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Ripening of banana fruit monitored by water relaxation and diffusion ^{1}H-NMR measurements

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Abstract

Water transverse relaxation times T_2 and self-diffusion coefficients D have been measured on banana samples at different ripening stages. Relaxation data have been interpreted on the basis of a chemical and diffusive exchange model proposed by Belton and Hills [Mol. Phys. 61(4) (1987) 999] and Hills et al. [Mol. Phys. 67(4) (1989) 903]. According to that model the observed increase of T_2 values of both cytoplasmatic and vacuolar water may be mainly attributed to the decrease of starch concentration during the ripening process. On the other hand, the observed water self-diffusion coefficient decrease is related to sugar accumulation as starch hydrolysis proceeds. At the early stages of ripening, the individual self-diffusion coefficient values of cytoplasmatic and vacuolar water differ from one another and have been calculated through the analysis of relaxation time-separated pulsed field gradient nuclear magnetic resonance experiments [J. Magn. Reson. A 112 (1995a) 237].

1. Introduction

Proton nuclear magnetic resonance (NMR) has been widely used to investigate the physicochemical properties of water in plant tissues and in foods (Belton & Colquhoun, 1989; Belton, Colquhoun, & Hills, 1993; Belton & Ratcliffe, 1985; Colire, Le Rumeur, Gallier, de Certaines, & Larher, 1988; Connelly, Lohman, Loughman, Quiquampoix, & Ratcliffe, 1987; Gil, Belton, & Hills, 1996; Ratković, Bačić, Radenović, & Vučinić, 1982). In particular, relaxation times (T_1 and T_2) and molecular self-diffusion coefficient (D) NMR measurements on intact tissue have been employed for studying, by a non-destructive technique, the dynamics of water molecules as well as the sub-cellular water distribution within plant tissues and water molecule transport properties in sub-cellular compartments (Callaghan, Jolley, & Lelièvre, 1979; Hills & Belton, 1989; Snaar & Van as, 1992).

Whereas NMR diffusion measurements provide direct information about translational mobility of water molecules (Tanner & Stejskal, 1968), proton relaxation times, especially in heterogeneous systems, are not simply related to the dynamic behaviour of water molecules. This makes it necessary to apply theoretical models capable of accounting for the various contributions to relaxation and, therefore, to adequately rationalise the experimental results (Hills, Takacs, & Belton, 1989; Hills, Takacs, & Belton, 1990; Hills, Wright, & Belton, 1989).

The transverse relaxation of water protons in plant tissues is multiexponential and much faster than in bulk water; the three decaying components have been commonly associated to water localized in different subcellular compartments (Bačić & Ratković, 1984; Stout, Steponkus, & Cotts, 1978). Hills and Duce (1990) proposed a simple cellular relaxation model based on chemical and diffusive exchange of water protons in order to account, semi-quantitatively, for the water proton transverse relaxation behaviour in parenchyma tissue of courgette, onion and apple. According to this approach, the intrinsic transverse relaxation rate of water in a particular compartment (the vacuole, the cytoplasm, the extra cellular space, or water associated with the cell wall) is controlled by proton chemical exchange between water and biopolymers and/or metabolites inside the compartment. Moreover, water

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molecules are in diffusive exchange between sub-cellular compartments and this can produce an averaging of the intrinsic relaxation times and of the observed relative amplitudes of water present at these different sites. The extent of this diffusive averaging depends on the exchange rates, the intrinsic relaxation times and the cell morphology, the exchange rates between the compartments being in turn controlled by membrane permeabilities and/or water diffusivity within each site. Therefore, the diffusive averaging can be misleading in the assignment of the various components of a multi-exponential transverse relaxation curve to different sub-cellular compartments.

In order to test the reliability of the former interpretation (a simple one to one correspondence between transverse relaxation components and water in distinct sub-cellular compartments), Snaar and Van as (1992) probed water distribution within cells of parenchyma tissue of apple by proton relaxation time measurements and, in particular, by following the uptake of Mn^{2+} ions; they concluded that, in that case, it was substantially correct to assign the three transverse relaxation components to water present, respectively, in the vacuole, in the cytoplasm and in the extra cellular space (or associated with the cell wall). In studies about water sub-cellular distribution in potato tissue and apple tissue (Hills & Le Floc'h, 1994; Hills & Remigereau, 1997), a tentative interpretation was made by assuming, as a first approximation, a simple correlation between the T_2 peaks of a continuous T_2 distribution within a individual sub-cellular compartment.

All these works show that relaxation studies represent a powerful tool for monitoring sub-cellular water compartmentations. On the other hand, NMR self-diffusion coefficient measurements, as measured by the traditional pulsed field gradient spin echo (PFG SE) pulse sequence (Stejskal & Tanner, 1965), only provide an average value of the diffusion coefficients, characteristic of each compartment. However, van Dusschoten, de Jager, and Van as (1995b) have recently proposed a new method for resolving diffusion coefficients in heterogeneous systems characterised by several water-containing compartments that differ in relaxation time values and diffusion coefficients; they demonstrated that the combination of T_2 and diffusion measurements at different echo times, based on the pulsed field gradient multiple spin echo (PFG MSE) sequence (van Dusschoten, de Jager, & Van as, 1995a), allows the determination of water diffusion constants associated with different transverse relaxation components and, hence, with distinct compartments. By the application of this approach (diffusion analysis by relaxation-time-separated – DARTS-PFG NMR) to water in apple parenchyma tissue, they separately determined the water diffusion coefficient in the cell vacuole and in the cytoplasm. In short, the combination of T_2 measurements, by the CPMG sequence, and self-diffusion measurements, by the PFG MSE sequence and the DARTS analysis, represents a valuable approach to the study of cellular systems, providing information not only about sub-cellular water distribution but also even dynamic properties of water in each sub-cellular compartment.

Changes in water NMR dynamic properties during ripening, processing and storage operations on fruits and vegetables can, in principle, reveal sub-cellular modifications and contribute to a microscopic understanding of these processes (Gil et al., 1996). Examples of the application of this approach are represented by NMR studies of non-freezing water in potato tissue on freezing and thawing and of sub-cellular water compartmentation in apple tissue during drying and freezing (Hills & Le Floc'h, 1994; Hills & Remigereau, 1997).

In the present work, a combination of transverse relaxation times and self-diffusion measurements has been used to observe the microscopic sub-cellular changes associated with banana ripening. Our aim was to test the potential of this approach for monitoring the changes in sub-cellular distribution, relaxation behaviour and translational mobility of water associated with the variations of chemical composition occurring during fruit ripening. To explore this possibility, we chose banana as a model system, because its ripening is a relatively rapid process, characterised by marked changes in chemical composition, particularly as concerns starch, present as insoluble granules, and its degradation to soluble sugars.

In addition to low-resolution NMR measurements, high-resolution ^{1}H-NMR spectra have been obtained on extracts from banana tissues in order to identify the individual sugars present in the cellular sap and to determine their relative concentrations during ripening.

High-resolution NMR techniques were applied to the study of banana ripening by Ni and Eads (1992, 1993a, 1993b); they determined the chemical composition of the liquid phase and obtained information about the composition of the solid phase by measurements on *intact* samples of edible banana tissue during ripening.

In our work, conventional spectra have been obtained on homogeneous samples prepared by simple extraction procedures, by an instrument operating at a ^{1}H frequency higher than that of spectrometers employed in the studies mentioned above, thus providing improved resolution.

2. Materials and methods

2.1. Sample preparation

Bananas (*Musa* sp.) purchased from a commercial warehouse at their very green stage were stored at room temperature (25 ± 2 °C) under normal fluorescent lights

for about 10 h per day. A traditional classification of ripening stages of banana, proposed by Von Loesecke (1950), defines nine consecutive stages, corresponding to the banana peel colour: stage 0, "green", corresponds to the freshly harvested fruit, not yet exposed to ripening gas, while stage 8 is characterised by a "yellow with many brown spots" peel. A less subjective characterisation of the ripening process is based on the internal concentration of ethylene and CO_2 measure; the starch conversion to soluble sugars starts, approximately, at the same time as the peak in ethylene synthesis and is completed in a period of 6–8 days (Beaudry, Severson, Black, & Kays, 1989). Our measurements began when bananas were at roughly stage 1–2 (green peel) according to the Von Loesecke (1950) classification and Beaudry et al. (1989) and lasted for the following 7–8 days.

For low-resolution measurements, samples were prepared by cutting a cylindrically shaped piece of edible tissue (approximately 600 mg), which was transferred to a 10 mm o.d. NMR tube and, to prevent water evaporation, a teflon insert was put into the tube, which was then sealed by a laboratory film. Samples used in the same set of experiments, but at different ripening stages, were cut from the same fruit.

For high-resolution measurements, slices were cut from two fruits of a single bunch, then weighed, ground and centrifuged for 15 min at high speed; approximately 0.5 ml of the liquid phase extracted was weighed. 0.2 ml of 0.1 M TSP and phosphate buffer (pH 7.4 in D_2O) were then added to a final volume 1 ml in order keep pH and ionic strength constant.

2.2. Low-resolution NMR measurements

Measurements were performed on a Minispec PC 120 pulsed NMR spectrometer (Bruker Spectrospin Company), with an operating frequency of 20 MHz for protons.

Transverse relaxation times were determined by the CPMG sequence (Meiboom & Gill, 1958) with a pulse spacing τ, between two following pulses, of 1 ms. 49 scans were acquired with a recycle delay of 10 s. The sample were thermostatted for 30 min in the NMR probe-head at (25 ± 1) °C before the experiments.

The amplitudes and relaxation times of the different components were extracted from multiexponential decay curves by means of a non-linear least-squares data fitting programme, based on the Marquardt algorithm (Marqardt, 1963).

Water self-diffusion (average) coefficient measurements were carried out by the standard PFG SE sequence (Stejskal & Tanner, 1965). At any given gradient amplitude G, and keeping fixed Δ, the time interval between the two gradient pulses of duration δ, the amplitude of the NMR signal at fixed echo time is given by

$$A_G = A_0 \exp(-kD), \quad (1)$$

where A_0 is the echo amplitude in absence of the pulsed gradients, and k is given by $k = (\gamma\delta G)^2(\Delta - \delta/3)$ (γ is the proton magnetogyric ratio). D values were determined by a monoexponential fitting of the echo amplitudes measured at different G values (from 0.541 to 0.673 T m^{-1}), while Δ was fixed at 20 ms and δ at 0.8 ms.

To resolve water self-diffusion coefficients contained in different sub-cellular compartments, experiments were performed by the PFG MSE sequence (van Dusschoten et al., 1995a, 1995b), depicted in Fig. 1. For each echo time (TE), eight A_G values were acquired by varying G between 0.214 and 0.712 T m^{-1}; δ was chosen as 1.732 ms, according to the formula $2\tau^2 = (2/3)\delta^2$, in order to reduce the background effect or in situ gradients, and Δ was 6 ms. From one experiment to another, the echo time, given by $2n\tau$ (n is the total number of π pulses), was varied by changing the number of π pulses before the first gradient pulse, while keeping Δ constant. Again, the diffusion coefficient D was calculated by a monoexponential fitting of the echo amplitude, according to Eq. (1); in this situation, an apparent value of D (D_{app}) is determined, because of the contribution of water of different compartments to the overall water signal. Since, in general, not only the D, but also the T_2 values are different for water molecules located in different compartments, the D_{app} values depend on TE, and are

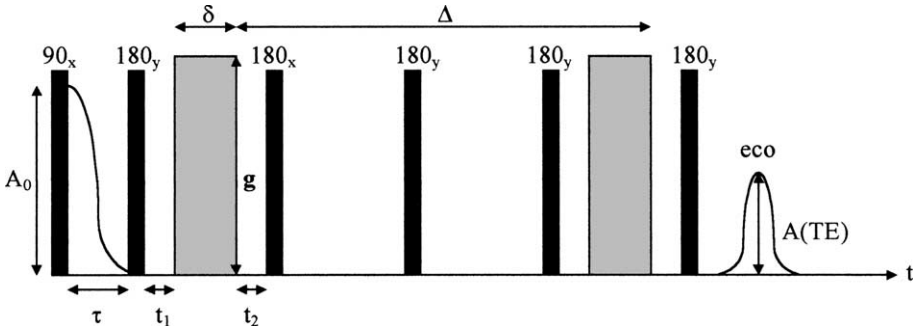

Fig. 1. Pulsed field gradient multiple spin echo (PFG MSE) sequence scheme (van Dusschoten et al., 1995a, 1995b).

related to the individual diffusion coefficients D_i through the following formula:

$$\sum_i f_i(\mathrm{TE}) kD_i = kD_{\mathrm{app}}(\mathrm{TE}). \quad (2)$$

The coefficients $f_i(\mathrm{TE})$ are the fractions of the signal amplitude related to different water components, respectively, at a given echo time. For each component, the fraction is given by

$$f_i(\mathrm{TE}) = \frac{A_i(\mathrm{TE})}{\sum_i A_i(\mathrm{TE})}. \quad (3)$$

The values of $A_i(\mathrm{TE})$ are calculated by the formula

$$A_i(\mathrm{TE}) = A_i(0) \exp\left(-\frac{\mathrm{TE}}{T_{2i}}\right) \quad (4)$$

and the values of $A_i(0)$ and T_{2i} are obtained by a CPMG experiment carried out with the same pulse spacing as PFG MSE. By performing experiments at different TEs, while maintaining the same Δ, it is possible to extract the self-diffusion coefficients of water related to different components if they are less than a factor of 2 apart. For this reason, van Dusschoten et al. (1995a, 1995b) named this approach "diffusion analysis by relaxation-time separated" (DARTS) PFG NMR. This data analysis method leads to errors in the diffusion coefficient below 10% in the case of two water fractions, while the standard approach of a biexponential fit to the diffusion data gives larger errors (>25%) in identical circumstances.

2.3. High-resolution NMR measurements

High-resolution experiments were performed on a pulse Fourier transform NMR spectrometer (Bruker, AM 500), operating at a magnetic field of 11.744 T and equipped with a two-channel probe $^1H/^{13}C$.

Water resonance was presaturated by irradiation with a weak rf field, using the decoupler channel in heteronuclear mode; the decoupler was switched off before the rf excitation pulse and acquisition. Experimental data were transferred to a PC and analysed with the WIN NMR data processing software. Quantitative analysis of the signals areas was carried out by comparison to the integral of TSP resonance. The variation of the soluble sugars amount was quantified by the integration of the following NMR resonances: the doublet at 4.22 ppm of sucrose (H-3′), the triplet at 3.25 ppm of glucose (β H-2), the doublet at 4.12 ppm of fructose (H-4′). The weight percentages of the different sugar components (glucose, fructose and sucrose) in the extracted liquid phase ($\%_{\mathrm{sug/liq}}$) were obtained by the following expression:

$$\%_{\mathrm{sug/liq}} = \frac{\frac{A_{\mathrm{sug}}(^1\mathrm{H})}{A_{\mathrm{TSP}}/9} \cdot 10^{-2} \cdot \mathrm{MW}_{\mathrm{sug}} \cdot 10^{-3}}{g_{\mathrm{liq}}} \cdot 100, \quad (5)$$

where $A_{\mathrm{sug}}(^1\mathrm{H})$ is the signal area of the considered sugar, normalized to one proton, and $\mathrm{MW}_{\mathrm{sug}}$ its molecular weight (180 g mol^{-1} for the hexoses, 342 g mol^{-1} for sucrose and other soluble sugars), A_{TSP} is the area of the TSP resonance (corresponding to 9 protons), whose concentration in the samples is equal to 10^{-2} M, and g_{liq} is the weight of liquid extracted from the fruit to prepare the sample. Total sugar content was estimated by the sum of signal areas of all sugars, from 3.38 and 3.96 ppm.

3. Results and discussion

3.1. Low-resolution NMR measurements

Cells from the edible banana tissue are characterised, as most of mature plant cells, by the presence of two principal sub-cellular compartments, the vacuole and the cytoplasm, a variety of organelles and the cell wall. The peculiarity of banana cells is represented by the existence of many membrane-bound starch granules that, in a fruit at the "green" stage, constitute ca. 20% of fresh weight and fill a considerable fraction of cellular volume, while soluble sugars content is relatively low, ca. 1–3% (Lii, Chang, & Young, 1982). Starch granule sizes range from ca. 5 to 80 μm and most of them fall within the range 20–60 μm (Beaudry et al., 1989). During the critical stage of the ripening process (the *climacteric peak*), a period of approximately eight days, starch is rapidly and almost completely converted to soluble sugars and, in a minor extent, to CO_2 (Beaudry et al., 1989; Cordenunsi & Layolo, 1995); at the end of this stage, starch content is approximately 1–3% of fresh weight while soluble sugars have accumulated to ca. 16–18%.

In order to monitor these marked chemical composition changes through the variations produced in water relaxation behaviour, proton transverse relaxation measurements were performed by the CPMG sequence on samples obtained from a single banana, over a period of seven days. The analysis of magnetisation decay curves showed the presence of three components. Table 1 shows the relaxation times and the relative amplitudes, of the three components at different ripening stages. The observed signal can be essentially attributed to water protons and exchanging protons on biomolecules, because non-exchanging protons in starch and in cell wall components have relaxation times which are too short to contribute to the signal on the time scale of our experiments; other low molecular weight metabolites do not significantly contribute either, owing to their relatively small amount.

The three components may not be directly related to water present in different sub-cellular compartments because contributions from distinct compartments may overlap and diffusive exchange may, to some unknown extent, average the magnetisation so that a simple

Table 1
Proton transverse relaxation times and signal percentages of the three components during seven days of storage. Data are the averages of five samples from the same section of the fruit[a]

Storage time (days)	Cellular wall		Cytoplasm		Vacuole	
	T_2 (ms)	%	T_2 (ms)	%	T_2 (ms)	%
0	14 ± 3	17 ± 3	90 ± 10	27 ± 4	320 ± 30	56 ± 4
1	21 ± 4	16 ± 3	100 ± 10	33 ± 5	370 ± 30	51 ± 6
2	18 ± 3	13 ± 3	110 ± 10	27 ± 5	410 ± 30	60 ± 6
3	20 ± 3	13 ± 3	140 ± 10	28 ± 5	470 ± 40	59 ± 6
4	21 ± 3	11 ± 1	170 ± 10	29 ± 4	510 ± 20	60 ± 5
7	27 ± 1	13 ± 2	190 ± 10	25 ± 5	610 ± 10	62 ± 5

All data were obtained from a single banana.
[a] Errors represent the standard deviations of the values of five samples.

correlation between components and sub-cellular compartments does not necessarily exist. However, neglecting these complications, and according to previous works about plant cells (Hills & Le Floc'h, 1994; Hills & Remigereau, 1997; Snaar & Van as, 1992), the following interpretation can be made. The shortest relaxation time component can be attributed to water strictly associated with cell walls or inside starch granules, as supposed in a study about starch-rich potato tissue cells (Hills & Le Floc'h, 1994). The relatively short value of T_2 can be explained on the basis of a chemical exchange effect produced by rapid proton exchange between water and polysaccharide hydroxyls, characterised by low intrinsic T_2 values (few microseconds). The intermediate T_2 component can be assigned to water located within the cytoplasm; in it the cytoskeleton constitutes a three-dimensional network of proteic structures that confers, to the compartment, a gel thickness; the high viscosity of the cytosol and proton chemical exchange between water and proteins (cytoskeleton, enzymes in solution), and components of other membrane-bound organelles, can account for the intermediate value of T_2. The third component may be attributed to water residing in the vacuole; the sap in this compartment can be essentially considered as a relatively dilute solution of various metabolites and, in particular, sugars; the reduction of T_2, compared with pure water, may be explained by considering the higher viscosity and proton chemical exchange between water and sugars.

Table 1 shows that, during seven days of storage, T_2 values attributed to cytoplasmatic and vacuolar water show a gradual, marked increase, $T_{2,\mathrm{cyt}}$ from 90 to 190 ms, and $T_{2,\mathrm{vac}}$ from 320 to 610 ms. In a qualitative interpretation it may be supposed that these trends are mainly correlated with the disappearance of starch, which acts as a relaxation sink, while the increasing sugar concentration produces only a minor effect on T_2 values.

In fact, proton chemical exchange between water and starch hydroxyls strongly decreases the water relaxation time inside starch granules and, to a lesser extent, cytoplasm and vacuolar water relaxation time, due to diffusive exchange of water molecules through the membranes bounding these compartments. The disappearance of starch granules during banana ripening reduces the cytoplasmatic and vacuolar water fractions that can be influenced by the chemical and diffusive exchange effect, and so produces an increase in water T_2 values of both cytoplasm and vacuole.

A similar increase of water T_2 values, corresponding to decreasing starch concentration, was observed by Callaghan, Jolley, Lelievre, and Wong (1983) on wheat starch pastes: decreasing starch amount from 8–2%, to 1%, gave place to T_2 values of ca. 300, 750 and 1250 ms, respectively. In a quantitative comparison, it can be seen that, in starch pastes, a smaller decrease of starch concentration (from 8% to 1%) produced a larger increase of T_2 value (approximately four times) if compared with our results on banana (a decrease from 16–18%, to 1–3%, produced, roughly, a doubling of T_2 values of vacuole and cytoplasm). Really the contribution to relaxation due to proton exchange with starch, that is probably the dominant effect in starch pastes, is only one among several contributions to relaxation in banana cells. It may be expected, indeed, that transverse relaxation in banana cells is significantly influenced, not only by proton chemical exchange with the structures of cytoskeleton membranes and sub-cellular organelles, but also by diffusion of water molecules through in situ field gradients; a quantitative interpretation has to take into account the effect of morphological factors and diffusive exchange of water molecules between membrane-bound compartments.

A semi-quantitative interpretation of T_2 data relative to a single compartment has been carried out by applying equations derived from the chemical exchange model proposed by Belton and Hills (1987), Hills et al. (1989) and Hills et al. (1990).

A proper equation to describe this situation is the following one:

$$T_{2,\mathrm{obs}}^{-1} = p_a T_{2a}^{-1} + \frac{f \cdot p_{b,\mathrm{starch}}}{T_{2b,\mathrm{starch}} + k_b^{-1}} + \frac{p_{b,\mathrm{sugars}}}{T_{2b,\mathrm{sugars}} + k_b^{-1}} \qquad (6)$$

where $T_{2\mathrm{a}}$ is the pure water relaxation time (ca. 2 s), $T_{2\mathrm{b}}$ is the intrinsic relaxation times of exchanging starch and sugar protons and p_a and p_b represent the fractions of

water and exchanging carbohydrate protons, respectively. The second term accounts for the exchange with starch protons characterised by $T_{2b,\text{starch}}$ (of the order of tens of microseconds) and the third term accounts for the exchange with sugars protons characterised by $T_{2b,\text{sugars}}$ (of about 1 s); k_b^{-1} is the mean lifetime of exchanging protons on the carbohydrate (ca. 10^{-3} s) and the f factor, in the second term, is the fraction of starch protons effectively accessible to exchange with water.

The third term is negligible, since it produces only a slight decrease of water relaxation time.

In fact, in the case of measurements performed at relatively low values of magnetic field intensity, as in our experiments, the contribution to relaxation due to chemical exchange with protons of, e.g. glucose at a concentration of $20\%_{w/w}$, can be accounted for by assuming that k_b^{-1} is approximately 10^{-3} s, T_{2a} is ca. 2 s, T_{2b} is ca. 1 s, and for a 20% glucose solution, $p_b = 0.045$ (p_a can be assumed equal to unity). It turns out that $T_{2,\text{obs}}$ is approximately 1.9 s and, hence, as expected, it shows only a slight decrease with respect to the value of pure water. It can be concluded that the contribution due to proton exchange with sugars plays only a minor role, with respect to exchange with starch, in determining water T_2 values in banana cells.

Moreover, in a semi-quantitative interpretation of T_2 values attributed to cytoplasm, by putting into Eq. (6) the T_2 values measured at extreme stages of storage (90 ms at day 0 and 190 ms at day 8) and the concentration of starch (ca. 20%, $p_{b,\text{starch}} = 0.0516$ and ca. 3%, $p_{b,\text{starch}} = 0.0053$), it is possible to determine the values of f corresponding to these fruit ripening stages. These are 0.09 and 0.81.

The value of f of 0.09 for the "green" banana shows that only a small fraction of starch exchangeable protons are accessible to water molecules within starch granules. On the other hand, the f value obtained for "yellow" banana shows that the polysaccharide hydroxyl groups, at the end of the ripening process, are almost completely exposed to water molecules, due to the action of hydrolytic enzymes on starch molecular chains.

As mentioned above, the disappearance of starch is accompanied by a consistent increase in concentration of soluble sugars, approximately from 1–3%, to 16–18% of fresh weight. This increased concentration can produce only a slight decrease in T_2 values. It was demonstrated in a previous work (Brosio, D'Ubaldo, & Verzegnassi, 1994) that, in this sugar solution concentration range, the non-exchanging sugar proton signal can be neglected, and that exchanging sugar and water proton T_2 values can be quantitatively interpreted by equations derived from the chemical exchange model proposed by Belton and Hills (1987) and Hills et al. (1989).

As regards the vacuole, the increase of water T_2 values cannot be ascribed to increasing sugars concentration but may be explained by taking into account the disappearance of starch occurring in other compartments and diffusive averaging effect. This contribution to vacuolar T_2 values cannot be simply evaluated by a semi-quantitative approach, similar to that used for cytoplasm, because the contribution of geometrical factors and membrane permeability to T_2 values cannot be evaluated (Hills, Belton, & Quantin, 1993).

Table 1 shows that the relative amplitude of signals attributed to different compartments remains substantially constant during the ripening period examined, and this may suggest that no marked changes in water distribution occur during this process. The slight reduction in the amplitude of the "fast" component (from 17% to 11–13%) may be ascribed to disappearance of starch granules and, hence, of the water fraction inside them.

In our study, besides relaxation measurements, diffusion experiments have been performed in order to evaluate the influence of the above-mentioned compositional changes on the translational mobility of water molecules within the cellular sap.

Experiments performed by PFG SE sequence were carried out to measure a single self-diffusion coefficient, D, of water in the samples, that represents *an average* of different coefficients associated with water molecules experiencing different chemical and physical environments within the cell. Table 2 shows the results obtained on tissue samples cut from a single fruit during 8 storage days. These D values were much lower than that of pure water ($D^{25\,^\circ\text{C}}_{\text{water}} = 2.30 \times 10^{-9}$ m^2 s^{-1}), and significantly lower than those observed in apple parenchyma tissue by Tanner and Stejskal (1968) ($D = 1.81 \times 10^{-9}$ m^2 s^{-1}). Moreover, the D values show a gradual decrease from $(1.21 \pm 0.04) \times 10^{-9}$ m^2 s^{-1}, for a banana at the "green" stage, to $(1.01 \pm 0.02) \times 10^{-9}$ m^2 s^{-1} for the fruit at full ripeness. The first observation can be, at least partly, explained by considering the smaller extent of the vacuolar water fraction in banana cell with respect to that in apple tissue, the vacuolar components being ca. 55–60% and 75–80%, respectively; in fact, it has been observed that this fraction of water is characterised by a

Table 2
Averaged water self-diffusion coefficient measured by PFG SE pulse sequence during seven days of storage

Storage time (days)	D (10^{-9} m^2 s^{-1})
0	1.21 ± 0.04
1	1.16 ± 0.02
3	1.08 ± 0.02
4	1.07 ± 0.02
5	1.07 ± 0.04
6	1.00 ± 0.04
7	1.01 ± 0.05
8	1.01 ± 0.02

larger translational mobility than cytoplasmatic or cell wall water (van Dusschoten et al., 1995a). The second observation may be discussed by taking into account the effect on the water D of the disappearance of starch and the increase of soluble sugar concentration. The influence of different starch concentration on water D was studied in wheat starch pastes in the above mentioned work (Callaghan et al., 1983); data obtained on a starch paste and on an amylopectin-water system showed an approximately linear decrease of D with increasing concentrations of starch, from 0% to 10%. On the other hand, in banana cell, a decrease of D was observed with the reduction of starch concentration during ripening. Whereas in pastes, gel particles of starch granule fragments are in direct contact with the aqueous phase, in banana cells, starch granules are separated from the cellular fluid by membranes. A more marked effect on water D values can be produced by the increased sugar concentration in cellular sap as ripening proceeds. Data reported in the literature (Brosio et al., 1994) and measurements carried out in our laboratory have shown a considerable reduction of D in solutions of increasing sucrose concentration (0, 10, 20%$_{w/w}$), from 2.29–2.14, to 1.28×10^{-9} m^2 s^{-1}: the smaller translational mobility of water molecules can be explained on the basis of the increase in solution viscosity associated with a solute-induced enhancement of the hydrogen bond formation (Brosio et al., 1994). It may be supposed, therefore, that the predominant effect in determining water D value in banana samples is that associated with the increase of sugar concentrations. Furthermore, the smaller D values observed in banana cells, with respect to simple sucrose solutions over the same concentrations range, may be ascribed to the complex internal cellular structure (the cytoplasm gel thickness, the presence of several organelles, starch granules and membranes).

In order to resolve the different cellular compartment water self-diffusion coefficients and to measure the water translational mobility in the vacuole and in the cytoplasm, the PFG MSE technique and the DARTS analysis (Barbieri, Quaglia, Delfini, & Brosio, 1998; Brosio & Barbieri, 1996; van Dusschoten et al., 1995a, 1995b) described in Section 2, was employed.

In biological compartmentalised systems, such as a cell, because of the small differences among the diffusion coefficients associated with each compartment and of the lack of resolution of the standard diffusion measurements (PFG SE), a single value of D is often observed and is considered sufficient to describe the water mobility in the system. The DARTS PFG NMR approach takes advantage of the possibility of simply and independently varying the echo time (TE) and the effective diffusion time ($\Delta - \delta/3$) in a CPMG-like sequence, such as PFG MSE. Thus, the overall echo amplitude is made dependent on the amplitude and the transverse relaxation times values of the different components. Consequently, the apparent diffusion constant (D_{app}) is affected by the time at which the echo amplitude is sampled, so that self-diffusion coefficients related to distinct relaxation components and, hence, to water located within different compartments, can be calculated. Measurements were performed by the PFG MSE sequence, at two different echo times (18 and 122 ms), on samples cut at different ripening stages. Different results were obtained, depending on the ripening stages: in the early stages, it was possible to show different D values for the two compartments, whereas in the following stages the D_{app} values, obtained at different echo times, were substantially identical (results not shown). Data obtained on the first two storage days are shown in Table 3. The obtained results of water diffusion coefficients in cytoplasm, ($D_{cyt} = 0.75 \pm 0.1 \times 10^{-9}$ m^2 s^{-1}), and in vacuole, ($D_{vac} = 1.1 \pm 0.1 \times 10^{-9}$ m^2 s^{-1}), showed a smaller mobility of water molecules located in the cytoplasm with respect to those in the vacuole, in agreement with the structural properties of the two compartments. The measured value of water translational diffusion coefficient in banana cytoplasm, ($D_{cyt} = (0.75 \pm 0.1 \times 10^{-9}$ m^2 s^{-1}), is in agreement with values reported for erythrocyte cytoplasm (0.89×10^{-9} m^2 s^{-1})

Table 3
Apparent self-diffusion coefficients (measured by PFG MSE), signal fractions (measured by CPMG) and calculated self-diffusion coefficients (by DARTS method) of cytoplasmatic and vacuolar water during the early days of storage

Storage time (days)	PFG MSE sequence		CPMG sequence	
	TE (ms)	D_{app} (10^{-9} m^2 s^{-1})	f_{cyt}	f_{vac}
1	18	1.05 ± 0.03	0.183 ± 0.002	0.817 ± 0.002
	122	1.11 ± 0.03	0.050 ± 0.002	0.950 ± 0.002
2	18	1.08 ± 0.01	0.155 ± 0.002	0.845 ± 0.002
	122	1.11 ± 0.02	0.054 ± 0.002	0.946 ± 0.002
Storage time (days)	DARTS method			
	D_{cyt} (10^{-9} m^2 s^{-1})	D_{vac} (10^{-9} m^2 s^{-1})		
1	0.7 ± 0.1	1.1 ± 0.1		
2	0.8 ± 0.1	1.1 ± 0.1		

and other cells (García-Martí, Ballesteros, & Cerdán, 2001). The D_{app} values tend to converge as ripening proceeds, so that, after the second storage day, there is no further difference between the D values of two compartments. This may be ascribed to changes in chemical composition associated with the disappearance of starch granules, that in turn can alter the translational mobilities of water in the two compartments in different ways, making them, no longer distinguishable. Furthermore, the greater difficulty in separating the D values may also be due to an enhanced diffusive exchange between compartments, associated with changes in sub-cellular morphology or membrane permeability. In fact, considerable changes of the cellular membrane permeability properties, occurring during the climacteric peak, have been observed in other studies (Lii et al., 1982). It is possible that, due to the small difference between the two measured D values (much smaller than that measured by van Dusschoten et al. (1995a, 1995b) for apple cytoplasm and vacuole respectively, $D_{cyt} = 1.0 \times 10^{-9}$ m^2 s^{-1} and $D_{vac} = 1.7 \times 10^{-9}$ m^2 s^{-1}), diffusive exchange plays a role, even in the samples at the early stages. Therefore, the D values shown in Table 3, calculated for vacuolar and cytoplasmatic water, may be subject, to some extent, to an averaging effect that make them more similar than the true values of the two compartments.

3.2. High-resolution NMR measurements

High-resolution ^{1}H-NMR spectra have been obtained on liquid extracts from banana tissue samples cut during seven days of storage. Fig. 2 shows a spectrum obtained by the water peak suppression pulse sequence described in Section 2.

The assignment of sugar resonance signals has been carried out on the basis of published spectra (Cho, Bellon, Eads, Stroshine, & Krutz, 1991; Eads & Bryant, 1986; Ni & Eads, 1993b) and by comparison with spectra obtained from sugar solutions under the same pH and temperature conditions. Several sucrose resonance signals are clearly identified: the doublet at 5.42 ppm is due to H-1 of sucrose (Suc); the doublet at 4.22 ppm is due to H-3′ of sucrose; the triplet at 4.05 ppm is due to H-4′ of sucrose; the triplet at 3.47 ppm is due to H-4 of sucrose; the resonance signals at 3.89, 3.82, 3.77, 3.68 and 3.56 ppm have been assigned to sucrose too. The doublets at 5.24 and 4.65 ppm are due to the anomeric protons of glucose (Glu), α-glucose H-1 and β-glucose H-1, respectively; the triplet at 3.25 ppm is due to the β-glucose H-2. The doublet at 4.12 ppm is the only resonance signal clearly assigned to fructose. The resonance signals in the region from 3.38 to 3.98 ppm are mainly due to overlapping resonances of ring protons of sucrose, fructose

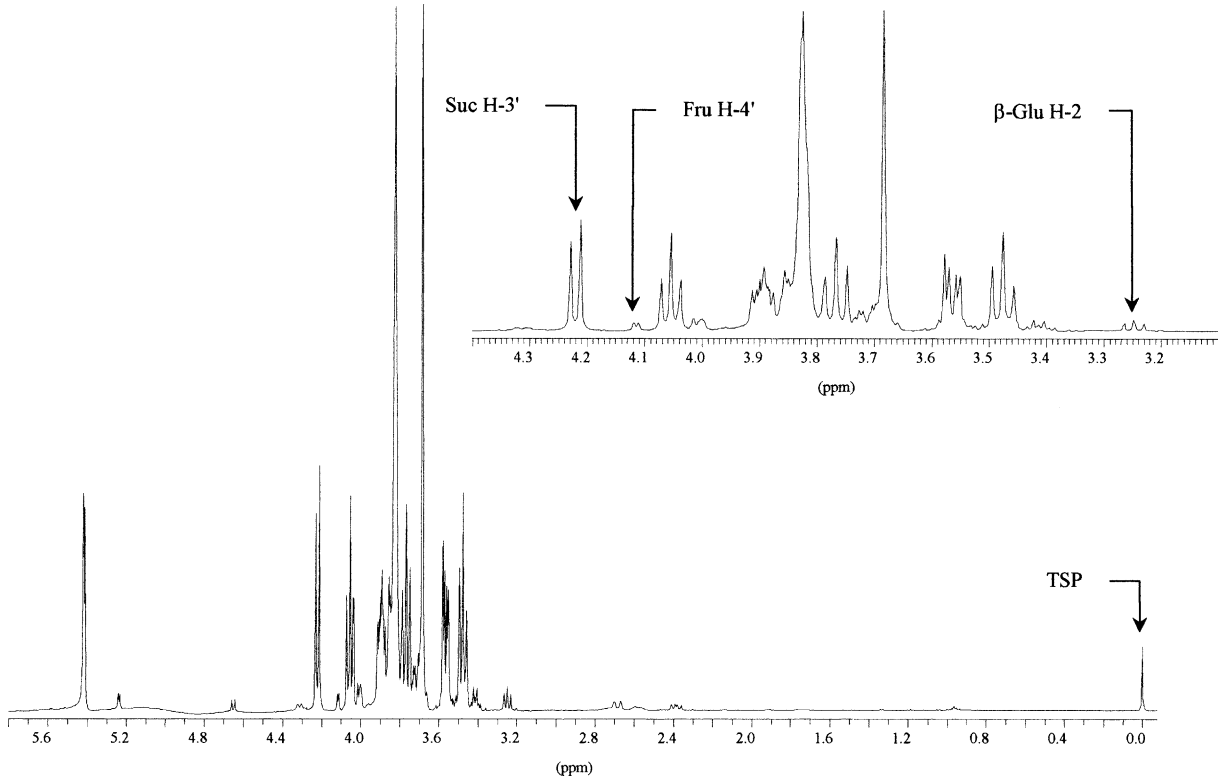

Fig. 2. High-resolution ^{1}H-NMR spectrum obtained, by water peak suppression pulse sequence, on liquid extracts of banana tissue samples cut at ripening stage 5 (third storage day). Bruker AM500 spectrometer ($T = 300 \pm 1$ K).

Table 4
Amount of individual sugars in the liquid extract (g/100 g) estimated by high-resolution ^{1}H-NMR, during seven days of storage

Storage time (days)	Suc	Glu	Fru[a]	Total sugars
3	20 ± 1	2.4 ± 0.1	4 ± 1	26 ± 1
4	47 ± 3	5.3 ± 0.3	10 ± 4	62 ± 4
5	44 ± 2	5.6 ± 0.2	8 ± 3	58 ± 3
6	40 ± 2	6.5 ± 0.3	8 ± 3	55 ± 3
7	42 ± 2	9.0 ± 0.5	14 ± 3	65 ± 3

[a] Determined by difference from total sugar integrals and sucrose and glucose integrals.

and glucose. Resonance signals below 3.00 ppm can be assigned to lipid methylene and methyl protons; the signal at 4.30 ppm is due to malic acid.

The increase of the three sugar resonance signals as total integral, reported in Table 4, is in agreement with the expected increase of sugar concentration, discussed above. The increase in sugars, quantified by other analytical methods (Beaudry et al., 1989; Cordenunsi & Layolo, 1995), during the climacteric peak, is, approximately, from 1–3% to 16–18%$_{w/w}$, and the fact that an increase of only 2.5 times of the total sugar integral is observed, indicates that, at the time of our first sampling, the conversion to sugars had already begun.

In order to determine the proportion of the three sugars in the liquid extract, selected resonance signals were integrated. However, quantitative information based on characteristic resonance signals is possible only for sucrose and glucose. The amount of sucrose was estimated on the basis of the doublet at 4.22 ppm (Suc H-3′). For glucose, the triplet at 3.25 ppm (β-Glu H-2) was selected: this signal is due only to the β-anomer of D-glucose that, at room temperature, in an aqueous solution, represents roughly 60% of the total D-glucose; normalization to 100 makes it possible to obtain an estimate of D-glucose present in the sample.

On the other hand, as regards fructose, which is also present in solution as a mixture of several anomers (the main anomers are β-D-fructopyranose, β-D-fructofuranose, α-D-fructofuranose), it is not possible to evaluate the total amount of this sugar only by the resonance signal at 4.12 ppm. Therefore, the fructose percentage has been determined by difference from the total sugar integral and sucrose and glucose integrals.

Data related to sucrose resonance signal integrals (Table 4) show a consistent increase, followed by a slight decline in the second period of sampling; this trend is in agreement with that observed in previous chemical analyses carried out by other methods, such as gas chromatography (Beaudry et al., 1989) or enzymatic methods (Cordenunsi & Layolo, 1995). According to these studies, the increase in sucrose concentration takes place immediately at the beginning of the climacteric peak, and the relatively high value observed for our first sample shows that probably, as already noted,the conversion to sugars had already begun at that time. On the other hand, the amounts of glucose and fructose show a gradual increase, in agreement with the results of previous studies. The sucrose/fructose/glucose molar ratio for the ripened banana is 4.5:1.7:1 (Table 4). Sucrose concentration, measured in our study, is somewhat higher than that measured by a high-resolution MAS ^{1}H-NMR technique on intact banana tissue (Ni & Eads, 1992, 1993a). In that paper the proportion 2.3:1.4:1 for sucrose/fructose/glucose concentration has been reported, but the obtained data refer to a single, not specific ripening stage so that the differences in the calculated proportion can be ascribed to a different storage time, as well as to natural variations due to differences in cultivars.

4. Conclusions

Proton nuclear magnetic resonance spectroscopy has been confirmed to be a powerful tool for studying microscopic changes of intact vegetable tissues during physiological or technological processes. In fact, an integrated approach, based on measurements and analysis of both relaxation times and self-diffusion coefficients, allowed description of the sub-cellular water distribution as well as monitoring of the changes of water dynamics associated with the variations of chemical composition occurring during banana ripening. In addition, in favourable cases, the translational mobility of water molecules in different cellular compartments (i.e. vacuolar and cytoplasm) can be measured. Furthermore high-resolution NMR spectra, obtained on extracts from banana tissues, allowed determination of the relative concentrations of the different soluble sugars in the cellular sap at different ripening stages.

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