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Authors	Boiani, Mattia;Sundekilde, Ulrik;Bateman, Lorraine M.;McCarthy, Daniel G.;Maguire, Anita R.;Gulati, Arunima;Guinee, Timothy P.;Fenelon, Mark A.;Hennessy, Deirdre;FitzGerald, Richard J.;Kelly, Phil M.
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Integration of high and low field ^{1}H NMR to analyse the effects of bovine dietary regime on milk metabolomics and protein-bound moisture characterisation of the resulting mozzarella cheeses during ripening

Mattia Boiani[a,b], Ulrik Sundekilde[c], Lorraine M. Bateman[d], Daniel G. McCarthy[e], Anita R. Maguire [f], Arunima Gulati[a,g], Timothy P. Guinee[a], Mark Fenelon[a], Deirdre Hennessy[h], Richard J. FitzGerald[b], Phil M. Kelly[a]*

[a] *Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland*

[b] *Department of Biological Sciences, University of Limerick, Castletroy, Limerick, Ireland*

[c] *Department of Food Science, Aarhus University, Kirstinebjergvej 10, DK-5792 Aarslev, Denmark*

[d] *School of Chemistry and School of Pharmacy, Analytical and Biological Chemistry Research Facility, University College Cork, Ireland, T12 K8AF*

[e] *School of Chemistry, Analytical and Biological Chemistry Research Facility, University College Cork, Ireland, T12 K8AF*

[f] *School of Chemistry and School of Pharmacy, Analytical and Biological Chemistry Research Facility, Synthesis and Solid State Pharmaceutical Centre, University College Cork, Ireland, T12 K8AF*

[g] *Department of Chemistry, Maynooth University, Maynooth, Co. Kildare, Ireland.*

[h] *Teagasc, Animal & Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland*

* Corresponding author. Tel.: +353877735352

E-mail address: phil.kelly@teagasc.ie (P. M. Kelly)

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ABSTRACT

The influence of dairy cow feeding regime was investigated using ^{1}H nuclear magnetic resonance (NMR). Two different NMR analytical systems were deployed: high field ^{1}H NMR to investigate the influence on milk metabolomics and low field NMR to characterise proton relaxation linked to changes in the state of mozzarella cheese moisture during ripening. The metabolomics results showed that grass-based feeding increased the concentration of a biological marker that signifies near-organic milk production conditions. On the other hand, the investigation of cheese moisture distribution showed that grass-based diets reached final moisture partitioning in a shorter time, which implied the formation of a more compact protein structure in the cheese matrix. These results indicate that pasture-based dairying may be differentiated in terms of the provenance of milk produced along with the accrual of additional benefits during ripening of the resulting mozzarella cheeses.

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1. Introduction

Milk production in temperate climates such as New Zealand (O'Brien et al., 1999) and Ireland relies substantially on grass growth as the principle bovine dietary source. Ideally matched to ruminant digestion needs, pasture grazing is valued at many levels such as animal welfare (Verkerk, 2003) and the promotion of natural animal foraging behaviour (Charlton, Rutter, East, & Sinclair, 2011; Legrand, von Keyserlingk, & Weary, 2009). However, this system is not practical in all other countries because of climatic extremes. Hence, an alternative milk production system widely practiced in mainland Europe utilises year-round indoor housing in which the dairy cows' dietary needs are addressed using a total mixed ration (TMR). It has been pointed out that in this system animal welfare presents some concern due to incidences of lameness and mastitis that diminish animal comfort and reduce milk output (Fregonesi, Veira, Von Keyserlingk, & Weary, 2007; Haskell, Rennie, Bowell, Bell, & Lawrence, 2006). On the other hand, this system protects animals from extreme climatic conditions (heat, cold and wetness) and allows better management of animal nutrition formulations.

The influence of different feeding systems on the milk production has been extensively studied over the years (Couvreur, Hurtaud, Lopez, Delaby, & Peyraud, 2006; Lee, Theobald, Tweed, Winters, & Scollan, 2009; White et al., 2001). A more recent study (O'Callaghan et al., 2016b) is of particular interest as it compared the influence of three different feeding regimes: TMR with outdoor grazing of perennial ryegrass (*Lolium perenne* L.; GRO), and outdoor grazing of perennial ryegrass/white clover (*Trifolium repens* L.; GRC) on milk raw composition and quality during lactation. The results show that, in general, the GRO feeding system increased milk quality, particularly higher concentrations of protein and fat, and improved the nutritional profile of milk fat. In a related study (O'Callaghan et al., 2016a), butters produced from GRO and GRC milks had a higher nutritional value and improved sensory scores compared with TMR butter.

However, while the milk composition from Irish dairy farming and its influence on the dairy processing has been extensively investigated (Kelly, O'Keeffe, Keogh, & Phelan, 1982; Keogh, Kelly, O'Keeffe, & Phelan, 1982; Phelan, O'Keeffe, Keogh, & Kelly, 1982), the influence that feeding has on metabolomics and on industrial processes such as the cheese making remain open. Increasingly, more advanced analytical instrumentation is being applied to probe in greater detail compositional differences and other markers in milks produced as a result of different feeding regimes, e.g., HR ^{1}H NMR spectroscopy has been already used successfully to investigate the influence of breeding on milk composition (Sundekilde, Frederiksen, Clausen, Larsen, & Bertram, 2011), milk somatic cells, usually correlate with mastitis, with the changes in metabolomics profile (Sundekilde, Poulsen, Larsen, & Bertram, 2013b).

In this study, a 600 MHz NMR installed with a cryoprobe was used to obtain spectra of milk ultrafiltrates from the already cited three feeding regimes. At the same time, a LF NMR was used to investigate the relaxation time of the proton of water present within the microstructure of mozzarella cheese prepared from the aforementioned milks, since it is known that LF-NMR is able to distinguish between three different populations of water based on binding to the cheese protein matrix (Gianferri, Maioli, Delfini, & Brosio, 2007; Kuo, Gunasekaran, Johnson, & Chen, 2001).

2. Materials and methods

2.1. Milk sampling

Milk sampling was conducted in mid-May (15 May 2016) from the afternoon lactation, at the period of maximum production. Sixty spring-calving Friesian cows were allocated to three groups (n = 20) at the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland. Groups were randomised based on milk yield, milk solids yield, calving date (mean calving date February 19, 2015), and lactation number. The feeding systems analysed

were the same as those used by O'Callaghan et al. (2016b) consisting of the first group housed indoors and fed a TMR diet, the second group was maintained outdoors on perennial ryegrass (*Lolium perenne* L.) pasture (GRO), whereas group 3 was also maintained outdoors on a perennial ryegrass/white clover (*Trifolium repens* L.) pasture (GRC). The daily feed dry matter allowance for the GRO, GRC and TMR herds was 18, 18 and 22.6 kg per cow, respectively. Dry matter consumption of TMR herd was obtained directly from the sum of dry matter of the grass silage, maize silage and concentrate used in the ration. Dry matter consumption for outdoor herds were measured by determining pre- and postgrazing sward heights daily using the rising plate meter (Jenquip, Feilding, New Zealand), while pregrazing herbage mass was measured with an Etesia mower (Etesia UK Ltd., Warwick, UK) (O'Callaghan et al. 2016b).

Sample of milk were collected from the individual cows, to which sodium azide was added (0.3 mg mL^{-1}) to prevent bacterial growing and stored at 4 °C overnight. On the following day, the samples were mixed and filtered to remove the fat and the protein phase. Five millilitres of sample was ultrafiltered using a Vivaspin® 6 10 kDa (Sartorius Stedim Ireland LTD, Dublin, Ireland) and centrifuged at 10000 × *g* for 30 min to separate the serum phase. The data from four of the sixty cows introduced at the beginning of the experiment were discarded from the sampling due to animal health issues, thus, resulting in a reduction of the total number of samples to fifty-six.

Cheese was made separately from the bulked milks of each of the three different dairy herds, which were feed on perennial ryegrass (GRO), perennial ryegrass and white clover (GRC) or total mix ration (TMR) on four separate occasions/days (trials), between October 10 and November 5, based on each of the three dietary treatments. Between 800 and 1000 kg of milk from the three different herds (GRO, GRC and TMR) was collected in three different tanks from both morning and afternoon milkings. Milk was then standardised to a protein:fat ratio of 1.2. After overnight storage at 4 °C the milk was pasteurised (72 °C for 15 s) to reduce bacterial load before cheesemaking.

2.2. ^{1}H Nuclear magnetic resonance

Prior to ^{1}H NMR analysis, sufficient D_2O was added to all filtered samples to produce a solution of composition 90:10 H_2O/D_2O (v/v). TSP (sodium 3-trimethylsilyl-2,2,3,3-tetra-^{2}H-propanoate) was employed as an internal chemical shift reference for each solution. ^{1}H NMR spectra of the samples were obtained using a Bruker Avance III 600 MHz NMR spectrometer (Bruker UK Ltd., Coventry, UK) equipped with a helium gas cooled 5 mm Bruker Dual C-H cryoprobe (CRYOPLATFORM™ technology) and a Bruker SAMPLEJET™ sample changer at University College Cork. Spectra were measured at a sample temperature of 300/303 K using Bruker TopSpin 3.2 software for spectrometer control, sample handling, NMR data acquisition and processing. For all samples, 16 scans were collected into 65.5 K data points using an excitation sculpting pulse sequence with gradients for water signal suppression and a spectral width of 12.33 KHz. Spectra were phased and baseline corrected in Topspin. Prior to Fourier transform, a line broadening function of 0.3 Hz was applied.

2.3. Multivariate data

The fifty-six ^{1}H NMR spectra obtained from the filtered milk were analysed using the method already described (Sundekilde et al., 2011) apart from some exceptions. All spectra were aligned using the *i*coshift tool (Savorani, Tomasi, & Engelsen, 2010) according to the signal of TSP and adjusted to 0.0 ppm. Alignment was performed in MATLAB 7.13 (MathWorks Inc., Natick, MA, USA).

The chemical shift region between 0.5 and 10 ppm (excluding residual water signal at 4.7–5 ppm) was subdivided into 0.01-ppm integral regions and integrated, reducing each spectrum into 950 separate variables in the region. Spectra were scaled using the Pareto scaling and meanscale. Principal component analysis (PCA) was used to provide a transformation of the original variables (NMR resonances) into a substantially reduced set of uncorrelated variables, the principal

components (PC). Furthermore, the data were analysed by a partial least squares (PLS) regression model coupled with a discrimination analysis (PLS-DA) to address the variation in metabolites specifically associated with the feeding system. PLS-DA models were cross-validated using segmented (n = 10) cross-validation. PLS-DA model robustness was valuated using correlation coefficient (Q2) and root mean square error of cross validation (RMSECV). PCA and PLS-DA were performed in PLSToolbox 7.9.3 (Eigenvector Research Inc., Manson, WA, USA).

2.4. Cheese manufacture

After pasteurisation, 460 kg of milk was cooled to 36 °C and pumped into the cheese vats (500-L; APV Schweiz AG, CH-3076 Worb 1, Switzerland). Mozzarella cheese manufacture was as previously described by Guinee, Feeney, Auty, and Fox (2002). In the specific instance, a full description of the prepared cheeses description may be found in Gulati et al. (2018), where their compositions (GRO, GRC and TMR) were investigated and compared in detail.

2.5. Relaxometry study

Water proton relaxometry was used to investigate the ripening of the mozzarella cheese. Samples were prepared for analysis by collecting a cylindrical plug of 1.5 cm of diameter and 5 cm of height from a cheese block. The samples were all collected using a cork borer from the bulk part of the cheese. Three samples were collected from any cheese block. The cheese samples were inserted in a NMR tube (diameter 1.5 cm) and closed with Parafilm-M to avoid dehydration during analysis.

Before commencing spectra collection, the temperature of the sample was equilibrated at 25 °C in a thermostatically-controlled unit (Techne Dri Block DB3) for 1 h. The analysis was performed using a MQC23-benchtop NMR analyser instrument (Oxford instrument, Abington,

Oxfordshire, UK), with an operating frequency of 23.4 MHz for protons. Spectra were conducted at the operating temperature of the instrument (40 °C). The samples were maintained inside the machine for as short a period as possible to minimise any structural modification arising from exposure to high temperature.

Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used to study the transverse relaxation times (T2). This pulse sequence reduces the influence of field inhomogeneity (Hills, Takacs, & Belton, 1990) for long relaxation times. A total of 1024 echos was collected, with a 90-180 Degree Pulse Gap (τ) value of 420μs. Eight scans were collected for each analysis. The resultant decays were analysed by tri-exponential fitting in the RI Win-DXP software (V. 1.2.3. Oxford Instruments, Abington, Oxfordshire, UK). Every sample was analysed in triplicate.

As in previous studies, four different populations of protons denoted as T4, T3, T2 and T1 according to decreasing order of relaxation time (Gianferri et al., 2007) were identified and monitored in the cheese samples over the course of ripening for 50 days. T4 relating to expressible serum showed a relaxation time close to that of free water. T3 was associated with the fat present in the cheese, while T2 was correlated with entrapped water–water that is in close vicinity to the protein matrix and its relaxation time is defined by the diffusion between the water and the protein phase. T1 was associated with junction water, i.e., the water directly bound to the protein matrix, with a relaxation time directly connected with the proton exchange within the protein proton (Gianferri et al., 2007).

2.6. Urea and lactose content

Analysis of lactose and urea contents was performed using a Milkoscan FT 6000 plus Fossomatic 300 (CombiFoss) (Foss Allé 1, DK-3400, Hilleroed, Denmark). Immediately after collection, samples were placed in a fridge at 4 °C during the course of transport to the laboratory. All samples were heated to 40 °C and mixed prior to testing on the same day of collection.

2.7. Moisture and fat content

Grated cheese samples were analysed in duplicate at one day (post production) for protein, moisture, fat, salt and pH. For the purpose of this paper, only selected results relating to moisture obtained by oven drying at 102 °C for 5 h (IDF, 1982) and fat determined by Röse-Gottlieb method (IDF, 1996) were shown. Additional details on production and composition of these cheeses are outlined in Gulati et al. (2018).

2.8. Expressible serum

Expressible serum of cheese was investigated by grating 120 g of cheese and centrifuging it at 12,500 × *g* for 75 min at 25 °C as described by Guo and Kindstedt (1995). The expressible serum was indicated as the weight of serum expressed for 100 g of cheese.

3. Results

3.1. NMR-based milk metabolomics

The influence of feeding system was investigated using spectra generated during ^{1}H NMR spectroscopy of milk sera, an example of which is illustrated in Fig. 1. The spectrometer was fitted with a cryo-probe to improve the signal to noise ratio. Spectra obtained from this analysis were in accordance with those reported in a previous studies (Sundekilde, Larsen, & Bertram, 2013a) and metabolomics identification was based on previous literature (Chenomx, 2018; Sundekilde et al., 2013a,b; Wishart et al., 2009, 2012). The list of the detected metabolites (Table 1) correspond with those of a previous study (Klein et al., 2010).

224 The influence of feeding regime on milk metabolites was first analysed using PCA. Lactose
225 dominated the ^{1}H NMR spectrum of milk. Consequently, the PC1 explained lactose variation
226 between samples. To identify milk metabolites other than lactose, resonances from lactose were
227 removed before PCA modelling. Resonances between 0.5 and 10 ppm were analysed by excluding
228 the main region of the lactose signals: 3.27–3.35, 3.51-4.01, 4.37-4.7 and 5.13-5.25 ppm. This
229 exclusion of lactose was done to remove any PC due to a change in this signal particularly in light
230 of the fact that no difference in lactose content was found between the three different feeding
231 systems (Table 2). The scores and loadings of a PCA model on ^{1}H NMR data (Fig. 2) showed a
232 clear distinction between the GRO and the TMR feeding regime (Fig. 2a). The PCA loadings
233 showed a tendency for GRO-based cow feeding to have a decreased milk urea content according to
234 its NMR spectrum (Fig. 2c). This lower urea content was confirmed by further analysis made on the
235 total sample (Table 2).

236 Further data investigation was conducted using PLS-DA statistical analysis. This analysis
237 showed a clear separation of the TMR feeding system from the GRO and GRC population (Fig 3d)
238 while separation between GRO and GRC results was compromised by the presence of some
239 samples appearing to belong to the other population (Fig 3b,c). This was also confirmed by the
240 value of RMSECV with TMR scoring a better value (0.232149) with respect to GRO (0.348863)
241 and GRC (0.36038). However, sufficient separation between all three groups of samples was
242 obtained to allow the interpretation of difference between them.

243 The results (Fig. 4a) confirm the lower content of urea in GRO samples, with the peak
244 related to the urea showing a higher presence in the GRC samples. Results also showed an increase
245 of orotate in the GRC samples that was not detected by PCA analysis. When contrasting TMR with
246 the two outdoor feeding regimes (Fig. 4b) it is possible to observe that these latter samples possess
247 higher concentrations of hippurate (7.8, 7.6 and 7.5 ppm), lactate (4.1 and 1.33 ppm), carnitine
248 (3.23 ppm), creatinine (3.05 ppm) and orotate (6.19 ppm). At the same time, however, TMR is
249 richer in citrate (2.72, 2.68, 2.54, 2.51 ppm) and choline (3.2 ppm).

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251 *3.2. Cheese microstructure*

252

253 Mozzarella cheeses produced from the three different milk batches were ripened at 4 °C for
254 50 days. Samples were collected after 1, 10, 20, 30 and 50 days following production and analysed
255 for expressible serum and proton transversal relaxometry profile. Further study on the cheeses was
256 done in parallel by Gulati et al. 2018.

257 The expressible serum and the total moisture contents are outlined in Table 3. No
258 differences in the total moisture content and expressible serum were detected between the cheeses
259 obtained from the three different feeding systems.

260 However, when the water transversal relaxometry signal of the cheeses was investigated it
261 was possible to monitor changes occurring in the nature of the moisture distribution during
262 ripening. Four different proton populations were identified from the spectra, namely T1, T2, T3 and
263 T4 (see Materials and methods, section 2.5) in order of increasing relaxation time. The relative
264 concentrations and relaxation times of these four moisture population changes during ripening may
265 be observed in Fig. 5. Only T2 and T3 were found at all sampling times, while T4 and T1 occurred
266 during the first and final stages of the ripening process, respectively.

267 With respect to the change of relaxation time and distribution of moisture populations (Fig.
268 6) over ripening duration, the second population (T2) increased in all cheeses whereas populations
269 three and four (T3 and T4) decrease. T4 was found only on the first day of the cheese obtained from
270 GRO, but was more persistent in GRC and TMR cheeses where it was present until day 20. The
271 first population (T1) was the more difficult to identify due to its low concentration, however it was
272 found after 20 days cheese ripening for the GRO-fed cows and after 10 days of ripening in the case
273 of the GRC-fed cows, and only on the Day 20 sample for the TMR cheese. GRO cheese showed a
274 higher content of this population (T1) even if this increase was not statistically significant with
275 respect to the values found in the other two cheeses ($p < 0.005$). The relaxation time was found to

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increase with ripening time for all the four populations, with GRO cheese showing a higher value compared with the other two cheeses for T1, T2 and T3 populations.

4. Discussion

The importance of dairy cow feeding system on milk composition has been recently studied (Gulati et al., 2018; O'Callaghan et al., 2016b). A unique Irish temperate climate condition favours a feeding regime based on outdoor grazing (O'Callaghan et al., 2016b). During a comparison of three different feeding regimes, sizeable changes to the fat content of the milks was observed (O'Callaghan et al., 2016b), as well as to the rumen metabolome of the individual lactating animals involved in the studies (O'Callaghan et al., 2018). In this paper, NMR-based analytical techniques are explored to examine milk metabolomics in the milks of the cows subjected to the feeding regimes already outlined.

4.1. Metabolomics

Using ^{1}H NMR spectra it was possible to identify the difference in the metabolomics profile of the filtered milks obtained from the three different feeding regimes. This technique has already been of value in previous published work (Sundekilde et al., 2011, 2013b). However, the NMR instrumentation used in this paper was furnished with a cryoprobe to reduce the background noise on the final spectra. While the use of the cryoprobe has a beneficial influence on the signal to noise ratio of the spectra, no difference in the milk metabolomics (Table 1) was detected when compared with previous work (Sundekilde et al., 2013a), suggesting that this specific probe does not increase the sensitivity of this technique.

Further investigation of the metabolomics data was explored using PCA and PLS-DA approaches. It was clear since the first PCA analysis (Fig. 2) that cows fed with GRO showed a

lower concentration of urea. This result was confirmed during PLS-DA analysis (Fig. 4a), where GRO showed a lower concentration compared with GRC samples. The difference in urea content was also confirmed by direct analysis on the sample (Table 2). This difference may be due to the presence of higher nitrogen in the GRC-feeding system compared with GRO (O'Callaghan et al., 2016a).

A PLS-DA comparison between the outdoor (GRO and GRC) and TMR showed an increase of hippurate, lactate, carnitine and orotate and a reduction of choline and citrate in the first group. On the other hand, TMR showed a higher content in choline and citrate (Fig.4b). These metabolites have different influences on milk's nutritional value and industrial application.

The hippurate metabolite was previously identified using gas chromatography in combination with mass spectrometry when comparing different brands of milk, where it was found to be in higher concentration in those brands associated with organic farming (Boudonck, Mitchell, Wulff, & Ryals, 2009). In addition, hippurate together with orotate, another metabolomic compound found at higher concentration in GRO and GRC samples, were associated with milk having a low concentration of somatic cells (Sundekilde et al., 2013b). Higher levels of lactate also occurred in GRO and GRC milks which raised a concern given that this metabolite was previously reported to be associated with elevated levels of somatic cells (Sundekilde et al., 2013b). However, it was not possible with the present set of results to associate positive or negative influences of feeding on somatic cell count of milk.

Both carnitine and choline are of importance nutritionally to milk (Campoy et al., 1998; Zeisel, Mar, Howe, & Holden, 2003). Choline is an important structural component in the formation of phospholipids, cell membrane signalling and lipid transporter. Its biosynthesis is insufficient to meet bodily needs, hence a reliance on supplementation from dietary sources. Carnitine plays an important role in the catabolism of long chain fatty acids, except that its biosynthesis during early life is less efficient. Hence carnitine deficiency may in extreme cases lead to infant mortality (Crill & Helms, 2007). Carnitine occurred at higher concentrations in GRO and GRC samples while

choline was more prominent in TMR-produced milk. Considering the high nutritional importance of both nutrients and their contrasting occurrences in both groups of samples, it is difficult to identify which feeding system has a higher positive influence on the nutritional value of milk.

Higher concentrations of citrate were found in the TMR milk. Those metabolomics were previously related to the coagulation properties of milk (Sundekilde et al., 2011) where a higher concentration of citrate reduces the storage module (G') during rennet coagulation. Impaired rennet gel properties as a result of a reduced storage module have a negative impact on cheese making and subsequent cheese quality.

4.2. Cheese microstructure

Relaxometry studies were previously done on freshly-produced mozzarella cheese prepared from buffalo milk (Gianferri et al., 2007) and also with mozzarella cheese over the course of a relatively short ripening period (Kuo et al., 2001). In the current study, the authors conducted their investigations over a longer cheese ripening period (50 days). The four different proton populations found in our results are similar to those found in previous work (Gianferri et al., 2007) and can be related to the same moisture and fat populations.

During the ripening of mozzarella cheese, the water contained in the serum-fat channel is absorbed by the cheese protein matrix (McMahon, Fife, & Oberg, 1999). This is visible with the reduction of the T4 population and the increase of T2 and T1, which is evident for all three feeding systems. In comparing the three different feeding systems it is possible to notice that the GRO cheese indicated the T4 population (expressible serum-related protons) only during first sampling while the other two cheeses continued to indicate this moisture population until 20 days of ripening in agreement with the results obtained with the expressible serum (Table 3). GRO also shows a faster increase of T2 (entrapped water-related protons) during the first period of ripening (20 days) and a higher content of T1 (protein-bound water).

These results suggest that in the GRO cheese the ripening time to reach the final structure is reduced to 40 days compared with 50 days in the case of TMR- and GRC-produced cheeses. It was also shown that the protein microstructure contained more bound water with respect to the other two cheeses, thus suggesting a different protein microstructure. The higher relaxation time value of GRO probably indicates a slower diffusion between protein matrix and water, hence suggesting a protein matrix that is more compact with respect to the other two moisture populations.

5. Conclusion

Using 1H NMR and Relaxometry instrumentation, it was possible to investigate the influence of the feeding regime on the metabolomics content of milk and on the microstructure of cheese, respectively. Both analytical techniques reveal useful scientific detail, which may be aligned with technological and commercial needs.

When identifying a feeding regime to promote the metabolomics value of milk from a nutritional perspective, GRO- and GRC-grass based diets stand out in terms of possessing higher concentrations of biological markers such as hippurate, which reflect near-organic milk production conditions. On the other hand, a lower citrate concentration in milk would contribute to an increase in storage module (G') during chymosin-induced gelation to the benefit of curd formation during cheesemaking. These differences in metabolomics for now are attributable to the effects of different feeding systems. However, it may be opportune in future work to combine NMR metabolomics with the potential of mid-infrared (MIR) spectroscopy because of its capability to monitor ruminant de novo fatty acid synthesis (Woolpert et al., 2016) provides additional information on the health status of the lactating cow and its impact on milk composition.

In relation to cheese ripening process, GRO-cheese shows that it needs a shorter ripening time to reach its final water distribution as defined by relaxometry. These findings add to advances in pastoral-based dietary regimes where applicable in dairy producing countries.

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Figure legends

Fig. 1. Solvent suppressed, 600 MHz ^{1}H NMR spectrum in 90:10 H_2O–D_2O of a milk sample.

Fig. 2. Results from the principal component analysis (PCA) obtained by comparing the three different feeding systems: (a) scores plot for the first and second principal component; (b) loading line plot of the first principal component of Pareto-scaled nuclear magnetic resonance (NMR) data (n = 56); (c) loading line plot of the second principal component of Pareto-scaled nuclear magnetic resonance (NMR) data (n = 56). Sample markers are coloured continuously according to their feeding regime: green, cows fed outdoor with grazing of perennial ryegrass (*Lolium perenne* L.); red, cows fed outdoor with grazing of perennial ryegrass/white clover (*Trifolium repens* L.); blue, cows fed indoor housing using a total mixed ration.

Fig. 3. Correlation coefficient and prediction graphic of the partial least squares discrimination analysis (PLS-DA) of the proton nuclear magnetic resonance (^{1}H NMR) metabolite profile of milk from different feeding regimes. Sample markers are coloured continuously according to their individual feeding regime: green, cows fed outdoor by grazing of perennial ryegrass (*Lolium perenne* L.); red, cows fed outdoor by grazing of perennial ryegrass/white clover (*Trifolium repens* L.); blue, cows fed indoor housing using a total mixed ration.

Fig. 4. Partial least squares discrimination analysis (PLS-DA) coefficient plots of the unit variance-scaled model back-transformed: (a) score of the comparison between grass feeding (GRS) and grass plus clover feeding (CLV) regimes – higher scores indicate higher concentrations within the CLV samples; (b) score of the comparison between the outdoor (GRS and CLV) and indoor feeding regimes (total mix ration, TMR) – numbers indicate different metabolomic signals: 1, hippurate; 2, orotate; 3, urea; 4, creatinine; 5, lactate; 6, choline; 7, citrate.

27

28 **Fig. 5.** Variation of the proton relaxation time of mozzarella cheese obtained from clover feeding
29 during ripening up to 50 days (──, day 1; − − −, day 10; - - - -, day 20; ·····, day 30;──, day 50).
30 Inset on the top left shows the zoom area within 100 and 3000 ms. The positioning of the different
31 water populations (T_1…T_4) according to signal intensity is indicated.

32

33 **Fig. 6.** Variation of (left column) moisture population detectable in the ^{1}H relaxation spectra during
34 the ripening of mozzarella cheese obtained from three different feeding systems and of (right
35 column) the relaxation time belonging to the different moisture populations: ─■─, GRO – cows
36 fed outdoor by grazing of perennial ryegrass (*Lolium perenne* L.); ···●···, GRC – cows fed outdoor
37 by grazing of perennial ryegrass/white clover (*Trifolium repens* L.); - -▲- -, TMR – cows fed
38 indoor housing using a total mixed ration.

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Table 1

List of chemical shift value and metabolomics detected in the ^{1}H NMR spectra of ultrafiltered permeate of milk.

Metabolomic	Chemical shift (ppm)	Metabolomic	Chemical shift (ppm)	Metabolomic	Chemical shift (ppm)	Metabolomic	Chemical shift (ppm)
3-Methylhistidine	2.25	Creatine	2.88	Hippurate	7.84	Lecithin	3.12
3-Methylhistidine	3.74	Creatine	3.79	Isobutyrate	1.16	Lecithin	3.75
3-Methylhistidine	3.97	Creatinine	3.05	Isoleucine	0.93	Lecithin	3.83
3-Methylhistidine	7.14	Creatinine	4.06	Lactate	1.33	Lecithin	4.18
3-Methylhistidine	8.09	Ethanolamine	3.15	Lactate	4.11	Lecithin	4.22
Acetate	1.92	Ethanolamine	3.83	Lactose (total)	3.54	Malonic acid	3.11
Acetone	2.24	Formate	8.45	Lactose (total)	3.67	Methionine	2.15
Adenine	8.12	Fucose	1.25	Lactose (total)	3.73	N-Acetylcarbohydrates	2.06
Adenine	8.13	Fumarate	6.52	Lactose (total)	3.78	Ornithine	1.8
Alanine	1.48	Galactose α	3.81	Lactose (total)	3.94	Orotate	6.2
Alanine	3.79	Galactose α	4.07	Lactose (total)	4.45	Phosphocholine	3.03
Betaine	3.26	Galactose β	3.49	Lactose α	3.59	Phosphocholine	3.18
Butyrate	0.9	Galactose β	4.57	Lactose α	3.66	Phosphocholine	3.58
Carnitine	2.44	Galactose-1-phosphate	5.38	Lactose α	3.84	Phosphocholine	3.93
Carnitine	3.21	Glucose	5.1	Lactose α	3.88	Phosphocholine	4.16
Carnitine	3.43	Glucose-1-phosphate	5.51	Lactose α	3.96	Taurine	3.27
Carnitine	4.57	Glutamate	2.36	Lactose α	5.23	Taurine	3.43
Choline	3.18	Glycerophosphocholine	3.65	Lactose β	3.29	Triethylamine-N-oxide	3.27
Choline	3.51	Glycerophosphocholine	4.32	Lactose β	3.6	Urea	5.79
Choline	4.06	Glycine	3.57	Lactose β	3.66	Valine	1.05
cis-Aconitate	3.15	Hippurate	7.54	Lactose β	3.81	β-Hydroxybutyrate	1.2
Citrate	2.52	Hippurate	7.64	Lactose β	3.84		
Citrate	2.72			Lactose β	3.96		
				Lactose β	4.67		

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Table 2

Average value of urea and lactose detected from the three different feeding regimes milk samples obtained from Milkoscan analysis.

Sample	Urea (mg L^{-1})	Lactose (g L^{-1})
Grass - clover	29 ± 4	4.8 ± 0.1
Grass	19 ± 3	4.7 ± 0.4
TMR	28 ± 2	4.8 ± 0.1

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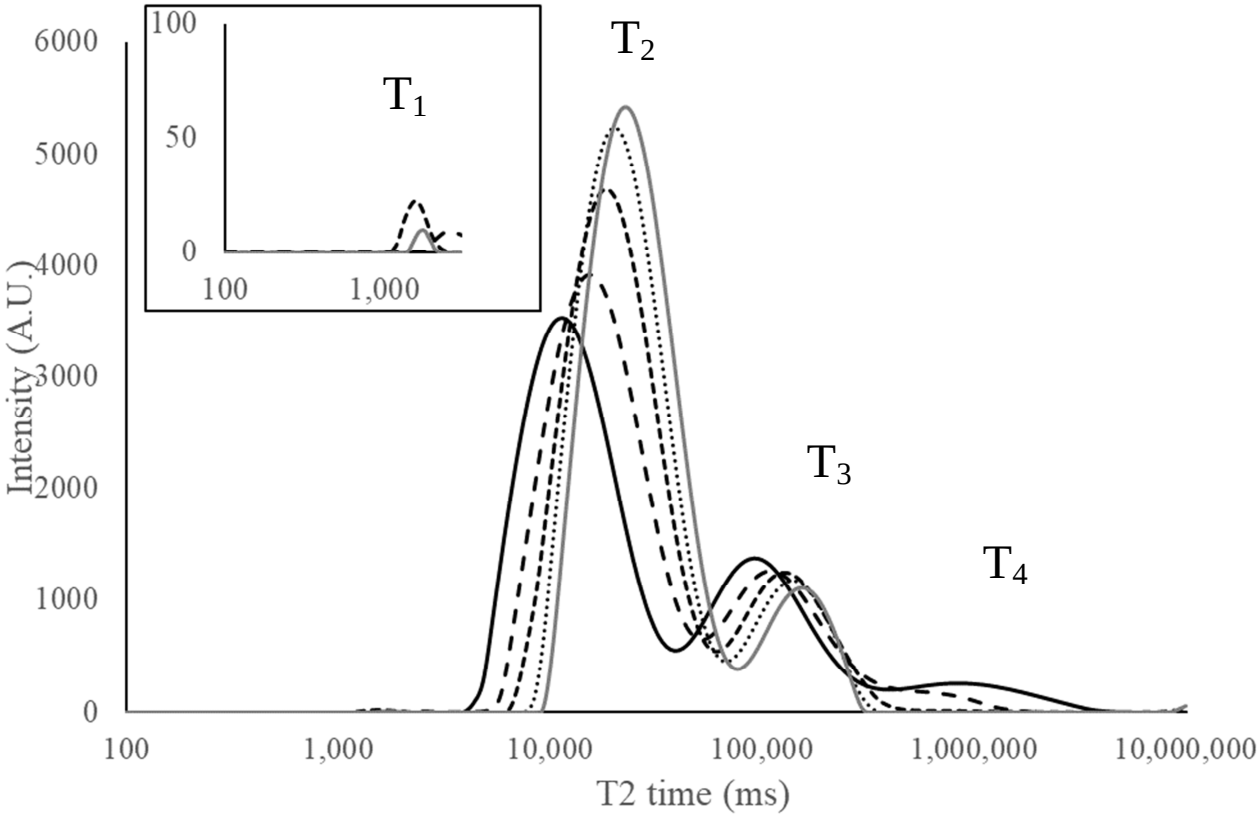
Table 3

Moisture content and expressible serum parameters of cheese made from milk from spring calved herds on 3 different feeding systems in late-lactation. [a]

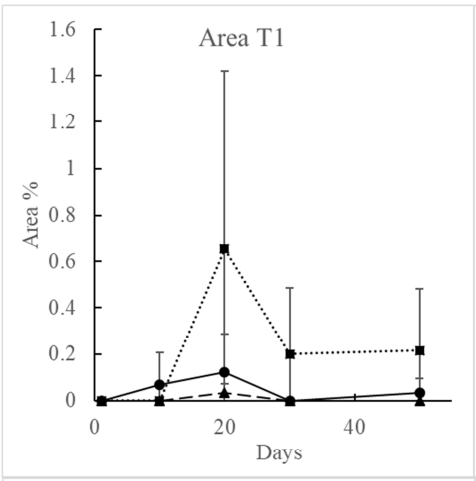
Parameter	Ripening time (days)	Feeding system		
		Grass	Grass-clover	TMR
Moisture (%, w/w)		48.41[a]	47.81[a]	47.12[a]
Expressible serum (% TM)	1	19.88[a]	17.98[a]	18.42[a]
	10	0.41[a]	6.77[a]	9.14[a]
	20	0	0	0
	30	0	0	0
	50	0	0	0

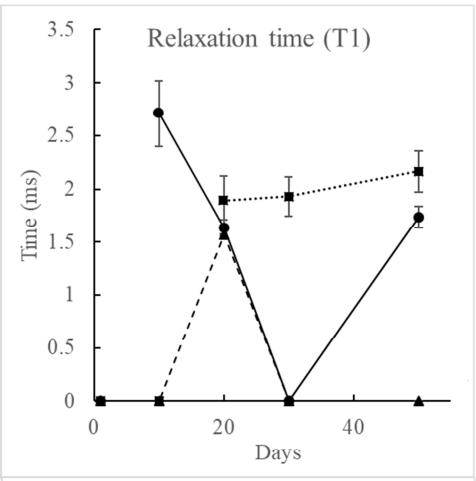
[a] Data are the mean values of four replicate trials in late-lactation (each made on a separate occasion); values within a row relating to Grass, Grass-clover or TMR diets and not sharing a common lowercase superscript letter differ significantly ($P < 0.05$) for the effect of feeding system. TM, total moisture.

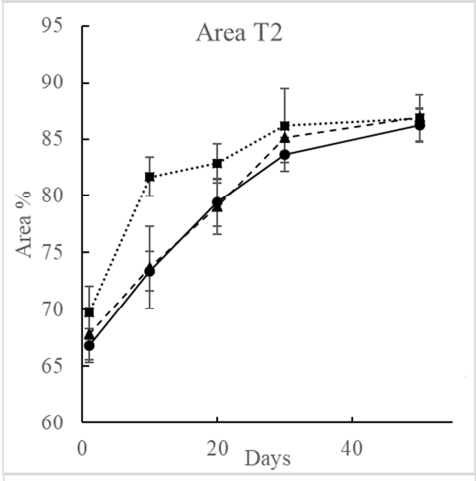
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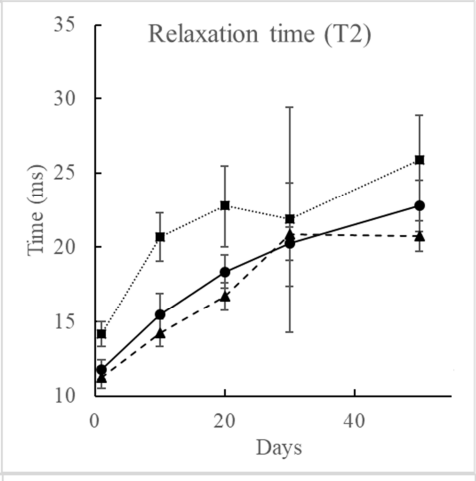


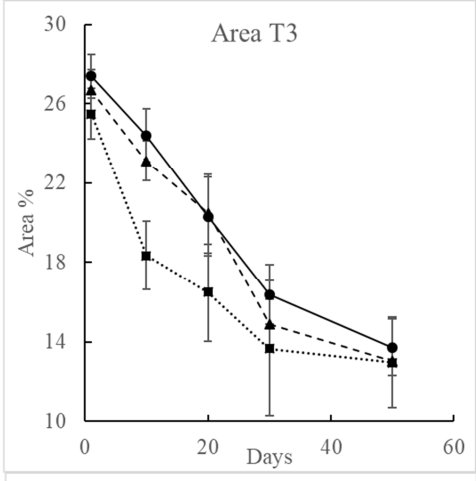
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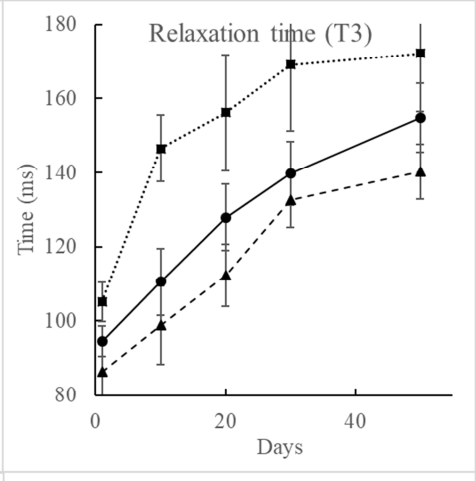

Area T1
Area %
Days

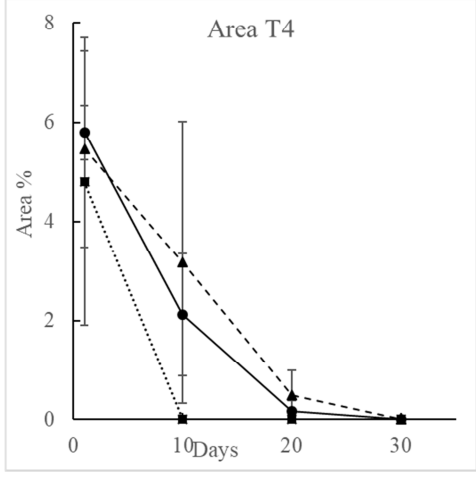

Relaxation time (T1)
Time (ms)
Days

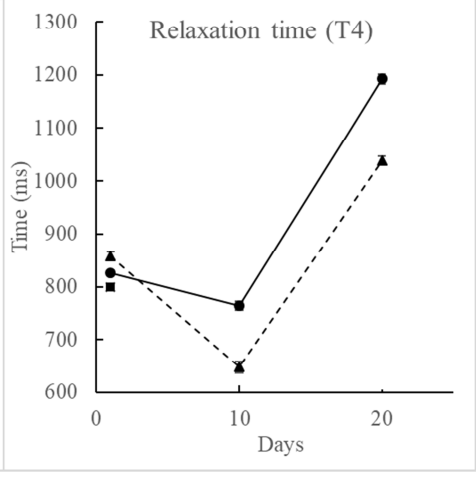

Area T2
Area %
Days

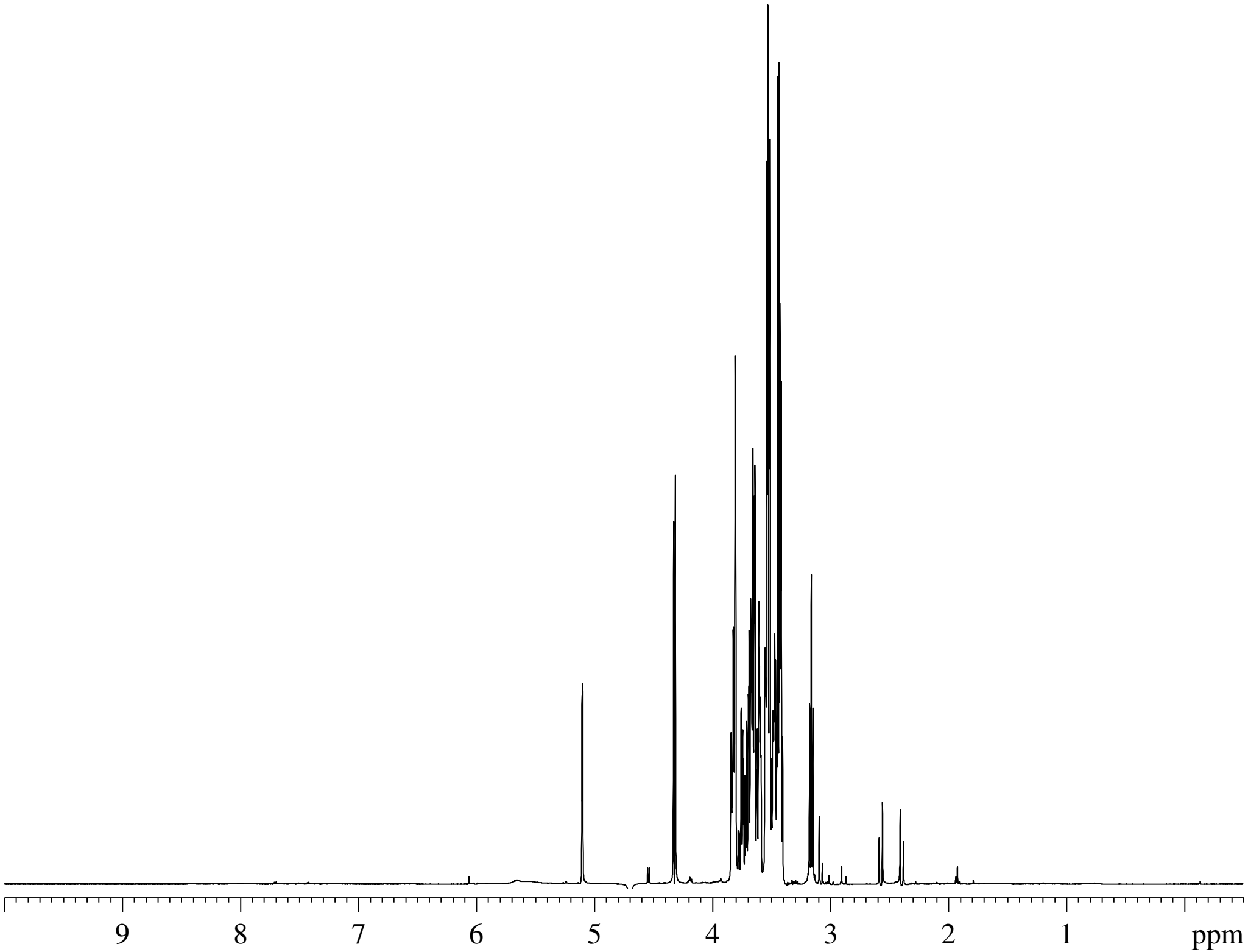

Relaxation time (T2)
Time (ms)
Days

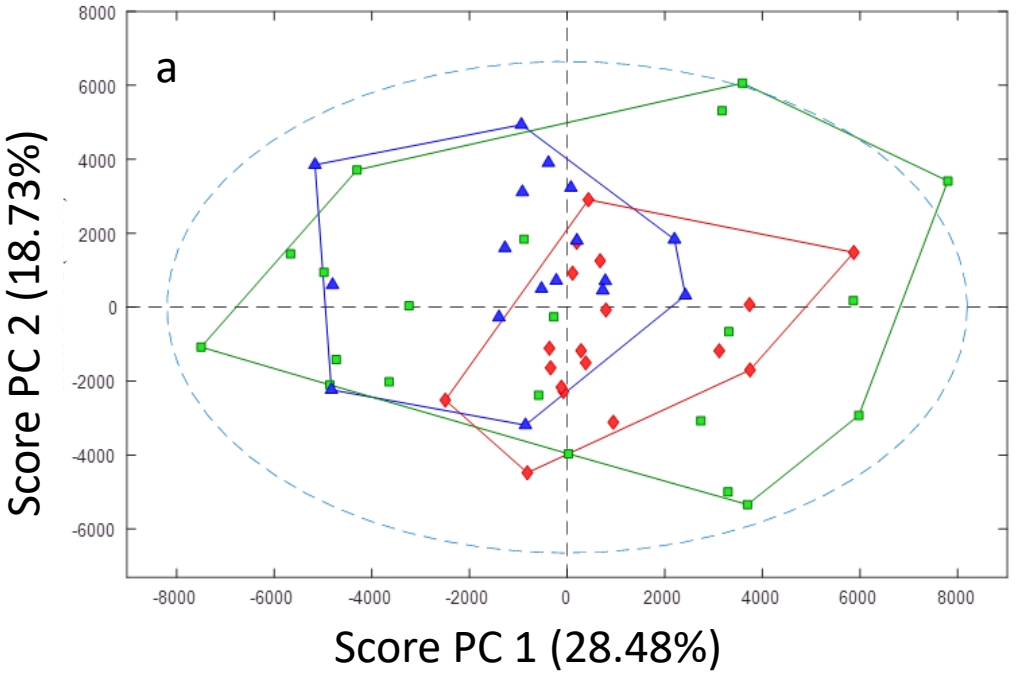

Area T3
Area %
Days

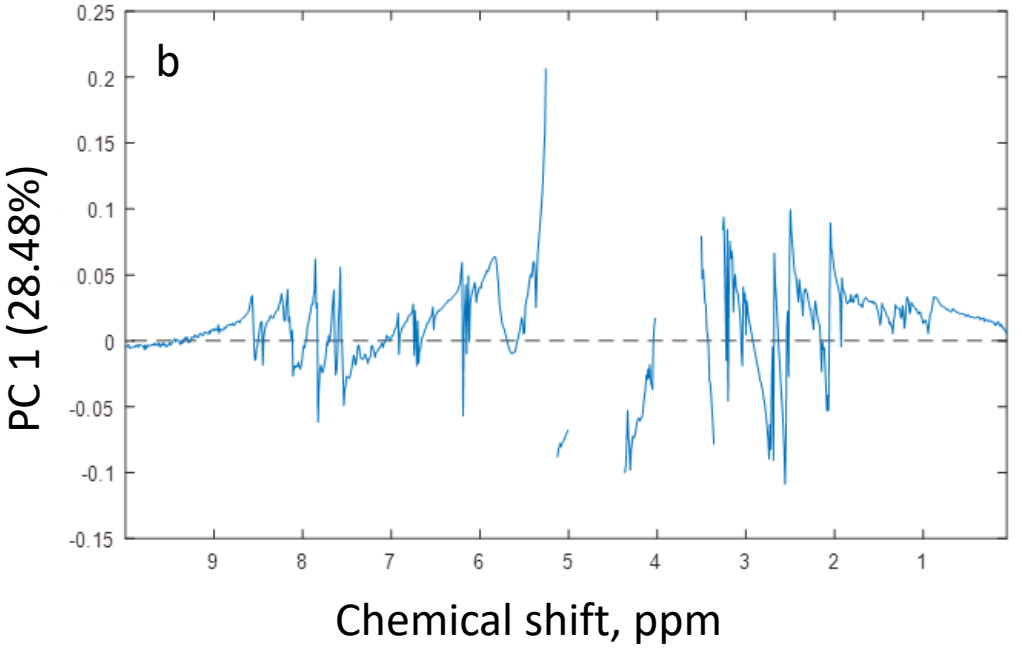

Relaxation time (T3)
Time (ms)
Days

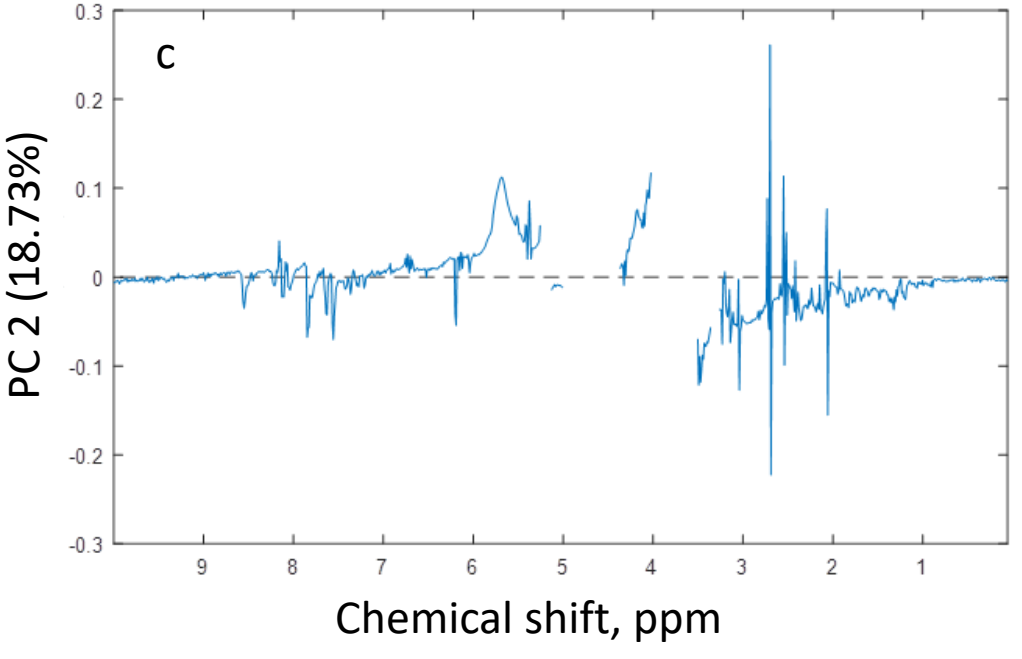

Area T4
Area %
Days

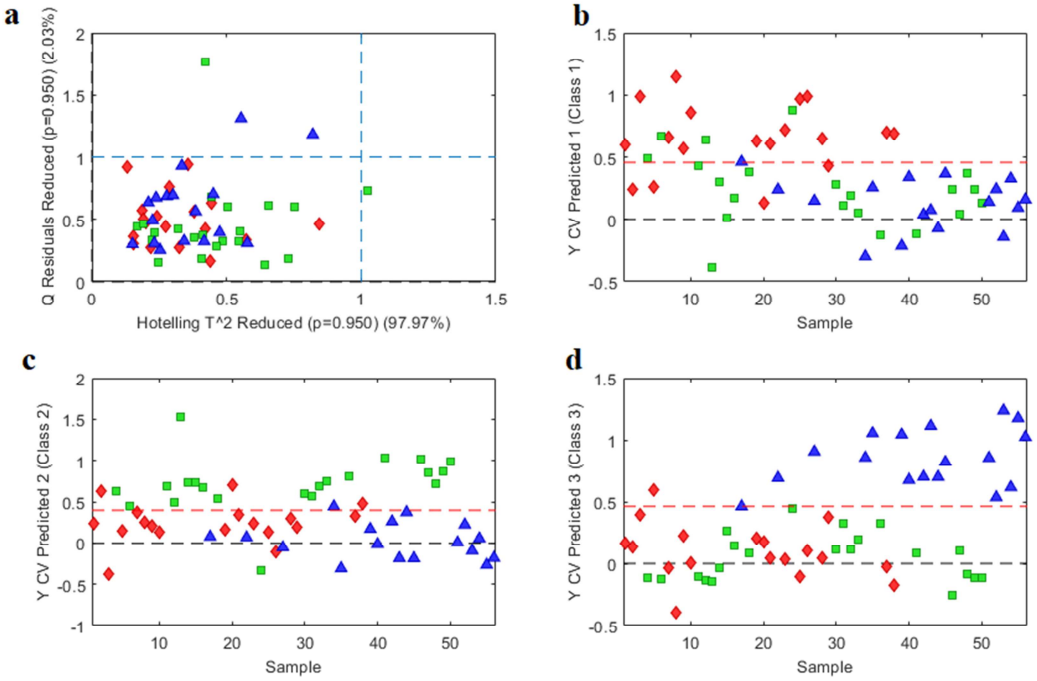

Relaxation time (T4)
Time (ms)
Days


9
8
7
6
5
4
3
2
1
ppm

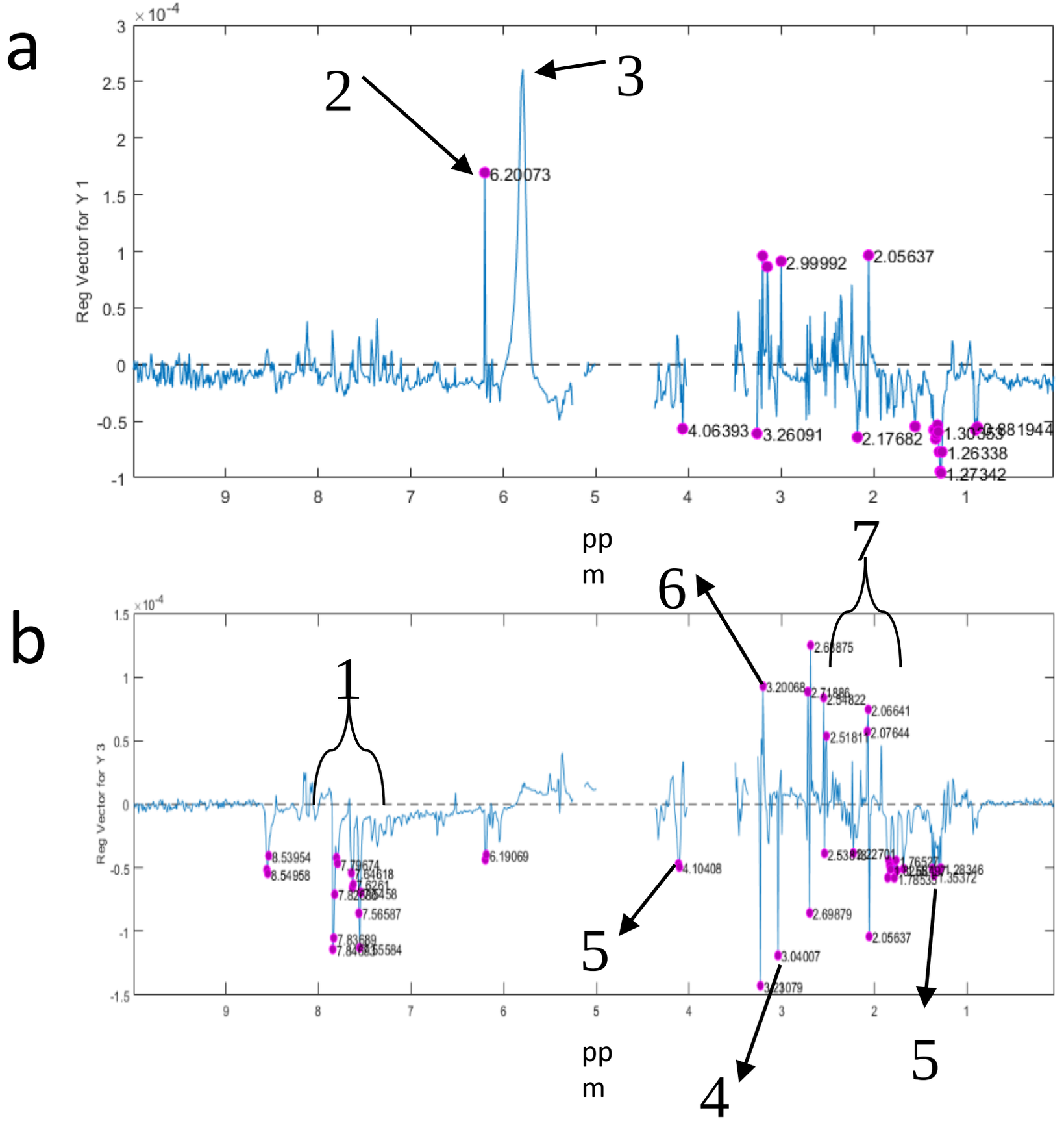

a
Score PC 2 (18.73%)
Score PC 1 (28.48%)


b
PC 1 (28.48%)
Chemical shift, ppm


c
PC 2 (18.73%)
Chemical shift, ppm



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a
×10^-4
Reg Vector for Y 1
2
3
6.20073
2.99992
2.05637
4.06393
3.26091
2.17682
1.26338
1.27342
ppm
b
×10^-4
Reg Vector for Y 3
1
6
7
5
4
5
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8.54958
6.19069
4.10408
3.20068
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ppm