Monitoring *pH* and lactic acid content during milk fermentation by *in situ* quantitative nuclear magnetic resonance spectroscopy (isq NMR)

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1. Introduction

During milk fermentation, micro-organisms produce several compounds, in particular they convert α- or β-lactose into D or L lactic acids [R]. The increase of D and L lactic acids content (*cLA*) during time is responsible of a *pH* decrease from milk *pH* (≈ 6.8) to a value below caseins' isoelectric point (≈ 4.6) [W]. The decrease of caseins micelle surface charges, in terms of absolute value, enhances interactions between these proteins: caseins micelles aggregate [T] and form a dairy gel (DG, a porous network of caseins).

Monitoring *pH* and *cLA* during fermentation is then essential to monitor DG formation and to evaluate strains efficiency. Several methods were developed and patented to evaluate *pH*, all being based on *pH*-meter measurements [C]. However, the use of *pH*-meter electrodes in a viscous and charged medium could raise the question of the precision of such methods [?]. No official method exists for measuring *cLA* but titrations using sodium hydroxyde are commonly used [T]. These uneasy-to-implement methods require manipulations.

Moreover, the added alkali modifies the medium and reacts with all acid species in the DG: the reliability of the measurment should be discussed.
In situ quantitative *(*isq) ^{1}H and ^{31}P nuclear magnetic resonance spectroscopies were used to correlate *cLA* and *pH. Isq* ^{1}H NMR showed promising results for the quantification of solutes in gels [A]. A validation of *cLA* by α- and β-lactose content (*cL*) was performed. *Isq* ^{31}P NMR was also used to measure intracellular *pH* following phosphate compounds chemical shift deviations, as phosp horic acid [P]. ????

2. Materials and Methods

5 solutions of D-L lactic acids in D^2O [7.425 g.L-1 to 74.25 g.L-1] (« solutions ol lactid acid standards, SLA_SD)
Racemic mix of D-L lactic acids from *PURAC, Spain / average concentration in DG:10.8 g.L-1* [B].
4 solutions (SL_SD) of α-lactose in D2O [14.82 g.L-1 to 142.8 g.L-1]
α-lactose (3% of β-lactose) from *Sigma Chemical*, USA / *average concentration in DG: 50 g.L-1* [B]

Isq ^{1}H NMR

***Superconducting Ultrashield 300 MHz** 7.05 T 54-mm magnet system NMR spectrometer Bruker Biospin*

32 scans of 32 K data points Automatic shimming and sample loading	
spectral width	6 kHz
acquisition time	2.7 s
recycle delay	25 s per scan
pulse angle	90°
internal lock	D2O

Fourier transformation with 0.3 Hz line broadening phase and baseline automatical correction by *XWINNMR 3.5* software (*Bruker Biospin*).
Temperature 20,00 °C (dev. < 0.01 K, *J* thermocouples)

Fermentation processes

Cow milk containing proteins (3.2%), saccharides (4.8 %) and lipids (<0.1%)
Inoculation by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*
Heating at 45 °C for 4 hrs (*J* thermocouples controls, deviation < 1 °C)
20 g sampling each 30 min for 4.5 hrs

- About exactly 0.7 g in a NMR tube (5mm glass, Wilmad Aldrich)*
- About 18 g sampling for *pH*-meter measurements

pH-meter measurements

3 determinations (stabilisation time: 3hrs) at 20.00 °C (*J* thermocouples)
pH-meter (*CyberScan pH* 1100, ATC probe, *EutechIntruments*, NL)
preliminary calibration (*pH* = 4.00, 7.03 and 10.06).

** Before Isq 1H NMR spectrum records, resp. Isq 31P NMR spectrum records, a homemade closed capillary tube containing a sodium salt of (trimethyl)propionic-2,2,3,3-d4 acid (TSP, Sigma Aldrich) dissolved in deuterium oxyde (D2O, Sigma Aldrich), resp. containing methylenediphosphonic acid (medronic acid, Sigma Aldrich) dissolved in D2O, was introduced into NMR tube, as internal reference. Every liquid or solid were weighed 3 times, means and standard deviations were considered. Integration was carried out 3 times using different integration intervals, and the mean and standard deviation were calculated using Maple 13 software.*

13 *pH*-controlled phosphoric acid (H3PO4) solutions (SPA_SD)
made from 50 g of a stock solution S0 (10.4 mmol.L-1 of H3PO4 in milli-Q water) and a calculated quantity of a stock buffer solution SC (10.0 mmol.L-1 of trisodium citrate in milli-Q water), chemicals from *Sigma Aldrich*. *pH* range : 3.00 - 6.42 (*pH*-meter measurements)

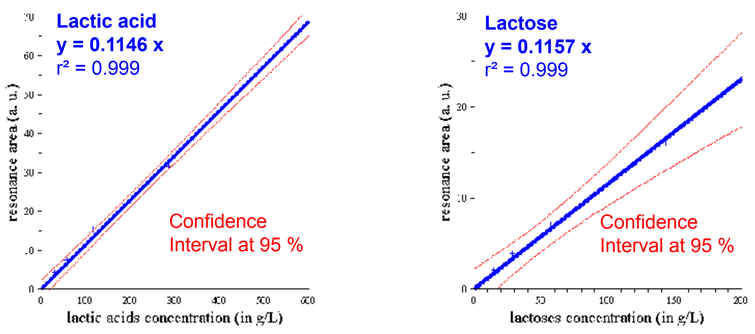
Isq ^{31}P NMR

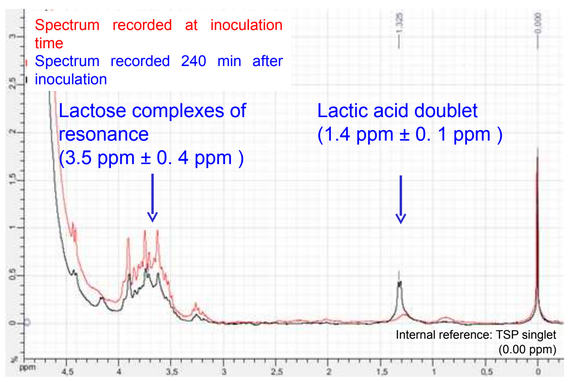
***Superconducting Ultrashield 300 MHz** 7.05 T 54-mm magnet system NMR spectrometer Bruker Biospin*

400 scans of 16 K data points Automatic shimming and sample loading	
spectral width	24 kHz
acquisition time	0.3 s
recycle delay	4 s per scan
pulse angle	90°
internal lock	D2O

Fourier transformation with 0.3 Hz line broadening phase and baseline automatical correction by *XWINNMR 3.5* software (*Bruker Biospin*).
Temperature 20,00 °C (dev. < 0.01 K, *J* thermocouples)

3. Results





Isq ^{1}H NMR spectra of milk recorded at lactic bacteria inoculation time (t = 0) and 240 min after lactic bacteria inoculation time: the area of the resonance of the doublet (d) at 1.4 ppm increases with time while the area of the resonance of the multiplet (cor) at 3.6 ppm ± 0.2 ppm decreases with time.

Calibration curves

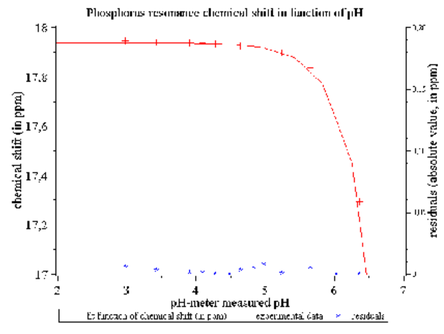
On ^{1}H NMR spectra of SLA_SD ???? solutions, besides water resonance, two resonances were observed: one at 1.4 ppm (d, *J* = 7.02 Hz) and one at 4.4 ppm (q, *J* = 7.03 Hz). Integration of resonances, performed using NMR Notebook software (NMR Notebook 2.50 build 0.0, 2000-2008), showed that their areas increases with *cLA*.
On ^{1}H NMR spectra of SL-SD solutions, besides water resonance, 5 other resonances (3.2 ppm, t, *J* = 8.24 Hz; 3.6 ppm ± 0.2 ppm, m; 4.4 ppm, d, *J* = 7.78 Hz; 4.6 ppm, d, *J* = 7.88 Hz; 5.157ppm, d, *J* = 3.73 Hz) were observed: their areas increased with *cL*.
Isq ^{31}P NMR spectra of SPA_SD solutions showed two resonances: a singlet and a triplet (*J* = 21.20 Hz). The difference between the chemical shifts of these two resonances decreases when the *pH*-meter measured *pH* increases.

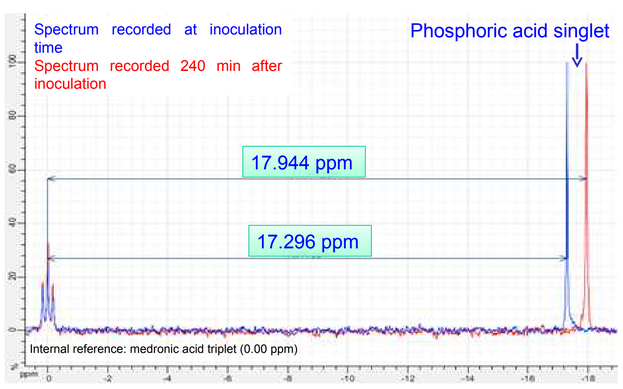
Isq ^{1}H NMR spectra

Spectra include the resonances obtained for both SLA_SD and SL_SD solutions. A large resonance at 4.8 ppm is observed, due to water; its area is about 10 times larger than d and cor areas, but it does not prevent the determination of their areas, around 1.4 ppm, respectively between 3.4 ppm and 4.0 ppm, where D-L lactic acids resonances, respectively α- and β-lactoses resonances, should appear []. ?????

Isq 31P NMR spectra

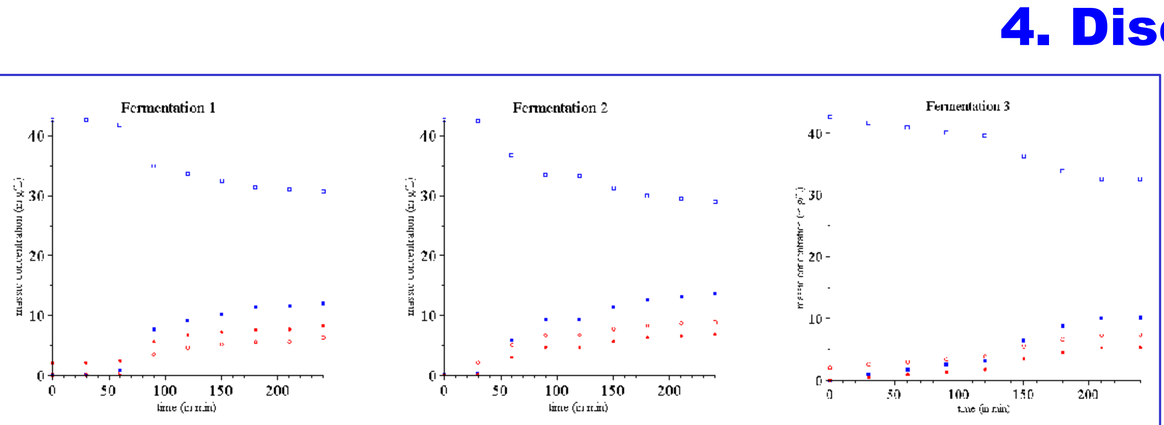
Spectra showed the resonances obtained for SPA_SD solutions . The difference between their chemical shifts (*Δst*), increases when the time of fermentation increases.



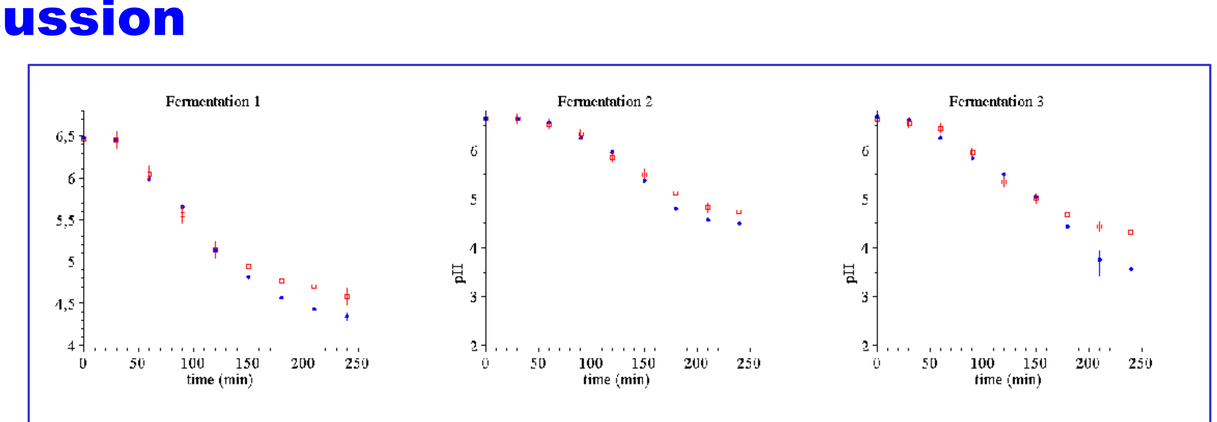


Superimposition of two *Isq* ^{31}P NMR spectra of milk recorded at lactic bacteria inoculation time (t = 0) and 240 min after lactic bacteria inoculation time (t = 240): a difference of chemical shifts between phosphoric acid singlet and medronic acid triplet (internal reference) is observed.

4. Discussion



Mass concentrations of lactic acids (○) and lactoses (□) in DG in function of time. Consumed lactoses (■) and produced lactic acids (●) are displayed. Uncertainities are too small too be visible.



pH measured by pH-meter (□) and by ^{31}P NMR (○). Uncertainities are displayed.

Lactic acids and lactoses initial contents and their variations are in accordance with literature [], differences between these 3 fermentations being explained by the non-repetability of fermentation processes (strains, experimental conditions…). The average ratio between produced lactic acids and consumed lactoses is 2.03 (standard deviation 0.05), which is a validation knowing that the involved lactic bacteria convert 1 molecule of lactose into 2 molecules of lactic acid *via* homofermentation through Embden-Meyerhof pathway [].

Isq ^{31}P NMR measured *pH* is 1,000 times more precise than *pH*-meter measured *pH* for *pH* > 4.1. The loss of precision for *pH* < 4.1 is coherent with the plateau observed on calibration curve. *pH*-meter measured *pH* and *Isq* ^{31}P NMR measured *pH* have the same variations, in accordance with literature []. However, significant differences are observed for *pH* below 5.0. The assumption of the inoculated milk viscosity increase can be made, as viscosity of the media highly increases around *pH* 5.0 [L].

5. Conclusions and Perspectives

Isq ^{1}H and ^{31}P NMR spectroscopies are useful tools for the monitoring of *pH* and for the quantification of lactic acids and lactoses, in particular during fermentation. These methods remains to be validated, but it appears clearly that a difference between *pH*-meter measured *pH* and *Isq* ^{31}P NMR measured *pH* appears when *pH* is below 5.0. The influence of viscosity and protein zeta potential can be assumed to be responsible of this difference. This work should go on with the investigation of relationships between *pH* measured *Isq* ^{31}P NMR and *cLA measured by Isq* 1H NMR. The understanding of textural properties of DG could be improved, as suggested by Allais [Al]. Stains efficiency could also be studied using these methods. Finally, this work could help to investigate acidity as a sensory descriptor.

References:

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