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## Investigation of water state during induced crystallization of honey

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### Abstract

This work studied water state of honey during crystallization, obtained statically and dynamically, by differential scanning calorimetry (DSC), water activity ( $a_w$ ) assessment and time domain nuclear magnetic resonance (TD-NMR).

Crystallization was induced by adding 5% of crystallized honey to three honey samples with different fructose/glucose ratio, the key characteristic for honey crystallization. Samples were stored at 14 °C. Dynamic crystallization was obtained by using an impeller. DSC showed that the dynamic crystallization was faster than the static one, the latter characterized by two phases, showing different rates. The crystallization rate did not affect  $a_w$ , that remained below 0.600. TD-NMR allowed to separately observe two kinds of protons, both pertaining to liquid sugars, one chemically exchanging with water and one not exchanging with it. The combination of techniques allowed speculating that the two crystallization methods led to crystals of different size and shape.

### Keywords

Differential scanning calorimetry; dynamic crystallization; Honey; static crystallization; time-domain nuclear magnetic resonance; water activity; water state

## 26 **Introduction**

27 Honey is a supersaturated solution that contains mainly glucose and fructose (70-80%) and only small  
28 amounts of other sugars. The crystallization, or granulation, of honey is a natural phenomenon that  
29 occurs during storage and involves only glucose, as fructose is characterized by a higher solubility  
30 value.

31 The rate of crystallization depends on many factors, among which amount of glucose, fructose and  
32 water, temperature, glucose supersaturation level, viscosity and presence of pre-formed crystals or  
33 impurities (Conforti, Lupano, Malacalza, Arias, & Castells, 2006; Venir, Spaziani, & Maltini, 2010).

34 Nucleation can be classified as primary or secondary. Primary nucleation occurs when the system does  
35 not contain any pre-formed crystal and an energy barrier has to be overcome for the formation of new  
36 nuclei. Collision among molecules in the solution leads to the formation of clusters that, if sufficiently  
37 big, can overcome the energy barrier and become stable (Hartel, 1993). Higher supersaturation levels  
38 increase the probability for clusters to overcome the critical dimension. The secondary nucleation can  
39 occur only when pre-existing crystals are present. A secondary crystal can be generated from dendritic  
40 growth on the surface of a primary nucleus or when a primary nucleus collides with another primary  
41 nucleus or with other components of the system, such as the walls of the vessel (Hartel, 1993).

42 Guided or induced static crystallization (1,987,893, 1935) is based on the secondary nucleation  
43 phenomena and involves the introduction of fine seed crystals that will act as primary crystallization  
44 nuclei into liquid honey. Such procedure on one side allows to obtain finely granulate honey, on the  
45 other side avoids both unpredictable changes in the texture of honey during storage and crystallization  
46 defects (Dettori, Tappi, Piana, Dalla Rosa, & Rocculi, 2018).

47 Dynamic crystallization (Gonnet, 1994) consists in carrying out the guided crystallization under a slow  
48 manual or automatic stirring of the mass for a few days, to impart creaminess and spreadability to the  
49 crystallized product. Honey obtained in this way is defined as creamy honey and its peculiar  
50 rheological characteristics are due to the formation of very small crystals (Karasu, Toker, Yilmaz,  
51 Karaman, & Dertli, 2015). The crystallization rate and rheological characteristics of honeys have been  
52 investigated by many works in relation to composition and crystallization levels (Slavomir Bakier,  
53 2007; Sławomir Bakier, Miastkowski, & Bakoniuk, 2016; Conforti et al., 2006; Dobre, Georgescu,  
54 Alexe, Escuredo, & Seijo, 2012; Venir et al., 2010). However, to the best of our knowledge, the  
55 differences between static and dynamic crystallization have never been investigated before.

56 In addition, the works published on honey have rarely addressed the behavior of water during  
57 crystallization, beyond the mere observation of water activity (Gleiter, Horn, & Isengard, 2006;  
58 Zamora & Chirife, 2006). Water activity determines honey microbiological stability during storage. It  
59 is deeply influenced by crystallization because glucose binds six molecules of water in liquid honey,  
60 but crystallizes mainly in the monohydrate form. Crystallization thus promotes water concentration in  
61 the liquid phase, leading in turn to an increase of water activity, allowing the growth of osmophilic  
62 yeasts (Gleiter, Horn, & Isengard, 2006).

63 DSC measurements have been successfully applied to evaluate the crystallization of honey by various  
64 authors (Al-Habsi, Davis, & Niranjana, 2013; Venir et al., 2010), who measured the amount of glucose  
65 crystals on the basis of their melting enthalpy. Transverse relaxation time ( $T_2$ ) of the protons observed  
66 by TD-NMR has been found able to give precious information about water interaction with solutes and  
67 sample's structures in several food matrices (Mauro et al., 2016; Petracchi et al., 2012). These  
68 interactions have been also studied in honey during crystallization, where they have even been used to  
69 assess product adulterations through water state (Ribeiro et al., 2014; Ribeiro et al., 2014).

70 The aim of the present study is to apply differential scanning calorimetry (DSC), water activity and  
71 time domain nuclear magnetic resonance (TD-NMR) measurements to investigate the behavior of  
72 water in honey during induced crystallization carried out in a traditional static manner or during a  
73 dynamic process, achieved through constant stirring of the mass at the optimal crystallization  
74 temperature (14 °C).

## 75 **Material and methods**

### 76 **Raw material and preparation of static crystallization samples**

77 The honey samples used in the present study were selected with the aim of having specific F/G ratios of  
78 approximately 1.05, 1.20 and 1.40, leading to fast (FC), medium (MC) and slow (SC) crystallization,  
79 respectively (Dettori et al., 2018). Before the experiment of crystallization kinetics, samples were  
80 gently heated up to 50 °C to melt any pre-formed crystal. The absence of glucose crystals was  
81 evaluated by optical microscopy. The crystal nuclei used were obtained by citrus honey finely  
82 granulated, added to the three samples so to reach 5% of the total mass. The so obtained mix was  
83 manually stirred with a spatula at room temperature for 10 min. Samples were analyzed for water (at 20  
84 °C with an Abbe refractometer) and sugars content (DIN-NORM-10758, 1997). During static  
85 crystallization, sampling itself could break the crystalline structure. To limit this confounding factor,

86 liquid honey added with crystal nuclei and stirred was poured into samples holders ready for each  
87 analytical determination. Samples, created in triplicate, were stored in a climatic chamber at 14 °C  
88 throughout the entire crystallization process. Storage time needed for complete crystallization of each  
89 sample was assessed by means of preliminary tests. Sampling intervals were then adjusted accordingly,  
90 as follows: 0, 2, 7, 9, 22, 43 and 50 days for the FCs samples; 0, 10, 15, 34, 51 and 62 days for the MCs  
91 samples; 0, 7, 14, 21, 28, 34, 41, 48, 63, 83 and 102 days for the SCs samples.

### 93 **Raw material and preparation of dynamic crystallization samples**

94 Samples were subjected to dynamic crystallization with an in-house made steel temperature controlled  
95 stirrer, equipped with a helical impeller having a diameter of 100 mm and rotating at 14 rpm. The  
96 mixing chamber (about 1.2 L of volume) was externally cooled with a flux of water/ethylene glycol  
97 fluid, so to grant a stirred sample temperature of 14 °C. To create the samples, liquid honey at room  
98 temperature was added with 5% (w/w) of crystallized honey and placed in the stirring chamber. Every  
99 sample took 3 to 4 hours to reach 14 °C. The moment when the 14 °C were reached was considered as  
100  $T_0$  for the analysis. Samples were collected from the stirring chamber without interrupting the process.  
101 Storage durations were determined for each sample through preliminary tests and were of 10, 16 and 32  
102 days for samples FCd, MCd and SCd, respectively. Sampling intervals were 0, 1, 2, 4, 7, 8 and 10 days  
103 for the FCd samples; 0, 1, 2, 3, 5, 7, 9, 12, 14 and 16 days for the MCd samples; 0, 2, 3, 4, 7, 9, 11, 14,  
104 18, 21, 25, 28 and 32 days for the SCs samples.

### 105 **Differential Scanning Calorimetry**

106 Thermal analysis was carried out by differential scanning calorimetry using a DSC Q20 (TA  
107 Instruments, Germany) equipped with a cooling unit (TA-Refrigerated Cooling System90). Heat flow  
108 and temperature calibration were performed with distilled water ( $T_m$  0.0 °C) and indium ( $T_m$  156.60  
109 °C) under a dry nitrogen flow of 50 mL min<sup>-1</sup>.

110 Honey samples were weighed in 50 µl aluminium DSC capsules and sealed. At each sampling time,  
111 three replicates were analyzed through temperature scanning at 5 °C/min from 14 to 100 °C.

112 Peaks were integrated with the Software TA-Universal analyzer, determining melting temperature ( $T_m$ )  
113 and enthalpy ( $\Delta H$ ) of the granulated honeys.

### 114 **Water activity ( $a_w$ )**

115 Water activity was measured with an ACQUA LAB Water Activity Meter, (Decagon Devices, US).

116 For statically crystallized honey, three samples holders were filled with liquid honey at the beginning  
117 of the storage, then measured at each sampling time. Between measurements, samples were covered  
118 with lids and protected with parafilm. For dynamically crystallized honey, samples were collected at  
119 each sampling time.

## 120 TD-NMR

121 The transverse relaxation time ( $T_2$ ) of protons was measured at 25 °C with the Carr–Purcell–Meiboom–  
122 Gill (CPMG) (Meiboom & Gill, 1958) pulse sequence ( Ribeiro et al., 2014; Ribeiro et al., 2014), using  
123 a Bruker Minispec PC/20 spectrometer (Bruker, Germany) working at 20 Hz. The exponential decay  
124 comprised 3200 echoes, an echo time (TE) of 0.080 ms, leading to a dead time of 167  $\mu$ s, and a recycle  
125 delay of 5 s. The number of scans and the amplification factor were chosen so that a S/N ratio value of  
126 300 was reached, while signal clipping was prevented. Data mining was performed in R computational  
127 language (R Development Core Team, 2011), by means of routines developed in-house. In order to  
128 treat honey as a two components system, by following de Ribeiro *et al.* (Ribeiro, Mársico, Carneiro,  
129 Monteiro, Júnior, et al., 2014), the phased experimental curves were fit towards the sum of two  
130 exponential decays, according to the equation:

$$S_{(t)} = \sum_{i=1}^N I_n e^{\left(\frac{-t}{T_{2,n}}\right)} + E_{(t)}$$

131 where  $I_n$  represents the intensity of each proton population and  $T_{2,n}$  its transverse relaxation time.

132 The presence of two water populations postulated by Ribeiro *et al.* could be doubted by the massive  
133 work by Brown (Brown, 1989), who demonstrated that the sum of two exponential curves fit nicely  
134 also the signal of a single water population. This happens when water covers a wide range of relaxation  
135 rates, a common case in food matrices (Iaccheri et al., 2015; Laghi et al., 2005; Petracci et al., 2014),  
136 thus giving the false impression of two water components. For this reason the  $T_2$  decays, centered and  
137 scaled to unity variance, were also employed to build a robust principal component analysis (rPCA)  
138 (Hubert, Rousseeuw, & Vanden Branden, 2005) model. This was done by setting an alpha value of  
139 0.75. For this model, we calculated the scoreplot, the projection of the samples in the PC space, tailored  
140 to highlight the underlying structure of the data. Besides, we calculated the Pearson correlation plot,  
141 relating the concentration of each variable to the model components.

## 142 **Statistics**

143 Differences among samples at specific time-points were looked for by anova test, with Tukey as a post-  
144 hoc test, by taking advantage of the aov function of the R package “stats” (Chambers, Freeny, &  
145 Heiberger, 1992).

146 For TD-NMR data, differences in the overall trends characterizing the FCs, MCs and SCs samples  
147 along storage time were looked for by two-way anova test, followed by Tukey as a post-hoc test. The  
148 limited number of samples per point was considered by applying the tests on ranks (Conover & Iman,  
149 1981). Intensity and  $T_2$  of each proton population and score values in the rPCA (Hubert et al., 2005)  
150 model registered along time were interpolated by means of non-parametric functions. For the purpose,  
151 a local regression model was applied, by taking advantage of the loess function (Cleveland, Grosse, &  
152 Shyu, 1992) of the R package “stats”, with degree of smoothing equal to 0.9 and the degree of the  
153 polynomial equal to 1.

## 154 **Results and discussion**

### 155 **DSC**

156 The precise composition (after crystal nuclei addition) of the samples fast (FCs), medium (MCs) and  
157 slow (SCs) static crystallization is reported in Table 1. Slight differences were observed between each  
158 of the couples of samples grouped as FC, MC or SC. However, the selection allowed to obtain very  
159 similar fructose/glucose ratio, the parameter the mostly affecting the crystallization rate.

160 The detailed evolution of the melting enthalpy during static and dynamic crystallization is reported in  
161 figure 1, while overall features of the crystallization processes are reported in table 2.

162 The melting enthalpy is proportional to the amount of crystallized glucose, so that at  $T_0$  its value  
163 reflects the amount of finely crystallized honey added as starter. FC, MC and SC samples stored  
164 statically reached the maximum crystallization values of 34.72, 27.62 and 21.68 J/g, respectively.  
165 These values were proportional to the glucose supersaturation level, hence to the amount of glucose  
166 that could crystallize (Dettori et al., 2018). Glucose supersaturation level determined also the  
167 crystallization rate, so that FCs, MCs and SCs samples reached the maximum melting enthalpy in 50,  
168 90 and 102 days, respectively.

169 Although dynamic crystallization is known to increase granulation rate, to our knowledge, no previous  
170 study has actually compared the crystallization behavior of honey according to static and dynamic  
171 crystallization process. Results obtained showed for the first time that the dynamic process boosted

172 significantly the crystallization rate, which was 5 to 6 fold faster than the static counterpart. In detail,  
173 full crystallization was reached in 10, 16 and 35 days for FCd, MCd and SCd samples, respectively.  
174 This was expected, as the nucleation process (both primary and secondary) is known to be strongly  
175 increased by inputs of energy into the system, here represented by the mechanical energy given by  
176 continuous mixing. According to Hartel (Hartel, 1993), the reason is that an external energy input  
177 promotes random energy fluctuations, represented by local concentrations of sugar exceeding the  
178 critical value for nucleation. Moreover, agitation promotes the forced migration of molecules, so to  
179 reduce the hindrance to mass transfer given by viscosity.

180 It is possible to notice from figure 1 that, in statically stored samples, the crystallization kinetic showed  
181 a linear trend along the entire process, but with an inflection occurring after 9, 15 and 21 days in FCs,  
182 MCs and SCs samples, respectively, corresponding to the 50-60% of the process. A similar result was  
183 observed by Venir *et al.* (Venir *et al.*, 2010) in *taraxacum* honey. In particular, they observed a change  
184 of slope after the crystallization of 15% of glucose, corresponding to the 60 % of the total glucose that  
185 could undergo crystallization.

186 Following Serra-Bovehì (Serra-Bonvehi, 1974), the two stages observed by us and Venir *et al.* (2010)  
187 could be ascribed to the alternation of nucleation and crystal growth. The two phenomena can occur  
188 simultaneously, but at different rates in relation to the supersaturation level. At the beginning of the  
189 process, when supersaturation is high, the formation of new crystals is faster than their growth. As the  
190 crystallization proceeds, the nucleation rate decreases exponentially, so that, in a second stage, the  
191 predominant process is the enlargement of the existing nuclei.

192 The two-phase behavior could not be observed in honey samples crystallized dynamically, that instead  
193 showed a linear increase of melting enthalpy along the entire storage time. This observation could be  
194 rationalized by considering that the energy input represented by the stirring promotes the formation of  
195 nuclei, while the constant movement of the mass inhibits the excessive growth of the crystals.

196 Hence, the different crystallization method adopted not only noticeably influenced the crystallization  
197 rate, but it also promoted changes in the formation of crystals that is at the basis of the difference in the  
198 rheological properties, as described by Gonnet (1994).

## 199 **Water activity**

200 Figure S1 reports the evolution of  $a_w$  in honey samples statically and dynamically stored as a function  
201 of melting enthalpy. Initial values ranged between 0.490 and 0.550. Differences were likely to be  
202 caused by the different concentration of sugars and water in the honey samples. Indeed, as shown in



203 table 1, water content varied in the 16.0 - 17.7% range. Values of  $a_w$  increased systematically with the  
204 amount of crystallized glucose. In detail, the observed changes in  $a_w$  were in the 0.3-0.6 range, in  
205 agreement with previous investigations (Zamora & Chirife, 2006) carried out on 49 different honey  
206 samples. However, in all cases, the final value never exceeded 0.60, the threshold usually considered  
207 for osmophilic yeast growth.

208 Contrarily to the melting enthalpy, the increase in  $a_w$  followed a linear trend for statically stored  
209 samples, with no evident change of slope. This could be explained by considering that water activity  
210 depends on the overall characteristics of the samples, failing to discriminate fine differences in the state  
211 of water throughout the sample itself.

### 212 **TD-NMR: two components model**

213 By following the works of Ribeiro *et al.* (Ribeiro et al., 2014; Ribeiro et al., 2014),  $T_2$  weighted TD-  
214 NMR signals were fit to a model postulating the possibility to separately observe two protons pools,  
215 that were named  $T_{21}$  and  $T_{22}$  (Fig. 2). Analysis of variance showed that, for both  $T_2$  and intensity of the  
216 two populations, level of supersaturation and time had in each case a statistically significant effect  
217 ( $p < 0.001$ ). To grab the trends of the two populations along storage in a non-parametric fashion,  
218 smoothing trends were calculated. The main features of the so evidenced trends are summarized in  
219 table 3.

220 In agreement with Ribeiro *et al.* works, the two protons pools had, at  $T_0$  in the statically crystalized  
221 samples,  $T_2$  values around 1.5 and 5 ms respectively. Their relative intensities were found in the  
222 present work to be around 55% and 45%. Ribeiro *et al.* ascribed the two populations exclusively to  
223 water differently interacting with crystals, but such ascription seems very unlikely, because it does not  
224 consider the remarkable number of protons of the sugars. A few qualitative considerations drive the  
225 point.

226 In liquid honey, each mole of water brings 0.11 moles of protons. Each mole of glucose or fructose is  
227 characterized by 0.028 moles of protons pertaining to -OH groups. Around 75% of them (0.021 moles)  
228 was found to be labile (Fabri, Williams, & Halstead, 2005). The exchange rate of these protons  
229 between water and sugars is expected (B. Hills, 1998; Venturi et al., 2007) to be much higher than the  
230 NMR signal registration rate, reasonably far above  $100 \text{ s}^{-1}$  (Fabri et al., 2005). In this regime, water and  
231 labile sugar protons are observed as a single population. Such population can be simply called  
232 “exchangeable”, as suggested by Petracci *et al.* (Petracci et al., 2014) on a different matrix. The

233 remaining sugar protons bound to carbons plus the non-labile –OH protons (accounting for 0.046  
234 moles) cannot exchange with water, so that they can be called “non-exchangeable”.

235 According to the above considerations, in the samples we have analyzed in the present work, non-  
236 exchangeable and exchangeable protons populations may contribute to  $T_2$  weighted TD-NMR signals  
237 with about a 50:50 relative intensity. Such qualitative consideration is in very good agreement with the  
238 ratio measured in SC samples (51.47:48.53), while it shows a 17.14% discrepancy in the case of FC  
239 samples.

240 The pool of exchangeable protons is expected to have longer  $T_2$  values, according to the following  
241 reasoning. The  $T_2$  of a proton pertaining to liquid sugar is reasonably in the range of milliseconds.  
242 When this proton is exchanged between sugar and water its  $T_2$  is the weighted average of the  $T_2$  of the  
243 two sites, according to the Carver and Richards (Carver & Richards, 1972), corrected by Hills and  
244 coworkers (B. P. Hills, Wright, & Belton, 1989). Such  $T_2$  is undoubtedly longer than the one of non-  
245 exchangeable protons, because water has been found liquid even in a glassy matrix (B. P. Hills &  
246 Pardoe, 1995), what translates into  $T_2$  in the range of hundreds of milliseconds. According to this  
247 consideration, we therefore suggest that the populations originally named  $T_{21}$  and  $T_{22}$  by Ribeiro *et al.*  
248 can be ascribed to non-exchanging and exchanging protons, respectively.

249 Along the entire storage period the two protons pools model was able to fit nicely the  $T_2$  weighted  
250 signals registered on every sample, even on those where crystallization has occurred massively. This  
251 suggests that the liquid fraction of honey had the major, if not the exclusive, contribution to the NMR  
252 signal. Indeed, the CPMG pulse sequence we employed had an unavoidable dead time of 167  $\mu$ s. The  
253 protons pertaining to the crystals were expected to be largely unobservable, because characterized by a  
254  $T_2$  of a few microseconds (B. P. Hills & Pardoe, 1995).

255 Interestingly, as already observed for the increase of melting enthalpy, for each of the studied samples  
256 two distinct stages of crystallization could be noticed from a  $T_2$  point of view. However, the change  
257 was observed at different times. The first stage could be considered as complete at days 30, 50 and 66  
258 for FCs, MCs and SCs samples, respectively. During this stage, the relative intensity of non-  
259 exchangeable protons population decreased by 8.7%, 7.6% and 4.6% in the FCs, MCs and SCs  
260 samples, respectively. In parallel, the  $T_2$  of non-exchangeable protons increased by 74% to 200%,  
261 while the  $T_2$  of exchangeable protons increased by 10% to 45%. The main contribution to the trends of  
262 both relative populations and  $T_2$  values in the first stage is very likely the subtraction of glucose from  
263 the liquid fraction due to crystallization, leading to an increased concentration of water, as noticed by  
264 Dettori *et al.* (Dettori *et al.*, 2018). Indeed, an increase in water concentration increases the amount of

265 the exchangeable protons in the system ( $\approx 53\%$ ). As confirmation, the FCs samples, with a higher  
266 supersaturation index, showed also the largest and quickest increase of exchangeable protons, followed,  
267 proportionally, by MCs and SCs samples. Moreover, the increase of water concentration in the liquid  
268 fraction has two further effects. First, it moves the weighted average of  $T_2$  of the exchangeable protons  
269 towards higher values. Second, it increases the tumbling rate of the molecules, leading to higher  $T_2$   
270 values for both exchangeable and non-exchangeable protons (Bordoni et al., 2014).

271 The second stage of static crystallization highlighted by  $T_2$  weighted signals started around day 30, 50  
272 and 66 for samples FCs, MCs and SCs, respectively. This stage was characterized by a partial inversion  
273 of the previous trends, with an increase of non-exchanging protons and with a shortening of the  $T_2$   
274 values of both populations. The most likely rationalization of this phenomenon is that the crystals were  
275 so densely spread across the entire honey volume that a high percentage of the still liquid molecules  
276 interacted with them. Even if it is not possible to describe rigorously such interaction, it probably  
277 comprised a reduced mobility, leading to shorter  $T_2$  values, and a less effective sugar-water protons  
278 exchange, that increased the number of non-exchanging protons. A confirmation seems to be devised in  
279 the fact that the highest reduction of  $T_2$  values and exchanging protons occurred in the FC samples,  
280 characterized by the highest amount of crystallized sugar, what translates into a higher solid-liquid  
281 interface. In addition, at the liquid-solid interface local gradients of the magnetic fields form, thus  
282 leading to shorter  $T_2$  values (Dunn, 2002), linked to the dimension and the shape of the crystals.

283 Dynamically crystallized samples did not show appreciable differences from the statically crystallized  
284 counterparts at  $T_0$ . The dynamic crystallization process showed the same overall features of the first  
285 stage of static crystallization, with an increased concentration of exchangeable protons and an increased  
286  $T_2$  values for both populations for each level of glucose supersaturation. The remarkable feature of  
287 these trends is the entity of the changes. While the first stage of static crystallization, when crystal  
288 nucleation is favoure, leads to a decrease in the pool of non-exchangeable protons of 8.7%, 7.6% and  
289 4.6% for FC, MC and SC samples respectively, dynamic crystallization leads in the same samples to a  
290 reduction of 9.8%, 15% and 5.8%. Differences even more clear could be noticed for  $T_2$  values. As an  
291 example, while the  $T_2$  of non-exchangeable protons increased for FC, MC and SC samples by 80.9%,  
292 56.9 and 26.4% as a consequence of static crystallization, the same values increased by 198.2%,  
293 114.3% and 74%, respectively, as a consequence of dynamic crystallization. Interestingly, stirring  
294 made the values of dynamically crystallized MC and SC samples change similarly to FC samples. This  
295 suggests that at the base of the phenomenon is the number of crystals, which it is higher in the samples  
296 crystallized dynamically.

297 **TD-NMR: model-free analysis**

298 In order to employ  $T_2$  weighed NMR signals to gain information on the samples without applying a  
299 priori determined model, robust principal component analysis (rPCA) (Hubert et al., 2005) was applied  
300 on the centered and scaled signals points (Figure 3). In the scoreplot (Figure 3A), the samples spread  
301 with storage time along PC 1, which represented 96.8% of the total samples variance.

302 The samples that the two components model identified as collected at the end of the first stage of static  
303 crystallization appeared at negative values along PCA, while samples freshly prepared or collected at  
304 the end of crystallization were characterized by high and intermediate scores, respectively. This made  
305 the pattern covered by the samples along PC 1 undoubtedly similar to those highlighted by the two  
306 protons pools model. Again similarly to the two protons pools model, the samples collected at the end  
307 of the dynamic crystallization were located at scores that were far lower than the corresponding created  
308 with static crystallization. The correlation between the points of the  $T_2$  weighted signals and their  
309 importance over PC1 (Figure 3C) showed, in agreement with the two components model, that in the  
310 fresh samples the non-exchangeable protons played the highest role, while the opposite was observable  
311 at the end of the first stage of crystallization.

312 The non-parametric approach constituted by the rPCA model directly calculated on the signals  
313 registered by TD-NMR confirmed, from a protons  $T_2$  point of view, that the static crystallization could  
314 be divided into two stages, the second of which partly reversing the effects of the first one. From a  $T_2$   
315 point of view, the interactions between crystals and liquid honey seemed of similar type but of different  
316 extent for statically and dynamically crystallized samples.

317 **Conclusions**

318 The water behavior in honey during induced crystallization according to static and a dynamic process  
319 was investigated.

320 DSC measurements confirmed that the constant movement of the honey during storage decreased the  
321 time necessary for the complete crystallization of all the honey types by 5-6 fold. Static crystallization  
322 showed two main phases of crystal genesis, identified both by DSC and TD-NMR measurements,  
323 characterized by different rates, probably related to the nucleation and crystal growth phases  
324 alternation. On the contrary, dynamic crystallization was characterized by a linear trend that was  
325 attributed to a prevalence of the nucleation phenomenon over the growth of crystals. Moreover, the  
326 crystallization rate did not influence the  $a_w$  increase, that remained below 0.600.

327 Through TD-NMR two populations of protons were identified and attributed to liquid sugars protons  
328 exchanging and non-exchanging with water. The interaction between crystals and liquid honey showed  
329 some differences according to the type of crystallization process adopted, that could be due to the  
330 different number and size of the crystals. However, further investigation is necessary to confirm this  
331 hypothesis.

332 In general, the described multi-analytical approach confirmed the suitability of the different techniques  
333 to study the water mobility in differently crystallized honey, giving integrative results able to increase  
334 the knowledge of these complex phenomena with a different level of detail.

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436 **Figures Captions**

437 Figure 1: Relationship between enthalpy and storage time for static (continuous lines) and dynamic  
438 (dashed lines) crystallization, for FC (points), MC (squares) and SC (triangles) samples.

439 Fig. 2. Relative concentration and  $T_2$  of the two protons populations non-exchangeable and  
440 exchangeable with water, as calculated from  $T_2$  weighted curves obtained by TD-NMR on samples  
441 stored statically (empty symbols) or dynamically (filled symbols) for fast (black circles), medium (dark  
442 gray squares) and slow (light gray triangles) crystalizing samples. To ease visual inspection of the data,  
443 trend dashed lines have been added for samples stored statically, while only samples at  $T_0$  and  $T_f$  have  
444 been represented for samples stored dynamically.

445 Figure 3: rPCA model calculated on the centered and scaled points of the  $T_2$  weighted TD-NMR  
446 signals. A) Scoreplot of samples stored statically (empty symbols) or dynamically (filled symbols) for  
447 fast (black circles), medium (dark gray squares) and slow (light gray triangles) crystalizing samples. To  
448 ease visual inspection of the data, for samples stored statically trend dashed lines have been added,  
449 while for samples stored dynamically only data from  $T_0$  and  $T_f$  have been represented. B) Example of  
450  $T_2$  weighted TD-NMR signals, registered on FC samples at the beginning (black) and at the end (gray)  
451 of the storage period. C) Correlation between the points of the signals and their importance over PC1

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Table 1. Composition (g/100 g) of samples.

|     | Fructose | Glucose | Sucrose | Turanose | Maltose | Water | F/G  |
|-----|----------|---------|---------|----------|---------|-------|------|
| FCs | 39.2     | 36.2    | < 0.5   | 0.8      | 1.4     | 17.7  | 1.08 |
| MCs | 38.6     | 32.5    | 0.5     | 0.8      | 1.1     | 16.5  | 1.19 |
| SCs | 42.8     | 31.0    | < 0.5   | 1.4      | 0.7     | 16.8  | 1.38 |
| FCd | 39.0     | 36.4    | <0.5    | 0.9      | 1.2     | 16.0  | 1.07 |
| MCd | 39.7     | 32.9    | <0.5    | 1.3      | 1.1     | 17.5  | 1.21 |
| SCd | 38.0     | 27.1    | <0.5    | 1.1      | 0.6     | 17.3  | 1.4  |

Table 2. Main features of the crystallization kinetics observed through melting enthalpy.

|    | T <sub>0</sub> static | T <sub>0</sub> dynamic | Inflection point static |      | T <sub>f</sub> static |      | T <sub>f</sub> dynamic |      |
|----|-----------------------|------------------------|-------------------------|------|-----------------------|------|------------------------|------|
|    | Enthalpy*             | Enthalpy               | Enthalpy                | Days | Enthalpy              | Days | Enthalpy               | Days |
| FC | 4.32 <sup>a</sup>     | 3.72 <sup>ab</sup>     | 25.05 <sup>a</sup>      | 9    | 34.72 <sup>ab</sup>   | 50   | 38.01 <sup>a</sup>     | 10   |
| MC | 3.84 <sup>ab</sup>    | 5.09 <sup>a</sup>      | 16.31 <sup>ab</sup>     | 15   | 27.62 <sup>bc</sup>   | 90   | 31.39 <sup>b</sup>     | 16   |
| SC | 2.62 <sup>b</sup>     | 3.6 <sup>b</sup>       | 14.77 <sup>b</sup>      | 21   | 21.68 <sup>c</sup>    | 102  | 21.85 <sup>c</sup>     | 35   |

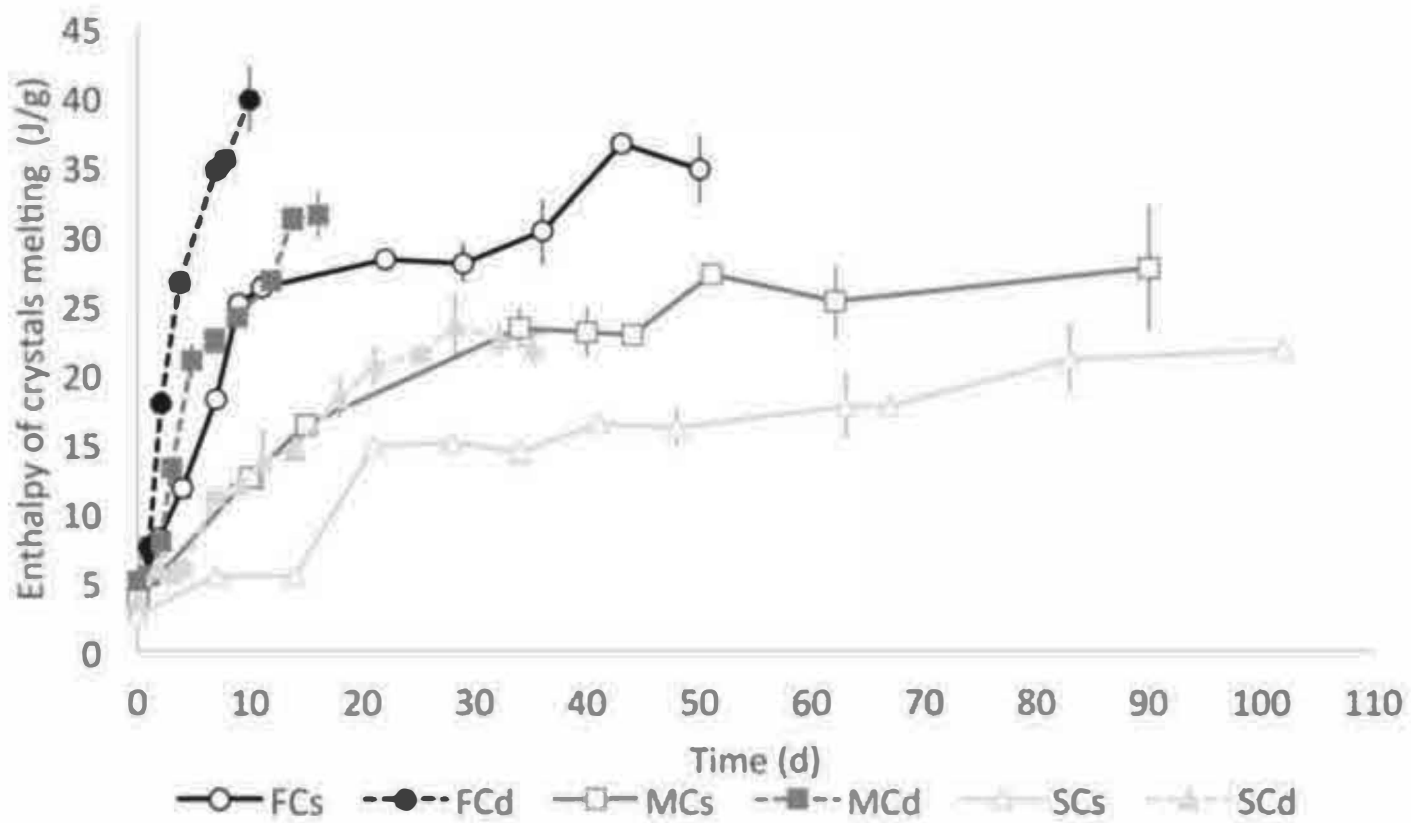
\* Enthalpy is expressed in J/g. At each time-point, different letters indicate significant differences between samples. For readability, data dispersion is not indicated.

Table 3. For TD-NMR data, main features of the trends evidenced by the non-parametric fitting (dashed lines of figure 2).

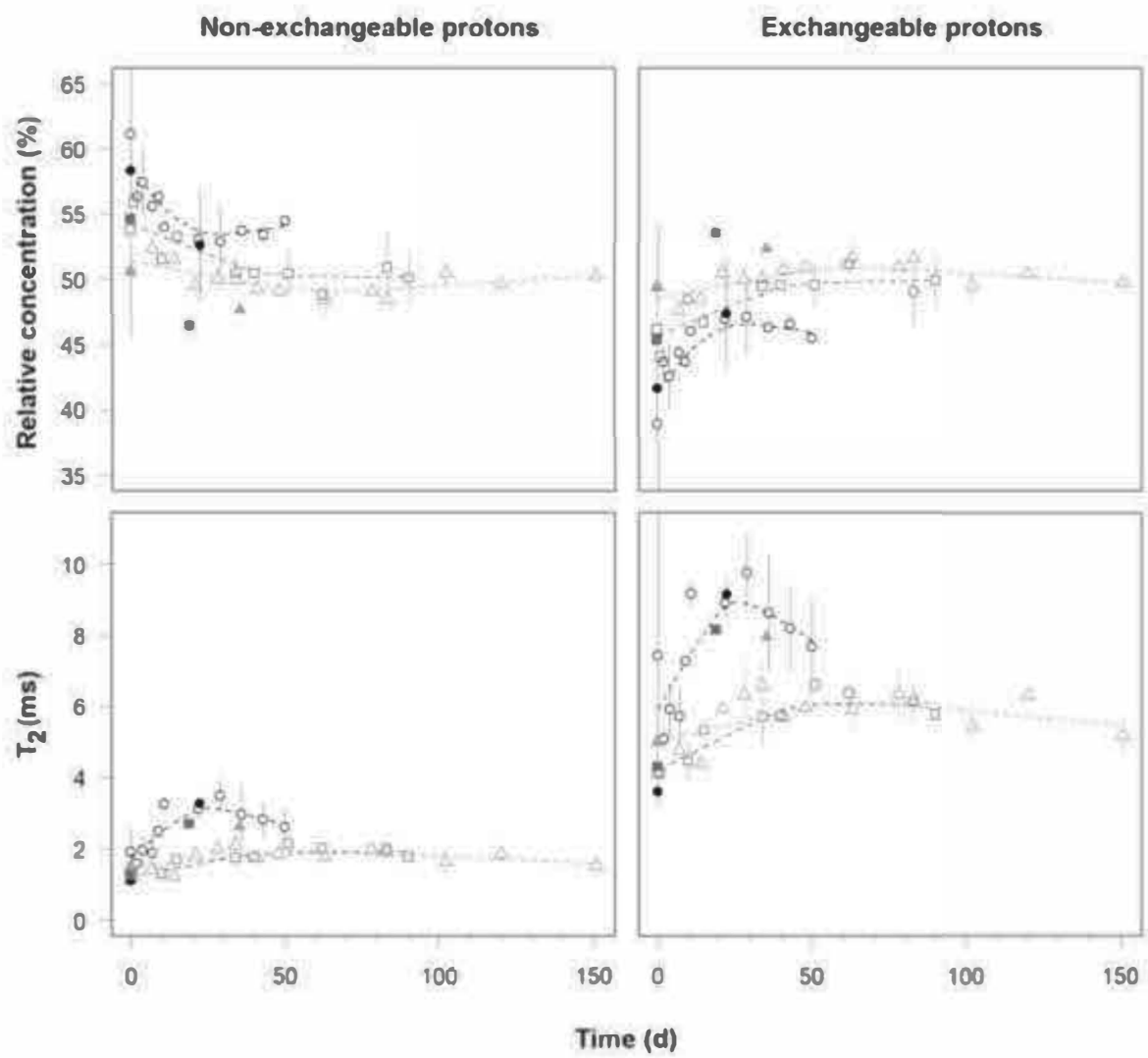
|                  |    | T <sub>0</sub> static |                   | T <sub>0</sub> dynamic |                   | Inflection point static |                   |      | T <sub>f</sub> static |                   | T <sub>f</sub> dynamic |                   |
|------------------|----|-----------------------|-------------------|------------------------|-------------------|-------------------------|-------------------|------|-----------------------|-------------------|------------------------|-------------------|
|                  |    | Int. %                | T <sub>2</sub> *  | Int %                  | T <sub>2</sub>    | Int. %                  | T <sub>2</sub>    | Days | Int. %                | T <sub>2</sub>    | Int. %                 | T <sub>2</sub>    |
| Non-exchangeable | FC | 58.57 <sup>a</sup>    | 1.73 <sup>a</sup> | 58.35 <sup>a</sup>     | 1.10 <sup>b</sup> | 53.46 <sup>a</sup>      | 3.13 <sup>a</sup> | 30   | 54.11 <sup>a</sup>    | 2.68 <sup>a</sup> | 52.63 <sup>a</sup>     | 3.28 <sup>a</sup> |
|                  | MC | 54.27 <sup>a</sup>    | 1.23 <sup>a</sup> | 54.65 <sup>ab</sup>    | 1.26 <sup>b</sup> | 50.15 <sup>b</sup>      | 1.93 <sup>b</sup> | 50   | 50.15 <sup>b</sup>    | 1.93 <sup>a</sup> | 46.47 <sup>b</sup>     | 2.70 <sup>b</sup> |
|                  | SC | 51.47 <sup>b</sup>    | 1.48 <sup>a</sup> | 50.63 <sup>b</sup>     | 1.50 <sup>a</sup> | 49.12 <sup>b</sup>      | 1.87 <sup>b</sup> | 66   | 50.28 <sup>b</sup>    | 1.59 <sup>a</sup> | 47.68 <sup>ab</sup>    | 2.61 <sup>b</sup> |
| Exchangeable     | FC | 41.43 <sup>b</sup>    | 5.91 <sup>a</sup> | 41.65 <sup>b</sup>     | 3.61 <sup>c</sup> | 46.54 <sup>b</sup>      | 8.92 <sup>a</sup> | 30   | 45.89 <sup>b</sup>    | 7.85 <sup>a</sup> | 47.37 <sup>b</sup>     | 9.15 <sup>a</sup> |
|                  | MC | 45.73 <sup>b</sup>    | 4.21 <sup>a</sup> | 45.35 <sup>ab</sup>    | 4.31 <sup>b</sup> | 49.85 <sup>b</sup>      | 6.10 <sup>b</sup> | 50   | 49.85 <sup>a</sup>    | 6.09 <sup>a</sup> | 53.53 <sup>a</sup>     | 8.15 <sup>b</sup> |
|                  | SC | 48.53 <sup>a</sup>    | 4.96 <sup>a</sup> | 49.37 <sup>a</sup>     | 5.01 <sup>a</sup> | 50.88 <sup>a</sup>      | 6.04 <sup>b</sup> | 66   | 49.72 <sup>a</sup>    | 5.48 <sup>a</sup> | 52.32 <sup>ab</sup>    | 7.95 <sup>b</sup> |

\* T<sub>2</sub> values are expressed in ms. For readability, data dispersion is not indicated.

Figure(s)



Figure(s)



Figure(s)

