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Volatile profile evolution of Reggianito cheese during ripening under different temperature–time combinations

Roberto J. Ceruti¹ · Susana E. Zorrilla¹ · Guillermo A. Sihufe¹

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Abstract Using solid-phase microextraction and gas chromatography–mass spectrometry procedure, volatile compounds were studied during ripening of Reggianito cheeses stored under different temperature–time combinations. Volatile profile was characterised for cheeses ripened under standard conditions at 0, 61, 124 and 180 days of ripening, and changes occurring between 61 and 180 days were evaluated for cheeses in which an elevated temperature of 20 °C was applied within the first month of maturation. A total of 41 volatile compounds including acids, ketones, aldehydes, esters, alcohols and hydrocarbons were identified. Despite the presence of other compounds of potential relevance for cheese flavour, carboxylic acids clearly dominated the volatile profile of Reggianito cheeses. The more notable variations in the volatile fraction of Reggianito cheeses occurred during the first 2 months of ripening and were mostly explained by changes observed for the predominant acid fraction. Significant changes in the quantities for some volatile compounds were observed for the different temperature–time combinations applied, though the balance between the different groups of compounds was not considerably affected and small changes were observed for total chromatographic area values. In this work, a first description of changes occurring during

ripening for the different volatile compounds present in traditional Reggianito cheese was provided.

Keywords Reggianito cheese · Solid-phase microextraction and gas chromatography–mass spectrometry (SPME–GC–MS) · Volatile compounds · Ripening · Elevated temperature

Introduction

The presence, content and composition of volatile substances in food are determinant for odour and aroma characteristics, which in turn have a substantial influence on quality. Each product has a characteristic and unique composition of volatile components [1]. In this context, each cheese variety has a particular volatile profile. Volatile compounds in different cheeses are often the same, although their ratio can vary considerably. The study of volatile compounds in each cheese variety is useful for both characterisation and quality definition purposes, linking it with the area and methodology of production [2]. There are many works in which cheese volatile fraction characterisation and studies of its evolution during ripening have been performed in both internationally recognised and rather local cheese varieties [1, 3–7]. The study of volatile fraction in cheese has been also useful for evaluating the effect of different changes made along cheese manufacture [8–11].

Reggianito is the major hard cheese variety produced in Argentina, in the vast central zone known as Pampa region. Its manufacture process is similar in many aspects to that of Parmigiano Reggiano and Grana Padano hard Italian cheeses, and includes the use of pasteurised bovine milk, natural whey starter and bovine chymosin, while a curd-cooking step up to 52 °C and brine salting are

✉ Susana E. Zorrilla
zorrilla@intec.unl.edu.ar

Roberto J. Ceruti
rceruti@santafe-conicet.gov.ar

Guillermo A. Sihufe
gsihufe@intec.unl.edu.ar

¹ Instituto de Desarrollo Tecnológico para la Industria Química (INTEC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) - Universidad Nacional del Litoral (UNL), Güemes 3450 (S3000GLN), Santa Fe, Argentina

applied. Cheeses (6–7 kg blocks of cylindrical geometry) are usually ripened at 11–13 °C for 6 months [12]. Several research works have been carried out to elucidate the effects of different technological changes applied during Reggiano cheese manufacturing [13–16]. In order to reduce the storage period, the use of elevated temperatures has showed to be an effective strategy. Sihufe et al. [17] carried out a study to assess the impact of an increased storage temperature of 18 °C and suggested an optimal ripening period between 2 and 3 months. Later, another study also showed encouraging results when elevated temperatures of 20 °C were applied during the first month of the ripening period, showing an important reduction in storage time of 2 months [18] with a milder development of the final sensory characteristics [19].

Regarding volatile compounds found in Reggiano cheese, Wolf et al. [20] made a volatile profile characterisation of fully ripened commercial Reggiano cheeses. The study was developed using 18 cheese samples from six different leader brands, purchased in local markets. A total of 53 volatile compounds were identified (including acids, alcohols, ketones, esters and aldehydes), despite only 40 of them were detected in at least 50 % of the samples analysed. The volatile profile was dominated by acidic volatile compounds (principally acetic and butyric acids), alcohols (principally ethanol) and ketones. Although many previous studies, those referring to the volatile profile composition for Reggiano cheese are scarce and no data have been published following the evolution of individual volatile compounds for this traditional cheese type during ripening. Therefore, this study was conducted with the aim of contributing to already existing characterisation of the volatile fraction of Reggiano cheese, describing its evolution over the ripening period, and assessing the impact of the different temperature–time combinations applied on this area of cheese chemistry.

Materials and methods

Cheese sampling and ripening conditions

Ripening conditions and sampling were previously described by Ceruti et al. [21]. Briefly, 20 cheeses (7.8 ± 0.1 kg weight, 23.7 ± 0.2 cm diameter, 15.3 ± 0.2 cm height) were manufactured at a local factory, using pasteurised milk from the same cheese vat and following a standard cheese manufacture procedure. Cheeses were brined during 7 days and brought to our laboratory immediately after. Two cheeses were used for initial composition determination, and 18 cheeses were kept under three different temperature–time combinations for 6 months. Six cheeses were ripened at 12 °C and 85 % relative humidity (cheeses C), whereas the other 12 cheeses

were stored at two different temperature–time combinations: six cheeses at 20 °C for 2 weeks followed by 12 °C up to 6 months (cheeses E₁) and six cheeses at 20 °C for 4 weeks followed by 12 °C up to 6 months (cheeses E₂), both at 85 % relative humidity. Cheeses in duplicate were sampled at 61, 124 and 180 days of ripening.

Analysis of volatile compounds by SPME–GC–MS

Sample preparation

Before chromatographic analysis, volatile compounds were both concentrated and isolated from a cheese matrix by a solid-phase microextraction (SPME) procedure. Five grams of grated cheese was placed in a 30-mL glass vial and sealed with 20-mm-diameter PTFE-silicone septa (Supelco, Inc., Bellefonte, PA, USA) and an aluminium crimp seal. For each cheese, two samples were prepared and subsequently analysed by GC–MS.

Headspace-SPME

A 1-cm-long DVB/CAR/PDMS 50/30 μm SPME fibre (Supelco, Inc., Bellefonte, PA, USA) housed in its manual holder (Supelco, Inc., Bellefonte, PA, USA) was used to concentrate volatile compounds on the vial headspace. The vial was kept at 40 °C for 10 min, and then the fibre was exposed to headspace during 30 min at 40 °C.

Gas chromatography–mass spectrometry

Compounds loaded onto the fibre were then analysed with a Clarus 600 gas chromatograph coupled to a Clarus 600T mass spectrometer (Perkin-Elmer, Shelton, CT, USA), equipped with a split/splitless injector and an Elite-5 (30 m \times 0.25 mm ID \times 0.25 μm film thickness) column (Perkin-Elmer, Shelton, CT, USA). Volatile compounds were desorbed from the fibre at the injection port of the chromatograph, splitless mode, during 5 min at 250 °C. The carrier gas was He at 1 mL min⁻¹ flow rate, oven temperature programme being 10 min at 40 °C, then 6 °C min⁻¹ to 150 °C, 10 °C min⁻¹ to 230 °C and 9 min at 230 °C. Transfer line temperature was set at 200 °C and source temperature at 180 °C. Peak detection was made by mass spectrometry on the total ion current obtained by electron impact at 70 eV, and data were acquired at a rate of 2 scans s⁻¹ over an m/z scan range of 40–500 amu. A C7–C40 *n*-alkanes mixture (Sigma-Aldrich, Inc., Saint Louis, MO, USA) was injected and run under the same chromatographic conditions, in order to obtain Kováts indices (KI) of detected compounds. KI were calculated according to Bianchi et al. [22]. Compounds were tentatively identified by computer matching of mass spectra with those of NIST2008 Mass Spectral Library (version 2.2). These identities were

also confirmed with bibliographical data and by comparison of their KI with those reported in the literature, collected by online databases [23–26]. Area counts of volatiles were provided by integration using TurboMass software version 5.4.2 (Perkin-Elmer, Shelton, CT, USA).

Statistical analysis

One-way ANOVA was performed with area values for control cheeses, considering ripening time as the analysis factor. When differences between treatment effects were significant ($P < 0.05$), the least significant differences (LSD) test was used for multiple comparison of means. Two-way ANOVA was also performed, considering ripening temperature and ripening time as main factors, for area values corresponding to *C*, *E*₁ and *E*₂ cheeses at 61, 124 and 180 days of ripening. Both statistical tests were performed using Statgraphics (Statgraphics Inc., Rockville, MD, USA). Principal component analysis (PCA) was performed taking into account information from the 41 volatile compounds identified for each cheese at 61, 124 and 180 days of ripening. PCA was applied to a correlation matrix, using Minitab (Minitab Inc., State College, PA, USA).

Results and discussion

Volatile profile composition

A total number of 41 volatile compounds were identified from SPME–GC–MS analysis of Reggianito cheese samples (Table 1). Peaks detected were grouped according to their structure in carboxylic acids (11 compounds), ketones (8), aldehydes (10), esters (1), alcohols (5) and hydrocarbons (6). All compounds found in this study have been previously described in other cheese varieties, and most of them (36) in Reggianito [20] or in related hard Italian cheese varieties [27–29].

Groups of volatile compounds observed in this study were the same found by Wolf et al. [20], and their contribution to total chromatographic area value was quite similar, with the exception of the alcohols fraction for which Wolf et al. [20] found a considerably higher proportion corresponding to alcohols, mostly explained by the high content of ethanol. Ethanol may have been present in our cheese samples but could not be detected in this work, probably due to its very high volatility. Nevertheless, particularly high odour threshold values have been reported for ethanol [28, 30], thus being improbable an important direct contribution to cheese flavour for this compound. Although acids were the predominant group in both studies, we found in this work a relatively higher contribution of this group to the volatile fraction of Reggianito cheese.

For control cheeses at 180 days of ripening (cheeses ripened under standard conditions), the volatile profile was dominated by acids, accounting for around 80 % of total area value. Among this group, seven even-chain FFA (C₂–C₁₄) were detected, as well as two uneven-chain FFA (heptanoic and nonanoic acids) and two branched-chain acids (isovaleric and 2-methylbutanoic acids) (Table 1). Acetic, butyric and caproic acids clearly predominated among volatile acids. In a second, yet important area value level was caprylic and capric acids, as well as isovaleric and 2-methylbutanoic acids, while minor quantitative contributions were observed for heptanoic, nonanoic, lauric and myristic acids. A similar composition of acidic fraction was observed in grana-type Trentingrana cheese, for which up to 90 % of total area value was due to acids [9]. All of these compounds have been reported to be odour-active compounds in cheese, and this fraction has been described as important or even predominant for flavour in many cheese types, including hard Italian cheeses [31]. Furthermore, acids are not only aroma compounds by themselves, but also serve as precursors of other volatile compounds as methyl ketones, alcohols, lactones and esters [32].

Butyric acid may play a major role in Reggianito cheese flavour, considering its higher concentration than caproic acid and relatively lower threshold value in relation to acetic acid [28–31]. Acetic, caproic, caprylic, capric, isovaleric and 2-methylbutanoic acids may also have influence on Reggianito cheese flavour characteristics due to the combination of the presence at relatively high proportions and the low odour thresholds values reported for these compounds [28–31]. Previous works in Italian grana cheeses reported a role as key odorants for butyric and caproic acids, whereas acetic, caprylic, capric, isovaleric and 2-methylbutanoic acids also were considered contributors to global flavour [5, 33, 34].

Ketones were the second group of importance, accounting for 8 % of total area value. As Table 1 shows, the most abundant compounds within this group were acetoin (3-hydroxy-butanone) and methyl ketones 2-heptanone and 2-nonanone, for which low odour thresholds have been reported [31]. Other ketones (2-pentanone, 2-undecanone, 2-hydroxy-3-pentanone, 6-methyl-5-hepten-2-one and 3,5-octadien-2-one) were present at considerably lower quantities. Methyl ketones, particularly 2-heptanone and 2-nonanone, are often described as the most abundant ketones found in cheese. These compounds are normally dominant in mould-ripened cheeses, but may also play a role in the aroma of grana-type cheeses, contributing to fruity and blue cheese-like aromas [28].

Aldehydes represented around 4 % of total area value, and higher amounts were observed for hexanal, heptanal and benzaldehyde, followed by nonanal, 3-methylbutanal, benzeneacetaldehyde, 3-hydroxy-butanal, octanal, decanal

Table 1 Average chromatographic area values and standard deviation ($\times 10^6$) for volatile compounds identified during Reggiano cheese ripening for control (C) and experimental (E_1 and E_2) cheeses

Compound	Rt (min)	KI	C	E_1			E_2			ANOVA				
				61 days	124 days	180 days	61 days	124 days	180 days	A	B	A*B		
Acids														
Acetic acid	2.62	637	109.50 ± 8.89	89.64 ± 9.00	78.27 ± 11.51	93.00 ± 5.74	81.11 ± 6.28	69.28 ± 12.11	81.94 ± 13.43	83.72 ± 7.13	77.92 ± 9.81	*	*	NS
Butyric acid	8.75	842	51.33 ± 3.61	61.47 ± 2.02	65.96 ± 9.49	45.39 ± 2.22	60.16 ± 3.56	60.36 ± 5.95	48.52 ± 8.65	60.38 ± 4.80	65.29 ± 5.47	*	NS	NS
Isovaleric acid	11.37	884	2.62 ± 0.66	3.36 ± 0.26	3.61 ± 0.70	3.83 ± 0.56	4.55 ± 0.64	3.56 ± 0.56	4.58 ± 0.57	4.07 ± 0.22	5.64 ± 0.33	NS	*	NS
2-Methylbutanoic acid	11.85	892	1.28 ± 0.12	2.27 ± 0.15	2.34 ± 0.35	1.76 ± 0.05	4.01 ± 0.66	2.88 ± 0.50	1.98 ± 0.19	2.84 ± 0.18	2.70 ± 0.38	*	*	NS
Caproic acid	17.53	1020	19.37 ± 0.93	24.73 ± 1.59	26.52 ± 2.56	17.82 ± 1.06	24.05 ± 1.30	27.06 ± 2.38	17.59 ± 1.86	26.41 ± 1.48	29.67 ± 2.41	*	NS	NS
Heptanoic acid	19.88	1092	0.11 ± 0.03	0.09 ± 0.01	0.11 ± 0.01	0.14 ± 0.03	0.09 ± 0.00	0.11 ± 0.02	0.08 ± 0.01	0.10 ± 0.02	0.14 ± 0.06	NS	NS	NS
Caprylic acid	22.56	1190	4.34 ± 0.26	4.65 ± 0.24	4.66 ± 0.41	3.50 ± 0.08	4.33 ± 0.36	4.39 ± 0.36	3.10 ± 0.33	4.83 ± 0.42	4.26 ± 0.45	*	*	*
Nonanoic acid	24.73	1278	0.11 ± 0.01	0.11 ± 0.03	0.50 ± 0.27	0.10 ± 0.03	0.22 ± 0.16	0.68 ± 0.29	0.09 ± 0.03	0.50 ± 0.17	0.56 ± 0.34	*	NS	NS
Capric acid	26.92	1376	1.11 ± 0.34	1.04 ± 0.18	1.44 ± 0.12	0.88 ± 0.17	1.15 ± 0.20	1.21 ± 0.31	2.92 ± 0.18	1.54 ± 0.28	1.18 ± 0.09	*	NS	*
Lauric acid	30.57	1570	0.07 ± 0.03	0.06 ± 0.02	0.09 ± 0.01	0.05 ± 0.01	0.07 ± 0.03	0.07 ± 0.01	0.04 ± 0.02	0.09 ± 0.02	0.07 ± 0.02	*	NS	NS
Myristic acid	33.22	1767	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.07 ± 0.09	NS	NS	NS
Ketones														
2-Pentanone	3.50	706	1.11 ± 0.53	0.10 ± 0.04	0.26 ± 0.06	0.51 ± 0.10	0.16 ± 0.02	0.13 ± 0.02	1.21 ± 0.16	0.21 ± 0.03	0.59 ± 0.56	*	NS	NS
Acetoin	3.82	717	14.44 ± 0.99	10.48 ± 1.24	7.42 ± 0.33	7.97 ± 0.02	7.23 ± 1.01	6.65 ± 1.07	7.14 ± 0.66	8.62 ± 0.25	6.60 ± 1.26	*	*	*
2-Hydroxy-3-pentanone	7.20	817	1.47 ± 0.03	1.09 ± 0.10	0.76 ± 0.05	0.75 ± 0.11	0.80 ± 0.03	0.82 ± 0.29	0.69 ± 0.21	1.09 ± 0.08	0.67 ± 0.26	NS	*	*
2-Heptanone	12.38	900	8.24 ± 0.38	7.62 ± 0.44	6.81 ± 0.26	7.50 ± 0.33	9.34 ± 0.44	9.21 ± 0.90	8.92 ± 0.30	11.61 ± 0.45	11.69 ± 1.99	NS	*	NS
6-Methyl-5-hepten-2-one	16.73	997	0.19 ± 0.12	0.33 ± 0.25	0.10 ± 0.05	0.02 ± 0.00	0.11 ± 0.08	0.18 ± 0.06	0.12 ± 0.08	0.05 ± 0.01	0.08 ± 0.05	NS	NS	NS
3,5-Octadien-2-one	19.53	1082	0.97 ± 0.11	1.21 ± 0.30	0.73 ± 0.09	0.75 ± 0.01	0.76 ± 0.04	0.69 ± 0.07	0.42 ± 0.11	0.64 ± 0.05	0.63 ± 0.21	NS	*	NS
2-Nonanone	20.08	1098	1.23 ± 0.06	1.86 ± 0.49	1.24 ± 0.07	1.43 ± 0.04	1.83 ± 0.29	1.85 ± 0.15	1.17 ± 0.05	2.12 ± 0.22	1.93 ± 0.16	*	NS	NS
2-Undecanone	25.23	1299	0.17 ± 0.01	0.18 ± 0.02	0.18 ± 0.01	0.14 ± 0.00	0.19 ± 0.01	0.21 ± 0.02	0.15 ± 0.01	0.23 ± 0.03	0.23 ± 0.01	*	*	*
Aldehydes														
3-Methylbutanal	2.90	662	1.28 ± 0.14	0.12 ± 0.06	0.31 ± 0.08	0.59 ± 0.10	0.09 ± 0.02	0.13 ± 0.03	0.59 ± 0.10	0.42 ± 0.10	0.16 ± 0.06	*	*	*
3-Hydroxybutanal	5.98	793	0.21 ± 0.04	0.15 ± 0.01	0.15 ± 0.01	0.10 ± 0.00	0.14 ± 0.01	0.18 ± 0.02	0.11 ± 0.03	0.18 ± 0.02	0.17 ± 0.02	NS	NS	*

Table 1 continued

Compound	Rt (min)	KI	C	E ₁			E ₂			ANOVA				
				61 days	124 days	180 days	61 days	124 days	180 days	A	B	A*B		
													61 days	124 days
Hexanal	6.63	807	5.24 ± 0.36	4.32 ± 0.43	3.76 ± 0.25	3.32 ± 0.07	4.08 ± 1.07	2.96 ± 0.41	2.92 ± 0.18	3.39 ± 0.18	5.60 ± 1.46	NS	NS	*
Heptanal	12.84	911	3.53 ± 0.19	2.10 ± 0.28	1.59 ± 0.15	1.98 ± 0.07	1.68 ± 0.19	1.39 ± 0.12	1.76 ± 0.21	1.56 ± 0.10	1.43 ± 0.22	*	*	*
Benzaldehyde	15.48	969	1.45 ± 0.55	3.76 ± 0.72	2.05 ± 0.31	0.82 ± 0.06	1.55 ± 0.47	1.44 ± 0.21	0.79 ± 0.17	1.55 ± 0.38	1.69 ± 0.65	*	*	*
Octanal	17.19	1010	0.16 ± 0.05	0.23 ± 0.07	0.12 ± 0.02	0.13 ± 0.03	0.14 ± 0.03	0.11 ± 0.00	0.09 ± 0.02	0.13 ± 0.02	0.11 ± 0.04	NS	*	NS
Benzeneacet- aldehyde	18.55	1052	0.27 ± 0.04	0.32 ± 0.07	0.24 ± 0.02	0.18 ± 0.01	0.19 ± 0.05	0.18 ± 0.01	0.19 ± 0.02	0.25 ± 0.04	0.24 ± 0.05	NS	*	NS
Nonanal	20.39	1110	0.87 ± 0.11	1.04 ± 0.05	0.79 ± 0.06	0.84 ± 0.27	0.78 ± 0.26	0.69 ± 0.12	0.61 ± 0.13	0.65 ± 0.10	0.67 ± 0.04	NS	*	NS
Decanal	23.10	1210	0.17 ± 0.04	0.16 ± 0.05	0.12 ± 0.02	0.11 ± 0.03	0.12 ± 0.02	0.12 ± 0.03	0.12 ± 0.06	0.10 ± 0.03	0.17 ± 0.08	NS	NS	NS
Dodecanal	27.74	1414	0.07 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.05 ± 0.00	0.07 ± 0.03	NS	NS	NS
Esters														
Ethyl butanoate	6.75	809	8.03 ± 0.32	5.73 ± 0.13	5.55 ± 0.40	4.77 ± 0.37	4.92 ± 0.14	4.65 ± 0.42	5.25 ± 0.56	5.79 ± 0.31	5.07 ± 1.71	NS	*	NS
Alcohols														
1-Butanol	3.06	676	0.24 ± 0.04	0.21 ± 0.13	0.23 ± 0.07	0.67 ± 0.05	0.30 ± 0.10	0.45 ± 0.12	1.06 ± 0.13	0.29 ± 0.09	0.77 ± 0.10	*	*	*
3-Methyl-3- buten-1-ol	4.38	737	0.23 ± 0.07	0.10 ± 0.03	0.19 ± 0.02	0.29 ± 0.02	0.19 ± 0.03	0.20 ± 0.06	0.27 ± 0.02	0.22 ± 0.05	0.28 ± 0.09	*	NS	NS
3-Methyl- 1-butanol	4.49	741	0.60 ± 0.09	1.35 ± 0.29	3.08 ± 0.48	1.54 ± 0.26	2.19 ± 0.17	2.30 ± 0.35	2.46 ± 0.17	2.91 ± 0.21	3.20 ± 0.79	*	*	NS
2-Heptanol	13.09	916	1.10 ± 0.02	1.27 ± 0.27	2.11 ± 0.08	0.25 ± 0.01	0.96 ± 0.18	1.02 ± 0.13	0.51 ± 0.12	0.92 ± 0.11	1.09 ± 0.22	*	*	*
2-Ethyl-1-hex- anol	18.20	1041	0.11 ± 0.03	0.86 ± 0.56	0.35 ± 0.08	0.18 ± 0.01	0.45 ± 0.14	0.53 ± 0.17	0.11 ± 0.04	0.45 ± 0.09	0.50 ± 0.21	*	NS	NS
Hydrocarbons														
Toluene	5.19	765	0.85 ± 0.16	1.84 ± 0.15	1.46 ± 0.12	0.92 ± 0.04	1.53 ± 0.17	1.52 ± 0.18	1.01 ± 0.90	1.93 ± 0.38	1.14 ± 0.27	*	NS	NS
Octane	6.41	804	0.80 ± 0.26	0.80 ± 0.19	0.66 ± 0.01	0.47 ± 0.01	0.55 ± 0.10	0.63 ± 0.11	0.64 ± 0.15	0.70 ± 0.06	0.73 ± 0.06	NS	NS	NS
Ethylbenzene	10.06	863	0.04 ± 0.01	0.54 ± 0.05	0.38 ± 0.06	0.08 ± 0.01	0.43 ± 0.08	0.47 ± 0.06	0.02 ± 0.01	0.44 ± 0.08	0.26 ± 0.24	*	NS	NS
Decane	16.94	1002	0.36 ± 0.04	2.50 ± 1.77	0.69 ± 0.22	0.26 ± 0.02	0.89 ± 0.37	1.17 ± 0.27	0.15 ± 0.05	0.86 ± 0.26	0.69 ± 0.67	NS	NS	NS
Limonene	17.91	1032	0.04 ± 0.01	0.86 ± 0.63	0.30 ± 0.09	0.16 ± 0.01	0.38 ± 0.16	0.48 ± 0.11	0.03 ± 0.01	0.36 ± 0.09	0.30 ± 0.27	NS	NS	NS
Decahy- dronaphtha- lene	18.69	1056	0.04 ± 0.04	1.76 ± 1.33	0.65 ± 0.17	0.58 ± 0.03	0.78 ± 0.30	0.95 ± 0.21	0.02 ± 0.02	0.77 ± 0.21	0.66 ± 0.62	NS	NS	NS
Total area			243.05 ± 13.74	240.38 ± 16.22	226.97 ± 23.30	203.84 ± 8.69	221.59 ± 10.65	211.15 ± 18.29	197.17 ± 24.01	232.97 ± 11.51	232.23 ± 17.70	NS	*	NS

Rt retention time, KI Kováts index on Elite-5 column, A ripening time factor, B temperature-time combination factor, A*B interaction

* Significant effect (P < 0.05), NS no significant effect (P > 0.05)

and dodecanal (Table 1). Almost all of these compounds, except for 3-hydroxy-butanal, were described to be present in grana-type cheese and to have low threshold values. Straight-chain aldehydes are associated with green, grass-like odours. Those with shorter chain are also described as pungent and malty, whereas those with longer chain as presenting fatty and citric notes [28]. Branched-chain aldehyde 3-methylbutanal is usually described with strong malty, green and cocoa-like aromas, while almond-like aroma is usually described for benzaldehyde and a strong, rosy and green aroma for benzeneacetaldehyde [28].

Ethyl butanoate was the only ester detected, but it represented around 2.5 % of total area value. For this compound, a particularly low odour threshold has been described [31], and due to the relatively high proportion in which it is present, it probably makes a contribution to Reggiano cheese aroma. Wolf et al. [20] found that ethyl butanoate accounted for higher mean area values and it was the only ester present in all Reggiano samples analysed. Ethyl esters are usually regarded as main contributors to the fruity character in cheese. Further, these compounds can contribute to the aroma of cheese by minimising the sharpness imparted by fatty acids. Ethyl butanoate has been identified as one of the most potent odorants in several cheese varieties, including Grana Padano, Pecorino [31] and Parmigiano Reggiano cheeses [35].

Minor area contributions were explained by the groups of alcohols and hydrocarbons, each one responsible of around 2 % of total area value. Within alcohols found, 3-methyl-1-butanol and 2-heptanol predominated, being also found in almost all samples by Wolf et al. [20], and 1-butanol, 3-metil-3-buten-1-ol and 2-ethyl-1-hexanol were detected in a lower proportion (Table 1). All of these compounds have been previously found in hard Italian cheeses [27], and particularly, branched-chain alcohol 3-methyl-1-butanol has been described as the alcohol probably making the highest contribution to Parmigiano Reggiano cheese flavour [36]. Hydrocarbons found were toluene, octane, ethylbenzene, decane, limonene and decahydronaftalene having all of them, except for limonene, relatively high thresholds values [30], thus making improbable an important contribution to cheese flavour by these compounds.

Volatile profile evolution during Reggiano cheese ripening

Except for heptanoic acid and 2-heptanol at day 0, detected peaks in this study were present in all samples from 0 to 180 days of ripening. Heptanoic acid and 2-heptanol appeared during the first 61 days, and no further peaks appeared or disappeared afterwards, and thus changes during ripening in the volatile profile of Reggiano cheese were caused by variations in individual quantities. Total

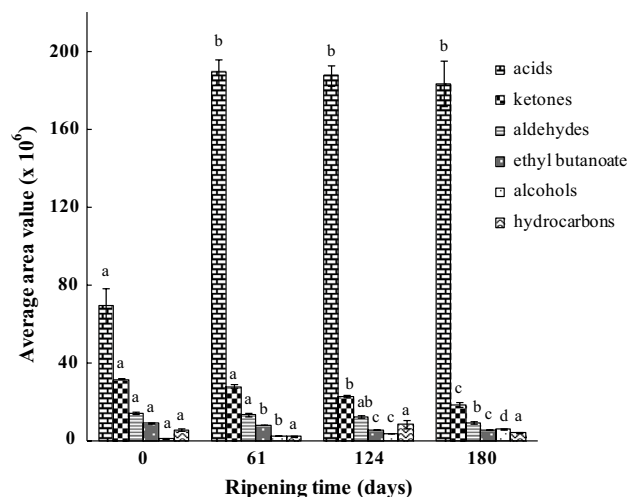


Fig. 1 Evolution during ripening of the main chemical groups of volatile compounds isolated in Reggiano cheese ripened under traditional conditions (12 °C for 180 days). Mean values with *different letters* within the same group of compounds mean significant differences ($P < 0.05$) at LSD test. *Error bars* indicate standard deviations between replicates

area value at day 0 was $130.8 \pm 20.7 \times 10^6$, while values towards 2 months of ripening were around 200×10^6 . A great increase in total area was then observed for cheeses in the first 2 months of ripening, while no significant differences were observed for this value in the following 4 months.

In Fig. 1, changes in the volatile fraction composition during ripening for control cheeses are shown. The increase in total area value during the first 2 months of ripening can be explained by the increase in the area values for acids during this period, while no significant changes were observed for this group in the following months. Because of this increase, the proportion of the remaining groups of compounds decreased notably between 0 and 61 days, regardless the change each had during this period. A tendency to increase for volatile acids during ripening has already been reported for various cheese varieties. Constant increases were observed for Nanos cheese [37] and Brie cheese [8]. For other cheese varieties, this change has been observed to occur during the first months of ripening, this quantity remaining constant thereafter, as reported for Iborea [38] and Montasio cheeses [6]. An important part of the increase observed in this work for volatile acids during the first 2 months of ripening was explained by the increment of FFA of 4 or more carbon atoms. The presence of these compounds in cheese is largely attributed to lipolysis [31]. In Reggiano cheese, lipolytic activity is explained by the presence of lipases and esterases from starter and NSLAB microflora, starter being composed by thermophilic lactobacilli *L. helveticus* (66 %) and *L. delbrueckii*

subsp. *lactis* (33 %) [16]. Lipases and esterases from *L. helveticus* and *L. delbrueckii* subsp. *lactis* strains are intracellular, and thus, cell lysis is important for lipolytic activity [39]. It was already described that thermophilic lactobacilli in Reggiano cheese, being at counts of 10^6 – 10^7 cfu g⁻¹ at the beginning of ripening, virtually disappeared within the first 2 months of ripening [19]. It can be stated therefore that liberation of intracellular lipolytic enzymes due to cell lysis may have been partially responsible of the increase in total area values for volatile acids.

Ketones and aldehydes decreased between 61 and 180 days of ripening, mainly due to decrease in areas for 2-pentanone, acetoin, 2-hydroxy-3-pentanone and 2-heptanone in the first case, and 3-methylbutanal, hexanal and heptanal in the second case. Net increases for both aldehydes and ketones throughout ripening have been observed for some varieties of cheese [8], while others reported a net increase over time for ketones and a decrease for total aldehydes [4, 40]. However, it was also referred an increase for ketones and aldehydes at early times during ripening, and a fast reduction for most of those compounds later [37, 38]. Aldehydes are considered as transitory compounds in cheese, as they can be rapidly reduced to primary alcohols or even oxidised to the corresponding acids [31]. In relation to this aspect, the increase for 3-methyl-1-butanol observed in this work may be attributed to the decrease in 3-methylbutanal. Once produced, ketones can also be precursors for other compounds. Methyl ketones can be converted to secondary alcohols [32], as it seems to be the case in this work for 2-heptanone, where a net decrease in this compound was simultaneous to the increase for 2-heptanol observed during ripening. Acetoin is usually referred, together with diacetyl, as responsible of milk or creamy attributes in cheeses. The decrease observed for acetoin may be related to the already observed diminution of the milk-creamy attribute in Reggiano cheese during ripening [14].

For ethyl butanoate, there was a reduction during the first 4 months of ripening. Commonly, esters increase during ripening, due to activity of esterase systems from lactic acid bacteria in cheese. Particularly, ethyl butanoate was found to increase over ripening in many cases [37, 38, 41, 42]. However, no changes have been reported in this group for Brie cheese during ripening [8] and esters decreasing with storage time were already found for Mahon cheese [43].

The fraction of alcohols, though being small, increased its area values throughout ripening, mainly because of increments for the two predominant compounds: 3-methyl-1-butanol and 2-heptanol. As previously stated, increases in 3-methyl-1-butanol and 2-heptanol can be explained by decreases in 3-methylbutanal and 2-heptanone, as this two carbonyl compounds are usually considered precursors of

the latter. No significant changes in the area values for the hydrocarbons family were observed.

Effect of the different temperature–time combinations applied during Reggiano cheese ripening on volatile compounds

According to ANOVA performed using chromatographic area values for the 41 compounds detected, different results were observed with respect to significance of the factors analysed (ripening time and temperature–time combination) as shown in Table 1.

A total number of 11 compounds were not significantly affected by any of the main experimental factors evaluated, whereas 11 were significantly affected by both of them. Between the remaining 19 compounds, 11 were significantly affected by ripening time and 8 by the temperature–time combination factor. Within those compounds that were significantly affected by temperature–time combination are included many of which had an important contribution to total area value (acetic, isovaleric, 2-methylbutanoic and caprylic acids, acetoin, ethyl butanoate, 2-heptanone, heptanal and benzaldehyde). As a consequence, there was a statistically significant effect of the temperature–time combination factor for total area values, but the importance of this fact may be limited, as no clear tendency was observed for the individual volatile compounds. In fact, these changes may have not altered Reggiano cheese flavour, as a characteristic sensory profile was described in a previous work for these cheeses under the experimental conditions mentioned [19].

Due to the number of detected compounds and the diversity of behaviours with respect to the factors evaluated, PCA was performed using the area values of all the 41 identified compounds for *C*, *E*₁ and *E*₂ cheeses at 61, 124 and 180 days of ripening (Fig. 2). Analysis showed that the first two principal components, PC1 and PC2, explained 52.9 % of total variability. From sample scores distribution, it can be inferred that PC1 (29.6 % VAR) can be associated with the ripening time factor, and a clear separation was observed for PC1 scores associated with samples at 61 days from those corresponding to 124 and 180 days of ripening. Second principal component PC2 (23.0 % VAR) was in relation to temperature–time combination.

Regarding loadings distribution in the biplot, it can be observed that among compounds in the left side of the graph (negative PC1 values) are included those with a decreasing tendency with time, as in the right side are included those increasing with time. At the same time, on the upper side of the graph were placed loadings for those compounds in which lower area values were observed for experimental cheeses with respect to control cheeses and in

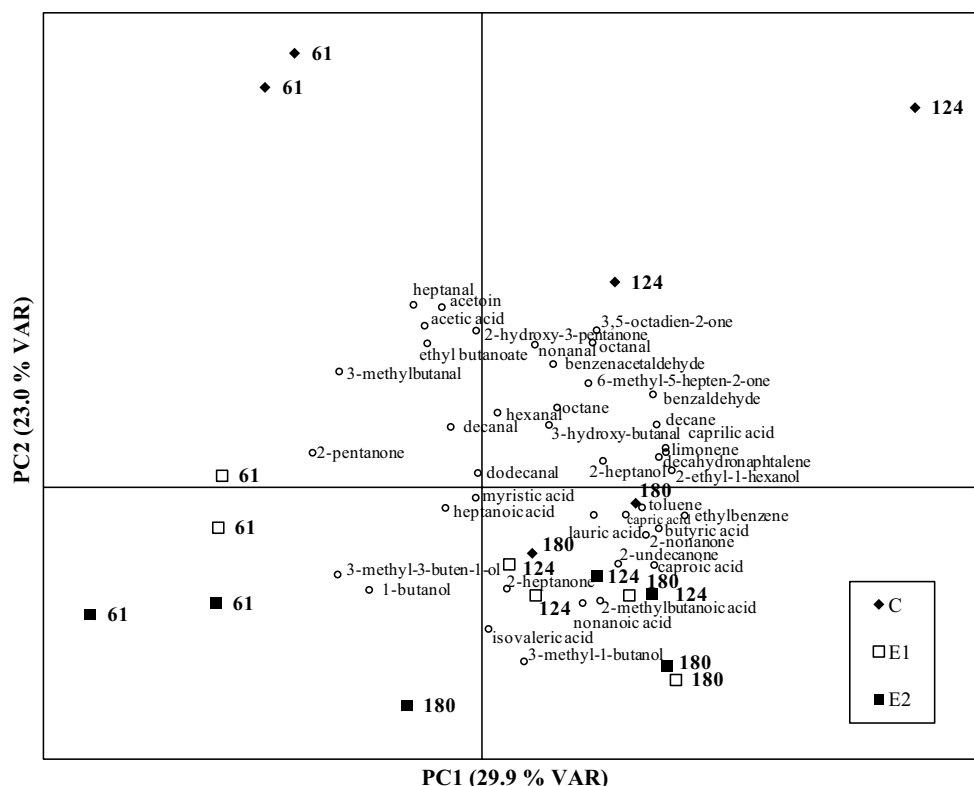


Fig. 2 Biplot of scores and loadings in the plane defined by the two first principal components after PCA of data obtained from volatile fraction analysis of Reggiano cheese samples. Numbers close to symbols indicate days of ripening

the lower side, those for which higher area values could be observed for experimental than for control cheeses.

Loadings for some aldehydes and ketones (3-methylbutanal, heptanal, 2-pentanone, 2-hydroxy-3-pentanone and acetoin), ethyl butanoate and acetic acid are localised in the left part of the graph, together with scores associated with C, E₁ and E₂ cheeses at 61 days of ripening, allowing to associate these compounds (that shows a tendency to decrease) with young cheeses. On the other side of the graph, most of acids and methyl ketones, 2-heptanone, 2-nonanone and 2-undecanone are placed near scores for cheeses at 124 and 180 days and may be associated with ripened cheeses.

Conclusions

From SPME–GC–MS methodology, volatile compounds profile in mature Reggiano cheese was studied, as well as its evolution during ripening and the influence of elevated temperatures applied at the beginning of the storage period. A total of 41 volatile compounds were identified and grouped as acids, ketones, aldehydes, esters, alcohols and hydrocarbons. The volatile profile of Reggiano cheese was clearly dominated by carboxylic acids

(especially short chain FFA), as reported in previous works for this and other grana-type cheeses. The more notable changes in the volatile compounds profile occur during the first 2 months of ripening, and can be mostly explained by the increase in carboxylic acids. Other changes with a potential impact on Reggiano cheese flavour were observed for ketones, aldehydes, ethyl butanoate and alcohols. The different temperature–time combinations applied during Reggiano cheese ripening significantly affected the quantities of some of the volatile compounds, but the balance between the different groups of compounds remained roughly the same, and changes in total chromatographic area were small. Finally, potential relationships were established between the presence of some volatile compounds and the age of cheeses.

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Compliance with ethical standards

Conflict of interest Roberto J. Ceruti, Guillermo A. Sihufe and Susana E. Zorrilla declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects performed by any of the authors.

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