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1 **Short communication**

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3 **Asparagine in plums detected by CEST – MRI**

4

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11

12 **Highlights**

- 13 ● CEST-MRI technique is applied for the first time in fruits
- 14 ● pH in plums can be monitored during the ripening process
- 15 ● Saturation transfer of amide protons from Asn can be followed by means of CEST-MRI
16 experiments at low magnetic field.

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18

19 **Abstract:**

20

21 Magnetic Resonance Imaging (MRI) relies on the topological distribution of the intense water NMR
22 signal and may be used to report about changes in the internal structures of fruits associated to
23 ripening, storing, pathogen infection. Herein the use of CEST- MRI (Chemical Exchange Saturation
24 Transfer) is introduced to show that in addition to structural information, insights into the presence
25 in the fruits of specific chemicals may be gained, Asparagine is present in plums at relatively high
26 concentration ($\approx 10 - 20$ mM) and owns two amide protons (at 2.1 and 2.8 ppm down field from
27 water) in slow exchange with water protons. By irradiating the amide resonances with a proper rf-
28 field it is possible to transfer saturated magnetization to the “bulk” water signal. The attained
29 change in signal intensity reflects the extent of prototropic exchange between amide and water
30 protons that is modulated by the local pH.

31

32 **1.Introduction**

33

34 Magnetic resonance imaging is extensively used because it can provide images of internal structures
35 with a good compromise between spatial and temporal resolution. Its wide range parameters linked
36 to water and nondestructive evaluation of various internal quality factors lead to innovative studies
37 in food chemistry [1]. Preliminary studies applied to fruits were focused on T_2 , T_1 , diffusion
38 coefficient assessment, for internal quality assessment during storage and ripening process studies
39 [2]. In damaged tissues, a contrast is immediately visible in the MR final image.

40 In this communication we report preliminary results obtained by applying the emerging procedure
41 named CEST-MRI [3]. It relies on the detection of molecules containing mobile protons in slow
42 exchange with water. Fruits may contain many molecules that respond to this requisite, namely
43 aminoacids, peptides, saccharides. CEST-MRI is an encoding frequency procedure that, in
44 principle, allows selecting the contrasting effect due to a specific molecule as the transfer of
45 saturated magnetization (ST) is associated to the irradiation of the mobile proton absorption of the
46 molecule of interest. As a first proof of concept of this approach the resonance of the amide moiety
47 of asparagine (Asn) have been exploited as this amino-acid is known to occur at relatively high
48 concentration in plums [4]

49

50 2. Results and discussion

51 Asparagine contains two water exchangeable amide protons that can be exploited for MRI via the
52 CEST effect. To assess the level of asparagine in plum pulp and to assess whether the pulp
53 contained significant sources of exchangeable protons in addition to those of asparagine, the
54 metabolite profile of plum pulp has been acquired by high resolution $^1\text{H-NMR}$ spectroscopy at 14T
55 (corresponding to 600 MHz proton Larmor frequency, Figure 1). The most intense signals in such
56 $^1\text{H-NMR}$ spectrum are due to sugars (contributing to the spectral region 4.2-3.2 ppm and 5.4-5.2
57 ppm), which are known to be very abundant in plum juice under the form of both monosaccharides,
58 disaccharides (mainly sucrose) and fructo-oligosaccharides (FOS) [5,6]. In addition to the
59 saccharide resonances, the resonance of Asn H_β (2.96 and 2.86 ppm) and Asn side chain amide
60 protons (7.60 and 6.88 ppm) are clearly detectable. No other significant sources of exchangeable
61 amide protons with chemical shifts close to the amide resonances were detected. A concentration of
62 Asn of 17.0 ± 0.5 mM could be determined by integrating the Asn H_β resonances with respect to that
63 of reference 60 mM TSP. The concentration of Asn did not change significantly in plums from
64 harvest up to 14-days ripening time (full ripening).

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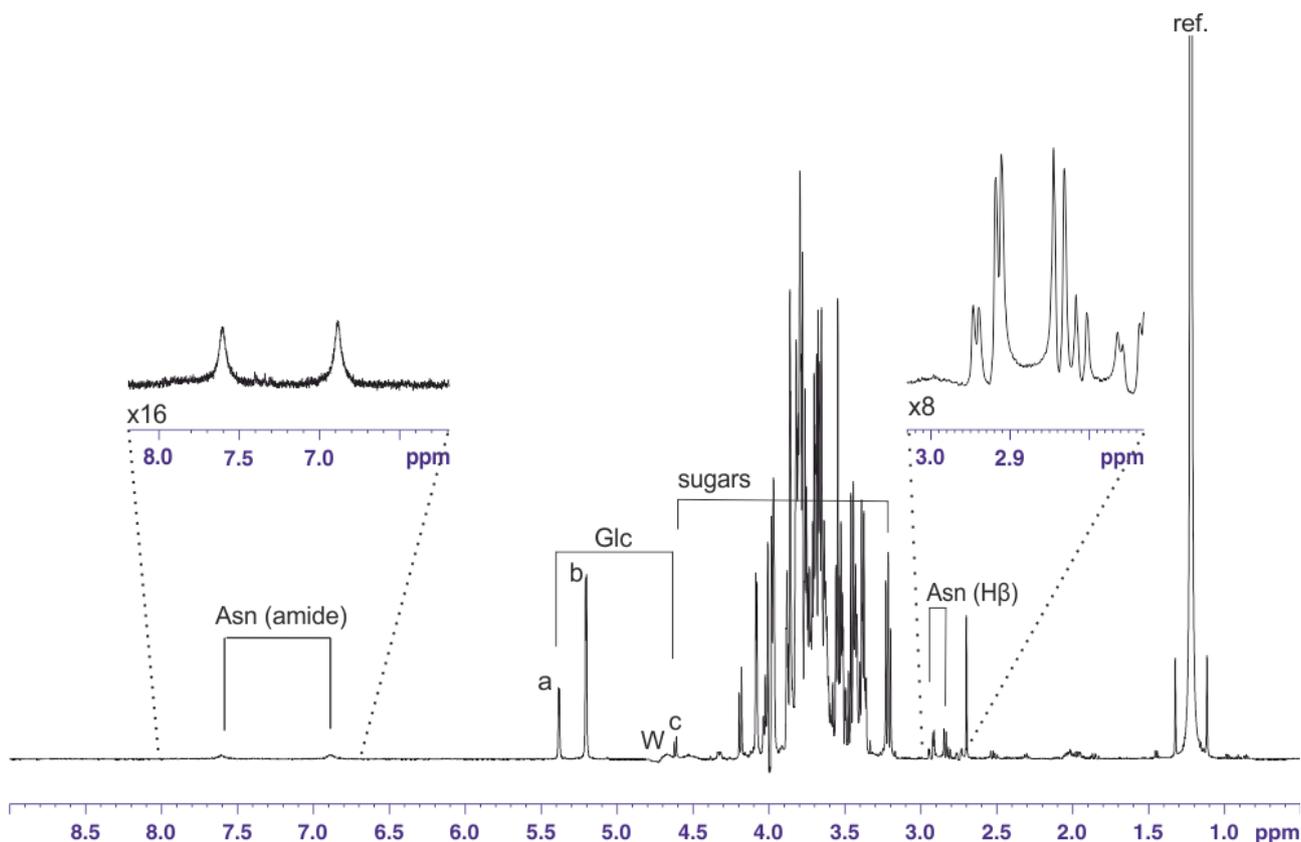
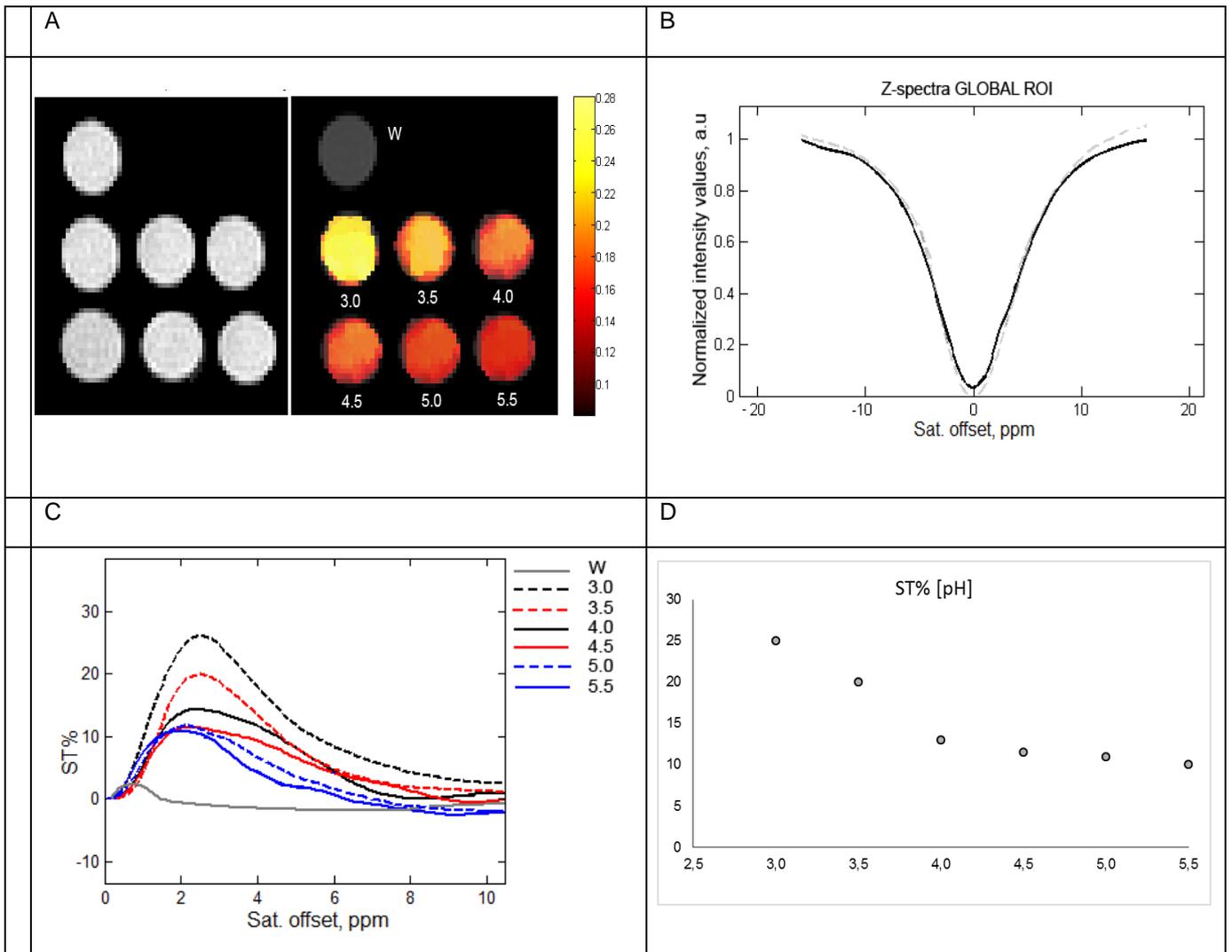


Figure 1. $^1\text{H-NMR}$ spectrum at 600 MHz of plum juice at 7-days post harvest (1:10 dilution with dH_2O , $\text{H}_2\text{O}/\text{D}_2\text{O}$ 95/5 v/v, pH 4.5, 25 °C). Water suppression was achieved by excitation sculpting with gradients to avoid saturation transfer of exchangeable protons. The resonances of asparagine amide and $\text{H}\beta$ are magnified in the inserts. **a**: anomeric proton resonance of sucrose/FOS; **b**: anomeric proton resonance of α -D-glucose, **c**: anomeric proton resonance of β -D-glucose; **R**: reference *tert*-butanol; **w**: residual water signal.

67

68 Next, CEST-MR images were acquired on a scanner working at a magnetic field of 1T. First, a
 69 phantom of six tubes containing 17 mM Asn (*i.e.* the concentration measured in the plum pulp) and
 70 at different pH values was assessed by CEST-MRI, as it is well-known that the exchange rate of
 71 amide protons with bulk water is base-catalyzed and rapidly increases with pH. Therefore, at the
 72 magnetic field of 1T, the pH dependent modulation of the exchange rate can significantly affect the
 73 saturation transfer efficiency. The dependency of ST on pH has been assessed within the pH
 74 interval 3.3–5.5, to include pH conditions of plum pulp juice after harvest (pH 3.3 for the cultivar
 75 considered) and at full ripening (pH 4.5). As expected (Figure 2), high ST% (35%) can be obtained
 76 from the solutions at the more acidic pH 3.3 (irradiation frequency offset at 2.1 ppm from the water
 77 resonance), while the CEST effect steadily drops to 10% at pH 5.5.



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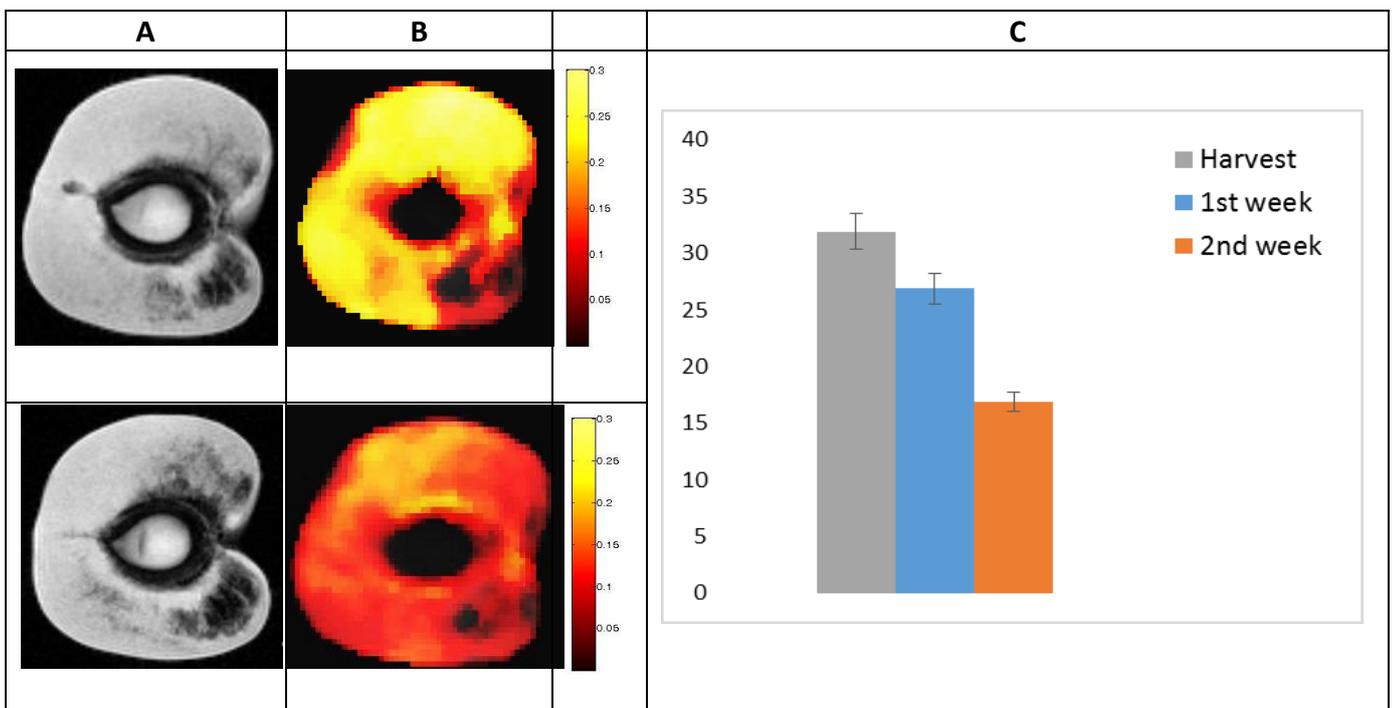
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Figure 2. CEST-MRI of asparagine solutions at different pH (3.3, 3.5, 4.0, 4.5, 5.0, 5.5), 1T. **A)** CEST-MR images of 17 mM Asn (irradiation offset at 2.1 ppm with respect to the water resonance). Pixels showing ST% above 10% (noise threshold) are represented in false color scale, else they are shown in gray **B)** Z-spectrum of 17 mM Asn (pH 3.3), water reference in gray line . By increasing pH, the amide protons exchange faster thus approaching the coalescence, resulting in the decrease of saturation transfer efficiency. **C)** ST% Asn 17 mM (with water reference, W). **D)** Plot of ST% (from images in B) versus pH.

91

92 On this basis, CEST-MR images of the whole plum were acquired (Figure 3). The irradiating rf
 93 offset was at 2.1 ppm and the images were acquired at 7 and 14 days after harvest. Conventional T₂-
 94 weighted MR images were also acquired to visualize the internal structure and texture of the fruits.
 95 At day 7 post harvest, a very high ST% (25%) was clearly detected all over the fruit. Conversely, at
 96 day 14, the ST% is reduced to 15%. The reasons for the observed behaviour may rely on different
 97 causes, namely changes in Asn concentration, change in pH or change in T₁ of water protons.
 98 Through the acquisition of the high resolution ¹H-NMR spectrum and T₁ measurements of the
 99 plum's pulp of these samples it assessed that Asn concentration and T₁ of water did not change with
 100 ripening. Conversely, the pH increased from 3.3 to 4.7. Thus we conclude that the change in pH is
 101 the main determinant for the observed change in CEST-MR images taken at day 7 and 14 after
 102 harvest.

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118 **Fig. 3 : plum fruit T₁-weighted images (A) and ST maps (B) at different chemical shift in thermal**
119 **scale, from acquired CEST spectra after 7 (left column) and 14 days (right column) post harvest. ST**
120 **maps were calculated at Δ ppm values of amide protons for Asparagine from bulk water (4.7 ppm) .**
121 **Mean values of ST% for the whole plum study (C)**

122

123 **3. Conclusions**

124 This is the first time that CEST-MRI technique has been used to food MRI research area. The
125 herein reported results show that CEST-MRI may be highly informative on the chemical
126 composition of a fruit. Actually the detection of ST% arising from sugar exchanging protons (5.0 –
127 5.5 ppm) may provide important insights into the ripening process. This task has not been possible
128 to be addressed on the MRI 1T scanner used to this work.

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132 **4. Materials and Methods Section**

133 4.1 Fruit imaging

134 Samples from *Prunus domestica* cv. TC Sun Angeleno were supplied by Sanifrutta fruit firm,
135 Saluzzo, Piedmont region. Healthy plums with an average diameter of 5 cm were chosen for
136 experiments. MRI and CEST scans were performed using an ICON Avance M2 (Bruker) with a
137 NdFeBmagnet (Aspect). Operative magnetic field B₀ was 1 T. T₁- and T₂-weighted plum images
138 were acquired for preliminar observation before CEST experiments. Fast low angle shot (FLASH)
139 and Rapid acquisition with Refocused echo (RARE) methods was used and for T₁- and T₂-

140 weighted images, respectively. FLASH parameters were set with echo time 5 s, repetition time
141 184.5 s, 4 averages pulse angle 30 degrees. RARE parameters were set with repetition time 6 s,
142 effective echo time 12.5 ms, rare factor 24. Geometry parameters were fixed for both sequences as
143 follows: 'field of view 50×50mm/slice thickness 2 mm/matrix 128*128px/ voxel size 0.3 mm³'.
144 Temperature was maintained at 6°C to prevent consistent tissue destruction and perform scans for 20
145 days.

146 All target compounds in this study has small $\Delta\omega$ offsets ranging from about 1 (hydroxyls group of
147 sugars) to 6 ppm (some amine groups). Spillover effect was minimized with low saturation
148 intensities. CEST effects was visualized by means of super-imposed map on a T₁ weighted image
149 with no magnetization transfer (MT) pulse. CEST MRI scans were performed using RARE method,
150 which parameters were defined as described above; MT module was active and impulse was a block
151 pulse set in length to 3 s and amplitude 3uT. Other setups were an echo time of 12 s, repetition time
152 10 s, one number of repetitions, refocusing flip angle 180 deg., rare factor 24 and one average. In
153 plane geometry was modified for single axial slices, 4 mm thickness, FOV 50×50 mm, matrix
154 64×64 px, voxel size 1.2 mm³.

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156 4.2 ¹H-NMR spectroscopy.

157 All ¹H-NMR spectra were acquired on a BrukerAvance III spectrometer operating at 14.1 T,
158 corresponding to a proton Larmor frequency of 600 MHz, equipped with an inverse Z-gradient 5
159 mm double resonance inverse probe. The temperature was controlled within ±0.1 K through the
160 BTO2000 VTU system. ¹H-NMR spectra aimed at assessing the presence of exchangeable protons
161 were carried out by diluting the plump juice 1:10 with dH₂O without pH adjustment. An aliquot of
162 540 μL of the diluted sample were added with 50 μL of D₂O for field/frequency lock and 1 μL of
163 *tert*-butanol as a secondary chemical shift reference. ¹H-NMR spectra were acquired with a pulse
164 sequence performing water suppression by excitation sculpting with gradients [7] to minimize
165 saturation transfer from water to exchangeable protons (Bruker pulprog zgesgp). Acquisition
166 parameters included 3 s relaxation delay, 64 K complex data points, 16 ppm spectral window, 128
167 scans, and 3 ms selective pulse length for water suppression. To quantify the metabolite levels and
168 to obtain metabolic profiles, the plum juice was diluted 1:10 with 100 mM phosphoric buffer pH
169 3.3 (final pH checked and eventually adjusted to to 3.35±0.05 pH units). A volume of 540 μL juice
170 was added with 60 μL of D₂O containing reference *tert*-butanol to yield a final concentration of
171 60±0.05 mM. ¹H-NMR spectra were acquired with the one-dimensional noesy pulse sequence with
172 pre-irradiation of the water signal for water suppression. Acquisition parameters included 5 s
173 relaxation delay, 128 scans, 4 dummy scans, 20.5 ppm spectral window, 64 K complex data points,

174 10 ms mixing time, and 25 Hz bandwidth of the water suppression pulse. The levels of asparagines
175 was evaluated by comparing the area of the Asn H β signal to that of reference TSP. Metabolite
176 signals were analyzed by the AMIX 3.9.2 software package implemented with the Bruker
177 BBIREFCODE 2.0.0 database and the BioMagResBank metabolomics database [8].

178 4.4 CEST analysis.

179 Raw MRI data were processed by means of custom-made software program implemented in
180 MATLAB (the Mathworks Inc., Natick, MA, USA) [9]. Z-spectra and saturation transfer (ST) maps
181 were corrected by means of interpolation method [10].

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