content	starting medium, g/L: glucose	starting medium, g/L: fructose	terminal medium: ethanol, % (v/v)	terminal medium: glycerol, g/L	fermentation time, h
1	Carignan	1079.8	198	95.4	102.6	12	7.0	144
2	Carignan	1082.8	203	95.2	107.8	11.8	6.9	183
3[b]	Macabeu	1078	201.5	90.3	111.2	6.4	7.4	65
4	Carignan	1078.4	205.5	94.6	110.9	11.1	5.8	162

[a] Temperature, 25 °C; initial yeast concentration, 20–30 g/hL. [b] This fermentation has been stopped before the total sugar consumption (residual content 103 g/L).

Table II. Relaxation Times and Signals with and without the Relaxation Reagent ($MnCl_2$)

	without $MnCl_2$				with $MnCl_2$			
	20% glucose (w/w)		10% ethanol (v/v)		20% glucose (w/w)		10% ethanol (v/v)	
	water	glucose[a]	water	ethanol[a]	water	glucose	water	ethanol
T_1,[e] ms	1700	2940	1900	4760	110	140	100	270
T_2,[e] ms	990	1240	1500	2850	30	110	28	260
E_0,[b] %	93.7	6.3	91.3	8.7	93.7	6.3	91.3	8.7
$E^n(1/2J)$,[c] %	93.6	6.4	91.1	8.9	74	26	54	46

[a] The relaxation times of nonexchangeable protons of glucose and ethanol molecules have been determined with solutions in 99% D_2O. [b] E_0 is the signal amplitude after a 90° pulse (%). [c] $E^n(1/2J)$ is the contribution to the 68th echo amplitude after a CPMG sequence at time $t = 1/2J = 68$ ms (%). $E^n(1/2J) = \sum_i a_i \exp[-t/T_2^i]$. [d] The transversal relaxation times T_2 are calculated owing to spin-echo sequence with 80 points. [e] The longitudinal relaxation times T_1 are calculated owing to the inversion–recovery sequence with 30 points.

at this value, the methyl (CH_3) and methylene (CH_2) protons no longer contribute to the total magnetization, $E(1/2J)$. After a Carr-Purcell multiecho sequence ($90°x-(\tau-180°y-\tau)$ acquisition), J modulation disappears and the magnetization ($E^n(1/2J)$) at $t = 2n\tau = 1/2J$ is proportional to the proton density of the medium. Therefore, at time $t = 1/2J = 68$ ms, the difference (Δ) between the modulated (E) and unmodulated (E^n) echos is proportional to the proton density of the CH_3 and CH_2 protons: in other terms, to the alcohol content of the solution.

Relaxation times, T_1 and T_2, were determined by the inversion–recovery sequence (Freeman and Hill, 1971) and the CPMG spin-echo sequence, respectively. In the case of a multiexponential relaxation rate, the different components were obtained from a smoothing program using the least-squares method. Relaxation times for the ethanol and glucose protons were determined using D_2O–99% CEA solutions.

The relaxation reagent, $MnCl_2$, used for additions, was always taken from a freshly prepared solution of $MnCl_2$ (10 mM) in water.

2.4. Statistics. For each fermentation, we attempted to establish a correlation between the concentration of alcohol (measured in percent v/v) or the concentration of sugars (g/L) and the corresponding NMR measurement. The correlation is characterized by r^2, the square of the linear regression coefficient obtained by the least-squares method. The standard deviation (σ), as an absolute value, between the experimental points and those given by the regression curve ($\hat{y} = a + bx$) expresses the precision of the method.

The confidence interval (S) with which the sugars (or ethanol) concentration x_0 of a sample can be given can then be written as

$$S = \pm \frac{t_{\alpha/2,n-2}}{b}\sigma\left[1 + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{\sum_{i=1}^{n}(y_i - \bar{y})^2}\right]^{1/2}$$

where y_0 is the value of the NMR measurement, y_i the NMR values obtained for the n points of the calibration range, and $\bar{y}$ their average; and $t_{\alpha/2,n-2}$ is the Student parameter for a confidence level of $1 - \alpha$ and $n - 2$ degrees of freedom. In practice, calculations were carried out for a confidence level of 95% ($\alpha = 0.05$).

3. RESULTS AND DISCUSSION

3.1. Attenuation of the Water Signal. In low-resolution NMR, the amplitude of the signal obtained immediately after a single pulse (90°) is directly proportional to the number of protons present in the sample. In the case of a fermentation broth, the protons originate mostly from the water (>90%) and to a much lesser extent from the residual sugars and the ethanol produced.

Thus, for an aqueous solution of glucose with a concentration of 200 g/L, the nonexchangeable glucose protons (7/mol) only account for 6% of the total number of protons in the solution.

In a 10% (v/v) aqueous solution of ethanol, the nonexchangeable protons (5/mol) are very much in the minority: 8.7% of the total number of protons.

For glycerol, the most abundant fermentation metabolite after ethanol, and for a concentration of 7 g/L, commonly reached by the final stage of fermentation, the contribution of nonexchangeable protons to the total signal at equilibrium is only 0.3%.

All other organic substances present in the must have a negligible proton density.

During a fermentation process, the concentration of residual sugars varies between 200 and 0 g/L and the alcohol content between 0 and 12% on average. The water and exchangeable OH protons always account for more than 90% of the signal. Therefore, if NMR signals are to be used to monitor sugar and alcohol contents accurately, then the signal from the water protons, and all exchangeable protons in the medium, must be suppressed or attenuated.

The absence of spectral resolution in an inhomogeneous field means that the classical methods of selective irradiation, used in high-resolution NMR, cannot be used to eliminate the water peak (Hore, 1983). Only spin-echo techniques, which exploit the difference in T_2 relaxation times, can be used (Rabenstein et al., 1985). T_2 relaxation times of glucose or alcohol protons, however, are not sufficiently different from those of water, and, at the sampling time necessary for the alcohol content determination ($\tau = 68$ ms), the contribution of the water protons to the total signal remains predominant (Table II). We therefore used a paramagnetic reagent ($MnCl_2$) able to affect selectively the transverse relaxation time of water (Lowman and Maciel, 1979; Eads et al., 1986). After several trial runs, it appeared that a $MnCl_2$ concentration of 1 mM was, for an echo time of 68 ms, the best compromise between loss of signal brought about by an overall decrease in T_2 relaxation times of all the protons ($E^n(t) = \sum_i a_i e^{-t/T_{2,i}}$) and the selective attenuation of the water peak.

Table III. Calibration Curves of the NMR Signal versus the Ethanol Content and the Residual Sugars Concentration[a]

		calibration curve for ethanol			calibration curve for sugars		
fermentation	points	$\Delta = \sigma(t_v) = a + bt_v$	r^2	σ/b, %	$E(1/2J) = f(d_s) = a + bd_s$	r^2	σ/b, g/L
1	16	$0.21t_v - 0.54$	0.996	0.048	$0.012d_s + 2.6$	0.996	4.5
2	12	$0.21t_v - 0.75$	0.994	0.064	$0.012d_s + 2.6$	0.993	5.7
3[b]	7	$0.19t_v - 0.50$	0.980	0.057	$0.012d_s + 2.1$	0.982	4.5
4[c]	16	$0.22t_v - 0.71$	0.982	0.102	$0.012d_s + 2.4$	0.986	7.7

[a] For each measurement, the NMR signals $\Delta(1/2J)$ and $E(1/2J)$ have been multiplied by $5/E^n(1/2J)$, $E^n(1/2J)$ being proportional to the proton density of the sample. [b] The fermentation has been stopped when $t_v = 6.4\%$ and $d_s = 103$ g/L. [c] The samples have not been taken regularly.

The T_2 relaxation time of water protons decreases more rapidly in the presence of $MnCl_2$ ($T_2/T_2^{Mn^{2+}} > 30$) than that of the ethanol or glucose protons (Table II). In fact, the accessibility of the Mn^{2+} ion decreases greatly with increasing molecular size. The efficiency of relaxation is therefore greater for the smaller water molecules. Under these conditions, the contribution to the total signal of the nonexchangeable glucose or alcohol protons is multiplied by 4 and 5, respectively, for an echo time of 68 ms.

At 20 MHz, the longitudinal relaxation of water and solutes is considerably affected by Mn^{2+}, making it possible to reduce the recovery time between each series of pulses and thus to shorten the total measurement time.

The presence of microorganisms in the fermentation broth considerably affects the action of the paramagnetic reagent on the water and solute relaxation times. A significant amount of Mn^{2+} is probably adsorbed or forms a complex at the surface or inside the yeasts, lowering the available concentration of paramagnetic ions.

The determination of ethanol or sugar concentrations of oenological fermentation mediums should therefore be carried out on a sample that has been previously filtered to remove any particles in suspension.

3.2. Calibrating the NMR Measurement as a Function of the Alcohol Content of a Fermentation Must. In a previous paper (Guillou and Tellier, 1988), we showed that a linear relationship exists between the percentage volume of alcohol (t_v, % (v/v)) and the NMR measurement, $\Delta(1/2J)$

$$\Delta(1/2J) = E^n(1/2J) - E(1/2J) = Kt_v \quad (1)$$

where $E^n(1/2J)$ is the signal amplitude after a CPMG multiple-echo sequence ($n = 68$) at time $t = 1/2J = 68$ ms and $E(1/2J)$ is the amplitude of the single echo obtained after a refocusing time of $1/2J = 68$ ms.

The addition of $MnCl_2$ (1 mM) to the fermentation medium sample does not alter the principle of this measurement at all. It only improves its precision by increasing the contribution of the CH_3 and CH_2 protons of the ethanol to the signal.

In the concentration range (0–12% (v/v)) encountered in fermentations, the linearity of the NMR measurement as a function of alcohol content is excellent ($r^2 > 0.99$) (Table III). For fermentation 1, $\sigma = 0.048$ and the confidence interval (S) with which the ethanol concentrations are determined is less than 0.5% (v/v) ($S = 0.23\%$ (v/v) for fermentation 1 in the middle of the range), disregarding the precision of the reference analytical methods.

The alcohol content determination is not hindered by the presence of sugars (glucose, fructose) or glycerol in the fermentation broth; these compounds possess a proton–proton coupling network with multiplicities and coupling constants different from those of the ethanol molecule.

3.3. Calibrating the NMR Measurement as a Function of the Sugar Concentration. When the paramagnetic reagent (Mn^{2+}, 1 mM) is added to the fermentation broth, the NMR signal obtained, after a single echo, $E(1/2J)$, arises from water protons and hydroxyl protons from the alcohol and sugars, in rapid exchange with the water protons, and nonexchangeable protons of the sugars present in the broth, glucose and fructose.

In fact, the contribution of the nonexchangeable ethanol protons to the echo is nil for a refocusing time of $t = 1/2J = 68$ ms

$$E(1/2J) \equiv I_W \exp(-t/T_2^W) + I_S \exp(-t/T_2^S) \quad (2)$$

where I_W and I_S are the proton concentrations of the OH protons of water and sugars and the CH and CH_2 protons of the sugars, respectively, T_2^W and T_2^S are their transverse relaxation times in the presence of $MnCl_2$; and $t = 1/2J = 68$ ms.

Now, $\exp(-68/T_2^W)$ remains approximately constant when the sugar concentration varies: $T_2^W \simeq 30$ ms for 100 or 200 g/L of glucose, and the amount of water varies little.

Of the 12 protons of each glucose molecule ($M = 180$ g), 5 are in rapid exchange with the water protons:

$$E(1/2J) \equiv k + \frac{5}{12}\frac{d_s}{180}\exp(-t/T_2^W) + \frac{7}{12}\frac{d_s}{180}\exp(-t/T_2^S) \quad (3)$$

Therefore, $E(1/2J)$ is related to the sugar content, d_s (g/L), by a relationship of the following type:

$$E(1/2J) = a + bd_s \quad (4)$$

This linear relationship was tested on the four fermentations studied, with good correlation coefficients (Table III). The excellent linearity confirms the hypothesis that neglects the interference with the measurement of the glycerol protons. Finally, the nonzero y intercept of the curve shows that in the presence of $MnCl_2$ (1 mM) the fermentation water signal, which is partially attenuated, still corresponds to half the total signal for a broth with a sugar concentration of 200 g/L. The proportion of the signal due to the sugars increases from 6% without paramagnetic reagent to approximately 50% (48–53%) in its presence, after the echo sequence. This increases the precision of the method considerably ($\sigma/b = 4.5$–7.7 g/L).

3.4. Monitoring the Fermentation Process Using Low-Resolution NMR. The calibration curves relating the NMR measurement to the ethanol and sugar concentrations were used to follow the kinetics of the fermentation of wine must (Carignan grape variety) by *S. cerevisiae*. Figure 1 shows the evolution of the sugar and ethanol concentrations as a function of time. The low-resolution NMR determinations can be superposed perfectly on the results obtained by chemical analysis, with a standard deviation of 3 g/L for the sugars and 0.2% (v/v) for the alcohol content.

Unlike the chemical analysis, which requires a lengthy preparation (>30 min) for each determination, low-resolution NMR provides, in one single measurement, both the sugar and alcohol concentrations. Since each measurement is stored after 100 accumulations, a result is obtained every

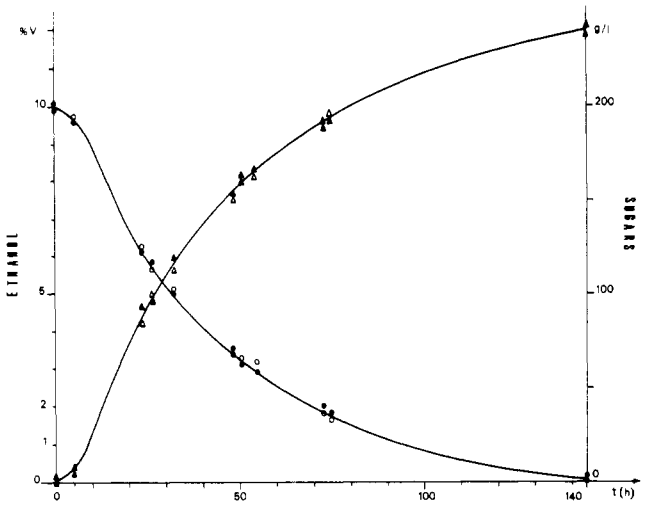

Figure 1. Fermentation kinetics (fermentation 1). Residual sugar concentration in the broth: chemical analysis (○), NMR determination (●). Alcohol content of the medium: chemical analysis (△), NMR determination (▲).

3.5 min for a sample volume not exceeding 2 cm^3.

During the fermentation process, the relative proportions of glucose and fructose change with time, the glucose being metabolized more quickly. However, this variation was not seen to affect the precision of the NMR determination of overall sugar content. Indeed, the NMR signal is proportional to the number of protons per unit volume, a quantity that is identical for glucose and fructose solutions with the same concentrations. The technique we propose is therefore valid for whichever sugar is metabolized, including saccharose with a proton density similar to that of glucose and fructose.

4. CONCLUSION

This low-resolution NMR technique provides simultaneous determinations of sugar and alcohol contents in a fermentation medium, in a short time (3.5 min). Sample preparation, simple filtration followed by addition of the relaxation reagent ($MnCl_2$, 1 mM), can easily be incorporated into an automated system (Marc et al., 1987). In addition, given the great similarity between the calibration curves obtained for the different vine varieties, it may be possible to use a single calibration scale to monitor the fermentation of all the grape musts.

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