The state of water and fat during the maturation of Cheddar Cheese

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13 Abstract

14 Cheddar cheese predicted to develop into different quality classes has been evaluated by
15 time domain Nuclear Magnetic Resonance, Thermogravimetric analysis and quantitative
16 sensory analysis. The water and fat proton signals in the transverse relaxation decay curves
17 have been deconvoluted. Proton transverse relaxation values for both the water and fat
18 fractions decrease and the relative %age of the proton peak area, predominantly from the
19 fat increases over a 450-day ripening period. The thermodynamic free water percentage
20 increases during maturation. Water and fat attributes can distinguish between Cheddar
21 cheese batches after 56 days. Cheese batches which have lower transverse relaxation
22 values for the water and fat proton fractions and a higher relative %age of the proton peak
23 area predominantly from fat at 56 days, mature after 270 days to be more yellow, rubbery
24 and smooth, have a less sour and lingering aftertaste and are also harder to form into a
25 cheese ball.

26 Highlights

27 Time domain water and fat proton Nuclear Magnetic Resonance signals are assigned.

28 Transverse relaxation times for water and fat protons decrease with cheese ripening.

29 Thermodynamic free water percentage increases with cheese ripening up to 450 days.

30 Water and fat state attributes can differentiate between batches of Cheddar cheese.

31 Batches with different water and fat attributes have distinctive sensory profiles.

32

33 **Keywords:** Cheddar cheese; Time domain NMR; TGA; Maturation; Sensory variation

34

35 1 Introduction

36 Cheddar cheese ripening is a complicated microbiological and biochemical transformation
37 involving glycolysis, lipolysis and proteolysis (Jiménez-Flores & Yee, 2007). All of these
38 biochemical processes contribute to the overall structure change during maturation. Cheese
39 structure is influence by factors that include casein-casein, casein-water and casein-fat
40 interactions; the state of water (bulk or bound to the casein matrix), the state of calcium
41 (ionic or bound to casein matrix); and the extent of proteolysis (Everett & Auty, 2017). Water
42 migration and binding capacity reflect structural properties such as casein matrix porosity
43 and tortuosity as well as textural properties. They are believed to be an important
44 consequence of ripening and determine many of the sensory and functional characteristics
45 of the cheese (Kuo, Gunasekaran, Johnson, & Chen, 2001; Saldo, Sendra, & Guamis,
46 2002). There are at least two types of water present in Cheddar cheese distinguished by
47 their extent of association with macromolecules. They are conveniently described as bound
48 and free water. Bound water is the fraction closely associated with the hydrophilic molecules
49 whilst free water is available for biological function (Hickey, Guinee, Hou, & Wilkinson, 2013;
50 Wang, Zheng, Li, Ma, Zhao, & Zhang, 2018). Accurate and detailed definition of these terms
51 depends on the methodology used to measure the free water content. For example, in
52 centrifugation methods, the amount of water removed depends on the conditions used to
53 expel the free water (Saldo et al., 2002). In a hydraulic pressing method, the amount of
54 expressible serum was used to describe water state of the cheese (Guinee, Auty, & Fenelon,
55 2000). The contribution of lipolysis to maturation in Cheddar cheese is not as significant as
56 in mould-ripened cheese. However, the texture, flavour and physico-chemical properties of
57 cheese are still largely governed by fat. The state of fat is also important in determining the
58 complex sensory properties of Cheddar cheese (Lopez, Camier, & Gassi, 2007).

59 Time Domain Nuclear Magnetic Resonance (TD-NMR) analysis is a rapid non-destructive
60 and solvent-free measurement which has been used in many studies to describe the
61 physical state and distribution of water and fat in materials (Oztop, Bansal, Takhar,
62 McCarthy, & McCarthy, 2014; Rudi, Guthausen, Burk, Reh, & Isengard, 2008; Yang, Zhang,
63 Li, Zheng, Khan, Xu, et al., 2016). The transverse relaxation decay time (T_2) is a
64 measurement of relaxation of protons towards an equilibrium state which reflects the
65 molecular dynamics in the neighbourhood of protons but can reflect other phenomena such
66 as chemical exchange and diffusion. It is associated with a particular frequency range and
67 is material specific (Rudi et al., 2008). NMR T_2 relaxometry combined with magnetic
68 resonance imaging (MRI) has been used to quantify spatial water and fat distribution and
69 mobility in methylcellulose coated chicken nuggets (Oztop et al., 2014). A T_2 relaxation
70 analysis has also been used to assess changes in the structure and the mobility of different
71 proton components in Mozzarella cheese during maturation and subsequent heating. A
72 Carr-Purcell-Meiboom-Gill (CPMG) sequence was used where the water and fat proton
73 signals were distinguished by Fourier transformation at specified points in the CPMG decay
74 (Smith, Vogt, Seymour, Carr, & Codd, 2017). However, the interpretation of such data can
75 often be difficult due to the existence of complex factors in addition to simple measures of
76 mobility, such as proton-proton exchange between water and the hydrophilic polymers
77 present in meat and plant-based products. In this work, TD-NMR was applied to explore the
78 casein-water and casein- fat interactions during maturation and batch variation. In a
79 heterogeneous system such as Cheddar cheese, each proton phase is affected by its
80 surrounding food matrix (Gianferri, D'aiuto, Curini, Delfini, & Brosio, 2007).

81 Thermogravimetric analysis is a technique that allows us to determine the loss of mass and
82 thermal response of a material subjected to a controlled heating rate. It is commonly used
83 in food and pharmaceuticals for moisture content analysis (Ducat, Felsner, da Costa Neto, &

84 Quinária, 2015; Pirayavaraporn, Rades, & Tucker, 2012; Silva, Silva, Andrade, Veloso, &
85 Santos, 2008). Thermogravimetric analysis (TGA) was also used to measure the distribution
86 of free and bound water and the interaction with the polymer in the food matrix (Roozendaal,
87 Abu-hardan, & Frazier, 2012; Saldo et al., 2002). Cheese thermo-physical properties can be
88 measured by TGA. They are influenced by the composition and interaction of the cheese
89 matrix and provide indirect evidence for structural interaction (Schenkel, Samudrala, &
90 Hinrichs, 2013).

91 Cheddar cheese texture and flavour do not depend exclusively on the ageing process.
92 Grading takes place during Cheddar cheese maturation and is used to inform the
93 manufacturer whether the cheese in question is suitable for extending the ripening period to
94 produce premium Cheddar cheese or must be sold quickly as a young cheese (Partridge,
95 2008). The Giles and Lawrence Cheddar cheese predictive grading model, which consists
96 of four chemical composition variables, is still used in Cheddar cheese manufacture to aid
97 the professional Cheddar cheese grader (Giles & Lawrence, 1973). After manufacture, the
98 four chemical composition variables used to grade Cheddar cheese as likely to be premium
99 or lower quality cheese are the percentage of salt in moisture, moisture in the fat-free-
100 substance, fat in the dry matter and pH.

101 Cheese quality prediction and the control of functional properties require an understanding
102 of the location of the components of cheese in relation to each other and how they interact
103 and change during ripening (Everett & Auty, 2017). Most of the research reported in the
104 literature to date concerning changes in the state of water and fat measured by NMR and
105 TGA during maturation, have concentrated on Mozzarella cheese rather than Cheddar
106 cheese, with little research focussing on same batch evolution of structure from early
107 through to late-maturation (Gianferri et al., 2007; Kuo et al., 2001; Luo, Pan, Guo, & Ren,
108 2013; Smith et al., 2017). Moreover, there is no work exploring the correlation between the

109 state of water and fat in Cheddar cheese with the sensorial profile and the variation seen
110 between batches.

111 The objective of this research was to study the change in the biochemical and biophysical
112 state of water and fat during Cheddar cheese maturation over a period from 56 to 450 days,
113 and to examine the potential of water and fat variation in different batches of cheese to
114 predict the differences in sensorial profile.

115 2 Material and methods

116 2.1 Materials

117 Six 20 kg blocks of Welsh Cheddar were obtained from a commercial Cheddar producer in
118 the UK. These blocks of Cheddar cheese were produced on the same day and production
119 line, namely labelled as batches A, B, C, D, E and F. All six batches were predictively graded
120 after manufacture based on Giles and Lawrence quality grading model (Giles et al., 1973).
121 All the grading composition attribute data were provided by the Cheddar cheese producer
122 and are available in Supplementary Table 1. The predicted Cheddar cheese quality outcome
123 is presented in Supplementary Figure 1 showing that batch B and F are predicted to mature
124 to premium quality, whereas batch A, C, D, E are predicted to mature to graded quality
125 Cheddar cheese due to the lower amount of fat in dry mass content and the pH.

126 All six blocks were cut into 14 pieces and re-vacuum packed in bags, further packaged in
127 cardboard and located in the factory warehouse for maturation at a constant controlled
128 temperature of 8 °C. Time-domain ¹H NMR relaxometry measurements, thermogravimetric
129 analysis (TGA) and descriptive sensory analysis were carried out at various times during the
130 ripening period, namely 56, 90, 180, 270, 360 and 450 days. At each time point, two bags
131 of cheese were removed at random from each block and placed in a 4 °C refrigerator before

132 measurement. One bag was used for instrumental measurements and the other for
133 descriptive sensory analysis.

134 A 180 g commercial package of Cheddar cheese from the same factory was delivered at
135 the same time. Half was freeze dried for three days; the other half was vacuum packed and
136 placed in a 4 °C refrigerator before testing. These commercial samples were for identification
137 of proton populations.

138 2.2 Time domain NMR measurement

139 For low resolution NMR measurements, cheese samples were moulded into cylindrical
140 shaped pieces cut from a central part of a block of Cheddar cheese. To prevent water
141 evaporation and reduce dead space, a glass rod wrapped with polytetrafluoroethylene
142 (PTFE) tape was inserted in the tube over the cheese and finally sealed by parafilm at the
143 top of the tube outside the magnet and NMR coil. All samples were measured in triplicate.

144 Measurements were performed with a benchtop NMR spectrometer operating at a proton
145 frequency of 21.23 MHz (AMR Ltd, Oxford, UK). The transverse relaxation curves were
146 measured using the Carr-Purcell-Meiboom-Gill (CPMG) sequence with a pulse spacing of 2
147 X tau between the two 180° pulses, where tau = 256 µs and recording at the echo peak
148 value. 1024 data points on the relaxation curve were acquired. The recycle delay (RD) time
149 was chosen as 10 s to avoid saturation of any bulk water fraction present. The relaxation
150 decay curves were fitted to distributed exponential decays based on zeroth order
151 regularisation using software supplied by AMR Ltd, Oxford, UK. A plot of relaxation amplitude
152 against relaxation time was obtained. T₂ values were measured from the peak position and
153 the area under each peak was determined by cumulative integration.

154 2.3 Thermogravimetric Analysis (TGA)

155 Thermogravimetric analyses were performed on a thermogravimetric analyser TGA/DSC 3+
156 (Mettler-Toledo GmbH Analytical, Switzerland). The measurements were carried out under
157 nitrogen with a gas flow of 50 cm³/min. Approximately 15 mg of fresh cheese from the centre
158 of a cheese cube were weighed in 100 µL aluminium crucibles with pierceable lids and
159 heated over the temperature range 25-450 °C, at a scanning rate of 10 °C/min. The analysis
160 was performed in duplicate for each sample. An empty aluminium crucible with a pierceable
161 lid was measured using the same heating program. The blank curve was subtracted from
162 the sample TGA curve in order to minimise the buoyancy effect on the empty crucible during
163 heating. Graphs of the sample weight as a function of temperature and its first derivative
164 (Derivative thermogravimetric (DTGA) with units of %/°C) were constructed and all analysis
165 was carried out using STARe software (Mettler-Toledo GmbH Analytical, Switzerland).

166 2.4 Differential scanning calorimetry (DSC) analysis

167 Differential scanning calorimetry was performed on DSC823^e (Mettler Toledo, Switzerland).
168 The instrument was calibrated for temperature and enthalpy using the onset transitions and
169 enthalpy values for Indium and cyclohexane. Typically, 10-13 mg of the sample taken from
170 the centre of cheese cubes were placed in aluminium pans and hermetically sealed. The
171 samples were heated from 20 to 60 °C at a heating rate of 5 °C/min.

172 2.5 Descriptive sensory analysis

173 Quantitative Descriptive Analysis was carried out at the Sensory Science Centre, University
174 of Nottingham (de Cássia Dos Santos Navarro Da Silva, Minim, Simiqueli, Da Silva Moraes,
175 Gomide, & Minim, 2012). Ten experienced trained panellists (3 males, 7 females) were
176 initially invited to participate in 2-hour training sessions to evaluate the sensory properties
177 of Cheddar cheese. Sensory panels were first presented with a wide range of commercial

178 Cheddar cheeses to generate a list of attributes that would describe the sensory properties
179 perceived. As a result of discussions within the panel and data from the literature, a final
180 descriptor list containing 19 sensory attributes covering appearance, aroma, taste, flavour
181 and mouthfeel properties was developed and is presented in Supplementary Table 2 (Drake,
182 McIngvale, Gerard, Cadwallader, & Civille, 2001; Kraggerud, Skeie, Høy, Røkke, &
183 Abrahamsen, 2008; Partridge, 2008). During this long period sensory study, the panel was
184 invited back every 3 months. Reference cheese samples were used in these subsequent
185 sessions to better qualitatively and quantitatively calibrate the panel. Other training sessions
186 were used to train sensory panels on standardising evaluation protocol and quantifying the
187 intensity of sensory attributes using the developed scales.

188 Reference samples were defrosted the previous night in a 4 °C walk in fridge. All the
189 reference and test sensory samples were equilibrated at 18 °C for 5 h before the sensory
190 sessions. The outer layer (5 mm) of each cheese was discarded and each sample cut into
191 2 cm×2 cm×2 cm cubes. Two cubes of each samples were presented to the assessors in a
192 sealed 60 mL plastic pot. All samples were numbered using random 3-digit codes in
193 randomised order. Due to the time limitation, only five batches of sample (A, B, C, D, E)
194 were used for sensory profiling.

195 Only the sensory analysis data for Cheddar cheese at 270 days are presented, which based
196 on previous studies was considered the most opportune time to reflect mature Cheddar
197 cheese (Hou, Hannon, McSweeney, Beresford, & Guinee, 2014). All data were collected
198 using Compusense Cloud (Compusense, Canada). A more complete set of data will be
199 presented in a future publication.

200 2.6 Statistical analysis

201 The differences in fat and water state during ripening and batch variations were analysed
202 using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant
203 difference (HSD) test. Agglomerative Hierarchical Clustering (AHC) was performed on
204 sensory attributes shown to be significant between batches. For modelling sensory profiles
205 from the water and fat state attributes measured by TD-NMR, Partial Least Squares (PLS)
206 was used. The X data matrix contained water and fat state attributes. The Y matrix contained
207 the results of sensory mean scores for all significantly different cheese attributes. All
208 statistical analysis was performed using XLSTAT (Addinsoft, France).

209 3 Results and discussion

210 3.1 Identification of proton populations and evolution in Cheddar cheese during 211 maturation

212 In Figure 1(a), the distribution of transverse proton relaxation times T_2 as determined by the
213 CPMG pulse sequence in fresh Cheddar cheese (non-freeze-dried Cheddar cheese) at
214 temperatures of 20 and 40 °C is presented. The amplitude of population A labelled in the
215 figure was higher than population B. The transverse magnetisation relaxation curves show
216 multi-exponential behaviour due to the presence of at least 2 components characterised by
217 different T_2 values and amplitudes. T_2 is directly proportional to the molecular mobility and
218 inversely proportional to the level of molecular interactions (Hindmarsh, Smith, Carr, &
219 Watkinson, 2019). For Cheddar cheese, the protons in the protein molecules which are in a
220 solid state will not contribute directly to the NMR signal that was acquired with a tau value
221 of 256 μ s. This setting will detect only comparatively mobile protons. However, the proton
222 signal will arise from a weighted average of the intensity and decay times of the water and
223 exchangeable protons on the polymer. The majority of the fat protons being directly

connected to the carbon backbone of the lipid fatty acid chains will be non-exchangeable and so will reflect the distinct properties of that phase. The proton signal obtained from Cheddar cheese can therefore be loosely regarded as coming from the water and fat protons. However, the NMR signals from water will interfere with the oil signal in sample at water contents higher than 15% due to the similarity of relaxation times of water and oil (Todt, Guthausen, Burk, Schmalbein, & Kamlowksi, 2006). Thus, the first part of this work had the aim of identifying the water and fat fractions in the relaxation curve.

The transverse relaxation curves of packaged commercial Cheddar cheese were obtained at temperatures of 20 and 40 °C. Figure 1(a) shows that when Cheddar cheese is heated, the relative amount of proton population B increased corresponding to the melting of solid fat. The Differential Scanning Calorimetry (DSC) curve in Figure 1(b) for the same cheese sample showed the melting of a fraction of the cheese fat between 20-40 °C. This supported the proposal that the proton signal of population B is predominantly from the fat fraction of Cheddar cheese. If the signal in population A were due to a solid component of the fat, we would expect the T_2 value to be in order of microseconds rather than the value of 10 – 20 milliseconds as shown. As heating proceeds, the peak values of the relaxation times of population A and B both increased which probably was related to the effect of heat treatment on the water binding capacity and general mobility in the cheese matrix and mobility of the proton fraction in the fat.

In order to determine the proton population assignment of peak A, Cheddar cheese drying experiments were performed. The same cheese sample after having been freeze dried for three days or room temperature dried for 3 hours were measured at 40 °C. As shown in Figure 1(c), a significant decrease in proton population A of room temperature dried and freeze-dried samples compared with non-dried fresh Cheddar was observed. This is associated with the loss of water during the drying process and further supports the

allocation of population A to the water protons. The maximum peak value relaxation times obtained for the 3 different treatments of Cheddar cheese are different, with the values for all the dried systems being less. This is probably due to the generalised decrease in mobility of the sample upon drying affecting all the material phases. A similar phenomenon was observed in dough and microwave expansion of imitation cheese where the relaxation time for the mobile proton population decreased with moisture decrease (Arimi, Duggan, O'Sullivan, Lyng, & O'Riordan, 2010; Assifaoui, Champion, Chiotelli, & Verel, 2006). Considering Figures 1(a) to (c), it is concluded that the protons in population A were primarily protons from the water fraction, whilst protons in population B were predominantly protons from the fat. However, we cannot ignore the possibility that population B contains a significant amount of free serum water protons. Water proton transverse relaxation time constants at 40 °C of about 20 ms and 400 ms were reported in the high field and sampled NMR spectrum of Mozzarella cheese (Smith, et al. 2017). As mentioned in the previous text, the relaxation time decay constants of water are similar to that of oil. Using a hydraulic pressing method, Cheddar cheese after 180 days of ripening was found to still have roughly 10 to 15 % expressible serum (Guinee et al., 2000). Previous research has confirmed that lipid in food samples exhibits relaxation times of a few hundred milliseconds. The value of T_2 for proton in solid fat is of the order of tens of μ s which cannot be observed on the timescale of this experiment. (Song, 2009; Todt et al., 2006).

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Only one main independent compartment of water was detected in Cheddar cheese. Three types of water were detected in Mozzarella di Bufala Campana which were allocated the following descriptions: junction zone water with a T_2 value of 7.3 ± 0.3 ms, entrapped water with $T_2 = 47 \pm 3$ ms and serum water with $T_2 = 951 \pm 36$ ms (Gianferri et al., 2007). This difference suggests that water distribution is homogenous in Cheddar cheese compared

274 with Mozzarella. This could also be because the size of different water compartments is
275 sufficiently small to allow the complete averaging of the two water pools by diffusive
276 exchange. The water relaxation would then be described by a single mono-exponential
277 curve (Mariette, 2003).

278 As shown in Table 1, as ripening proceeds from 56 days to 450 days, the T_2 value for both
279 fractions decrease significantly. This indicates that protons from both the water and fat
280 fractions become less mobile. As both fractions are affected this suggests that protons from
281 the water and fat are binding to or immobilised by, for example, the casein rather than any
282 effect on the exchange mechanism which would predominantly affect the water fraction. The
283 decrease in T_2 value for water fractions indicated that increased hydration of casein during
284 ripening may lead to enhanced casein-water interactions. This is in agreement with
285 McMahon's findings. He concluded that serum along with the protein contained therein is
286 absorbed into protein matrix and becomes an integral part of protein matrix by day 21
287 (McMahon, Fife, & Oberg, 1999). A similar phenomenon was reported during Mozzarella
288 maturation, the free water surrounding the fat channels gradually being reabsorbed into the
289 protein matrix (Kuo et al., 2001). There are several possible reasons which could explain
290 this phenomenon. Firstly, during the maturation process, as proteolysis progresses, more
291 α -carboxylic acid and α amino groups are produced by the cleavage of peptide bonds
292 producing soluble peptides. They increase the water-binding capacity of casein and restrict
293 proton mobility (Smith et al., 2017; Upadhyay, McSweeney, Magboul, & Fox, 2004). The
294 second mechanism behind the phenomenon could be the pH change and molecular
295 interaction. During the ripening process, pH increases gradually, when it is higher than
296 casein isoelectric point, the casein molecules have a net negative charge and while
297 hydrophobic interactions persist, the ionic interactions between the molecules change from
298 attractive to repulsive. Thus, the tight casein aggregates absorb water, partly to solvate the

299 non-neutralized ionic charges. A contributing factor is also the partial exchange of Na^+ for
300 Ca^{2+} in Cheddar cheese further weakening the aggregates (Abdelsalam, Alichanidis, &
301 Zerfiridis, 2012). As a consequence, the casein water binding capacity increases and the T_2
302 value decreases significantly. A similar phenomenon was reported by Lucey, Johnson, and
303 Horne (2003) in that young Camembert readily “waters off”, but once the pH increases, no
304 watering-off is observed.

305 There is another similar mechanism taking place in Cheddar cheese. It is widely accepted
306 that the interaction in the Cheddar cheese curd formation after manufacture involves a
307 casein calcium phosphate bridge linkage as shown in Supplementary Figure 2. As ripening
308 proceeds, acid levels increase resulting in the solubilisation of the calcium which in turn
309 causes the phosphate ion to be protonated, resulting in a tighter association of the water
310 with the casein. Consequently, the proton mobility in the water fraction decreases as
311 ripening proceeds (Lucey et al., 2003).

312 One other explanation for the reduction in water mobility during ripening is the decrease in
313 water content. Thermogravimetric Analysis (TGA) data shows that the Cheddar cheese
314 water content decreased from 38.44% to 34.48% from 56 days to 450 days. The proton
315 mobility decreased linearly as the water content decreased (Arimi et al., 2010; Assifaoui et
316 al., 2006). Water within the cheese matrix became less mobile with decreasing moisture
317 content which suggested that the protons in water were bound more tightly to the cheese
318 matrix or that the exchange mechanism became more effective.

319 The peak T_2 value of population B decreased during ripening. A similar phenomenon was
320 found by Gianferri et al. (2007), namely that the fat proton T_2 and the serum water proton T_2
321 value showed a clear and gradual reduction from day 1 to day 14 in Mozzarella. Some of
322 the free serum water is absorbed by the protein matrix during ripening which may contribute
323 to the decrease of T_2 value from population B. This may also have led to an increase of

324 surface contact between fat and protein and reduced mobility (Karami, Reza Ehsani,
325 Ebrahimzadeh Mousavi, Rezaei, & Safari, 2008). The fat also progressively became more
326 solid, reducing both diffusion and T_2 with aging (Smith et al., 2017). Lopez et al. (2007)
327 working on Emmental cheese microstructure, suggested that during maturation pockets of
328 serum, which were surrounded by pools of fat, disappeared. The casein matrix then
329 expanded into the fat phase and broke the fat pool into smaller regions causing a possible
330 reduction in mobility of the fat fraction and a decrease in the T_2 proton value. Consequently,
331 there are several possible contributions to the overall decrease in the T_2 value of population
332 B during ripening.

333

334 3.2 Thermal measurements on the state of water in Cheddar cheese during 335 maturation

336 The thermogravimetric curves describe Cheddar cheese thermal stability which is related to
337 the water holding capacity. Examination of the thermogravimetric and first derivative curves
338 shows at least four different weight loss steps. Water release over the range 25-200 °C is
339 described by points A, B, C in the first derivative curve on Figure 2 (de Angelis Curtis, Curini,
340 amp, Amp, Apos, Ascenzo, et al., 1999; Ducat et al., 2015). From a thermal perspective,
341 there are at least two types of water present in Cheddar cheese. The initial shoulder at
342 approximately 92 °C labelled A can be attributed to the water loosely bound to the cheese
343 matrix and is lost at relatively low temperatures, namely free water. Peak B and C can be
344 attributed to water relatively tightly bound to the Cheddar cheese matrix and is lost at a
345 higher temperature (de Angelis Curtis, et al., 1999). This is rather imprecisely defined here
346 as bound water. The state of water as defined by these thermal measurements during
347 maturation, are described by the three attributes; total water content, free water ratio (free
348 water/ total water) and bound water ratio (bound water/total water). Although these three

349 attributes describe primarily the water state, they also provide an indication of the
350 thermodynamic properties of cheese matrix structure.

351 In Table 2, during the maturation process, total water content drops from 38.44% at 56 days
352 ripening to 36.68 % at 450 days ripening. Free water and bound water ratio changes more
353 significantly than the overall total water content. As ripening proceeds free water ratio
354 increases from 56 days to 270 days. A dramatic change occurs between 270 days and 450
355 days where the free water ratio jumps steeply from 28.78 % to 42.65 %.

356 During heating in the TGA experiment, the first stage of water release is the diffusion of the
357 free water through the cheese matrix which is related to the integrity of cheese matrix (Smith
358 et al., 2017). This is followed by the melting of solid fats and the dissolution of the calcium
359 linkage which holds the casein molecules together reducing the strength of the casein-
360 casein interaction (Lucey et al., 2003). Finally, the protein structure collapses and liquefies.
361 Water in the Cheddar cheese and associated with these components is progressively
362 liberated as the microstructure changes. TGA data therefore reflect the thermal stability of
363 the Cheddar cheese microstructure as well as the water release.

364 The increase of free water percentage during maturation is most likely due to the loss of
365 integrity of the Cheddar cheese matrix which facilitates the water diffusion and release. The
366 calcium is replaced by hydrogen under acidic conditions which causes casein breakdown
367 resulting in the increase of meltability of Cheddar cheese (Johnson & Lucey, 2006).

368 This conclusion from TGA appears superficially to conflict with the TD-NMR data. Definitions
369 of free and bound water from the thermal perspective are in a thermodynamic sense. We
370 use the description “thermodynamic” in a loose sense as a way of distinguishing it from NMR
371 definitions. A fraction of the water can be considered to be thermally transient in that it
372 migrates from the “bound” water state to a “free” water state. Therefore, there can be a
373 constant thermodynamic fraction of water which is nevertheless continually exchanging with

374 water in other states. The water is able to move more freely throughout the cheese structure
375 when heated, for cheese of greater maturity, due to the decrease of the tortuosity of cheese
376 matrix during ripening (Smith et al., 2017). The NMR measurements of bound and free water
377 where they are not compromised by exchange phenomena, are fundamentally different from
378 thermal measurements which in the case of TGA is the determination of the temperatures
379 at which various fractions are released.

380 3.3 Batch to batch variation in the state of water and fat

381 Variations in the state of water and fat have also been detected in different batches of
382 cheese. Thermal measurements on the state of water using TGA did not detect batch to
383 batch variations. However, TD-NMR was capable of distinguishing the behaviour of different
384 batches. Table 3 shows the distribution of transverse proton relaxation times T_2 from the
385 water and fat fractions and peak area proportion predominantly from the fat fraction at 56
386 days after Cheddar cheese manufacture for five different batches of Cheddar cheese. At all
387 stages in the ripening process, the same variation trend among batches was observed,
388 however here we present only data for the 56-day time point.

389 The T_2 value for the water fraction protons for batch C is significantly lower ($P<0.05$)
390 compared with the other batches. The proton peak area %age predominantly from the fat
391 fraction for batch C is significantly higher ($P<0.05$) than all the others. The T_2 value
392 predominantly from the fat fraction protons for batch C is lower than the other batches but
393 at a lower significance level compared with the previous parameters.

394 Four batches of Cheddar cheese including batch C were predictively graded at an early
395 stage of maturation to produce graded quality cheese according to the Giles and Lawrence
396 model. Only batch C however was found to be significantly different in terms of the state of
397 fat and water. The lower T_2 value for the water fraction protons for batch C indicated a tighter

398 binding of the water to the casein matrix resulting in lower the water availability in the
399 entrapped water. Bacteria will therefore be more prone to plasmolysis and cell lysis and
400 therefore less biological activity will be present (Hickey et al., 2013). This could account for
401 the different values measured in the case of batch C.

402 3.4 Batch to batch variation in sensorial profile and correlation with state of water 403 and fat

404 A significant batch to batch variation was observed in terms of the state of fat and water at
405 56 days. As Cheddar cheese matured to 56 days has a bland sensorial profile, the same
406 named batches of Cheddar cheese at 270 days ripening were chosen for analysis and the
407 data presented in this section. Cheddar cheese is commonly sold as mature cheese
408 commercially after 270 days ripening and has a more distinctive sensory character. Although
409 the physical measurements at 270 days could have been chosen, the values at 56 days
410 were used in order to create a link to the predictive properties of the grading and the 270
411 days sensory profile.

412 Cluster analysis was carried out to understand the variation among the batches of Cheddar
413 cheese based on sensory attributes. Five batches Cheddar cheese can be distributed in two
414 main clusters (Figure 3a). One cluster contained only batch C; the other cluster comprised
415 batches A, B, D, and E. Batch C sensorial attributes were characterised by a significantly
416 ($P<0.05$) higher yellow colour and break down but relatively lower cohesiveness and sour
417 and lingering aftertaste (data shown in Supplementary Table 2).

418 Partial least squares regression (PLSR) analysis was used to determine the relationship
419 between fat and water state attributes measured by NMR and sensory attributes. A biplot of
420 two latent variables is presented in Figure 3c and the Pearson correlation coefficient among
421 all the variables is shown in Supplementary Table 3. The T_2 value for water and fat fraction

422 protons were highly positively correlated with cohesiveness, sour, lingering aftertaste and
423 negatively correlated with yellow (all Pearson coefficients higher than 0.9). The peak area
424 %age predominantly from the fat fraction protons behaves in the opposite sense compared
425 with these two attributes in terms of sensorial profile.

426 At the same ripening stage, batch C which had a lower T_2 value for protons predominantly
427 from the fat fraction, suggesting that the fat was in a different state, was less prone to
428 dissolve and absorb organic flavour compounds, and so had a less lingering aftertaste due
429 to the influence of flavour release during consumption (Lawlor, Delahunty, Wilkinson, &
430 Sheehan, 2001). Batch C also had a higher colour yellow score which was explained by the
431 diffraction of light in the altered fat fraction of the cheese (Madsen, Reinbold, & Clark, 1966).
432 Cohesiveness in this sensory evaluation was evaluated by how the cheese formed a ball
433 under finger pressure. The lower cohesiveness of batch C meant that it was harder to make
434 a smooth ball and displayed seams on the ball indicating that the sample exhibited a rubbery
435 and curdy body. The lower proton T_2 value predominantly from the fat fraction in Batch C
436 implied that the fat fraction was more strongly bound to cheese casein matrix resulting in a
437 tighter microstructure of the casein network (Ardo, 1997). This microstructure may result in
438 less susceptibility to deformation and contribute to the rubbery texture of young Cheddar
439 cheese. The sensory profile of batch C is similar to low fat content Cheddar Cheese which
440 has a more intense yellow colour and rubbery body.

441 4 Conclusions

442 The population with the shorter relaxation time is attributed to water and the longer relaxation
443 component to oil, or oil with a component of modified free serum water. Both transverse
444 relaxation times decrease and the relative peak area predominantly from fat protons and
445 free water increases during Cheddar cheese ripening up to 450 days. The transverse

446 relaxation values for water and fat fraction protons after 56 days ripening were highly
447 positively correlated with cohesiveness, sour and lingering aftertaste and negatively
448 correlated with yellow (related to particle size) sensorial attributes after 270 days ripening.
449 The NMR determined attributes of the fat and water state can be used to monitor Cheddar
450 cheese maturity and predict sensory profile.

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456 Ethical statement

457 The sensory ethics in this project have been approved by the University of Nottingham,
458 School of Biosciences Research Ethical Committee. The approval code is SBREC160127A.

459 Conflict of interest

460 The authors have no conflict of interest to declare

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Figure 1 (a) The distribution of transverse proton relaxation times T_2 as determined by the CPMG pulse sequence in fresh Cheddar cheese (non-freeze-dried Cheddar cheese) upon heating from 20 to 40 °C. The different proton populations are indicated with capital letters A and B. (b) DSC curve showing the melting of fresh Cheddar cheese over the range of 4°C to 60 °C at a heating rate of 5 °C/min. (c) The distribution of transverse proton relaxation times T_2 as determined by the CPMG pulse sequence in fresh Cheddar cheese, room temperature dried Cheddar cheese and freeze-dried Cheddar cheese all at 40 °C.

Table 1 The distribution of transverse proton relaxation times T_2 from water and fat fractions and proton predominantly from fat fraction peak area proportion at different ripening times in days after Cheddar cheese manufacture.

Ripening days	Proton from water fraction (ms)	Proton T_2 predominantly from fat fraction (ms)	Proton T_2 predominantly from fat fraction peak area proportion (%)
56	30.27±2.3 ^a	209.33±14.29 ^a	30.43±2.41 ^e
90	28.17±2.71 ^b	196.23±16.63 ^b	31.94±3.33 ^d
180	27.57±1.89 ^b	192.36±15.16 ^b	33.70±3.22 ^c
270	24.85±2.88 ^c	181.62±18.56 ^c	35.64±3.00 ^b
360	24.65±1.91 ^c	175.98±14.33 ^c	36.17±2.89 ^b
450	21.59±1.45 ^d	164.60±11.28 ^d	40.43±2.42 ^a

For each time point the data represents three measurements on the six batches of the cheese and so is an average of 18 samples. Errors present the standard deviation for the eighteen values. Different superscripts in the same column indicate significant statistical difference (Tukey’s test $P<0.05$)

Figure 2 Mass loss (solid line) and first derivative (dotted line) TGA curves for a cheese sample heated at a rate of 10°C/min. The water loss peaks in the 1st derivative plot are indicated by the capital letters A, B, C. Three weight loss values ($\Delta W1$ - $\Delta W3$) are indicated.

Table 2 Thermal properties of Cheddar cheese at different stages of maturation. Total water content is calculated by $\Delta W1 + \Delta W2$. Free water ratio (Free water /total water) is calculated by $\Delta W1$ divided by the sum of $\Delta W1$ and $\Delta W2$. Bound water ratio (Bound water/ Total water) is calculated by $\Delta W2$ divided by the sum of $\Delta W1$ and $\Delta W2$.

Ripening Days	Total Water Content (%)	Free water/Total water (%)	Bound water/Total water (%)
56	38.44±1.19 ^a	25.26±1.95 ^d	74.74±1.95 ^a
90	38.31±0.97 ^a	25.64±1.3 ^d	74.36±1.30 ^a
180	37.98±1.58 ^a	29.34±3.01 ^c	70.66±3.01 ^b
270	35.13±1.22 ^{bc}	28.11±2.80 ^c	71.89±2.80 ^b

360	36.27±1.32 ^b	36.84±6.34 ^b	63.16±6.34 ^c
450	34.68±2.54 ^c	42.65±4.85 ^a	57.35±4.85 ^d

Each time point represents two measurements on the six batches of the cheese and so is a mean of twelve measurements. Errors represent the standard deviation. Different superscripts in the same column indicate significant statistical difference (Tukey's test $P < 0.05$).

Table 3 Peak values of T_2 for water and fat fractions taken from the distribution of transverse proton relaxation times. Also shown are peak area proportions for five batches of Cheddar cheese. These are predominantly from the fat fraction at 56 days after Cheddar cheese manufacture which occurred on the same day and in the same factory.

Batch Number	Proton T_2 (ms) for water fraction	Proton predominantly from fat fraction T_2 (ms)	Proton predominantly from fat fraction peak area proportion (%)
A	29.17±1.19 ^b	205.76±8.38 ^{ab}	31.93±1.13 ^b
B	30.54±0 ^{ab}	210.6±8.38 ^{ab}	29.89±0.30 ^c
C	26.56±0 ^c	187.38±0 ^b	34.09±0.55 ^a
D	30.54±0 ^{ab}	210.6±8.38 ^{ab}	29.10±0.05 ^c
E	31.27±1.27 ^a	215.79±15.05 ^a	30.63±0.98 ^{bc}

Data are the average of 3 measurements on different samples. Errors present the standard deviation of the three values. Different superscripts in the same column indicate significant statistical difference (Tukey's test $P < 0.05$)

622 **Figure 3** (a) Dendrograms representing the five batches of Cheddar cheese. The grouping
623 is based on the 270 days sensory profile. (b) Partial Least Squares observations on axes t1
624 and t2. The coordinates of the different predictive qualities of Cheddar cheeses in t
625 coordinate space. Batch C is distinct from the other batches (c) Partial Least Squares
626 correlation biplot of sensory attributes evaluated at 270 days and water and fat state
627 attributes measured by TD-NMR for Cheddar cheese of different predictive qualities. (d)
628 Partial Least Squares correlation biplot for cheese batches A, B, C, D, E showing the
629 loadings of sensory descriptors measured at 270 days and water and fat state attributes
630 measured by TD-NMR at 56 days.

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