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1 **Evolution of chemico-physical characteristics during manufacture and ripening of**
2 **Castelmagno PDO cheese in wintertime.**

3 Marta Bertolino¹, Paola Dolci, Manuela Giordano, Luca Rolle, Giuseppe Zeppa

4 Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Settore di Microbiologia Agraria e

5 Tecnologie Alimentari, Facoltà di Agraria, Università degli Studi di Torino, Via Leonardo da Vinci 44 Grugliasco

6 (TO), Italy

7

8

9 **Abstract**

10 Biochemical, volatile and textural profiles during manufacture and ripening were determined in
11 samples of Castelmagno PDO cheese obtained from three different batches in the main artisan
12 cheese plant of Castelmagno PDO production area. At the end of manufacture, samples were
13 characterized by a pH of 6.57 and 52.4% moisture content. The HPLC analysis of organic acids
14 and sugars showed the exhaustion of lactose content, while the Urea-PAGE indicated extensive
15 primary proteolysis on both β -casein and α_{s1} -casein. During ripening, cheeses were characterized
16 by high degradation of β -casein and α_{s1} -casein due to bacterial action. RP-HPLC profiles showed
17 a high production of peptides eluted between 20 and 30 minutes. A total of 92 volatile compounds
18 were identified in cheese headspace. Texture profiles showed an increase in hardness, gumminess,
19 chewiness and adhesiveness values as well as a decrease in cohesiveness during ripening.

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¹ Corresponding author: Tel.: +39-0116708705; fax: +39-0116708549

email address: marta.bertolino@unito.it

1 **Keywords:** Castelmagno PDO cheese, chemistry, proteolysis, volatile compounds, texture,
2 evolution, ripening.

3 **1. Introduction**

4 Castelmagno PDO cheese is one of the most important Italian hard cheeses and was given the
5 Protected Denomination of Origin label (PDO) in 1996. It takes its name from the homonymous
6 small town in Piedmont (North West Italy) where it was originally produced. Currently, the
7 production area is limited to three municipalities (Castelmagno, Pradleves and Monterosso Grana)
8 in the Province of Cuneo in Piedmont. The cheese is produced by six manufacturers (2 industrial
9 and 4 artisanal dairy plants). It is usually made from raw cow milk obtained from two consecutive
10 milkings. The evening milk may be partially skimmed after overnight creaming at 15°C in
11 shallow and large diameter tanks. Such semi-skimmed milk is mixed at a 1:1 ratio with the whole
12 milk collected during the successive morning milking. A small percentage of ewe or goat's milk
13 may be added to cow's milk, although such practice is not currently in use. Production technology
14 does not allow the use of starter cultures, so acidification is due to indigenous lactic acid bacteria
15 and milk is coagulated with liquid calf rennet at 32-38°C. The curd is transferred to molds and
16 harvested for at least 18 h for complete whey elimination. Then the curd is left at 10°C for a
17 period of 2-4 days under the whey obtained from previous cheesemaking. The curd is then milled,
18 dry-salted and strongly pressed. Finally, the cheese is placed in natural caves where ripening takes
19 place at 10-12°C and 85-90% humidity for at least 60 days. The cheese has a cylindrical shape,
20 measuring 12-20 cm high and 15-25 cm in diameter, and weighing 2-7 kg. *Penicillium* spp. from
21 the environment occasionally colonizes the interior part of the cheese during the final phase of
22 ripening. Due to the presence of this colonization, the Castelmagno PDO cheese is usually
23 considered a hard blue cheese variety (Ottogalli, 2001; Gobbetti & Di Cagno, 2002; Gobbetti,
24 2004) but nowadays the cheese is marketed before the appearance of mould.

1 Although a little is known about the microbiology of Castelmagno PDO cheese (Dolci,
2 Alessandria, Rantsiou, Rolle, Zeppa & Cocolin, 2008; Dolci, Alessandria, Rantsiou, Bertolino &
3 Cocolin, 2010) there are no studies on the technology, gross composition, glycolysis, proteolysis,
4 lipolysis, volatile and textural profiles of this cheese. Therefore, the aim of this research was to
5 determine the biochemical, volatile and textural profile of Castelmagno PDO cheese. Since its
6 production process consists of 4-5 days, it influences the biochemical pathways that determine the
7 final characteristics of the cheese. As a consequence, it was also necessary to analyze the samples
8 during Castelmagno PDO cheese manufacture and not only during ripening.

9

10 **2. Materials and methods**

11 *2.1. Materials*

12 Samples were taken from three batches of Castelmagno PDO cheese produced in the main
13 artisanal dairy plant in the town of Castelmagno (Piedmont, Italy) during the wintertime. The
14 three batches were produced on different consecutive days (A, B, C) by using milk from the same
15 farm. The cow milk used in cheese production had a pH of 6.59 ± 0.01 , and contained $4.51 \pm$
16 0.17% lactose, $3.45 \pm 0.07\%$ protein and $3.40 \pm 0.32\%$ fat. For each batch the milk (A1, B1,
17 C1), the curd after the cut (A2, B2, C2), the curd after 24 hours (A3, B3, C3), the curd after 3
18 days under the whey (A4, B4, C4), and the cheese after 3 (A5, B5, C5), 30 (A6, B6, C6), 60 (A7,
19 B7, C7), 90 (A8, B8, C8), 150 (A9, B9, C9) days of ripening were sampled. Samples were
20 transferred to the laboratory in refrigerated conditions and milk was immediately analysed for pH
21 and gross composition. Cheesemaking samples (A2, B2, C2, A3, B3, C3, A4, B4 and C4) were
22 analysed immediately for pH and an aliquot was also frozen and subsequently used for
23 compositional, glycolysis, proteolysis and volatile analysis. Cheese ripening samples (from A3 to

1 C9) were immediately analysed for pH and texture profile and an aliquot was also frozen and
2 subsequently used for compositional, glycolysis, proteolysis and volatile analysis.

3

4 *2.2. Methods*

5

6 *2.2.1. Compositional analysis*

7 Milk samples were analysed for lactose, protein and fat content by using a Milko ScanTM FT 120
8 (Foss, Padova, Italy). Cheese samples during manufacturing and ripening were analysed for:
9 moisture by the oven drying method at 102°C (IDF, 1982), salt by titration with AgNO₃ (IDF,
10 1988), total protein and pH 4.6-soluble nitrogen by Kjeldhal method (IDF, 1993), and fat by the
11 FIL-IDF Standard 5A method (1969). The pH was determined with a Portamess 913 pHmeter
12 (Knick, Berlin, Germany) placing the penetration electrode in contact with the sample mass. All
13 analyses were performed in triplicate.

14

15 *2.2.2. Assessment of proteolysis*

16 The pH 4.6-insoluble and -soluble extracts were prepared according to the method of Kuchroo
17 and Fox (1982), which was slightly modified as outlined by Hayaloglu, Guven, Fox, Hannon and
18 McSweeney (2004). Urea-polyacrylamide gel electrophoresis (Urea-PAGE) was performed on
19 the insoluble fraction using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd.,
20 Watford, UK) according to the method of Shalabi and Fox (1987). The gels were stained directly
21 with Coomassie Brilliant Blu G-250 using the method of Blakesley and Boezi (1977) and
22 destained using distilled water. After destaining, gel slabs were digitised by a scanner (Epson
23 Perfection 1650, Seiko Epson Corporation, Nagano, Japan). Scans of the electrophoretograms
24 were used to quantify bands using densitometric software (Image Master TotalLab 1D Gel

1 analysis v 1.11 software, Nonlinear Dynamics Ltd, Newcastle-upon-Tyne, UK). Similar bands
2 were recognized visually as described by McSweeney, Poochet, Fox and Healy (2004) and peak
3 volumes of corresponding bands were quantitatively determined.

4 Peptides of the pH 4.6-soluble fraction of cheeses were determined by RP-HPLC using the
5 method described by Hayaloglu *et al.* (2004) utilising a HPLC system (Thermo Electron
6 Corporation, Waltham, MA, USA) equipped with a isocratic pump (P1000), and a multiple
7 autosampler (AS3000) fitted with a 20 µL loop a UV detector (UV100) set at 214 nm.

8 Individual free amino acids (FAA) of the pH 4.6-soluble fractions of cheeses were prepared and
9 analysed according to the method of Bertolino, Zeppa, Gerbi and McSweeney (2008).

10

11 *2.2.3. Assessment of organic acid, sugars, diacetyl and acetoin*

12 Organic acids (citric, orotic, pyruvic, lactic, oxalic, hippuric, isobutyric, valeric and isovaleric),
13 sugars (lactose, glucose and galactose), diacetyl and acetoin were determined by high
14 performance liquid chromatography according to the method of Zeppa and Rolle (2008). Five
15 grams of sample were added to 25 mL of 0.013N H₂SO₄ (mobile phase) and homogenised for 10
16 min with a Stomacher blender (PBI, Milano, Italy). The extract was subsequently centrifuged for
17 5 min at 2500 g and the supernatant was filtered through a PTFE 0.20 µm disposable syringe
18 membrane filter (Sartorius AG, Göttingen, Germany). The HPLC system (Thermo Electron
19 Corporation, Waltham, MA, USA) was equipped with an isocratic pump (P1000), a multiple
20 autosampler (AS3000) fitted with a 20 µL loop, a UV detector (UV100) set at 210 and 290 nm
21 and a Refractive Index detector (RI-150). The analyses were performed isocratically at 0.8
22 mL/min and 65°C with a 300 × 7.8 mm i.d. cation exchange column (Aminex HPX-87H)
23 equipped with a Cation H⁺ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA).
24 Three replicates for each sample were analysed. The data treatments were carried out using the

1 ChromQuest™ chromatography data system (ThermoQuest, Inc., San Jose, CA, USA).
2 Analytical grade reagents were used as standards (Sigma-Aldrich Corporation, Milan, Italy).

3

4 *2.2.4. Volatile compounds analysis*

5 Grated homogenized sample (5g) was placed in a 40 mL vial fitted with a PTFE silicone septa
6 (Supelco, Bellefonte, PA, USA), through which the SPME syringe needle fitted with a Stable
7 Flex 2cm-50/30 µm divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS) fiber
8 (Supelco, Bellefonte, PA, USA) was introduced. The internal standard was methyl nonanoate
9 (Sigma Aldrich) at a final concentration of 80.4 µg/kg in the sample (Katechaki, Panas, Rapti,
10 Kandilogiannalis & Koutinas, 2008). The vial was placed in a heat/stir plate at 80°C for 35 min
11 for the absorption phase. After exposure in the headspace (HS), the fibre with the analytes was
12 retracted and transferred to the injector, which was operated in the splitless mode at a temperature
13 of 280°C for 4 min. Compound identification was achieved with a Shimadzu GC-17A gas
14 chromatograph (GC) coupled with a Shimadzu QP5000 quadrupole mass spectrometer (Shimadzu
15 Corporation, Kyoto, Japan). The GC was equipped with a DB-wax column (30 m, 0.25 mm i.d.,
16 and 0.25 µm film thickness) and a split/splitless injector. The carrier gas was ultrahigh purity
17 (99.999%) helium with a flow rate of 1 mL/min. The following column temperature programming
18 sequence was used: an initial temperature of 35°C for 3 min, increased to 110°C at a rate of
19 5°C/min, increased to 240°C at a rate of 10°C/min and a final extension at 240°C for 10 min.
20 Mass spectra were recovered in the electron impact mode at an ionisation voltage of 70 eV. The
21 ion source and the interface were maintained at 250°C. Identification was achieved by
22 comparison to standard compounds where available, or/and using the NIST 12 and the NIST 62
23 data base (National Institute of Standards and Technology, Gaithersburg MD, USA).

24

1 2.2.5. *Texture analysis*

2 For Texture Analysis, samples were cut with a thin blade in 20 mm squared cubes and
3 immediately analyzed. The TPA test was carried out using a Universal Testing Machines (UTM)
4 TA-XT2i Texture Analyzer[®] (Stable Micro System, UK) equipped with a 25 kg loadcell and
5 HDP/90 platform. Samples were compacted in height to 30% of the original using a crosshead
6 speed of 0.8 mm/s and a P-35 DIA cylinder stainless flat probe (Kapoor, Metzger, Biswas &
7 Muthukummarappan, 2006; Blazquez *et al.*, 2006). Each sample was subjected to a two-cycle
8 compression with 5 s between cycles (Drake, Gerard, Truong & Daubert, 1999). For the
9 acquisition of the force-time curve, a Texture Export Exceed software rel. 2.54 (Stable Micro
10 Systems, Godalming, UK) was used. According to Gunesakaran and Mehemet Ak (2003), the
11 following parameters were measured from the force-time curves (Figure 1): hardness (N, as F_1
12 maximum force), cohesiveness (adimensional, as $(A_2)/(A_1+A_{1w})$), adhesiveness (mJ, as A_3),
13 gumminess (N, as hardness \times cohesiveness), springiness (mm, as d_2), chewiness (mJ, as
14 gumminess \times springiness) and resilience (adimensional, as (A_{1w}/A_1)). For each batch and point of
15 ripening, five analyses were performed.

16

17 2.2.6. *Statistical analysis*

18 The distribution and the differences in the compositional parameter, organic acids, sugars,
19 diacetyl, acetoin, free amino acids, and textural parameters of Castelmagno PDO samples were
20 analysed using Brown-Forsythe test of homogeneity of variance, ANOVA and the Duncan mean
21 comparison test respectively to underline the normal distribution of the data and differences
22 during the manufacture and ripening of cheeses. Calculation was performed by Statistica 7.0
23 Software (Statsoft, Tulsa, USA).

24

1

2 **3. Results and discussion**

3 *3.1. Compositional analysis*

4 The average of pH, moisture, salt, fat, protein and pH 4.6-soluble nitrogen contents of
5 Castelmagno PDO samples during manufacturing and after 3, 30, 60, 90, 150 days of ripening are
6 shown in Table 1. The pH of cheeses was between 6.57 to 4.71 during manufacturing and
7 between 4.71 to 5.02 during ripening due to the microbial ecosystem evolution as reported by
8 Dolci, Alessandria, Rantsiou, Bertolino & Cocolin, (2010). The pH average value during
9 Castelmagno PDO market life (after 60 days of ripening), was 4.94 lower than that reported by
10 Gobbetti and Di Cagno (2002). According to moisture data after 60 days of ripening (period after
11 which the cheese can be sold), Castelmagno PDO cheese can be categorized as a hard cheese with
12 an average value of 35.5% (McSweeney, Ottogalli & Fox, 2004). After 3 days under the whey the
13 curd is grinded, pressed and formed with a high loss of whey. As a consequence, samples at 3
14 days of ripening showed a high decrease in moisture content.

15 Low salt levels found during Castelmagno PDO manufacturing (0.75%) was due to the fact that it
16 is a dry-salted cheese and salt is added during the curd grinding at the end of cheesemaking.
17 During ripening, salt levels increased to an average value of 2.46%, which was in line with data
18 reported by Delforno (1960).

19 Fat content increased from an average value of 23.2% during the manufacture period to an
20 average of 31.1% after 60 days. The second one was lower than that reported by Gobbetti (2004)
21 and Merlo (2001) but within the range reported by Delforno (1960).

22 Protein content increased from an average value of 19.31 % during manufacture to an average of
23 26.56 % after 60 days, whilst the pH 4.6-soluble nitrogen rose from 7.13% to 13.65% as a

1 consequence of the decrease of moisture. Protein content during market life was higher than that
2 reported by Gobbetti (2004).

3

4

5 *3.2. Assessment of proteolysis*

6 The data of pH 4.6-SN level in Castelmagno PDO samples are shown in Table 1. During cheese
7 manufacturing pH 4.6-SN decreased as a consequence of its use as a nutritional requirement by
8 LAB (Monnet, Condon, Cogan & Gripon., 1996) and due to its diffusion in whey as a
9 consequence of the attainment of equilibrium of soluble constituents into two solutions as
10 reported for the cheese ripening in brine (Abd El Salam and Alichanidis, 2004). Contrarily, pH
11 4.6-SN increased during the ripening period due to the breakdown of casein into peptides and
12 amino acids by the action of chymosin, plasmin, and bacteria.

13 Urea-PAGE electrophoretograms of the pH 4.6insoluble fractions of Castelmagno PDO cheese
14 (batch A) during manufacture and ripening are shown in Figure 2. Bands in the
15 electrophoretograms and the densitometric analysis (data not reported) showed that the
16 degradation of β - and α_{s1} -caseins occurs early, already during cheese manufacturing due to the
17 rennet and plasmin activity effects. However it can be seen that neither β - nor α_{s1} -casein were
18 totally degraded at the end of ripening but α_{s1} -casein hydrolysis rate was greater than that of
19 β -casein during all stages of ripening. As a consequence of α_{s1} -casein hydrolysis, in all
20 electrophoretogram samples the band corresponding to α_{s1} -I-casein (α_{s1} -CN f 24-199) which is
21 the first product of rennet action on α_{s1} -casein, was present; from the third day of ripening other
22 bands corresponding to other peptides appeared (marked as z α_{s1} -CN) these are characterised to
23 have faster mobility than α_{s1} -I-casein (α_{s1} -CN f 24-199) which are degradation products of α_{s1} -

1 casein due to rennet and indigenous milk proteinases action. From the thirtieth day of ripening,
2 cheeses showed a band corresponding to the peptide α_{s1} -I-casein (α_{s1} -CN f 102-199). Concerning
3 γ -caseins (the polypeptides produced by the action of plasmin on β -caseins), the γ_2 -casein [β -
4 casein (f106-209)] was present at the highest concentration followed by γ_3 - [β -casein (f108-209)]
5 and γ_1 - [β -casein (f29-209)] caseins. The RP-HPLC profiles of the pH 4.6-soluble fractions of
6 Castelmagno PDO cheese (batch A) are shown in Figure 3. To compare the chromatographic data
7 obtained by RP-HPLCs, visual identification of similar peaks were evaluated. Common peaks
8 were evident in the region 5-8 min in the chromatograms of all with an increase in concentration
9 during ripening. Similar peptides eluted with retention times of 11-18 and 24-29 mins were
10 observed in samples 24 hours after manufacturing until the 150th day of ripening, with increased
11 concentration during ripening. All these regions were composed principally of amino acids and
12 hydrophilic peptides (Gonzalez del Llano, Polo & Ramos, 1995; Pavia, Trujillo, Guamis &
13 Ferragut, 2000). However, qualitative and quantitative differences were observed in the region
14 included between 30 and 50 minutes considered to be composed mainly of hydrophobic peptides
15 (Gonzalez del Llano *et al.*, 1995; Pavia *et al.*, 2000). Cheese proteolysis was also monitored by
16 determining the levels of individual free amino acids (FAAs). These data are reported in Table 2.
17 Overall, the total concentration of FAAs increased considerably from the end of manufacture
18 (14.78 ± 4.89 mg/g of cheese) to the end of ripening 98.56 ± 14.13 mg/g of cheese. Glutamic
19 acid, valine, leucine, phenylalanine and lysine were the FAAs characterised by the highest
20 concentration during cheese manufacturing whilst the most common FAAs during ripening were
21 aspartic acid, glutamic acid, valine, leucine and phenialanine. Most of these amino acids were
22 previously found to be present at high concentration in several hard or extra-hard Italian cheese
23 varieties (Resmini, Pellegrino, Hogenboom & Bertuccioli, 1988; Albenzio *et al.*, 2001; Gobbetti,
24 2004).

1

2 3.3. Assessment of organic acids, sugars, diacetyl and acetoin composition

3 Organic acids, sugars, diacetyl and acetoin concentrations of Castelmagno PDO samples are
4 reported in Table 3.

5 Lactose metabolism was totally complete at the end of manufacture but already after just 24 hours
6 most of the lactose had been converted into lactate by the growth of starter bacteria or by its loss
7 into whey as reported by McSweeney (2004). Glucose and galactose were also present at very
8 low concentration (0.01 ± 0.01 mg/g of cheese and 0.11 ± 0.02 mg/g of cheese respectively) and
9 they were already absent in curd after 3 days under the whey samples and 3-day-old cheeses
10 respectively due to their use by lactic acid bacteria (LAB) and non starter lactic acid bacteria
11 (NSLAB) as substrate of growth (Michel & Martley, 2001). During ripening, lactic acid was the
12 main organic acid in all samples, representing approximately 95% of the total organic acid
13 content in 3-day-old cheeses and 78% of total organic acid content in 150-day-old cheeses. The
14 mean lactic acid concentration during Castelmagno market life was similar to that observed for
15 Cheddar and Colby cheeses (Mullin & Emmons, 1997) but higher than that already reported for
16 Castelmagno PDO cheese (Dolci *et al*, 2008; Zeppa & Rolle, 2008). Citric acid was present with
17 the highest concentration (1.32 ± 0.11 mg/g of cheese) in 24hour-old cheeses; it then decreased to
18 0.03 ± 0.02 mg/g of cheese in 3-day-old cheeses due to its metabolization by Cit⁺ strains of LAB
19 or NSLABS into acetate, acetoin and diacetyl (McSweeney & Fox, 2004). In particular, all
20 Castelmagno PDO samples demonstrated a higher concentration of diacetyl than acetoin, which
21 can also be derived from the metabolism of pyruvate by NSLAB. The acetic acid concentration
22 increased during manufacture to a final level of 0.81 ± 0.08 mg/g of cheese in samples at 3 days
23 under the whey; it then decreased during the ripening phase. In 150-day-old cheeses it was found
24 at a concentration of 0.50 ± 0.03 mg/g of cheese. Acetate is produced from lactose, lactic acid or

1 citric acid metabolisms or from the catabolism of amino acids. Many authors have reported that
2 its concentration in different PDO cheeses such as Cheddar, Camembert, Beaufourt, Canestrato
3 Pugliese, Murazzano, Raschera, Robiola di Roccaverano and Toma Piemontese ranged from 0.18
4 to 1.89 mg/g of cheese. (Bouzas *et al.*, 1991; Mullin & Emmons, 1997; Faccia, Gambacorta,
5 Lamacchia & Luccia, 2004; Zeppa & Rolle, 2008). The propionic acid concentration increased
6 from manufacture to the end of ripening where it was found to be 1.43 ± 0.14 mg/g of cheese,
7 representing 7.3 % of total organic acid content. The propionic acid is produced from lactic acid
8 metabolism by *Propionobacterium* spp. as reported by McSweeney (2004) or from the lypolitic
9 activities of starter and secondary microflora as reported by Collins, McSweeney and Wilkinson
10 (2004). Iso-butyric acid was detected only in samples after 90 days of ripening with a mean
11 concentration of 0.68 ± 0.18 mg/g of cheese. Iso-valeric acid concentration increased during
12 Castelmagno PDO production and in the 150-day-old cheeses it was detected at a concentration of
13 0.69 ± 0.28 mg/g of cheese, representing 3.5 % of total organic acid content.

14

15 3.4. Volatile compound analysis

16 Volatile compounds identified in Castelmagno PDO samples by HS-SPME-GC/MS during
17 manufacturing and ripening are shown in Table 4. A total of 92 compounds were detected: 15
18 acids, 28 esters, 13 ketones, 12 aldehydes, 13 alcohols, 3 lactones, 3 hydrocarbons, and 6
19 compounds which could not be classified in these chemical groups. Acids constituted the main
20 chemical class during manufacturing with a mean concentration of 88.61% w/w of total volatile
21 compounds and during ripening (77.92% w/w of total volatile compounds concentration). Acids
22 can originate from three biochemical pathways: lipolysis, proteolysis and glycolysis (Curioni &
23 Bosset, 2002). During manufacturing the most abundant acids were acetic, decanoic, dodecanoic,
24 hexanoic and octanoic acids. The acetic acid increased its concentration from the beginning to the

1 end of manufacture and could have a microbial origin as a product of lactose fermentation due to
2 the growth of lactic and propionic bacteria (McSweeney & Fox, 2004), which are abundant in this
3 cheese as shown by microbiological data (Dolci, Alessandria, Rantsiou, Bertolino & Cocolin,
4 2010). The other acids were derived from the action of esterases and lipases present in raw milk
5 used for Castelmagno PDO cheesemaking. During cheese ripening, the highest acid
6 concentrations were found for acetic, butyric, decanoic, docecanoic, hexanoic and octanoic acids.
7 Esters are important common constituents of the volatile fraction of cheese. Different esters have
8 been reported, such as methyl, ethyl, propyl and butyl esters as a reaction of free fatty acid with
9 ethanol, methanol, propanol and butanol in different cheese varieties (Liu, Holland & Crow,
10 2004). Esters formation is correlated to the growth of lactic acid bacteria and *Micrococcaceae*
11 (Gripon, Monnet, Lambert & Desmazeaud 1991). In Castelmagno PDO samples, esters
12 concentration represented 1.41% of the total volatile compounds concentration during
13 manufacturing and 5.64% during cheese ripening. Ethyl esters were the predominant esters in
14 analysed samples due to the high concentration of ethanol arising from lactose fermentation or
15 amino acid catabolism. Among esters during Castelmagno PDO manufacturing, ethyl hexanoate,
16 ethyl octanoate and ethyl decanoate concentrations represented 72.29% w/w of total ester
17 concentration and ethyl butanoate and ethyl acetate represented 8% w/w of total ester
18 concentration. The concentration of all esters identified in Castelmagno PDO samples increased
19 during ripening and ethyl hexanoate, octanoate and decanoate represented 74% w/w of total esters
20 concentration during this time. The increase of these esters could be associated to the decrease in
21 corresponding acids. Ethyl hexanoate was also identified as the most abundant ester in other PDO
22 cheeses such as Grana Padano (Moio & Addeo, 1998), Parmigiano Reggiano (Bellesia *et al.*,
23 2003) and Pecorino Romano (Di Cagno *et al.*, 2003).

1 Ketones were the second most abundant compounds isolated in Castelmagno PDO samples with a
2 mean percentage of 2.04% (w/w of total volatile compounds concentration) during manufacturing
3 and 8.25% (w/w of total volatile compounds concentration) during ripening. They are formed by
4 enzymatic oxidation of free fatty acids to β -ketoacids and their consequent decarboxilation to
5 ketones. They are very important compounds for dairy products because they have very particular
6 odours and low perception thresholds (McSweeney & Sousa, 2000; McSweeney, 2004). A total
7 of 12 ketones were identified in Castelmagno PDO samples - 2-butanone, 2-pentanone, 2-
8 heptanone were the most abundant. Acetoin originates from citrate metabolism as a reduction of
9 diacetyl by the action of lactic acid bacteria (McSweeney & Fox, 2004). The highest
10 concentration of acetoin was detected during cheesemaking at the cut of the curd (44.37 ± 75.69
11 $\mu\text{g}/\text{kg}$ of cheese); its concentration then decreased until the 90th day of ripening. In 150-day-old
12 cheeses, its concentration was $3.25 \pm 2.59 \mu\text{g}/\text{kg}$ of cheese and this decrease could be due to its
13 reduction to butanone as reported by Urbach (1993).

14 Aldehydes were present with the highest concentration (4.50% w/w of total volatile compounds
15 concentration) at the end of manufacture of Castelmagno PDO cheeses. This concentration
16 decreased to a mean value of 0.18% w/w of total volatile compounds concentration during
17 ripening because they were rapidly converted to the corresponding alcohols or acids (Lemieux &
18 Simard, 1992). During manufacturing, hexanal, heptanal and 2-nonenal were the aldehydes with
19 the highest concentration and represented 37%, 15% and 30% (w/w) respectively of the total
20 aldehydes concentration of the curd after 3 days under whey. During ripening, the aldehydes with
21 the highest concentration were acetaldehyde, *trans* 2-hexenal and hexanal with a concentration
22 that represented 45%, 17% and 13% w/w of total aldehydes concentration. Acetaldehyde, which
23 represented nearly half the concentration of total aldehydes during ripening, could derive from the

1 breakdown of threonine, from the lactose metabolism, or by the oxidation of ethanol (McSweeney
2 & Sousa, 2000).

3 Alcohols were abundant during Castelmagno PDO manufacturing with a mean percentage of
4 3.56% w/w of total volatiles detected, whilst these levels increased during ripening to a mean
5 percentage of 6.18% (w/w of total volatile compounds concentration). Ethanol was the most
6 abundant. It is a product of lactose fermentation or amino acid catabolism and it is the alcohol that
7 contributes to the formation of ethyl esters. Primary alcohols are produced by the reduction of
8 aldehydes derived by the catabolism of the amino acids (Moio & Addeo, 1998) and were present,
9 during manufacture, to a final concentration of $7.11 \pm 1.31 \mu\text{g/kg}$ of cheese and a final
10 concentration at the end of ripening of $56.87 \pm 15.69 \mu\text{g/kg}$ of cheese. Instead, secondary alcohols
11 formed by enzymatic reduction from the corresponding methyl ketones which are produced from
12 fatty acids (Collins *et al.*, 2004) were not detected during manufacture but only during ripening.
13 The branched-chain alcohols detected (2-methyl-1-butanol and 3-methyl-1-butanol) derived from
14 the reduction of aldehydes produced from the catabolism of isoleucine and leucine respectively
15 (Yvon & Rijnen, 2001), and were present at a final concentration of 14.79 ± 6.68 and $18.62 \pm$
16 $9.68 \mu\text{g/kg}$ of cheese at the end of manufacture and ripening respectively.

17 Among the lactones family, three δ -lactones (δ -octalactone, δ -decalactone and δ -dodecalactone)
18 were detected. Lactones are cyclic compounds formed by the intramolecular esterification of
19 hydroxyl fatty acids through the loss of water (Molimard & Spinnler, 1996). Lactones were
20 present with a mean percentage of 0.82% w/w of total volatile compounds concentration during
21 manufacture of cheese and a mean percentage of 1.33% w/w of total volatile compounds
22 concentration during ripening. Lactones represented only a very small portion (c.a. 0.1% of total
23 volatile compounds concentration) in other cow's milk PDO cheeses like Grana Padano (Moio &
24 Addeo, 1998) but a higher portion in other ewes' milk PDO cheeses like Canestrato Pugliese (c.a

1 7.8% w/w of total volatile compounds concentration) or Fiore Sardo (c.a. 8.1% of total volatile
2 compounds concentration) or Pecorino Romano PDO (c.a. 9.2% w/w of total volatile compounds
3 concentration) (Di Cagno *et al.*, 2003).

4

5 3.5. Textural analysis

6 The mean values obtained for texture parameters of TPA obtained during ripening are shown in
7 Table 5.

8 Texture profile analysis presented changes for all variables during ripening, in particular in the
9 first 30 days and between 90 and 150 days. In general, hardness, adhesiveness gumminess and
10 chewiness values increased up to 3 days while an inverse behaviour was identified for
11 cohesiveness and resilience parameters.

12 Hardness, the force necessary to attain a given deformation (Szczesniak, 2002) increased
13 particularly at the beginning of ripening (first 30 days), was maintained for up to 90 days of
14 ripening and increased significantly in the last 60 days (about 18 N, from 25.88 ± 4.13 to $43.15 \pm$
15 5.50). Similar behaviour were also found in gumminess and chewiness properties (respectively
16 defined as the force and energy required to masticate cheese into a uniform state before
17 swallowing) . Also adhesiveness, (the work necessary to overcome the attractive forces between
18 the cheese and the contact surfaces of the universal testing machine probe) (Tunick, 2000),
19 showed a high increase in the last two months of ripening (0.45 ± 0.15 mJ). On the other hand,
20 small differences in springiness (measure of the distance recovered by the cheese sample during
21 the time between the end of the first bite and start of second bite), were registered throughout the
22 entire ripening period. Instead the parameters of cohesiveness (measure of the strength of the
23 internal bonds of the protein mycelium) (Tunick, 2000), and resilience, (a dimensionless
24 parameter which represents the ability of the cheese to regain its original position after the first

1 compression) (Chevanan, Muthukumarappan, Upreti & Metzger, 2006), were characterized by a
2 strong decrease in the values between 3 and 30 days, respectively -0.07 ± 0.01 and -1.51 ± 0.63 .
3 The values of these parameters also showed a decrease between 90 and 150 days.
4 Therefore, three distinct phases in texture development took place during Castelmagno PDO
5 cheese ripening: the first between 3 and 30 days, the second, from 30 to 90 days and the third,
6 from 90 to 150 days.

7

8 **4. Conclusions**

9 During the manufacture of Castelmagno PDO cheese, it was possible to detect the conclusion of
10 lactose metabolism with the total conversion of lactose into lactate and the commencement of the
11 primary proteolyses. The volatile profile was characterised by a high level of acids, in particular of
12 hexanoic, octanoic and decanoic acids, which are the primary products of lipolysis metabolism.

13 During ripening of Castelmagno PDO cheeses, it is possible to observe high degradation of α -
14 casein with an increase of all its degradation products, an evolution of the hydrophilic peptides
15 associated also to the highest concentration of glutamic acid, valine, leucine phenylalanine and
16 lysine. The volatile profiles of Castelmagno PDO cheese during ripening are characterised by a
17 decrease in acid compounds and an increase in ketones and alcohols as a consequence of free
18 fatty acids metabolism. Texture profiles underline an increased of hardness, gumminess,
19 chewiness and adhesiveness properties, and a diminution of cohesiveness.

20 However, to fully characterize the Castelmagno PDO cheese it will also be necessary to analyze
21 samples of Castelmagno PDO during the summer period, when the producers transfer the herd to
22 grassland where cows eat fresh forage that both directly and indirectly influences the organic acid
23 and volatile profile of the obtained products. At the same time, the producers change the caves, an

1 act that can influence microbiological effects and, as a consequence, the biochemical pathways of
2 Castelmagno PDO cheese during ripening.

3

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- 14

1 Table1: Mean value \pm standard deviation for the gross composition of Castelmagno PDO cheese
 2 during its production and its ripening and result of variance analysis.

	Manufacture of cheese			Days of ripening					Statistic al signific ance
	Cut of the curd	After 24 hours	Curd after 3 days under whey	3	30	60	90	150	
pH:	6.57 \pm 0.01 ^a	5.06 \pm 0.02 ^a	4.71 \pm 0.07 ^a	4.74 \pm 0.02 ^b	4.71 \pm 0.08 ^b	4.80 \pm 0.11 ^c	5.02 \pm 0.10 ^c	4.99 \pm 0.05 ^c	***
Moisture (% w/w):	54.6 \pm 1.35 ^a	53.4 \pm 1.34 ^b	52.4 \pm 0.34 ^b	45.5 \pm 0.65 ^c	40.1 \pm 0.99 ^d	37.7 \pm 0.79 ^e	35.2 \pm 0.31 ^f	33.5 \pm 0.82 ^g	***
NaCl (% w/w):	0.08 \pm 0.01 ^a	0.07 \pm 0.03 ^a	0.08 \pm 0.03 ^a	2.14 \pm 0.24 ^b	2.50 \pm 0.40 ^{b,c,d}	2.75 \pm 0.54 ^{c,d}	2.24 \pm 0.35 ^{b,c}	2.69 \pm 0.14 ^{c,d}	***
Fat (% w/w):	22.5 \pm 0.44 ^a	23.9 \pm 0.21 ^b	23.3 \pm 0.46 ^b	25.8 \pm 0.76 ^c	29.0 \pm 1.03 ^d	29.9 \pm 1.09 ^d	31.4 \pm 0.57 ^e	31.9 \pm 0.79 ^e	***
Protein (% w/w):	18.44 \pm 0.07 ^a	19.52 \pm 0.20 ^b	19.97 \pm 0.08 ^b	20.86 \pm 0.02 ^c	23.29 \pm 0.02 ^d	25.97 \pm 0.08 ^c	27.05 \pm 0.09 ^{e,f}	26.67 \pm 0.01 ^{e,f}	***
pH 4.6-solubleN (% total N)	8.09 \pm 0.15 ^a	7.72 \pm 0.90 ^a	5.59 \pm 0.19 ^b	5.53 \pm 0.30 ^b	7.85 \pm 0.22 ^c	9.72 \pm 0.10 ^d	12.36 \pm 0.24 ^e	14.95 \pm 0.28 ^f	***

5 Mean data for the three batches of Castelmagno PDO cheeses analysed in triplicate.

7 a, b, c, d, e, f: Different letters in the same row indicate significant statistical differences (Duncan Test,
 8 $p < 0.05$).

9 Statistical significance: ***= $P < 0.001$; **= $P < 0.01$; * = $P < 0.05$; ns= not significance.

1 Table2: Mean value \pm standard deviation of free amino acids composition of Castelmagno PDO
 2 cheese (mg/g) during its manufacture and ripening.

	Manufacture of cheese			Days of ripening					Statistic al signific ance
	Cut of the curd	After 24 hours	Curd after 3 days under whey	3	30	60	90	150	
Aspartic acid	0.07 \pm 0.06 ^a	0.29 \pm 0.05 ^a	0.65 \pm 0.19 ^a	0.74 \pm 0.03 ^a	2.05 \pm 0.55 ^b	4.45 \pm 0.25 ^c	5.24 \pm 0.47 ^c	7.98 \pm 0.30 ^d	***
Threonine	0.02 \pm 0.01 ^a	0.15 \pm 0.03 ^a	0.44 \pm 0.12 ^{a,b}	0.55 \pm 0.02 ^{a,b}	0.85 \pm 0.20 ^b	1.70 \pm 0.29 ^c	1.90 \pm 0.43 ^c	2.67 \pm 0.22 ^d	***
Serine	0.02 \pm 0.01	0.14 \pm 0.05	0.38 \pm 0.19	0.45 \pm 0.18	0.58 \pm 0.22	1.00 \pm 0.56	1.19 \pm 0.51	1.65 \pm 0.72	ns
Glutamic acid	0.45 \pm 0.26 ^a	1.55 \pm 0.14 ^{a,b}	2.69 \pm 1.10 ^{a,b}	3.80 \pm 0.09 ^{a,b}	4.02 \pm 1.14 ^{a,b,c}	7.94 \pm 1.63 ^{b,c}	6.74 \pm 1.16 ^{c,d}	11.41 \pm 1.96 ^d	***
Glycine	0.04 \pm 0.03 ^a	0.04 \pm 0.02 ^a	0.12 \pm 0.03 ^{a,b}	0.32 \pm 0.02 ^{a,b}	0.57 \pm 0.11 ^b	1.29 \pm 0.24 ^c	1.58 \pm 0.01 ^c	2.66 \pm 0.51 ^d	***
Alanine	0.03 \pm 0.01 ^a	0.22 \pm 0.01 ^a	0.87 \pm 0.18 ^a	1.20 \pm 0.02 ^a	1.56 \pm 0.24 ^{a,b}	2.74 \pm 0.15 ^b	3.01 \pm 0.09 ^b	5.66 \pm 1.10 ^c	***
Cysteine	0.04 \pm 0.02 ^a	0.06 \pm 0.02 ^a	0.14 \pm 0.07 ^a	0.32 \pm 0.03 ^{a,b}	0.44 \pm 0.01 ^{a,b}	0.63 \pm 0.04 ^{b,c}	0.91 \pm 0.08 ^{c,d}	1.29 \pm 0.66 ^d	***
Valine	0.16 \pm 0.05 ^a	0.50 \pm 0.04 ^a	1.02 \pm 0.28 ^{a,b}	1.76 \pm 0.09 ^{a,b}	2.54 \pm 0.09 ^b	4.83 \pm 0.05 ^c	6.28 \pm 0.04 ^c	9.78 \pm 2.54 ^d	***
Methionine	0.02 \pm 0.02 ^a	0.13 \pm 0.02 ^a	0.27 \pm 0.09 ^a	0.80 \pm 0.06 ^a	1.43 \pm 0.22 ^b	2.91 \pm 0.53 ^c	3.47 \pm 0.21 ^d	4.89 \pm 0.20 ^e	***
Isoleucine	0.01 \pm 0.02 ^a	0.19 \pm 0.06 ^a	0.40 \pm 0.14 ^a	0.70 \pm 0.04 ^a	1.00 \pm 0.05 ^{a,b}	2.28 \pm 0.30 ^{b,c}	3.37 \pm 0.07 ^c	5.46 \pm 1.45 ^d	***
Leucine	0.12 \pm 0.08 ^a	0.70 \pm 0.12 ^{a,b}	2.09 \pm 0.54 ^{a,b}	4.61 \pm 2.81 ^b	7.50 \pm 1.36 ^c	13.57 \pm 1.81 ^d	15.99 \pm 1.11 ^e	20.15 \pm 0.33 ^f	***
Tyrosine	0.10 \pm 0.06 ^a	0.42 \pm 0.12 ^{a,b}	0.65 \pm 0.27 ^b	0.58 \pm 0.05 ^{a,b}	0.80 \pm 0.28 ^b	1.33 \pm 0.09 ^c	1.55 \pm 0.10 ^c	1.50 \pm 0.07 ^c	***
Phenylalanine	0.10 \pm 0.07 ^a	0.71 \pm 0.14 ^{a,b}	1.81 \pm 0.44 ^b	3.35 \pm 1.71 ^b	4.82 \pm 0.42 ^c	7.76 \pm 0.31 ^d	9.26 \pm 0.35 ^e	11.88 \pm 1.84 ^f	***
Histidine	0.14 \pm 0.07 ^a	0.60 \pm 0.13 ^{a,b}	1.00 \pm 0.25 ^b	1.82 \pm 0.09 ^b	2.44 \pm 0.08 ^c	3.31 \pm 0.05 ^d	3.57 \pm 0.23 ^d	4.03 \pm 0.83 ^d	***
Lysine	0.13 \pm 0.05	0.71 \pm 0.38	1.15 \pm 0.65	1.66 \pm 0.56	2.46 \pm 0.41	2.49 \pm 0.80	4.51 \pm 0.03	3.98 \pm 0.25	ns
Arginine	0.01 \pm 0.01	ND	0.07 \pm 0.09	0.02 \pm 0.01	0.05 \pm 0.11	ND	ND	ND	ns
Proline	0.11 \pm 0.14 ^a	0.64 \pm 0.18 ^a	1.03 \pm 0.26 ^a	1.03 \pm 0.28 ^a	1.05 \pm 0.23 ^a	1.19 \pm 1.26 ^a	1.41 \pm 0.01 ^a	3.57 \pm 1.15 ^b	***
Total free amino acids	1.57 \pm 0.97	7.05 \pm 1.51	14.78 \pm 4.89	23.71 \pm 6.09	34.16 \pm 5.72	59.42 \pm 8.36	69.98 \pm 4.90	98.56 \pm 14.13	

4
 5 Mean data for the three batches of Castelmagno PDO cheeses analysed in triplicate.

6 a, b, c, d, e, f: Different letters in the same row indicate significant statistical differences (Duncan Test,
 7 $p < 0.05$).

8 ND: not detected.

9 Statistical significance: ***= $P < 0.001$; **= $P < 0.01$; * = $P < 0.05$; ns= not significance.

10

1 Table 3: Mean value \pm standard deviation of organic acids, sugars, diacetyl and acetoin
 2 concentrations of Castelmagno PDO (mg/g) cheese during its manufacture and ripening.

	Manufacture of cheese			Days of ripening					Statistic al signific ante
	Cut of the curd	After 24 hours	Curd after 3 days under whey	3	30	60	90	150	
Lactose	36.65 \pm 2.06 ^a	6.13 \pm 1.71 ^b	0.72 \pm 0.49 ^c	ND	ND	ND	ND	ND	***
Glucose	0.01 \pm 0.01 ^a	0.01 \pm 0.01 ^b	ND	ND	ND	ND	ND	ND	***
Galactose	0.10 \pm 0.01 ^a	0.11 \pm 0.02 ^b	0.12 \pm 0.03 ^b	0.03 \pm 0.01 ^c	ND	ND	ND	ND	***
Lactic acid	0.90 \pm 0.90 ^a	20.31 \pm 2.68 ^{b,c}	31.06 \pm 3.54 ^d	30.57 \pm 2.58 ^d	24.96 \pm 2.41 ^b	19.08 \pm 0.90 ^c	17.73 \pm 2.07 ^c	15.28 \pm 1.45 ^c	***
Diacetyl	0.04 \pm 0.01 ^a	0.20 \pm 0.13 ^a	0.66 \pm 0.10 ^{a,b,c}	0.52 \pm 0.08 ^{a,b}	0.74 \pm 0.25 ^{a,b,c}	1.17 \pm 0.39 ^{b,c,d}	1.39 \pm 0.35 ^{c,d}	1.59 \pm 0.20 ^d	***
Acetoin	ND	ND	0.02 \pm 0.01 ^a	0.02 \pm 0.01 ^a	0.02 \pm 0.00 ^a	0.04 \pm 0.00 ^b	0.06 \pm 0.01 ^c	0.06 \pm 0.02 ^d	***
Citric acid	1.24 \pm 0.04 ^a	1.32 \pm 0.11 ^b	0.30 \pm 0.17 ^c	0.03 \pm 0.02 ^d	0.03 \pm 0.01 ^d	0.04 \pm 0.02 ^d	0.07 \pm 0.03 ^d	0.09 \pm 0.02 ^d	***
Pyruvic acid	0.04 \pm 0.02 ^a	0.12 \pm 0.06 ^a	0.43 \pm 0.17 ^b	0.41 \pm 0.11 ^b	0.12 \pm 0.04 ^a	0.04 \pm 0.03 ^a	0.03 \pm 0.02 ^a	0.02 \pm 0.01 ^a	***
Formic acid	0.03 \pm 0.03	0.04 \pm 0.04	0.02 \pm 0.01	0.03 \pm 0.01	0.02 \pm 0.00	0.02 \pm 0.01	0.02 \pm 0.01	0.02 \pm 0.01	ns
Acetic acid	ND	0.13 \pm 0.05 ^a	0.81 \pm 0.08 ^b	0.77 \pm 0.06 ^c	0.64 \pm 0.02 ^d	0.57 \pm 0.05 ^{d,e}	0.51 \pm 0.03 ^e	0.50 \pm 0.03 ^e	***
Propionic acid	ND	0.08 \pm 0.02 ^a	0.08 \pm 0.03 ^a	0.09 \pm 0.03 ^a	0.54 \pm 0.06 ^b	0.83 \pm 0.08 ^c	1.23 \pm 0.13 ^d	1.43 \pm 0.14 ^e	***
Oxalic acid	0.42 \pm 0.05 ^a	0.56 \pm 0.07 ^b	0.18 \pm 0.09 ^c	0.03 \pm 0.01 ^d	ND	ND	0.01 \pm 0.01 ^d	0.01 \pm 0.01 ^d	***
Orotic acid	0.04 \pm 0.02 ^a	0.02 \pm 0.01 ^b	0.01 \pm 0.01 ^c	0.01 \pm 0.01 ^{c,d}	ND	ND	0.01 \pm 0.01 ^{c,d}	0.01 \pm 0.01 ^{c,d}	***
Iso-butyric acid	ND	ND	ND	ND	ND	ND	0.19 \pm 0.28 ^a	1.17 \pm 0.09 ^b	***
Butyric acid	0.06 \pm 0.05 ^b	0.01 \pm 0.01 ^a	0.15 \pm 0.02 ^{c,d}	0.14 \pm 0.03 ^{c,d}	0.11 \pm 0.02 ^{b,c}	0.15 \pm 0.01 ^{c,d}	0.19 \pm 0.04 ^d	0.19 \pm 0.04 ^d	***
Iso-valeric acid	ND	0.01 \pm 0.01 ^a	0.01 \pm 0.01 ^a	0.11 \pm 0.06 ^a	0.43 \pm 0.09 ^b	0.44 \pm 0.08 ^b	0.48 \pm 0.06 ^b	0.69 \pm 0.28 ^b	***
n-Valeric acid	ND	0.01 \pm 0.00	0.01 \pm 0.00	ND	ND	ND	0.01 \pm 0.02	0.03 \pm 0.02	ns
Hippuric acid	0.01 \pm 0.01	ND	ND	ND	ND	ND	ND	ND	***
Uric acid	0.02 \pm 0.00 ^a	0.02 \pm 0.00 ^a	0.03 \pm 0.00 ^b	ND	ND	ND	0.04 \pm 0.01 ^c	0.05 \pm 0.01 ^d	***

3 Mean data for the three batches of Castelmagno PDO cheeses analysed in triplicate.

4 a, b, c, d, e: Different letters in the same row indicate significant statistical differences (Duncan Test, $p <$
 5 0.05).

6 ND: not detected.

7 Statistical significance: ***= $P < 0.00$; **= $P < 0.01$; * = $P < 0.05$; ns= not significance.

1 Table 4: Mean value \pm standard deviation of volatile compound concentrations of Castelmagno

2 PDO ($\mu\text{g}/\text{kg}$) cheese during its manufacture and ripening.

3

Compounds	L RI w	Manufacture of cheese			Days of ripening				
		Cut of the curd	After 24 hours	Curd after 3 days under whey	3	30	60	90	150
Acids									
	14	28.97 \pm	17.94 \pm	124.53 \pm	136.21 \pm	248.66 \pm	136.43 \pm	103.20 \pm	142.06 \pm
Acetic acid	38	38.79	8.61	64.67	13.29	92.53	71.41	24.08	60.07
Propanoic acid	15 28	ND	ND	ND	ND	4.85 \pm 4.93	12.73 \pm 14.34	11.67 \pm 4.95	21.77 \pm 7.87
Isobutyric acid	15 60	0.18 \pm 0.32	ND	ND	0.48 \pm 0.05	3.43 \pm 0.58	2.70 \pm 2.73	1.17 \pm 0.60	6.76 \pm 4.44
Butyric acid	16 20	141.32 \pm 82.97	8.02 \pm 2.57	178.29 \pm 89.78	166.79 \pm 8.63	397.15 \pm 115.75	235.34 \pm 119.82	162.74 \pm 50.05	278.45 \pm 165.54
Isovaleric acid	16 65	1.43 \pm 2.48	0.60 \pm 0.17	1.90 \pm 0.73	1.18 \pm 0.20	16.33 \pm 3.82	10.30 \pm 9.50	4.90 \pm 1.79	59.35 \pm 46.83
Valeric acid	17 36	2.11 \pm 1.46	0.26 \pm 0.04	0.75 \pm 0.33	0.52 \pm 0.05	2.97 \pm 0.61	1.69 \pm 0.51	1.49 \pm 0.65	3.62 \pm 2.30
Hexanoic acid	18 42	328.85 \pm 187.73	309.78 \pm 7.13	780.34 \pm 343.41	541.58 \pm 176.69	1341.57 \pm 678.09	1001.54 \pm 357.95	733.01 \pm 251.50	1094.37 \pm 698.89
Heptanoic acid	19 50	10.19 \pm 6.34	2.59 \pm 0.69	4.25 \pm 2.35	1.58 \pm 0.59	11.21 \pm 5.38	9.36 \pm 5.46	6.93 \pm 4.68	15.25 \pm 12.85
Octanoic acid	20 56	522.77 \pm 248.85	516.19 \pm 16.28	155.89 \pm 80.77	102.97 \pm 38.57	637.05 \pm 198.77	509.07 \pm 285.30	380.47 \pm 163.66	720.76 \pm 517.91
Nonanoic acid	21 63	19.00 \pm 11.23	4.03 \pm 0.94	9.13 \pm 2.73	1.47 \pm 2.31	11.71 \pm 3.41	10.02 \pm 4.77	9.47 \pm 6.06	22.43 \pm 18.38
Decanoic acid	22 68	594.32 \pm 335.82	280.39 \pm 26.15	259.90 \pm 114.41	128.06 \pm 59.15	692.78 \pm 203.65	570.27 \pm 329.06	448.79 \pm 197.36	842.10 \pm 553.94
Undecanoic acid	23 78	14.66 \pm 13.42	0.43 \pm 0.46	ND	ND	1.94 \pm 3.36	ND	7.61 \pm 5.79	15.18 \pm 19.91
Benzoic acid	-	3.44 \pm 2.53	1.30 \pm 0.41	5.41 \pm 2.05	3.95 \pm 0.63	8.56 \pm 1.21	4.78 \pm 3.03	3.88 \pm 1.99	9.61 \pm 15.60
Dodecanoic acid	-	246.21 \pm 154.89	20.77 \pm 8.41	54.22 \pm 26.77	18.86 \pm 16.25	188.25 \pm 44.75	164.06 \pm 106.76	148.29 \pm 83.84	290.84 \pm 218.32
Tetradecanoic acid	-	1.72 \pm 2.99	ND	ND	ND	ND	ND	5.71 \pm 6.30	1.65 \pm 2.85
Total Percentage (%)^x		1915.17 \pm 1089.80	1162.30 \pm 71.86	1574.62 \pm 728.01	1103.65 \pm 316.41	3566.45 \pm 1356.85	2668.30 \pm 1310.64	2029.33 \pm 803.30	3524.21 \pm 2345.72
		93.35	86.99	85.48	88.13	75.89	70.70	76.20	78.70
Esters									
Ethyl acetate	88 0	0.13 \pm 0.14	0.30 \pm 0.07	1.12 \pm 0.59	0.60 \pm 0.18	2.05 \pm 1.06	1.88 \pm 1.81	0.97 \pm 0.21	1.42 \pm 0.04
Methyl butanoate	97 8	0.03 \pm 0.05	0.22 \pm 0.20	0.96 \pm 0.55	ND	ND	1.14 \pm 1.25	0.30 \pm 0.53	1.03 \pm 1.04
Ethyl butanoate	10 30	0.72 \pm 0.21	1.34 \pm 1.72	2.27 \pm 0.66	1.09 \pm 0.30	6.89 \pm 4.26	11.52 \pm 15.82	5.92 \pm 1.37	7.76 \pm 3.89
Butyl butanoate	11 20	ND	ND	ND	ND	ND	ND	0.22 \pm 0.20	0.35 \pm 0.60

Butyl 3-methylbutanoate	12 57	ND	ND	ND	0.07 ± 0.12	1.95 ± 0.50	1.90 ± 2.10	1.09 ± 0.31	1.61 ± 0.68
Ethyl hexanoate	12 26	0.95 ± 0.87	3.09 ± 4.11	22.89 ± 13.20	16.11 ± 1.51	120.11 ± 5.30	111.11 ± 10.86	75.50 ± 1.49	89.12 ± 44.10
<i>n</i> -Hexyl acetate	12 65	ND	ND	ND	ND	ND	0.25 ± 0.29	0.26 ± 0.17	0.52 ± 0.13
Propyl hexanoate	13 12	ND	ND	ND	ND	8.09 ± 8.15	12.21 ± 13.79	15.11 ± 8.63	14.85 ± 5.03
Ethyl heptanoate	13 25	ND	0.22 ± 0.38	ND	ND	0.40 ± 0.68	0.90 ± 1.56	0.25 ± 0.22	0.78 ± 0.87
Ethyl octanoate	13 36	2.96 ± 2.04	4.47 ± 6.60	1.87 ± 0.70	1.25 ± 0.65	36.98 ± 45.36	44.73 ± 69.44	22.45 ± 9.58	17.96 ± 6.98
Allyl caproate	13 64	ND	ND	ND	ND	0.07 ± 0.13	0.05 ± 0.08	0.23 ± 0.05	0.49 ± 0.32
<i>n</i> -Heptyl acetate	13 66	ND	ND	ND	ND	ND	1.09 ± 1.88	0.25 ± 0.23	0.32 ± 0.28
Methyl octanoate	13 80	0.33 ± 0.57	1.46 ± 2.53	ND	ND	0.81 ± 1.41	3.04 ± 5.27	1.15 ± 2.00	1.48 ± 1.54
<i>n</i> -Hexyl butanoate	14 08	ND	ND	ND	ND	0.43 ± 0.52	0.70 ± 0.56	0.69 ± 0.14	0.93 ± 0.30
Propyl octanoate	15 13	ND	ND	ND	ND	3.20 ± 4.43	2.67 ± 3.83	3.07 ± 2.13	2.51 ± 1.18
Ethyl nonanoate	15 29	0.19 ± 0.19	ND	ND	ND	0.99 ± 1.71	ND	0.08 ± 3.48	ND
Methyl decanoate	15 88	2.83 ± 1.52	2.10 ± 3.38	2.91 ± 1.48	0.66 ± 0.14	12.01 ± 8.68	11.72 ± 9.08	10.50 ± 4.85	17.94 ± 15.42
<i>n</i> -Hexyl hexanoate	16 05	ND	ND	ND	ND	2.45 ± 1.32	1.89 ± 0.74	1.72 ± 0.59	2.83 ± 1.63
Ethyl decanoate	16 36	6.71 ± 7.03	5.84 ± 10.12	3.09 ± 2.69	2.26 ± 1.33	56.20 ± 63.10	52.21 ± 80.43	46.14 ± 30.37	65.86 ± 72.29
Ethyl 9-decanoate	16 87	0.82 ± 0.43	0.53 ± 0.91	ND	ND	5.88 ± 6.49	5.86 ± 8.68	5.47 ± 3.46	7.79 ± 9.55
Methyl undecanoate	16 94	ND	ND	ND	ND	0.06 ± 0.10	ND	0.03 ± 0.05	0.23 ± 0.41
Propyl decanoate	17 24	ND	ND	ND	ND	5.79 ± 5.08	3.51 ± 4.76	3.38 ± 2.21	3.61 ± 1.85
Ethyl undecanoate	17 41	ND	0.07 ± 0.12	ND	ND	0.55 ± 0.95	ND	0.45 ± 0.78	ND
Methyl dodecanoate	17 99	0.67 ± 1.16	0.42 ± 0.73	ND	ND	ND	ND	1.09 ± 1.88	ND
2-Phenyl ethyl acetate	18 08	ND	ND	ND	0.05 ± 0.08	9.18 ± 6.55	2.78 ± 4.82	3.54 ± 1.15	2.44 ± 2.11
Propyl dodecanoate	19 27	ND	ND	ND	ND	0.57 ± 0.52	0.60 ± 1.04	0.74 ± 0.19	0.48 ± 0.51
Ethyl tetradecanoate	20 52	ND	0.17 ± 0.29	ND	ND	5.37 ± 1.62	0.10 ± 0.18	5.62 ± 4.00	1.06 ± 1.04
Methyl tetradecanoate	20 08	ND	0.08 ± 0.14	ND	ND	ND	ND	0.40 ± 0.44	0.33 ± 0.35
Total Percentage (%)		16.33 ± 14.21	20.31 ± 31.31	35.10 ± 19.88	22.09 ± 4.31	280.03 ± 167.93	271.88 ± 238.28	208.559 ± 80.71	243.72 ± 172.18
		0.80	1.52	1.91	1.76	5.96	7.20	7.83	5.44

Ketones

Acetone	810	0.81 ± 0.38	0.32 ± 0.14	0.40 ± 0.10	0.73 ± 0.32	6.81 ± 6.85	4.72 ± 5.04	1.28 ± 1.14	2.42 ± 1.04
2-Butanone	896	0.17 ± 0.17	0.24 ± 0.21	0.55 ± 0.29	0.53 ± 0.37	32.00 ± 26.60	73.64 ± 85.31	58.17 ± 10.68	46.88 ± 25.99
2-Pentanone	965	0.22 ± 0.24	ND	2.75 ± 3.15	0.94 ± 1.33	82.05 ± 9.89	54.72 ± 76.05	13.97 ± 14.25	21.27 ± 15.86
Diacetyl	970	3.97 ± 6.56	ND	ND	2.75 ± 1.62	0.67 ± 1.16	ND	ND	ND
2-Hexanone	1069	ND	ND	ND	ND	0.43 ± 0.74	1.74 ± 2.75	0.28 ± 0.49	0.81 ± 0.18
2-Heptanone	1171	0.68 ± 1.18	ND	ND	1.60 ± 0.80	25.03 ± 14.39	77.63 ± 13.89	13.05 ± 5.19	41.36 ± 23.75
Acetoin	1270	44.37 ± 75.69	10.82 ± 6.82	27.76 ± 23.59	27.04 ± 15.52	11.06 ± 6.17	3.86 ± 2.40	1.84 ± 1.05	3.25 ± 2.59
2-Octanone	1274	ND	ND	ND	ND	0.70 ± 0.63	1.85 ± 2.64	0.38 ± 0.12	1.16 ± 0.70
2-Nonanone	1379	2.27 ± 1.21	0.10 ± 0.18	0.99 ± 0.13	1.03 ± 0.20	34.20 ± 25.70	65.44 ± 82.77	24.41 ± 10.56	57.71 ± 26.26
8-Nonen-2-one	1432	0.11 ± 0.19	ND	ND	ND	5.99 ± 5.93	11.00 ± 14.40	3.18 ± 1.16	10.11 ± 4.88
3,5-Octadien-2-one	1506	1.88 ± 1.09	3.66 ± 1.65	10.48 ± 4.43	ND	ND	ND	ND	ND
Benzyl methyl ketone	1717	ND	ND	ND	ND	0.23 ± 0.40	ND	ND	0.42 ± 0.46
2-Tridecanone	1809	ND	ND	ND	ND	256.62 ± 44.48	0.76 ± 1.14	ND	2.09 ± 2.97
Total Percentage (%)		54.47 ± 86.70	15.14 ± 9.00	42.92 ± 31.68	34.62 ± 20.16	455.78 ± 142.94	525.26 ± 341.69	208.72 ± 69.99	316.12 ± 146.04
		2.66	1.13	2.33	2.76	9.70	13.92	7.84	7.06

Aldehydes

Acetaldehyde	-	ND	0.35 ± 0.25	1.61 ± 1.79	2.79 ± 1.61	4.24 ± 1.79	2.62 ± 0.36	0.20 ± 0.17	1.11 ± 1.36
3-Methyl butanal	911	0.54 ± 0.87	0.25 ± 0.24	1.68 ± 0.95	0.56 ± 0.15	1.20 ± 0.87	0.21 ± 0.24	0.14 ± 0.12	0.70 ± 0.61
Hexanal	1071	1.31 ± 0.60	26.43 ± 10.52	30.20 ± 26.51	1.51 ± 0.17	0.98 ± 0.85	0.06 ± 0.10	0.27 ± 0.47	0.42 ± 0.72
Heptanal	1171	ND	4.81 ± 4.66	12.43 ± 9.10	ND	ND	ND	ND	ND
trans-2-Hexenal	1203	ND	2.42 ± 1.20	2.80 ± 2.51	ND	4.11 ± 7.12	ND	ND	ND
Octanal	1272	ND	4.13 ± 3.09	ND	ND	ND	ND	ND	ND
2-Heptenal	1307	ND	1.96 ± 1.78	2.90 ± 2.19	ND	ND	ND	ND	ND
Nonanal	1383	ND	6.57 ± 2.97	ND	0.15 ± 0.27	ND	ND	ND	ND
2-Octenal	1412	ND	4.56 ± 2.37	5.03 ± 4.77	ND	ND	ND	ND	ND
Benzaldehyde	1504	0.37 ± 0.35	0.80 ± 0.27	1.39 ± 1.72	ND	ND	ND	ND	0.59 ± 1.03
2-Nonenal	1524	1.06 ± 0.96	19.03 ± 11.40	24.93 ± 22.92	0.62 ± 0.15	ND	0.04 ± 0.07	0.08 ± 0.14	0.09 ± 0.15
Decanal	1767	ND	ND	ND	ND	0.88 ± 1.53	ND	0.12 ± 0.21	0.61 ± 0.15
Total Percentage (%)		3.28 ± 2.78	71.31 ± 38.75	82.96 ± 72.47	5.64 ± 2.34	11.42 ± 12.16	3.00 ± 0.91	0.81 ± 1.11	3.52 ± 4.02
		0.16	5.34	4.50	0.45	0.24	0.08	0.03	0.08

Alcohols

Ethanol	93 0	8.19 ± 8.15	42.99 ± 42.87	57.18 ± 39.87	27.83 ± 13.12	30.18 ± 7.50	62.37 ± 59.46	45.95 ± 39.57	157.26 ± 106.51
2-Butanol	10 25	ND	ND	ND	ND	9.84 ± 6.95	35.95 ± 45.99	50.30 ± 12.31	40.14 ± 6.98
Isobutanol	10 97	0.14 ± 0.24	ND	ND	1.44 ± 0.43	2.08 ± 1.61	1.26 ± 0.81	0.80 ± 0.49	0.96 ± 0.99
2-Pentanol	11 23	ND	ND	ND	0.11 ± 0.19	33.58 ± 9.21	40.97 ± 47.22	14.61 ± 10.42	16.14 ± 11.63
1-Butanol	11 44	ND	ND	ND	0.53 ± 0.09	1.92 ± 0.93	2.29 ± 1.95	3.59 ± 0.83	3.61 ± 1.40
Isopentanol	12 06	6.94 ± 12.03	2.38 ± 2.06	14.79 ± 6.68	10.64 ± 2.22	47.35 ± 24.66	20.95 ± 19.26	13.40 ± 4.88	17.66 ± 8.69
2-Heptanol	13 20	ND	ND	ND	ND	32.84 ± 32.10	36.63 ± 44.28	15.57 ± 7.88	22.58 ± 4.29
1-Hexanol	13 51	0.36 ± 0.34	1.28 ± 0.55	3.30 ± 1.31	1.80 ± 0.58	6.19 ± 3.99	6.07 ± 2.05	8.56 ± 3.34	8.47 ± 2.76
2-Nonanol	15 19	ND	ND	ND	ND	45.74 ± 70.44	23.83 ± 22.64	15.98 ± 2.02	28.60 ± 7.87
2,3- butanediol <i>d,l</i>	15 35	6.65 ± 11.52	ND	ND	1.70 ± 1.05	9.52 ± 3.83	7.55 ± 7.81	4.04 ± 1.81	5.08 ± 0.91
1-Octanol	15 55	ND	2.17 ± 1.99	ND	0.07 ± 0.12	ND	0.19 ± 0.19	0.34 ± 0.12	0.78 ± 0.33
2,3- butanediol <i>meso</i>	15 73	13.63 ± 23.61	5.51 ± 3.92	14.28 ± 5.18	19.43 ± 1.60	40.47 ± 20.40	15.11 ± 6.43	4.95 ± 4.31	5.69 ± 3.53
Benzyl alcohol	18 83	ND	ND	ND	ND	1.43 ± 1.60	0.53 ± 0.92	ND	ND
Total Percentage (%)		35.92 ± 55.89	54.31 ± 51.39	89.56 ± 53.05	63.55 ± 19.40	261.15 ± 183.21	253.71 ± 259.01	178.09 ± 87.97	306.96 ± 155.89
		1.75	4.07	4.86	5.06	5.56	6.72	6.69	6.85

Lactones

δ- Octalactone	16 09	2.63 ± 0.99	0.86 ± 0.30	3.90 ± 2.26	1.81 ± 0.51	3.73 ± 1.67	2.68 ± 1.24	1.90 ± 0.69	2.88 ± 1.50
δ- Decalactone	21 57	8.81 ± 4.35	6.65 ± 3.29	3.61 ± 6.26	12.58 ± 11.14	80.09 ± 48.88	23.07 ± 6.18	16.62 ± 0.52	41.87 ± 42.80
δ- Dodecalactone	-	8.26 ± 6.37	3.82 ± 4.67	4.23 ± 1.62	1.51 ± 1.84	15.94 ± 7.84	9.77 ± 5.82	8.20 ± 3.74	13.78 ± 12.17
Total Percentage (%)		19.70 ± 11.71	11.32 ± 8.26	11.74 ± 10.13	15.91 ± 13.49	99.76 ± 58.40	35.52 ± 13.25	26.72 ± 4.95	58.54 ± 56.46
		0.96	0.85	0.64	1.27	2.12	0.94	1.00	1.31

Hydrocarbons

Decane	10 00	0.62 ± 0.22	0.45 ± 0.04	1.89 ± 0.77	1.45 ± 0.10	2.21 ± 1.04	1.35 ± 0.73	1.50 ± 1.20	0.24 ± 0.28
Undecane	11 00	ND	ND	ND	2.26 ± 1.32	0.86 ± 0.89	ND	ND	0.08 ± 0.14
Dodecane	12 00	ND	ND	ND	0.36 ± 0.37	2.87 ± 3.23	2.77 ± 2.16	1.83 ± 0.89	0.79 ± 0.95
Total Percentage (%)		0.62 ± 0.22	0.45 ± 0.04	1.89 ± 0.77	4.07 ± 1.78	6.04 ± 5.33	4.12 ± 2.89	3.33 ± 2.09	1.11 ± 1.36
		0.03	0.03	0.10	0.32	0.13	0.11	0.12	0.02

Other compounds

Phenolic compounds

1,3-

Dimethoxybenzene	17402000	ND	ND	ND	ND	0.80 ± 1.38	ND	0.32 ± 0.55	1.12 ± 1.50
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Phenol	00	ND	ND	ND	0.26 ± 0.02	0.21 ± 0.20	ND	ND	8.86 ± 9.74
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Terpenes

Limonene	1181	ND	ND	ND	ND	0.10 ± 0.17	ND	ND	0.70 ± 1.22
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N-compounds

N,N-Dibutylformamide	1773	0.18 ± 0.30	ND	ND	ND	ND	ND	ND	ND
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Aromatic alcohol

Phenyl ethyl alcohol	1907	5.84 ± 9.56	0.98 ± 0.13	3.35 ± 1.61	2.51 ± 0.73	17.78 ± 6.63	12.49 ± 13.39	7.44 ± 2.36	10.21 ± 4.14
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S-compounds

Dimethyl disulfide		ND	ND	ND	ND	ND	ND	ND	2.21 ± 2.87
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Total		6.01 ± 9.87	0.98 ± 0.13	3.35 ± 1.61	2.78 ± 0.75	18.89 ± 8.38	12.49 ± 13.39	7.76 ± 2.91	23.10 ± 19.47
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Percentage (%)

		0.29	0.07	0.18	0.22	0.40	0.33	0.29	0.52
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2 Mean data for the three batches of Castelmagno PDO cheese analysed in triplicate

3 ^w LRI: linear retention index.

4 ND: not detected.

5 ^z Percentage (%): percentage of volatile compounds of each chemical group in each step of manufacture
6 and ripening analysed.

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1 Table 5: Mean value \pm standard deviation of TPA parameters of Castelmagno PDO cheese during
 2 its ripening.

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	Days of Ripening					Statistical significance
	3	30	60	90	150	
Hardness (N)	12.44 \pm 2.79 ^a	23.21 \pm 4.46 ^b	24.07 \pm 3.36 ^b	25.88 \pm 4.13 ^b	43.15 \pm 5.50 ^c	***
Cohesiveness (-)	0.28 \pm 0.06 ^a	0.21 \pm 0.05 ^b	0.25 \pm 0.04 ^a	0.20 \pm 0.06 ^b	0.17 \pm 0.03 ^b	***
Adhesiveness (mJ)	- 0.04 \pm 0.04 ^a	-0.21 \pm 0.13 ^b	-0.13 \pm 0.08 ^{a,b}	-0.12 \pm 0.05 ^{a,b}	-0.57 \pm 0.20 ^c	***
Gumminess (N)	3.51 \pm 1.34 ^a	4.91 \pm 1.75 ^{a,b}	6.09 \pm 1.41 ^{b,c}	5.24 \pm 1.70 ^b	7.51 \pm 1.77 ^c	***
Springiness (mm)	3.27 \pm 0.23 ^a	3.31 \pm 0.23 ^a	3.58 \pm 0.18 ^b	3.57 \pm 0.24 ^b	3.18 \pm 0.42 ^a	***
Chewiness (mJ)	11.53 \pm 4.40 ^a	16.41 \pm 6.34 ^{a,b}	21.91 \pm 5.29 ^{b,c}	18.92 \pm 6.47 ^{b,c}	24.00 \pm 7.69 ^c	***
Resilience (-)	3.15 \pm 2.26 ^a	1.64 \pm 1.63 ^b	1.63 \pm 1.15 ^b	1.41 \pm 1.30 ^{b,c}	0.79 \pm 0.31 ^c	***

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 5 Mean data for the three batches of Castelmagno PDO cheese analysed in quintuplicate

6 a, b, c: Different letters in the same row indicate significant statistical differences (Duncan Test, $p < 0.05$).

7 Statistical significance: ***= $P < 0.001$; **= $P < 0.01$; * = $P < 0.05$; ns= not significance.

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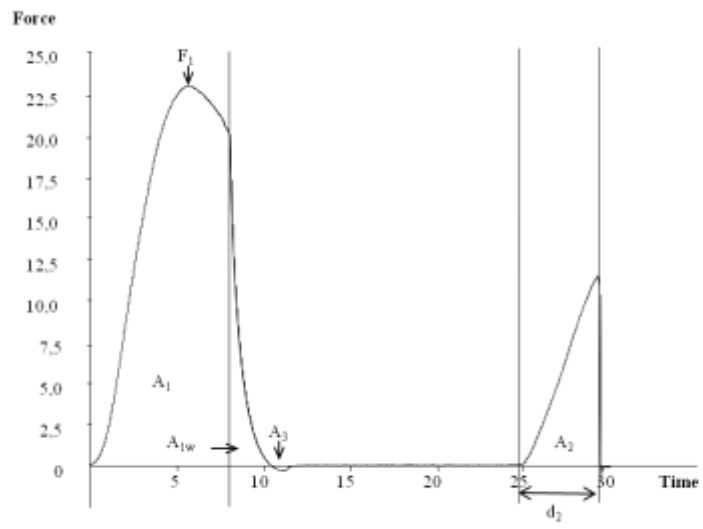
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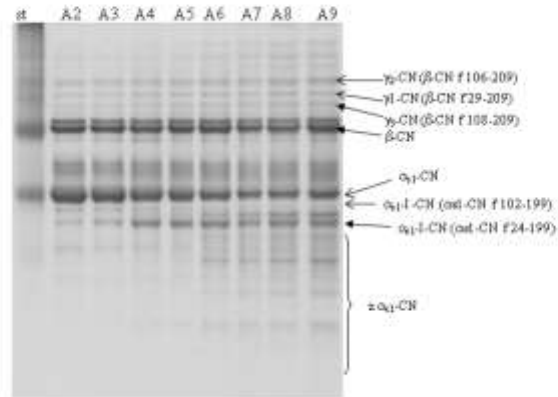
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2 Figure 1: Example of a TPA profile of Castelmagno PDO cheese.

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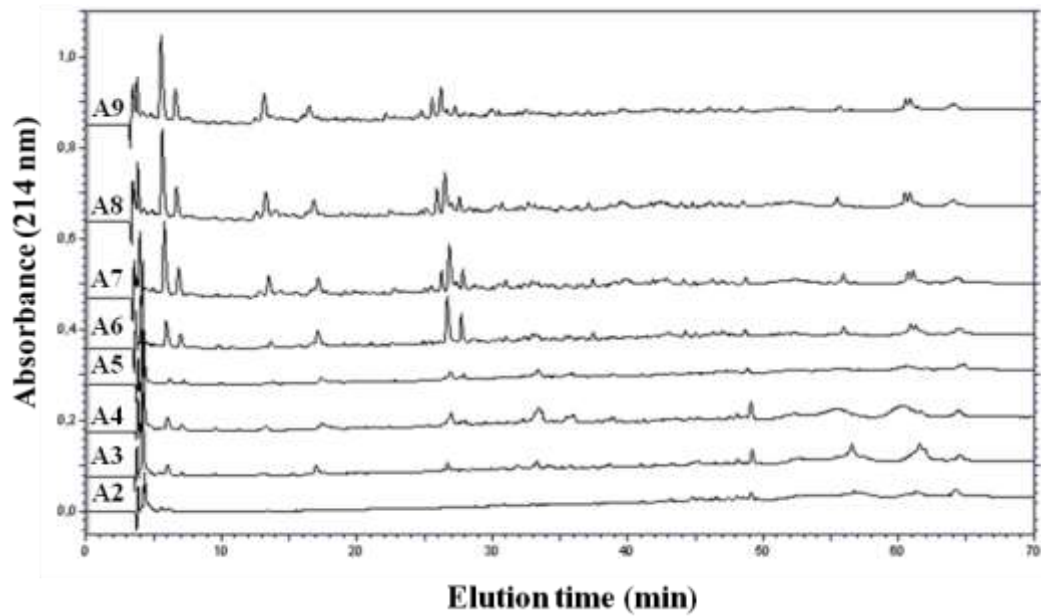
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2 Figure 2: Urea-polyacrylamide gel electrophoretograms of Castelmagno PDO cheese (batch A)
 3 during its production (A2= cut of curd; A3=after 24hours; A4= curd after 3 days under whey) and its
 4 ripening (A5= 3 days of ripening; A6= 30 days of ripening; A7= 60 days of ripening; A8= 90 days of
 5 ripening; A9= 150 days of ripening). Lane st = Na-caseinate.

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2 Figure 3: RP-HPLC chromatograms of the pH 4.6-soluble fraction of Castemagno PDO cheese
3 (batchA) during the manufacture (A2= cut of the curd; A3= curd after 24 hours; A4=curd after 3 days
4 under the whey) and the ripening (A5= 3 days of ripening; A6= 30 days of ripening; A7= 60 days of
5 ripening; A8=90 days of ripening; A9=150 days of ripening).

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