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Study of natural mango juice spoilage and microbial contamination with *Penicillium expansum* by high resolution ^{1}H NMR spectroscopy

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Abstract

High resolution ^{1}H NMR has been applied to monitor the changes in the composition of natural mango juice subjected to spoilage and to microbial contamination with *Penicillium expansum*. A vast number of compounds undergoing changes upon these processes have been identified and their variations followed throughout time (132 h). Besides the formation of typical fermentation products (e.g. acetate, lactic acid, acetoin and isopropanol/2,3-butanediol) and the utilization of the major sugars (sucrose, glucose and fructose), there were changes in organic acids (e.g. decreases of quinic and shikimic acids with formation of 3,4,5-trihydroxycyclohexane acid in spoiled juice, and decreases of citric and malic acids in contaminated juice), amino acids (decreases of alanine, leucine, isoleucine and valine), and less abundant components such as oligosaccharides and aromatic compounds. The possibility of using these changes as early indicators of natural spoilage or *P. expansum* contamination is discussed.

Keywords: Mango juice; Spoilage; *Penicillium expansum*; Spectroscopy; NMR

1. Introduction

Microbial growth in fruits and fruit products is of serious concern, as it causes decay, loss of nutritive and organoleptic properties and production of toxic substances. Due to the low pH of most fruit products, fungi are often the predominant microorganisms while only a few bacteria are sufficiently aciduric to be important (Frazier & Westhoff, 1988; Splittstoesser, 1996). The traditional methods of detecting microbial growth in fruit and fruit products consist of plating procedures based on culturing the fruit sample in adequate medium, incubating and counting the microbial populations after 3–5 days (Beuchat, 1992). This is often too long for quality control management to initiate prompt corrective action if product contamination is detected. Therefore, research efforts have focused on developing more rapid methods for detecting microbial contamination (De Bower & Beumer, 1999). Examples of these methods are the bioluminescent procedure that measures the quantity of adenosine triphosphate (ATP) in microbial cells (Littel & LaRocco, 1986; Takahashi, Nakakita, Watari, & Shinotsuka, 2000), the impedimetric detection which correlates the microbial population with the time required to produce a change in impedance (Schaertel, Tsang, & Firstenberg-Eden, 1987; Kleiss, Albrecht, Putallaz, & Cordier, 1995), immunological methods which rely on the specific binding of an antibody to an antigen (Lin, Lister, & Cousin, 1986; Notermans, Heuvelman, Van Egmond, Paulsch, & Besling, 1986) and

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nucleic acid-based methods, such as the polymerase chain reaction (PCR) technique (Casey & Dobson, 2004; Liming & Bhagwat, 2004). Another approach is to detect specific metabolic products of microorganisms. For instance, the degree of mouldiness in grapes has been related to the glycerol content of the must, and the amounts of ethanol have long been used to monitor yeast growth in bulk-stored grape juice (Splittstoesser, 1996). Acetylmethyl carbinol (acetoin) and diacetyl have been suggested as indicators of growth of certain fungi and bacteria, conferring characteristic off-flavours to the contaminated fruit products (Singhal, Kulkarni, & Rege, 1997). Ethylene has been proposed to be an early indicator of mould infection in tomato fruits (Polevaya, Alkalai-Tuvia, Copel, & Fallik, 2002). Organic acids are also usual indicators of microbial growth. Succinic, acetic, formic and lactic acids have been found to be produced in tomatoes by mould and bacteria, whereas citric acid has been seen to be used up (Singhal et al., 1997). The profile of volatile amines in apple fruits has also been reported to be a good indication of contamination by different moulds. It has been observed that butylamine and isobutylamine, not present in healthy fruits, occur in contaminated fruits and could, therefore, be used as an index of mould contamination (Singhal et al., 1997). Another common response of plant tissue

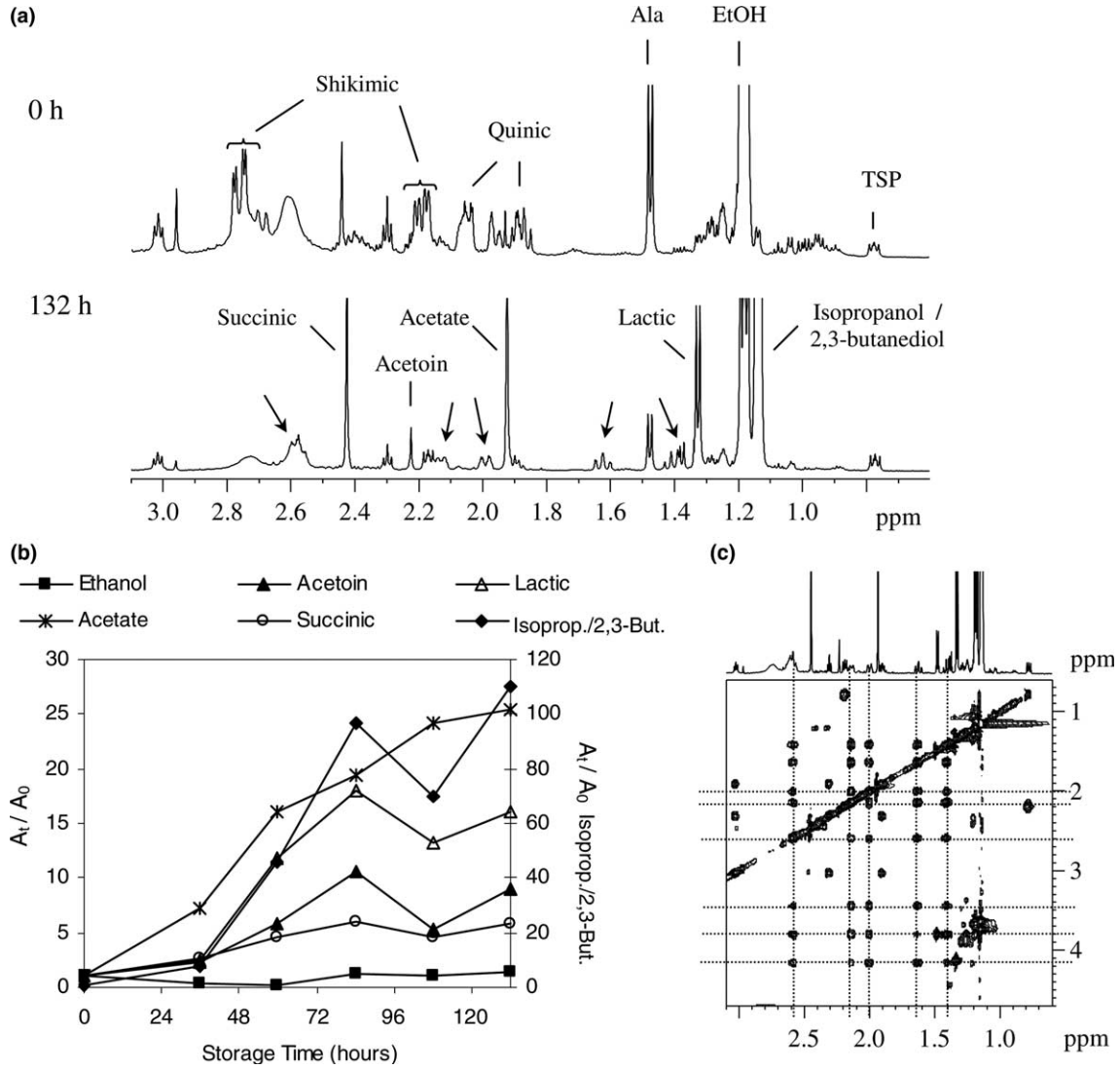

Fig. 1. (a) High-field region of the 600 MHz ^{1}H NMR spectra of natural mango juice, freshly prepared (time 0) (top) and after storage at 25 °C during 132 h (bottom), NS 64; the arrows indicate the signals of 3,4,5-trihydroxycyclohexane carboxylic acid (Fig. 4); (b) Relative variations of some compounds determined by NMR integration (A_t = area at time t, A_0 = area at time 0): ethanol – 1.17 ppm, acetoin – 2.22 ppm, lactic acid – 1.32 ppm, acetate – 1.92 ppm, succinic acid – 2.41 ppm and isopropanol/2,3-butanediol-1.14 ppm; (c) expansion of the TOCSY spectrum of the non-treated juice stored for 132 h at 25 °C; the spin system of 3,4,5-trihydroxycyclohexane carboxylic acid is highlighted by dashed lines.

to infection by microorganisms is an increase in the synthesis of characteristic phenolic compounds (Singhal et al., 1997).

High resolution NMR spectroscopy has been shown to be a valuable method for the analysis of low molecular weight compounds in fruit juices, allowing the simultaneous identification of several sugars, organic acids, amino acids and other minor components such as phenolic compounds (Belton et al., 1996; Belton et al., 1997; Gil et al., 2000; Le Gall, Puaud, & Colquhoun, 2001; Sobolev, Segre, & Lamanna, 2004). The technique has also been successfully applied to follow the fermentation process of lactic bacteria on sorghum (Correia, Nunes, Duarte, Barros, & Delgadillo, 2005), whereas in vivo ^{1}H, ^{13}C and ^{31}P NMR methods have been used to study the metabolic pathways of these microorganisms (Santos, 1995; Ramos & Santos, 1999). The strength of NMR within this area is its non-invasive nature and its ability to simultaneously detect a wide range of species and potentially enable the identification of novel, unexpected compounds.

The present work makes use of high resolution NMR to follow the compositional changes of mango juice subjected to natural spoilage and to inoculation with *Penicillium expansum*. This mould is the most common apple-rotting fungus and can be commonly found in the processing lines of fruit products. It contaminates the intact fruit through wounds caused during handling and packaging operations and is responsible for high post-harvest losses. In this work, the application of high

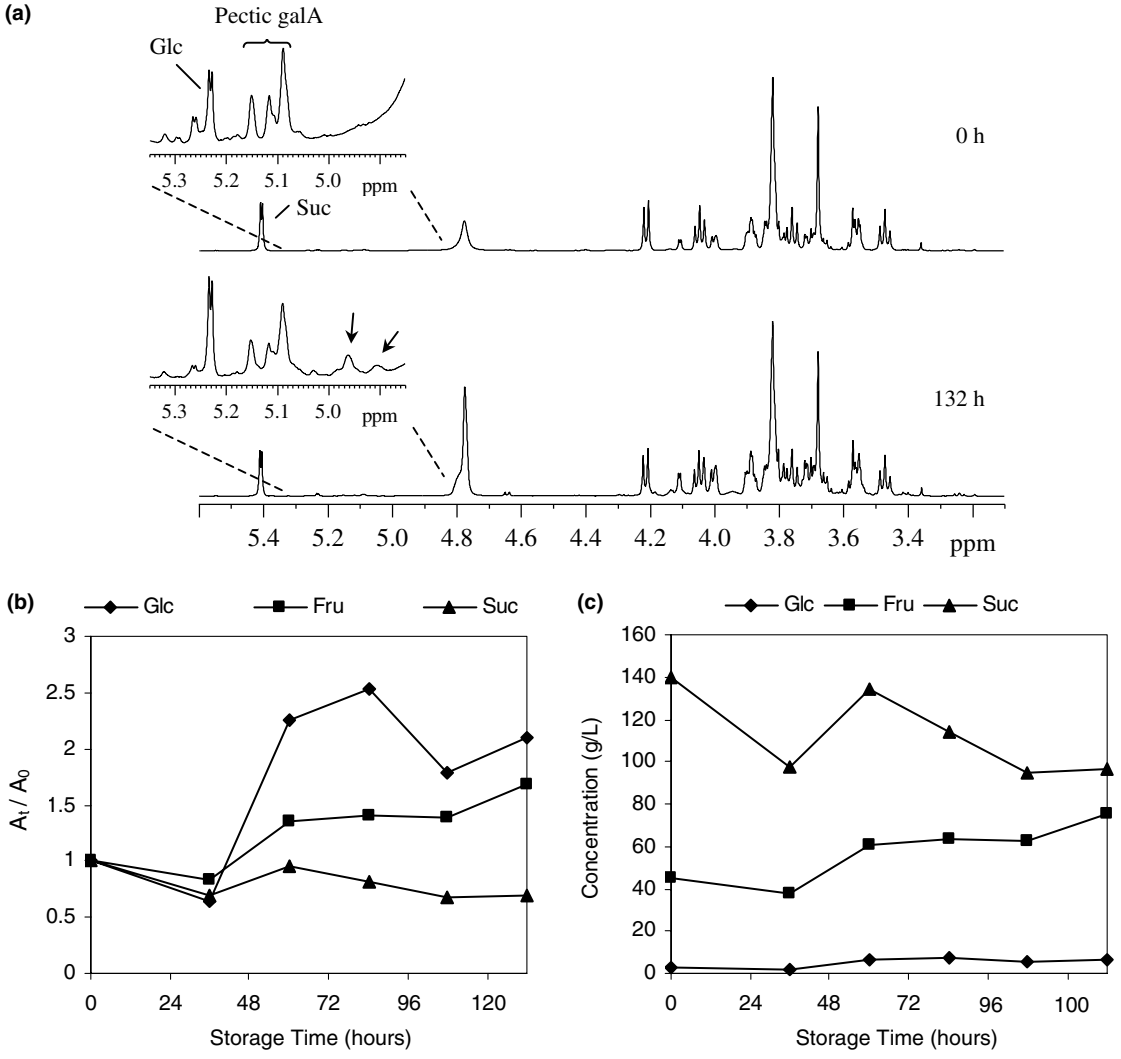

Fig. 2. (a) Mid-field region of the 600 MHz ^{1}H NMR spectra of natural mango juice, freshly prepared (time 0) (top) and after storage during 132 h (bottom), NS 64. Peaks indicated with arrows correspond to unassigned compounds showing significant variation (Table 1); (b) Relative variation of sugars determined by NMR integration (A_t = area at time t, A_0 = area at time 0): glucose – 5.23 ppm, fructose – 4.11 ppm and sucrose – 5.41 ppm; (c) Absolute concentrations of sugars as a function of storage time, determined enzymatically for time 0 and estimated for subsequent storage times (see text).

resolution ^{1}H NMR to mango juice undergoing natural spoilage or the effects of *P. expansum* inoculation aims at characterising the biochemistry of both situations and identifying suitable indicators of their detection in natural fruit juice, that is in the juice without industrial processing. In a subsequent article, the use of NMR to detect *P. expansum* and *Neosartorya fischeri* contaminations in commercial mango juice will be described in order to account for post-processing spoilage.

2. Materials and methods

2.1. Preparation of spore suspensions

The mould *Penicillium expansum* used in this study was kindly provided by the 'Instituto Nacional de Engenharia e Tecnologia Industrial', in Lisbon, from its culture collection (strain 310 isolated from apple juice). In order to produce sufficient mature spores, *P. expansum* was inoculated into slants of potato dextrose agar (PDA) and incubated at 25–28 °C for 5 days. Spores were harvested from the slants by flooding with 5–6 ml of sterile deionised water containing 0.1% Tween 80 and collecting the aliquots to an Erlenmeyer flask, until 20–30 ml were obtained. A 10 min sonication treatment was followed by filtration of the suspension through sterile glass wool to remove hyphae (branching formations in fungi). Microscopic examination of the filtrate revealed free spores. The suspension was then diluted to give spores concentrations of 10^5/ml and 10^4/ml, measured by using a Newbauer chamber for counting spores.

2.2. Preparation and Inoculation of mango juice

Ripe edible mangoes of the cultivar Haden grown in Brazil and obtained from a commercial source were used for this study. The fruits were washed, peeled and their pulps macerated in a domestic juice extractor. The resulting puree was centrifuged (at 15000 r.p.m. during 15 min) to give the juice. Aliquots of 20.0 ml were poured into 22 sterile bottles of 100 ml, which were treated and stored for 132 h (5.5 days) in the following conditions: one group of six bottles was stored at 25 °C without any treatment, in order to follow the changes occurring during natural spoilage of the juice, while the remaining 16 bottles were heated in a water bath at 80 °C during 15 min, in order to inactivate enzymes and to eliminate most of the juice natural microflora (Ejechi, Souzey, & Akpomedaye, 1998), so that the effects of inoculation could be investigated separately. Ten bottles of heated juice were inoculated with spores of *P. expansum*: five bottles were inoculated with 0.500 ml of suspension containing 10^5 spores per ml, and five bottles were inoculated with 0.500 ml of suspension containing 10^4 spores per ml, to give spores concentrations in the juice of about 2500/ml (batch A) and 250/ml (batch B), respectively. The remaining six bottles of heated juice were stored at 25 °C and used as control samples.

During a period of 132 h, one bottle was withdrawn every day from each group. The juice was filtered through a glass microfiber filter under vacuum and through a 0.45 μm filter with a syringe, and both pH and soluble solids (%SS) were measured. For NMR analysis, the filtered juice was prepared to contain 10%

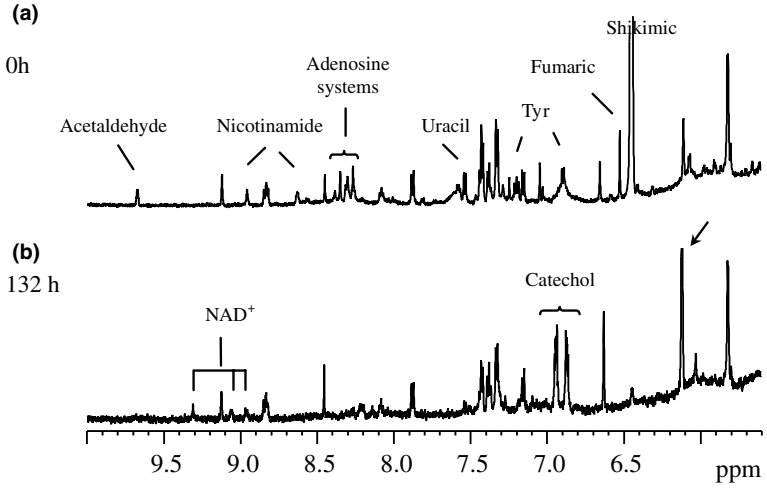

Fig. 3. Low-field region of the 600 MHz ^{1}H NMR spectra of natural mango juice, freshly prepared (time 0) (a) and after storage during 132 h (b), NS 64. The peak indicated with an arrow corresponds to an unassigned compound showing significant variation (Table 1).

D_2O (field-frequency lock), 0.02% 3-(trimethylsilyl)propionate sodium salt (TSP, chemical shift and intensity reference) and 0.05% sodium azide (to prevent microbial growth in the stored samples). The pH of the juices was adjusted to 7.00 ± 0.05 and samples were frozen in liquid nitrogen and stored at −20 °C until their analysis.

2.3. NMR measurements

The NMR spectra were recorded on a Bruker Avance DRX-600 spectrometer, operating at 599.87 MHz for proton and 150.85 MHz for carbon. Typically, each ^{1}H 1D spectrum consisted of 64 scans of 65536 data points with a spectral width of 12019.23 Hz, an acquisition time of 2.73 s, and a relaxation delay of 7 s. A presaturation sequence was used to suppress the water signal by applying low-power selective irradiation at the water frequency during 3 s of the relaxation delay (Bax, 1985). All the FIDs were Fourier transformed with 0.3 Hz line broadening, phased and baseline corrected using the Bruker XWIN-NMR software. Two-dimensional Total Correlation Spectroscopy (TOCSY) spectra were also recorded for a few samples to aid spectral assignment. These spectra were acquired in the phase sensitive mode using time proportional phase incrementation (TPPI), and the MLEV17 pulse sequence was used for the spin-lock (Bax & Davis, 1985). 2048 data points with 16 transients per increment and 200 increments were acquired with a spectral width of 6613.76 Hz in both dimensions. The relaxation delay

Table 1
Changes in the ^{1}H NMR spectra of natural mango juice, during storage at 25 °C for 132 h

Compound	δ^1H ppm (multiplicity)	Type of variation ↑: increase ↓: decrease	Time when variation is first noted
High-field region (0–3 ppm)			
Acetate	1.92 (s)	↑	36 h
Acetoin	1.37 (d) 2.22 (s) 4.42	↑	36 h
Alanine	1.47 (d) 3.79	↓	36 h
Cyclic acid[a]	1.38 1.62 1.99 2.13 2.58 3.43 3.79 4.13	Appears	36 h
Isoleucine	0.93 (t) 1.00 (d)	↓	36 h
Isopropanol/2,3-butanediol	1.14 (d) 3.67	↑	36 h
Lactic acid	1.32 (d) 4.12	↑	36 h
Leucine	0.95 (t) 1.72	↓	36 h
Quinic acid	1.87 195 2.06 3.55 4.02 4.14	↓	36 h
Shikimic acid	2.19 2.77 3.70 3.98 4.40 6.45	↓	36 h
Succinic acid	2.41 (s)	↑	36 h
Valine	0.98 (d) 1.04 (d)	↓	36 h
Unassigned:			
Compound 1	2.96 (s)	↓	36 h
Mid-field region (3–5.5 ppm)			
Fructose	4.11	[c]	
Glucose	4.64, 5.23	[c]	
Sucrose	5.41	[c]	
Unassigned			
Compound 2	4.90 [3.95]	Appears	60 h
Compound 3	4.96 [3.75 4.01]	Appears	60 h
Low-field region (5.5–10 ppm)			
Acetaldehyde	9.67	Disappears	36 h
Adenine/Adenosine[b]	8.30 (s), 8.31 (s), 8.35 (s), 8.38 (s)	Disappear	36 h
Adenosine (ribose moiety)[b]	6.07 (d) [4.43]	Disappears	36 h
Catechol[b]	6.86 6.94	Appears	60 h
Fumaric acid[b]	6.52 (s)	Disappears	36 h
Gallic acid[b]	7.04 (s)	Disappears	36 h
Nicotinamide	7.57 8.27 8.63 8.96	Disappears	36 h
NAD^+	8.99 9.09 9.34	Appears	36 h
Shikimic acid	6.45	↓	36 h
Tyrosine	6.89 (d) 7.19 (d)	Disappears	36 h
Uracil[b]	5.80 (d) 7.54 (d)	↓	36 h
Unassigned			
Compound 4	6.11 (d) [3.77 4.35]	↑	36 h
Compound 5	7.24 (s)	Disappears	36 h

The values in square brackets correspond to signals belonging to the same spin system.
[a] Possibly 3,4,5-trihydroxycyclohexane carboxylic acid.
[b] Tentative assignment.
[c] Additional information provided in Fig. 2(b) and 2(c).

between successive pulse cycles was 2 s and the mixing time of the MLEV spin lock was 80 ms. The data were zero-filled in the f_1 dimension to 2048 data points and a sine-bell apodization function was applied prior to FT.

For quantification purposes, peak areas were measured by conventional integration of the 1D spectra using the same chemical shift limits for each signal. Coefficients of variation, obtained from measurements made in three replica spectra of some mango juice samples, were found to be lower than 5%.

3. Results and discussion

3.1. Effect of natural spoilage

Figs. 1(a), 2(a) and 3 show, respectively, the high-field, mid-field and low-field regions of the 1D spectra of natural juices, freshly prepared (time 0), and after storage during 132 h. Many differences may be observed in the whole spectral range, reflecting drastic changes in the juice composition (Table 1). In order to aid spectral interpretation, the information provided by 1D and 2D TOCSY spectra was combined and resonances were assigned by comparison of ^{1}H chemical shifts and spin–spin coupling constants with reference spectra of some commercial standards and literature values of metabolites commonly found in biological and food samples (Nicholson, Foxall, Spraul, Farrant, & Lindon, 1995; Fan, 1996; Gil et al., 2000). The pH of the samples was seen to decrease from 5.1 to 4.1 at 132 h, whereas soluble solids showed a slight increase from 14% to 16%.

In the high-field region (Fig. 1(a)), prominent increases are detected for several signals, namely a doublet at 1.14 ppm (isopropanol or 2,3-butanediol), a doublet at 1.32 ppm (lactic acid), singlet at 1.92 ppm (acetate), signals at 1.37 ppm and 2.22 ppm (acetoin), and singlet at 2.41 ppm (succinic acid). The relative variations of these compounds were measured by dividing the areas of each signal at different storage times (A_t) by the area measured at time 0 (A_0). Absolute quantification was not carried out because, although the same amount of TSP was added to all samples, the area of its signal showed significant variations probably due to physical and/or chemical interactions with the juice components. As shown in Fig. 1(b), the largest increase is registered for isopropanol/2,3-butanediol (100× at 84 h). Acetate increases 25× at 132 h, while lactic acid, acetoin and succinic acid increase, respectively, 18×, 10× and 6× at 84 h. All these compounds are typical products of different types of fermentation undergone by microorganisms present in the juice. In addition, alcoholic fermentation, typical of yeasts, does not seem to be important here since the amount of ethanol does not increase.

Other changes in the high-field region of the spectra (Fig. 1(a)) regard some amino acids, namely valine, leucine, isoleucine and alanine, and quinic and shikimic acids, which progressively decrease to residual amounts. Interestingly, this is accompanied by the formation of a new compound which gives rise to the signals marked with arrows in Fig. 1(a). The spin system of the new compound includes signals at 3.43, 3.79 and 4.13 ppm (Fig. 1c), which is consistent with a structure based on that of 3,4,5-trihydroxycyclohexane carboxylic acid (Fig. 4). The similarity of this structure with those of quinic and shikimic acids (Fig. 4) suggests that these compounds may indeed be biochemically correlated.

The mid-field regions of the spectra (Fig. 2(a)) show a sucrose decrease of 1.5×, while glucose and fructose increase 2× and 1.7×, respectively (Fig. 2(b)). Knowing

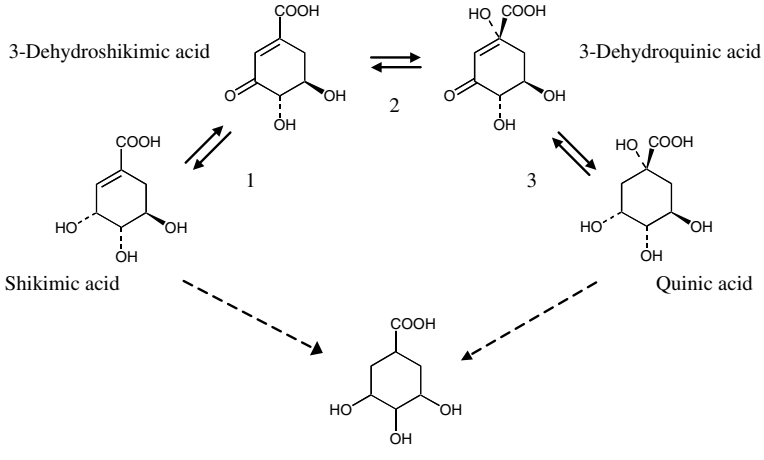

Fig. 4. Scheme showing the pathway for the inter-conversion of quinic and shikimic acids (Dewick, 1998) and the relationship proposed here with the formation of 3,4,5-trihydroxycyclohexane carboxylic acid. Enzymes involved in steps 1, 2 and 3 are, respectively, shikimate dehydrogenase, 3-dehydroquinate synthase, and quinate dehydrogenase.

that the initial concentrations at the time 0 are 136 g/L of sucrose, 40 g/L of fructose and 3 g/L of glucose, as determined by specific enzymatic tests, sugar amounts may be estimated for each storage time (Fig. 2c). This gives an approximate decrease of 45 g/L for sucrose and estimated increases of 28 g/L and 3 g/L for fructose and glucose, respectively. These numbers show that sucrose hydrolyses into fructose and glucose and that, as expected, most glucose is metabolised, resulting in a residual amount of this sugar.

In addition to the major sugars variations, the appearance of signals at 4.90 and 4.96 ppm is noted (arrows in Fig. 2(a)), indicating the formation of new carbohydrates in the juices stored for more than 60 h. The broad nature of these signals suggests that the corresponding carbohydrates are relatively large molecules, possibly pectin derived.

In the low-field region, a large number of variations occur (Fig. 3), but given the structural similarity between compounds and the singlet nature of some NMR signals, spectral assignment is not straightforward. For instance, the singlets at 8.3–8.4 ppm, seen to disappear with storage time, may only be tentatively assigned to protons H2 and H8 of adenine/adenosine, either in free form or in mono-, di-, or tri-phosphate derivatives. This is accompanied by the disappearance of a small doublet at 6.07 ppm, which may arise from a ribose moiety of an adenosine system. Other interesting changes regard the disappearance of the {7.57, 8.27, 8.63, 8.96 ppm} spin system, concomitantly with the appearance of that at {8.99, 9.09 and 9.34 ppm}. The former may arise from nicotinamide, while the latter is assignable to NAD^+ (nicotinamide adenine dinucleotide). Additional changes in the aromatic spectral regions reflect decreases in shikimic acid, tyrosine, uracil and acetaldeyde. On the other hand, a significant increase is noted for the unassigned doublet at 6.11 ppm probably arising from a sugar moiety (arrow in Fig. 3(b)), and for the multiplets at 6.86 and 6.94 ppm arising from an aromatic ring, possibly in catechol.

Given the vast number of changes noted at the earliest storage time studied (36 h), it is difficult, at this stage, to select peaks that might be used as early indicators of natural mango juice spoilage. The use of the observed changes for this purpose would require a more detailed study of their timescale prior to 36 h of storage and this is the scope of future work.

3.2. Effect of inoculation with penicillium expansum

Prior to inoculation with *P. expansum*, the juice was heated in order to inactivate most enzymes and natural microflora, thus providing a sample not affected by spoilage and, thus, suitable as control for the effects of *P. expansum* alone. Indeed, the heated juice showed practically no changes in composition during the 132 h of the experiment, the only changes noted being the gradual decreases of tyrosine, the singlet at 7.04 ppm (possibly gallic acid) and the unassigned singlet at 7.24 ppm (Fig. 5(b) and (c)). It is also interesting to note

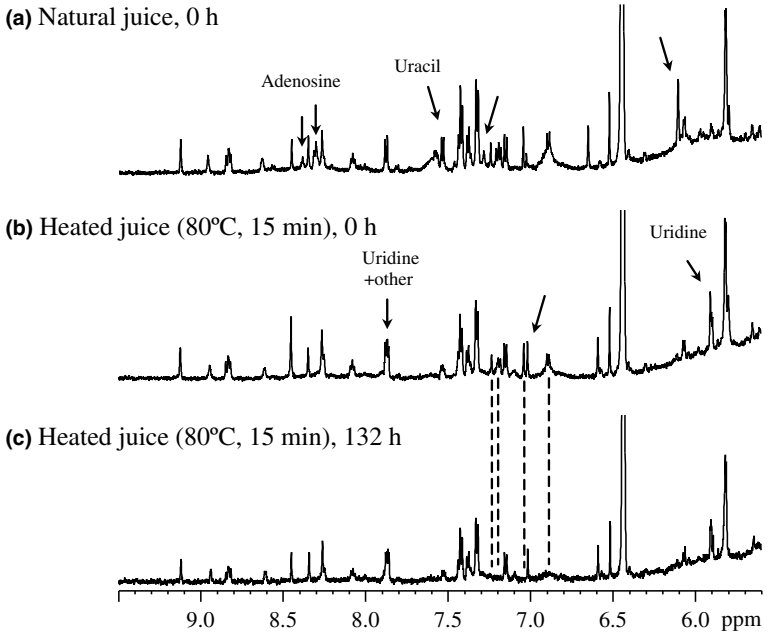

Fig. 5. Low-field region of the 600 MHz ^{1}H NMR spectra of: (a) natural juice collected at time 0; (b) natural juice pre-heated at 80 °C for 15 min, at time 0; (c) sample in (b) after storage at 25 °C for 132 h; NS 64. Arrows and dashed lines indicate signals undergoing significant changes.

the occurrence of slight changes in the aromatic spectral region as a result of sample heating (Fig. 5(a) and (b)). These changes are, however, not relevant at this stage of the study since both control and inoculated juices were heated and only relative changes will be noted and discussed.

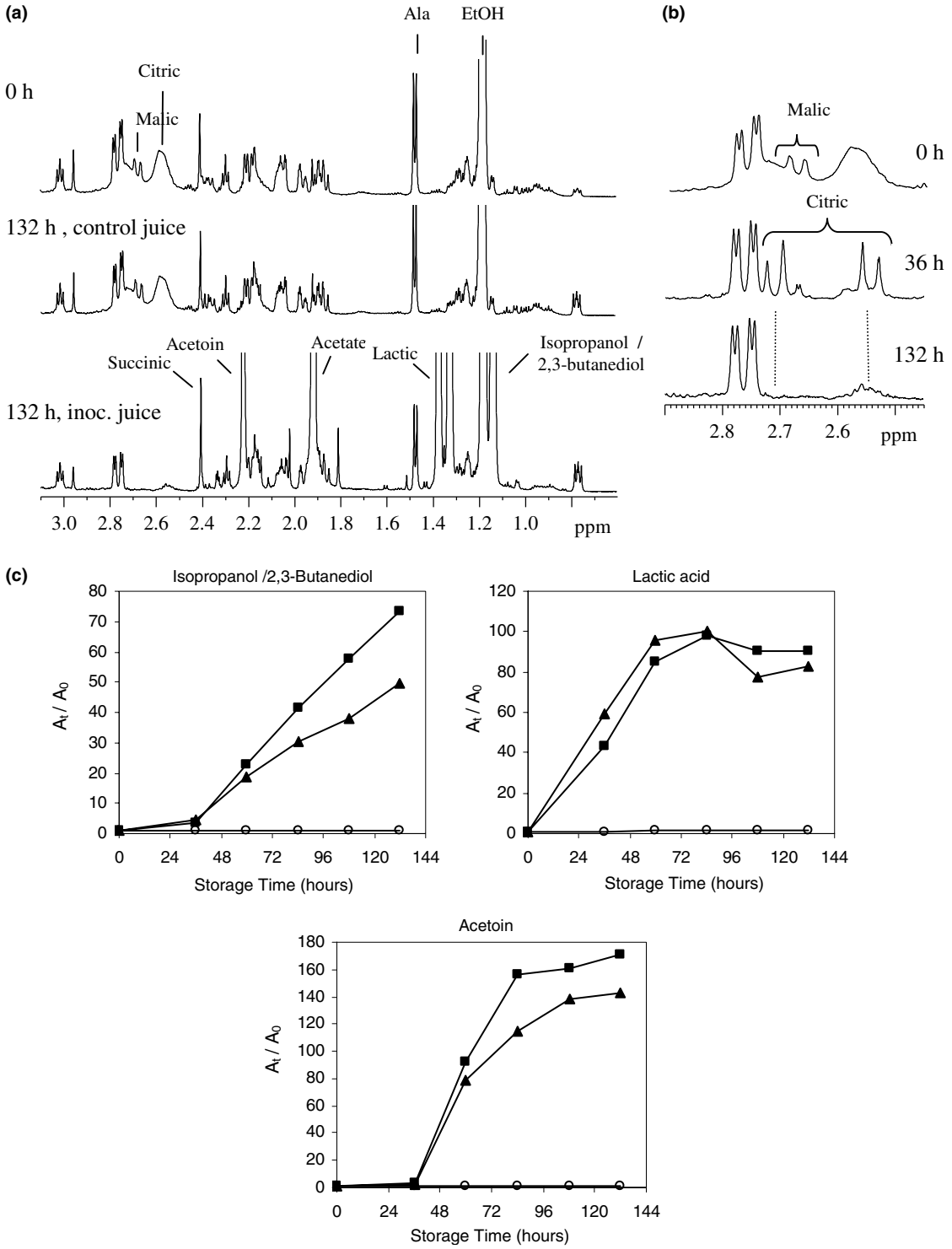

Fig. 6. (a) High-field region of the 600 MHz ^{1}H NMR spectra of control juice at time 0 (top) and at 132 h of storage (middle), and of the juice inoculated with *P. expansum* at 132 h of storage (bottom), NS 64; (b) 2.45–2.90 ppm region of inoculated juices spectra at time 0 (top), 36 h (middle) and 132 h (bottom); (c) Relative variation of some compounds determined by NMR integration (A_t = area at time t, A_0 = area at time 0): isopropanol/2,3-butanediol-1.14 ppm, lactic acid-1.32 ppm and acetoin – 2.22 ppm; (○) control juice, (■) inoculated juice – batch A and (▲) inoculated juice – batch B.

Figs. 6(a), 7(a) and 8 show, respectively, the high-field, mid-field and low-field regions of the 1D spectra of the control and of the juice inoculated with 2500 spores/ml (batch A), after 132 h of storage. The spectral variations observed for batch A are summarised in Table 2. The juices of batch B, inoculated with 250 spores/ml, have shown the same qualitative changes as batch A, simply occurring at a slower rate. Regarding

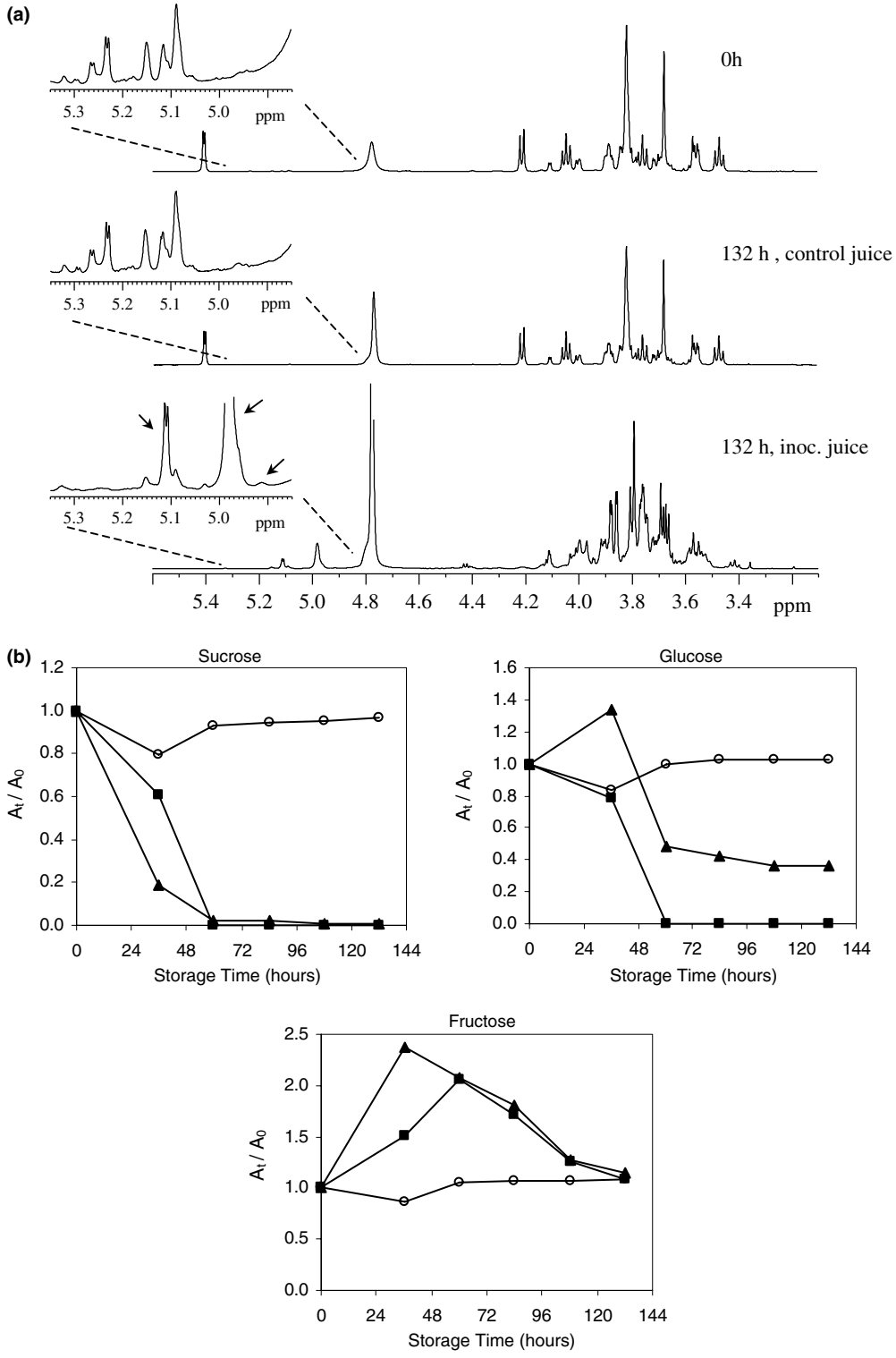

Fig. 7. (a) Mid-field region of the 600 MHz ^{1}H NMR spectra of control juice at time 0 (top) and at 132 h of storage (middle), and of the juice inoculated with *P. expansum* at 132 h of storage (bottom), NS 64. Peaks indicated with arrows correspond to unassigned compounds showing significant variation (Table 2); (b) relative variation of sugars determined by NMR integration (A_t = area at time t, A_0 = area at time 0): glucose – 5.23 ppm, fructose – 4.11 ppm and sucrose – 5.41 ppm. (○) control juice, (■) inoculated juice – batch A, (▲) inoculated juice – batch B.

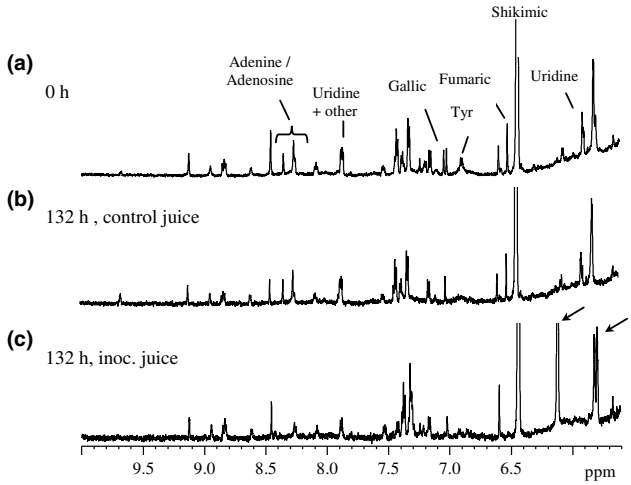

Fig. 8. Low-field region of the 600 MHz ^{1}H NMR spectra of control juice at time 0 (a) and at 132 h of storage, (b) and of the inoculated juice at 132 h of storage, (c) NS 64. Peaks indicated with arrows correspond to unassigned compounds showing significant variation (Table 2).

pH variation, both batches of inoculated juice showed a decrease from 5.1 to 4.1, whereas soluble solids showed no significant variation.

In the high-field region (Fig. 6(a)), the largest increases are noted for acetoin (120–160× at 84 h), lactic acid (85–95× at 60 h), and isopropanol/2,3-butanediol (50–75× at 132 h) (Fig. 6c). Acetate and succinic acid are also seen to increase. As expected, these metabolites are formed at higher rate in the juices of batch A, except for lactic acid, which increases at approximately the same rate independently of the inoculum concentration. All these compounds have also increased as a result of natural spoilage, however, the magnitude of their increase is quite distinct in the inoculated juice.

In the same spectral region, citric and malic acids are seen to decrease with time. Interestingly, citric acid peaks are significantly broad at 0 h (Fig. 6(b)), giving place to well-resolved signals at 36 h and disappearing thereafter. The initial broad nature of the signals suggests some sort of interaction involving citric acid, possibly complexation. As time proceeds, citric acid becomes free and is then used up promptly. In addition to citric and malic acids, several amino acids are seen to decrease (Table 2), probably reflecting their use as organic sources.

The sugar profile found for the contaminated juice is significantly different from that of the control juice (Fig. 7(a)), the drastic decreases of glucose and sucrose contributing largely for this difference. The relative variations of these sugars and of fructose are shown in Fig. 7(b). The results show that sucrose is hydrolysed to glucose, which is then rapidly used up, and to fructose, which also decreases after 36–60 h, when glucose levels are already very low. These observations support the preferential utilization of glucose by the mould (Moore-Landecker, 1982). Besides the variations mentioned above, many other changes are noted in the anomeric region of the spectra, most of which are still unassigned (Table 2). Among the new signals detected, the broad signal at 4.98 ppm, possibly arising from a relatively large carbohydrate, and the doublet at 5.11 ppm, also belonging to a sugar spin system, are the most intense (arrows in Fig. 7(a)). In the low-field region (Fig. 8), signals tentatively assigned to adenine/adenosine, fumaric and gallic acids, as well as signals of uridine, tyrosine and acetaldehyde are seen to disappear. Gallic acid and tyrosine variations also occur in the control juice, and, thus, are likely to be unrelated to *P. expansum*. On the other hand, the unassigned signals at 5.79 ppm and at 6.12 ppm (arrows in Fig. 8) register clear increases, only for the contaminated juice.

In summary, the variations seen in the NMR spectra which could correspond to specific indicators of *P. expansum* growth, since they were not detected in control or in spoiled juices, are the decreases of citric and malic acids, and the increases of a few unassigned signals, marked in Table 2. The assignment of such signals by improved strategies (multidimensional and hyphenated NMR) is an important task to be carried out in the future. All these signals are potential useful indicators of *P. expansum* contamination in mango juice, however, as was the case for natural spoilage, the selection and use of some of these signals as early indicators require a more detailed study of their time dependence prior to 36 h.

4. Conclusions

High resolution ^{1}H NMR showed promising results in the detection of indicators of mango juice degradation, caused either by natural spoilage or by deliberate contamination with the mould *Peniccilium expansum*.

Table 2
Changes in the ^{1}H NMR spectra of mango juice inoculated with *P. expansum* (batch A) and stored at 25 °C for 132 h

Compound	^{1}H ppm (multiplicity)	Type of variation ↑: increase ↓: decrease	Time when variation is first noted
High-field region (0–3 ppm)			
Acetate	1.92 (s)	↑	36 h
Acetoin	1.37 (d) 2.22 (s) 4.42	↑	36 h
Alanine	1.47 (d) 3.79	↓	36 h
Isoleucine	0.93 (t) 1.00 (d)	↓	36 h
Isopropanol/2,3-butanediol	1.14 (d) 3.67	↑	36 h
Lactic acid	1.32 (d) 4.12	↑	36 h
Leucine	0.95 (t) 1.72	↓	36 h
Citric acid[a]	2.57 2.70	Disappears	60 h
Malic acid[a]	2.38 2.68 4.29	↓	36 h
Succinic acid	2.41 (s)	↑	36 h
Valine	0.98 (d) 1.04 (d)	↓	36 h
Unassigned:			
Compound 1[a]	0.83 (d) 0.96	Appears	36 h
Compound 2[a]	1.43 (d) 4.11	Appears	36 h
Compound 3[a]	1.51 (s)	Appears	36 h
Compound 4	2.96 (s)	↓	36 h
Mid-field region (3–5.5 ppm)			
Fructose	4.11	[c]	
Glucose	4.64, 5.23	[c]	
Sucrose	5.41	[c]	
Unassigned:			
Compound 5	4.91 [3.71 3.95]	Appears	36 h
Compound 6	4.98 3.57 3.71 3.89]	Appears	36 h
Compound 7[a]	5.11 [3.41 3.54 3.77 3.89]	Appears	36 h
Compound 8	5.37 [3.80 4.01]	Appears	36 h
Low-field region (5.5–10 ppm)			
Acetaldehyde	9.67	Disappears	36 h
Adenine/Adenosine[b]	8.26 (s), 8.35 (s)	Disappear	36 h
Adenosine (ribose moiety)[b]	6.07 (d) [4.43]	Disappears	36 h
Fumaric acid[b]	6.52 (s)	Disappears	60 h
Gallic acid[b]	7.04 (s)	Disappears	36 h
Tyrosine	6.89 (d) 7.19 (d)	Disappears	36 h
Uridine	5.90 7.86 (d)	Disappears	36 h
Unassigned:			
Compound 9[a]	5.79 [3.71 4.26]		60 h
Compound 10	6.12 (d) [3.73 4.35]	↑	36 h
Compound 11	7.24 (s)	↓	60 h

The values in square brackets correspond to signals belonging to the same spin system.
[a] Variation specific of *P. expansum* growth (compared to natural spoilage).
[b] Tentative identification.
[c] Additional information provided in Fig. 7(b).

Besides the utilization of the main sugars and the formation of typical fermentation products (namely acetate, lactic acid, acetoin and isopropanol/2,3-butanediol), changes in organic acids, amino acids, and less abundant components such as oligosaccharides and aromatic compounds were viewed. In particular, the naturally spoiled juice showed the unexpected formation of 3,4,5-trihydroxycyclohexane carboxylic acid (or a similar derived compound) which is, to our knowledge, a newly found product, possibly resulting from quinic and shikimic acids catabolism. In the *P. expansum* contaminated juice, specific changes regarded the utilization of citric and malic acids and the formation of a few minor compounds, still unassigned at this stage.

The practical use of the above changes as early indicators of mango juice natural spoilage or *P. expansum* contamination requires further work involving the collection and analysis of samples at shorter intervals prior to 36 h of storage, and systematic comparison of the NMR timescale with that of selected alternative methods. Nevertheless, compared to the typical 3–5 days required by traditional plating procedures, it is clear that high resolution ^{1}H NMR enables earlier detection of the juice compositional degradation. This result, along with the ability to allow for structural characterisation of novel, unexpected compounds enlightening specific aspects of fruit biosynthesis, shows the potential usefulness of NMR in the area of food microbiological control.

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