Characterization of Italian Cheeses Ripened Under Nonconventional Conditions

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ABSTRACT

Four Italian cheeses (Casciotta di Urbino, Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso) nonconventionally ripened under different plant materials (walnut leaves, herbs, hay, and wine by-products, respectively) were compared for compositional, microbiological, biochemical, and volatile profile characteristics. Mean values for gross composition were rather similar. Because primary starters were not used for manufacture, the endogenous lactic acid bacteria were mainly present (7.0 to 9.0 log₁₀ cfu/g). Except for *Lactobacillus paracasei* and *Leuconostoc mesenteroides*, which were commonly identified in 3 cheeses, *Lactococcus lactis*, *Enterococcus sanguincola*, *Lactobacillus brevis*, *Enterococcus durans*/*Enterococcus faecium*, *Lactobacillus plantarum*, and *Weissella cibaria*/*Weissella confusa* were variously found in the 4 cheeses. Random amplification of polymorphic DNA-PCR analysis showed the biodiversity among the strains, and the species of lactobacilli were in part grouped according to their origin. As shown by the principal component analysis of reverse-phase fast protein liquid chromatography data for the pH 4.6-soluble fractions and by the determination of free AA, the secondary proteolysis of Barricato San Martino and Vento d'Estate mainly differed from the other 2 cheeses. Purge-and-trap and solid-phase microextraction were coupled with gas chromatography-mass spectrometry to determine volatile compounds. Vento d'Estate showed the highest levels of almost all chemical classes, and Casciotta di Urbino was characterized by a very low level of volatile components. Esters, ketones, and terpenes were the chemical classes that mainly differentiated the cheeses. Several volatile compounds seemed to be released directly from the plant materials used for ripening, especially terpenes for Vento d'Estate cheese. The lowest level of volatile free fatty acids was found in Casciotta

d'Urbino, in which rennet paste was not used during manufacture. The highest concentration of free fatty acids, especially butyric and caproic acids, was found in Vento d'Estate cheese.

Key words: barrique cheeses, endogenous lactic acid bacteria, proteolysis, volatile compound

INTRODUCTION

Italy is ranked fourth in the world for cheese production (approximately 1,000,000 t/yr; Fox and McSweeney, 2004). It has a very large diversity of traditional cheeses, 31 of which have a Protected Denomination of Origin (PDO) status. In addition to PDO cheeses, many varieties contribute markedly to the total production, having typical features and originating from limited geographical areas. Nevertheless, most of these cheeses, including those ripened under nonconventional conditions (also defined as “barrique” cheeses), have not been characterized.

Overall, cheese ripening takes place under well-defined environmental conditions that may differ in temperature, relative environmental humidity, and especially time, depending on the cheese variety, and which may differently influence the biochemical changes in the cheese (McSweeney, 2004). Other nonconventional conditions may include the use of mainly plant materials to cover cheese curds during ripening. The popularity of some Italian cheeses ripened under nonconventional conditions is increasing (production of approximately 350 t/yr; www.inea.it). Curds of these cheeses are covered with plant materials throughout ripening, and sometimes the cheeses are sold with these materials covering the rind. Casciotta di Urbino (Marche region, center of Italy) is ripened under walnut leaves, Barricato San Martino is ripened under herbs (mainly mint, thyme, and rosemary), Vento d'Estate is ripened under hay, and Ubriaco di Raboso is ripened under wine by-products (grape marc, Veneto region, Northern Italy). These cheeses are considered to be the most popular Italian cheeses belonging to this category. Although a particular cheese may be consumed after a

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different period of ripening, the above-mentioned non-conventionally ripened cheeses belong to the semihard or hard categories (usually 3 to 4 mo of ripening), are manufactured from raw cows' milk using rennet paste (except for Casciotta di Urbino), do not use a primary natural or commercial starter culture, and contain adventitious or endogenous microbial flora that are responsible for most of the biochemical changes during ripening. To the best of our knowledge, no studies have been carried out on cheeses that are ripened under these conditions. Plant materials are used in an effort to influence cheese flavor directly by releasing volatile compounds, but also indirectly by influencing the microbiological and biochemical changes that occur during ripening.

Purge-and-trap (**PT**) and solid-phase microextraction (**SPME**) techniques (Mallia et al., 2005) have been used in a number of studies to characterize the volatile compounds that affect the flavor of Italian (Di Cagno et al., 2003; Horne et al., 2005; Coda et al., 2006), Spanish (Fernández-García et al. 2004; Barron et al., 2005), Portuguese (Pinho et al., 2004), and Brazilian cheeses (Nogueira et al., 2005). Comparative studies on the compositional, microbiological, biochemical, and volatile profile characteristics of several cheeses belonging to the same category may be helpful for: 1) differentiating the cheeses; 2) establishing the effect of selected technological parameters (e.g., source of plant material used for cheese ripening) on specific differences in the microbial flora and related biochemical activities; and, in general, 3) identifying the most appropriate characteristics suitable for obtaining a "denomination of origin" designation, which may increase the market popularity of individual cheeses.

This study describes the compositional, microbiological, biochemical, and volatile profile characteristics of Casciotta di Urbino, Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso cheeses, which are ripened under nonconventional conditions.

MATERIALS AND METHODS

Cheese Samples

Samples of Casciotta di Urbino (ripened under walnut leaves), Barricato San Martino (ripened under herbs, mainly mint, thyme, and rosemary), Vento d'Estate (ripened under hay), and Ubriaco di Raboso (ripened under wine by-products, i.e., grape marc) were supplied in triplicate at the end of ripening (different batches made from different batches of milk) by local cheese markets and were stored at 4°C for a few hours before analyses. All the compositional, microbiological, biochemical, and volatile profile analyses were carried out at least in duplicate for each batch of cheese. Analy-

ses were carried out on 2 different sections of each cheese (under the rind, ~1 cm; core, ~2 cm) and the values were averaged. Therefore, a total of 12 analyses were carried out per type of cheese. The protocols for manufacture are shown in Figure 1. All cheeses had a cylindrical shape with a diameter of 16 to 21 cm, a height of 7 to 10 cm, and a weight of 2 of 3 kg. All plant materials were added at the beginning of ripening. Except for hay, all plant materials used to cover the curd were fresh. The fresh curd of Casciotta d'Urbino was gently covered with walnut leaves (a layer of 0.2 to 0.3 cm), which were renewed at least 3 times during ripening. Spicy grass herbs and hay were pressed manually over the fresh curds of Barricato San Martino and Vento d'Estate to form a thin (~0.1 to 0.2 cm) layer. The fresh curd of Ubriaco di Raboso was completely submerged in a layer (height of ~12 cm) of wine by-products.

Compositional Analysis

Moisture, NaCl, and pH were determined as described by the International Dairy Federation (IDF 1970, 1988, 1989). Protein and fat were determined, respectively, by the micro-Kjeldahl method (IDF, 1964) and the Soxhlet method using diethyl ether (Gobbetti et al., 1999).

Microbiological Analysis

Samples (20 g) of cheese were diluted in 180 mL of sodium citrate (2%, wt/vol) solution and homogenized with a Stomacher Lab-Blender 400 (PBI International, Milan, Italy). Serial dilutions were made in quarter-strength Ringer's solution and plated on specific media for viable counts. Total mesophilic bacteria (plate count agar, Oxoid Ltd., Basingstoke, UK) at 30°C for 48 h; presumptive mesophilic lactic acid bacteria [de Man, Rogosa, Sharpe (**MRS**) agar, Oxoid Ltd.] at 30°C for 48 to 72 h; and molds (wort agar, Oxoid Ltd.) at 30°C for 72 h were enumerated. The enumeration of molds was carried out on the cheese surface also.

At least 15 colonies, possibly with different morphologies, were isolated from the MRS plates of the highest dilution of each type of cheese. Gram-positive, catalase-negative, nonmotile rod and cocci isolates were cultivated in MRS broth (Oxoid Ltd.) at 30°C for 24 h, and restreaked onto MRS agar. All isolates considered for further analyses could acidify the culture medium and grow at 15°C but not at 45°C. Stock cultures were stored at -20°C in 10% (vol/vol) glycerol.

Figure 1. Protocols for the manufacture of the 4 Italian cheeses ripened under nonconventional conditions. C, Casciotta di Urbino; B, Barricato San Martino; V, Vento d'Estate; U, Ubriaco di Raboso.

Genotypic Identification by 16S rRNA Gene Sequence Analysis

Genomic DNA from each strain was extracted as described by De Los Reyes-Gavilán et al. (1992). Two primer pairs (Invitrogen Life Technologies, Milan, Italy), LacbF/LacbR and LpCoF/LpCoR (De Angelis et al., 2006), were used to amplify the 16S rRNA gene fragment of lactic acid bacteria. Fifty microliters of each PCR mixture contained 200 μ M of each 2'-deoxynucleoside 5'-triphosphate, 1 μ M of both forward and reverse primer, 2 mM $MgCl_2$, and 2 U of *Taq* DNA polymerase (Invitrogen Life Technologies) in the supplied buffer, and ~50 ng of DNA. The expected amplicons of approximately 1,400 and 1,000 bp (after amplification with primers pairs LacbF/LacbR and LpCoF/LpCoR, respec-

tively) were eluted from the gel and purified by the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ). Taxonomic strain identification was performed by comparing the sequences for each isolate with those reported in the Basic BLAST database (Altschul et al., 1997). Strains showing homology of at least 97% were considered to belong to the same species (Goebel and Stackebrandt, 1994).

Random Amplified Polymorphic DNA-PCR Analysis

Genomic DNA was extracted as reported above, from 2 mL of overnight cultures grown in MRS at 30°C. Three primers (Invitrogen Life Technologies), with arbitrarily chosen sequences (P4, 5'-CCGCAGCGTT-3'; P7, 5'-AGCAGCGTGG-3'; and M13, 5'-GAGGGTGGCGGTTCT-

3'; De Angelis et al., 2001; Rossetti and Giraffa, 2005), were used singly in 3 series of amplifications. The reaction mixture contained 200 μM of each 2'-deoxynucleoside 5'-triphosphate, 1 to 2 μM primer, 1.5 to 3 μM MgCl_2 , 1.25 U of *Taq* DNA polymerase (Invitrogen Life Technologies), 2.5 μL of PCR buffer, 25 ng of DNA, and sterile double-distilled water to 25 μL . For amplifications with primers P4 and P7, the PCR program comprised 45 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 35°C, and elongation for 2 min at 72°C; the cycles were preceded by denaturation at 94°C for 4 min, followed by elongation at 72°C for 5 min. For primer M13, amplification reactions were performed according to the protocol described by Giraffa et al. (2000): one cycle at 94°C for 60 s (denaturing), 42°C for 20 s (annealing), and 72°C for 2 min (elongation). Polymerase chain reaction products were separated by electrophoresis (2 h at 130 V) on 1.5% (wt/vol) agarose gel (Invitrogen Life Technologies), and the DNA was detected by UV transillumination after staining with ethidium bromide (0.5 $\mu\text{g/mL}$). The molecular weight of the amplified DNA fragments was estimated by comparison with 1 Kb Plus DNA Ladder (Invitrogen Life Technologies) ranging from 100 to 12,000 bp. Under our experimental conditions, primer P4 gave only one or a few bands despite extended annealing under modified conditions. Therefore, it was excluded from the characterization.

The reproducibility of random amplified polymorphic DNA (RAPD) fingerprints was assessed by comparing the PCR products obtained from 3 separate cultures of the same strain. Ten strains were studied, and the patterns for the same strain were ~95% similar (data not shown). Three type strains from the American Type Culture Collection (ATCC; *Lactobacillus plantarum* ATCC 14917, *Lactobacillus brevis* ATCC 14869, and *Lactobacillus paracasei* ssp. *paracasei* ATCC 25302) were also included.

Assessment of Proteolysis

The pH 4.6-insoluble and pH 4.6-soluble fractions of the cheeses were prepared as described by Kuchroo and Fox (1982) and analyzed by urea-PAGE, using an SE 600 electrophoresis unit (Hoefer, Amersham Biosciences) and the stacking gel system described by Andrews (1983). The gels were stained directly by the method of Blakesley and Boezi (1977) with Coomassie Brilliant Blue G250.

The peptide profiles of the pH 4.6-soluble fractions were determined by reverse-phase fast protein liquid chromatography (RP-FPLC) using a Resource RPC column and Äkta FPLC equipment with a UV detector operating at 214 nm (Amersham Pharmacia Biotech

AB, Uppsala, Sweden). For each cheese, aliquots (1 mL) of the pH 4.6-soluble fraction, containing 1.5 to 3 mg of peptides, as determined by the *o*-phthaldialdehyde method (Church et al., 1983), were added with 0.05% (vol/vol) trifluoroacetic acid, and centrifuged at 10,000 $\times g$ for 10 min. The supernatant was filtered through a Millex-HA 0.22 μm pore size filter (Millex-HA, Millipore S.A., Saint Quentin, France) and loaded onto the column. Gradient elution was performed at a flow rate of 1 mL/min using a mobile phase composed of water and acetonitrile containing 0.05% trifluoroacetic acid. The CH_3CN content was increased linearly from 5 to 46% between 16 and 62 min, and from 46 to 100% between 62 and 72 min. The peptide profiles of the pH 4.6-soluble fractions were analyzed in duplicate for each batch of cheese (total of 6 analyses per type of cheese) and subjected to multivariate statistical analysis. The data for factor reduction analysis were obtained by FPLC/Unicorn software (Amersham Pharmacia Biotech AB) identification of the peaks, taking peak heights as variables. Factor reduction analysis was performed on the data by the covariance matrix for the determination of principal components (PC; Pripp et al., 1999) using the statistical software Statistica for Windows (Statistica 6.0 per Windows 1998, StatSoft France, Maisons-Alfort, France).

Total and individual free AA (FAA) in the pH 4.6-soluble fraction were analyzed using a Biochrom 30 series AA analyzer (Biochrom Ltd., Cambridge Science Park, UK) with a Na cation-exchange column (20 $\times$ 0.46 cm i.d.). A mixture of AA at known concentration (Sigma Chemical Co., St. Louis MO), containing cysteic acid, methionine sulfoxide, methionine sulfone, Trp, and Orn, was used as the standard. Proteins and peptides in the samples were precipitated by adding cold solid sulfosalicylic acid to 5% (vol/vol), holding at 4°C for 1 h, and centrifuging at 15,000 $\times g$ for 15 min. The supernatant was filtered through a 0.22 μm pore size filter and diluted, when necessary, with sodium citrate (0.2 M, pH 2.2) loading buffer. Amino acids were postcolumn derivatized with ninhydrin reagent and detected by absorbance at 440 (Pro and hydroxyproline) or 570 nm (all the other AA).

Determination of Volatile Components

Volatile components were determined after 2 methods of extraction, PT and SPME. Both methods were coupled with GC-MS (PT-SPME/GC-MS). Prior to PT analysis, 10 g of cheese was cut into 12 cubes and placed in a glass extractor connected to the PT apparatus (Tekmar 3000, Agilent Instruments, New York, NY). Extraction was performed by helium at a flow rate of 40 mL/min on a Tenax trap at 37°C. Trap desorption was performed at 225°C, and injection into the chroma-

tograph was performed with a crio-cooldown. For SPME extraction, 2 g of grated cheese was placed in a 10-mL sealed flask and placed in a bath at 40°C for 30 min. An SPME polydimethylsiloxane fiber (Supelco, Sigma Chemical Co.) was introduced into the flask and held in the headspace for 30 min, then removed and desorbed for 5 min in a splitless chromatograph injector at 250°C. The chromatograph (Agilent Instruments) was equipped with a DB5-like capillary column (RTX5 Restek, Agilent Instruments), 60 m length, 0.32 μm i.d., and 1 μm thickness. The helium flow rate was 2 mL/min; the oven temperature was 40°C during the first 6 min and was then increased at 3°C/min to 230°C. The mass detector (MSD5973, Agilent Instruments) was used in scan mode, from 29 to 206 atomic mass volts at 70 electron volts. Quantification of compounds was expressed in log arbitrary units of area.

For extraction of volatile FFA, 15 g of grated cheese was mixed with valeric acid as the internal standard and 50 mL of 10% sulfuric acid (vol/vol), homogenized using an UltraTurrax homogenizer, and poured into a Jalade apparatus, to the bottom of which was attached a balloon containing 60 mL of diethyl ether (Carlo Erba, Val de Reuil, France) and 60 mL of petroleum spirits (40 to 60°C, Normapur Prolabo, Fontenay S/Bois, France) and at the top was a refrigerator. Extraction was performed for 6 h by ebullition of the solvent. The solvent phase was separated from water, mixed with 50 mL of a solution of ethanol and water (4:1, vol/vol) and 2 drops of 1% phenolphthalein. The volatile FFA were saponified by addition of 1% NaOH (wt/vol). The water phase was kept and desiccated by heating at 103°C. Twenty milligrams of volatile fatty acid soaps was dissolved in 0.5 mL of 12% TCA in ethanol. Injection was by a split injector at 240°C, 10 mL/min split in a GC apparatus (CE8160 Thermoquest, Agilent Instruments) equipped with an FFAP column (Restek Stabilwax DA, 30 m length, 0.53 μm diameter, and 1 μm thickness, Agilent Instruments) and a flame-ionization detector. The helium flow rate was 6 mL/min; the oven temperature was 120°C for 1 min, then increased to 240°C at 10°C/min, held at 240°C for 5 min, and then increased to 250°C at 2°C/min.

Statistical Analysis

Data from microbiological, physicochemical, and volatile compound analyses were subjected to one-way ANOVA (SAS Institute, 1985); pair-comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$ using the statistical software Statistica for Windows (Statistica 6.0 per Windows 1998, StatSoft France).

RESULTS

Compositional Analysis

All nonconventionally ripened cheeses had values for moisture in the range of 29.7 (Barricato San Martino) to 31.8% (wt/wt; Ubriaco di Raboso) and were considered as semihard cheeses (Table 1). The values for fat and protein varied from 29.8 (Ubriaco di Raboso) to 34.5% (wt/wt; Casciotta di Urbino), and from 27.5 (Barricato San Martino) to 29.7% (wt/wt; Ubriaco di Raboso), respectively. The pH at the end of ripening was in the range of 5.05 (Casciotta di Urbino) to 5.32 (Barricato San Martino). The concentration of NaCl in Vento d'Estate (0.3%, wt/wt) was significantly ($P < 0.05$) different from the other cheeses. No differences were found for the other dry (0.6%, Casciotta di Urbino) or brine-salted cheeses (0.49 to 0.5%, Barricato San Martino and Ubriaco di Raboso, respectively). Overall, for this and further analyses, the same cheese variety manufactured from different batches of milk did not differ significantly ($P > 0.05$).

Microbiological Analysis, Identification, and Typing of Endogenous Lactic Acid Bacteria

The number of total mesophilic bacteria was rather similar in the 4 cheeses, ranging from 7.0 (Casciotta di Urbino) to 8.5 $\log_{10}$ cfu/g (Vento d'Estate). No molds were found in the interior and surface of the 4 cheeses at the end of ripening. As estimated by plating on MRS agar, cell numbers of presumptive mesophilic lactic acid bacteria varied from 7.0 to 7.5 (Casciotta di Urbino and Ubriaco di Raboso, respectively) to 8.5 to 9.0 $\log_{10}$ cfu/g (Barricato San Martino and Vento d'Estate, respectively).

Gram-positive, catalase-negative, nonmotile cocci and rods able to grow at 15°C and to acidify MRS broth, isolated from the plates of the highest dilution of each type of cheese, were identified by partial sequencing of the 16S rRNA. Cheeses of the same type manufactured from different batches of milk showed a similar microbial composition at the species level. The number of isolates indicated below refers to the average from the 3 batches. The following species were identified for each cheese: Casciotta d'Urbino, *Lactococcus lactis* (12 isolates) and *Enterococcus sanguinicola* (3); Barricato San Martino, *E. sanguinicola* (5), *Leuconostoc mesenteroides* (5), *L. paracasei* (3), *L. brevis* (1), and *Enterococcus durans/Enterococcus faecium* (1); Vento d'Estate, *L. plantarum* (7), *L. paracasei* (5), *Weissella cibaria/Weissella confusa* (1), *Leuc. mesenteroides* (1), and *Lactobacillus* spp. (1); and Ubriaco di Raboso, *L. paracasei* (13) and *Leuc. mesenteroides* (2).

Table 1. Mean values¹ for the gross composition of the 4 Italian cheeses ripened under nonconventional conditions

Item	Casciotta di Urbino	Barricato San Martino	Vento d'Estate	Ubriaco di Raboso
Moisture, % (wt/wt)	31.2 ± 0.5 ^a	29.7 ± 1.2 ^a	30.5 ± 0.8 ^a	31.8 ± 0.5 ^a
Fat, % (wt/wt)	34.5 ± 0.2 ^a	32.1 ± 0.4 ^a	31.2 ± 0.5 ^a	29.8 ± 0.6 ^b
NaCl, % (wt/wt)	0.6 ± 0.1 ^a	0.49 ± 0.5 ^a	0.3 ± 0.3 ^b	0.5 ± 0.1 ^a
Protein, % (wt/wt)	28.5 ± 0.5 ^b	27.5 ± 0.6 ^b	28.2 ± 0.3 ^b	29.7 ± 0.7 ^a
pH	5.05 ± 0.2 ^c	5.32 ± 0.1 ^a	5.11 ± 0.3 ^{bc}	5.19 ± 0.2 ^b
pH 4.6-soluble N, % of total N	18.1 ± 1.1 ^c	22.4 ± 0.8 ^b	24.5 ± 0.8 ^a	22.2 ± 1.0 ^b

^{a-c}Means within a row with different superscript letters are different ($P < 0.05$).

¹Mean value ± standard deviations for 3 batches of each type of cheese, analyzed in duplicate.

Genotypic typing by RAPD-PCR was carried out by primers M13 and P7, which generated the most diverse patterns (2 to 8 bands ranging from 5,000 to 200 bp). Almost all the isolates effectively corresponded to different biotypes (data not shown). An example of RAPD-PCR used to find relationships with the cheese sources is shown in Figure 2 for lactobacilli (the most numerous group). At a similarity level of 80%, type strains were separated and the highest percentage of the *Lactobacillus* isolates was grouped in 6 clusters (clusters I to VI). *Lactobacillus brevis* ATCC 14869, *L. brevis* B1, *Lactobacillus* spp. V14, and *L. paracasei* U11, U7, and U14, which gave unique RAPD patterns, did not belong to any cluster. *Lactobacillus paracasei* strains were sep-

arated into 5 clusters, including isolates from Barricato San Martino in cluster I; from Ubriaco di Raboso, Vento D'Estate, and type strain ATCC 25302 in cluster II; and only isolates from Ubriaco di Raboso in clusters IV, V, and VI. All *L. plantarum* isolates from Vento d'Estate cheese, including the type strain ATCC 14917, were grouped in cluster III.

Proteolysis

The level of pH 4.6-soluble nitrogen, expressed as a percentage of total nitrogen in the cheeses, was in the range of 18.1 (Casciotta di Urbino) to 24.5% (Vento d'Estate; Table 1). Urea-PAGE of the pH 4.6-insoluble

Figure 2. Dendrogram obtained by combined random amplification of polymorphic DNA (RAPD) patterns for the lactobacilli isolated from 3 Italian cheeses ripened under nonconventional conditions using primers P7 and M13. Isolates are indicated according to the cheeses: B, Barricato San Martino; V, Vento d'Estate; U, Ubriaco di Raboso. Cluster analysis was based on the simple matching coefficient and unweighted pair group method, arithmetic average. ATCC = American Type Culture Collection.

fractions (Figure 3A) showed that primary proteolysis of Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso was rather similar. The profile of Casciotta di Urbino cheese differentiated it from the others. Overall, α_{s1} -CN was degraded almost completely in all cheeses. β -Casein was found to be slightly more persistent, and the formation of protein bands with low electrophoretic mobility, which presumably corresponded to γ -CN, was evident in Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso. The urea-PAGE electrophoregram of the pH 4.6-soluble fractions (Figure 3B) showed differences between the cheeses. In particular, Barricato San Martino and Ubriaco di Raboso cheeses showed the most complex profiles. The pH 4.6-soluble fractions were analyzed by RP-FPLC also (Figure 4). Quantitative, and especially qualitative, differences were evident. Barricato San Martino differentiated from the other cheeses especially because of the absence of polypeptide peaks in the late zone of the acetonitrile gradient. Principal component analysis (PCA) was applied to the RP-FPLC data. The score plot and loading plot of the first and second PC after PCA, based on 17 variables (peaks) of the 4 cheeses, are shown in Figure 5 (panels A and B). These 2 PC explained approximately 81% of the total variance. As also shown by the urea-PAGE electrophoregram of the pH 4.6-soluble fractions (Figure 3B), Casciotta di Urbino was located in a zone of the plane separated from the other cheeses, thus indicating a different secondary proteolysis. As analyzed by PCA, the peptide profiles of Barricato San Martino and Vento d'Estate cheeses differed only slightly. The concentrations of total FAA (Table 2) of the 4 cheeses were in substantial agreement with the level of pH 4.6-soluble nitrogen, expressed as a percentage of total nitrogen. The highest concentration of FAA was found for Vento d'Estate (246.0 $\mu\text{g/g}$), followed by Barricato San Martino (216.7 $\mu\text{g/g}$) and Ubriaco di Raboso (202.6 $\mu\text{g/g}$). Very low concentrations of FAA were found for Casciotta di Urbino (8.64 $\mu\text{g/g}$). Except for Met, Tyr, and Arg, no other FAA were found at concentrations higher than 1 $\mu\text{g/g}$ in Casciotta di Urbino cheese. Although Ser, Glu, Gly, Tyr, and Arg were generally found at the highest concentrations, the concentration of Cys, Ser, Glu, Lys, and Pro statistically ($P < 0.05$) differentiated Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso cheeses.

Volatile Components

Two methods were used to extract volatile compounds from nonconventionally ripened cheeses: PT, which is more effective for extracting the highly volatile compounds, and SPME, which is better for extracting medium- and high-boiling compounds (Mallia et al., 2005).

Figure 3. Urea-PAGE of the pH 4.6-insoluble (A) and pH 4.6-soluble (B) fractions of the 4 Italian cheeses ripened under nonconventional conditions. Lane: C, Casciotta di Urbino; B, Barricato San Martino; V, Vento d'Estate; U, Ubriaco di Raboso; St, cows' milk CN.

Figure 4. Reverse-phase fast protein liquid chromatography of the pH 4.6-soluble fractions of the 4 Italian cheeses ripened under nonconventional conditions. Chromatograms: Casciotta di Urbino (C), Barricato San Martino (B), Vento d'Estate (V), and Ubriaco di Raboso (U).

Table 3 shows 145 volatile components with molecular masses of 40 to 200 Da, as identified by PT, and grouped according to chemical class. The variability of the volatile components among the 3 batches of each cheese was not significant ($P > 0.05$). In contrast, all the volatiles shown, except for 10 components, differentiate the cheeses with various degrees of statistical significance.

Figure 5. Score plot (A) and loading plot (B) of the first and second principal components (PC) after PC analysis based on individual peaks obtained from reverse-phase fast protein liquid chromatograms of the pH 4.6-soluble fractions of the 4 Italian cheeses ripened under nonconventional conditions. C, Casciotta di Urbino; B, Barricato San Martino; V, Vento d'Estate; U, Ubriaco di Raboso.

Overall, Vento d'Estate showed the highest levels for almost all chemical classes and Casciotta di Urbino seemed to be characterized by a very low level of volatile components (Table 3).

Table 2. Concentration ($\mu\text{g/g}$ of cheese)¹ of individual and total free AA of the 4 Italian cheeses ripened under nonconventional conditions

AA	Casciotta di Urbino	Barricato San Martino	Vento d'Estate	Ubriaco di Raboso
Cys	0.059 ^d	1.15 ^c	3.33 ^b	8.60 ^a
Asp	0 ^b	0 ^b	0.56 ^a	0 ^b
Thr	0.11 ^c	6.20 ^b	15.89 ^a	15.46 ^a
Ser	0.11 ^d	14.29 ^b	21.37 ^a	11.81 ^c
Glu	0.019 ^d	35.26 ^a	17.49 ^b	6.53 ^c
Gly	0.42 ^c	20.19 ^b	28.22 ^a	21.40 ^b
Ala	0.11 ^b	10.89 ^a	12.98 ^a	10.95 ^a
Cys	0.78 ^b	14.46 ^a	15.95 ^a	13.46 ^a
Val	0.14 ^b	0.78 ^a	0.69 ^a	0.99 ^a
Met	1.27 ^b	13.36 ^a	14.37 ^a	13.24 ^a
Ile	0.20 ^d	6.97 ^b	9.19 ^a	8.38 ^{ab}
Leu	0.092 ^c	15.85 ^{ab}	17.55 ^a	12.43 ^b
Tyr	1.08 ^b	18.34 ^a	19.63 ^a	18.74 ^a
Phe	0.24 ^c	1.06 ^b	12.10 ^a	2.16 ^b
His	0.74 ^b	13.83 ^a	15.71 ^a	14.31 ^a
Trp	0.089 ^b	3.63 ^a	4.07 ^a	0.44 ^b
Orn	0.035 ^b	4.37 ^a	5.32 ^a	0.59 ^b
Lys	0.54 ^d	8.29 ^b	2.64 ^c	11.88 ^a
Arg	2.81 ^c	27.76 ^b	26.72 ^b	30.70 ^a
Pro	0 ^d	0.025 ^c	2.23 ^a	0.61 ^b
Total	8.64 $\pm$ 1.11 ^d	216.70 $\pm$ 7.34 ^b	246.01 $\pm$ 5.34 ^a	202.68 $\pm$ 8.97 ^c

^{a-d}Means within a row with different superscript letters are different ($P < 0.05$).

¹Averaged data from 3 batches of each variety of cheese analyzed.

Esters (44 compounds identified) were the most numerous chemical class (Table 3). The levels of numerous esters (22) statistically ($P < 0.001$) differentiated Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso cheeses. In particular, they concerned methylbutanoate to methyl-octanoate, methyl 2-methylbutanoate, ethyl-pentanoate to ethyl-heptanoate, ethyl 2-methylbutanoate and 3-methylbutanoate, and other propyl-, butyl-, hexyl-, methylpropyl-, and methylbutyl-esters. Ethyl-acetate, ethyl-butanoate, ethyl-propanoate, and ethyl-hexanoate were found at the highest levels in all 3 cheeses. Ethyl-hexenoate, propyl-pentanoate, pentyl-acetate, 1-methylbutyl-butanoate, and 1-methylhexyl-butanoate were identified in Vento d'Estate only. The levels of propyl-, butyl-, hexyl- and 3-methylbutyl acetate statistically ($P < 0.001$) differentiated Ubriaco di Raboso from the other cheeses. Only 5 esters were found in Casciotta di Urbino.

Twenty-four ketones were identified (Table 3). The levels of 10 ketones (mainly 2-alkanones, 2,3-butanedione, and 2,3-pentanedione) statistically ($P < 0.001$) differentiated the 4 cheeses. Some components were present in only one cheese: 2,3-hexanedione and 3-hydroxy-2-butanone (acetoin) in Casciotta di Urbino, 4-methyl-2-hexanone in Barricato San Martino, and 3-methyl-2-pentanone and 5-hepten-2-one in Vento d'Estate. 2-Propanone, 2-butanone, and 2-pentanone were found at the highest levels in all cheeses.

After esters, alcohols (26 compounds identified) were quantitatively the most abundant chemical class of vol-

atiles in Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso cheeses (Table 3). Nevertheless, the levels of only 6 alcohols (propanol, 1-pentanol, 2-propen-1-ol, 2-propanol, 2-pentanol, and 3-methyl-2-butanol) statistically ($P < 0.001$) differentiated the 4 cheeses. 3-Octanol was found in Barricato San Martino only. Primary (e.g., 1-butanol and 1-pentanol), secondary (e.g., 2-propanol, 2-butanol, 2-pentanol, and 2-heptanol) and branched-chain alcohols (e.g., 2-methylpropanol, 3-methyl-1-butanol, and 2-methyl-1-butanol) were found at the highest levels in Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso cheeses.

Compared with the previous chemical classes, none of the 15 aldehydes identified statistically ($P < 0.001$) differentiated the 4 cheeses (Table 3). 2-Methylpropanal, 3-methylbutanal, and 2-methylbutanal statistically ($P < 0.001$) differentiated Barricato San Martino and Vento d'Estate from the other 2 cheeses. The number of aldehydes identified in Casciotta d'Urbino was higher than that of esters. Hexanal was found only in this cheese.

Among the 8 sulfur compounds identified, only S-methylthiocyanate statistically ($P < 0.001$) differentiated the 4 cheeses (Table 3). Except for methylthioethane, identified only in Casciotta di Urbino, the level of sulfur compounds was always lowest in this cheese. Dimethyl sulfide, carbon disulfide, dimethyl disulfide, and dimethyl trisulfide were the principal sulfur compounds found. Several furans, alkanes, alkenes, and

Table 3. Volatile components¹ [log(arbitrary units of area)] found by purge-and-trap extraction in the 4 Italian cheeses ripened under nonconventional conditions

Chemical class	Casciotta di Urbino	Barricato San Martino	Vento d'Estate	Ubriaco di Raboso	Statistical significance ²
Esters					
Methyl-acetate	2.22 ^c	4.49 ^b	4.79 ^a	4.67 ^a	***
Methyl-propanoate	ND ^c	3.34 ^b	4.05 ^a	3.36 ^b	***
Methyl-butanoate	2.01 ^d	4.89 ^b	5.84 ^a	4.74 ^c	***
Methyl-pentanoate	ND ^d	2.84 ^b	3.83 ^a	2.62 ^c	***
Methyl-hexanoate	ND ^d	3.84 ^c	5.41 ^a	4.44 ^b	***
Methyl-heptanoate	ND ^c	ND ^c	3.11 ^a	2.18 ^b	***
Methyl-octanoate	ND ^d	2.32 ^c	3.98 ^a	3.60 ^b	***
Methyl 2-methyl-butanoate	ND ^d	0.98 ^c	2.44 ^a	1.81 ^b	***
Ethyl-acetate	3.12 ^c	5.97 ^b	6.08 ^a	5.87 ^b	***
Ethyl-propanoate	ND ^b	5.51 ^a	6.03 ^a	5.71 ^a	***
Ethyl-butanoate	2.67 ^c	6.12 ^b	6.48 ^a	6.10 ^b	***
Ethyl-pentanoate	ND ^d	4.24 ^b	4.85 ^a	4.19 ^c	***
Ethyl-hexanoate	0.12 ^d	5.25 ^c	6.22 ^a	5.87 ^b	***
Ethyl-heptanoate	ND ^d	2.88 ^c	4.15 ^a	3.81 ^b	***
Ethyl-octanoate	ND ^c	3.96 ^b	4.89 ^a	5.01 ^a	***
Ethyl 2-methyl-butanoate	ND ^d	2.89 ^c	3.78 ^a	3.37 ^b	***
Ethyl 3-methyl-butanoate	ND ^d	3.06 ^c	4.27 ^a	3.40 ^b	***
Ethyl 4-methyl-pentanoate	ND ^c	ND ^c	2.07 ^a	0.61 ^b	***
Ethyl 2-hexenoate	ND ^b	ND ^b	2.92 ^a	ND ^c	***
Ethyl-benzoate	ND ^b	2.14 ^a	2.19 ^a	2.43 ^a	***
Propyl-acetate	ND ^c	4.43 ^b	4.37 ^b	4.87 ^a	***
Propyl-propanoate	ND ^c	2.88 ^b	3.31 ^a	3.31 ^a	***
Propyl-butanoate	ND ^c	3.95 ^b	4.58 ^a	4.54 ^a	***
Propyl-pentanoate	ND ^b	ND ^b	3.27 ^a	ND ^b	***
Propyl 2-methyl-propanoate	ND ^c	ND ^c	1.59 ^a	0.89 ^b	***
Isopropyl-acetate	ND ^d	3.20 ^b	4.77 ^a	2.49 ^c	***
Butyl-acetate	ND ^d	4.00 ^c	4.31 ^b	4.59 ^a	***
Pentyl-acetate	ND ^b	ND ^b	3.06 ^a	ND ^b	***
Hexyl-acetate	ND ^d	1.93 ^c	2.39 ^b	2.79 ^a	***
1-Methylethyl-butanoate	ND ^c	2.55 ^b	4.91 ^a	2.66 ^b	***
1-Methylpropyl-acetate	ND ^c	3.67 ^a	3.36 ^a	2.80 ^b	***
1-Methylpropyl-propanoate	ND ^c	0.37 ^c	2.14 ^a	1.25 ^b	***
1-Methylpropyl-butanoate	ND ^c	3.23 ^b	3.61 ^a	3.46 ^{ab}	***
1-Methylpropyl-hexanoate	ND ^c	1.26 ^b	2.49 ^a	2.71 ^a	***
2-Methylpropyl-butanoate	ND ^d	2.85 ^c	3.97 ^a	3.52 ^b	***
2-Methylpropyl-hexanoate	ND ^c	ND ^c	2.73 ^a	2.35 ^b	***
3-Methylbutyl-acetate	ND ^c	3.51 ^b	3.79 ^b	4.60 ^a	***
2-Methylbutyl-acetate	ND ^c	2.57 ^b	3.09 ^a	3.29 ^a	***
3-Methylbutyl-butanoate	ND ^d	2.53 ^c	3.72 ^a	3.45 ^b	***
2-Methylbutyl-butanoate	ND ^c	1.75 ^b	3.47 ^a	3.06 ^a	***
1-Methylbutyl-hexanoate	ND ^b	ND ^b	2.93 ^a	ND ^b	***
1-Methylhexyl 2-methyl-propanoate	ND ^d	1.31 ^c	4.33 ^a	2.52 ^b	***
1-Methylhexyl-butanoate	ND ^b	ND ^b	2.27 ^a	ND ^b	***
3-Hexenyl-acetate	ND ^b	2.24 ^a	2.30 ^a	2.13 ^a	***
Total esters	10.14	112.95	168.14	135.07	
Ketones					
2-Propanone	4.73 ^c	5.36 ^b	5.92 ^a	3.54 ^d	***
2-Butanone	3.96 ^d	6.22 ^a	5.78 ^b	4.71 ^c	***
2-Pentanone	4.55 ^c	6.17 ^b	6.83 ^a	4.61 ^c	***
3-Pentanone	1.98 ^c	2.75 ^b	3.53 ^a	0.32 ^d	***
2-Hexanone	2.47 ^c	3.79 ^b	4.83 ^a	2.30 ^c	***
3-Heptanone	0.83 ^b	2.58 ^a	2.93 ^a	2.03 ^a	**
2-Heptanone	3.70 ^c	4.88 ^b	5.82 ^a	3.36 ^d	***
3-Octanone	ND ^c	3.24 ^b	4.55 ^a	0.41 ^c	***
2-Octanone	2.31 ^c	2.86 ^b	3.76 ^a	2.03 ^d	***
2-Nonanone	2.63 ^d	3.69 ^b	4.51 ^a	3.06 ^c	***
2,3-Butanedione	5.33 ^a	4.09 ^b	3.73 ^c	2.90 ^d	***
2,3-Pentanedione	3.54 ^a	2.72 ^c	3.24 ^b	2.38 ^d	***
2,3-Hexanedione	0.74 ^a	ND ^b	ND ^b	ND ^b	***
3-Hydroxy-2-butanone	4.13 ^a	ND ^b	ND ^b	ND ^b	***
3-Methyl-2-butanone	0.45 ^b	2.65 ^a	3.09 ^a	0.25 ^b	***
4-Methyl-2-pentanone	1.56 ^b	0.67 ^c	2.68 ^a	ND ^d	***
3-Methyl-2-pentanone	ND ^b	ND ^b	3.62 ^a	ND ^b	***
2-Methyl-3-hexanone	ND ^c	2.03 ^b	2.64 ^a	ND ^c	***

Continued

Table 3 (Continued). Volatile components¹ [log(arbitrary units of area)] found by purge-and-trap extraction in the 4 Italian cheeses ripened under nonconventional conditions

Chemical class	Casciotta di Urbino	Barricato San Martino	Vento d'Estate	Ubriaco di Raboso	Statistical significance ²
4-Methyl-2-hexanone	ND ^b	2.48 ^a	ND ^b	ND ^b	***
6-Methyl-5-hepten-2-one	ND ^c	1.93 ^{ab}	2.17 ^a	1.62 ^b	***
5-Hepten-2-one	ND ^b	ND ^b	4.26 ^a	ND ^b	***
8-Nonen-2-one	ND ^c	2.18 ^b	3.39 ^a	0.11 ^c	***
Cyclopentanone	1.54 ^b	2.50 ^a	2.45 ^a	0.85 ^b	**
1-Phenyl-ethanone	1.73	2.40	2.43	2.31	NS
Total ketones	46.18	65.20	82.16	36.79	
Alcohols					
Ethanol	5.24 ^b	7.17 ^a	6.69 ^a	6.86 ^a	***
Propanol	3.19 ^d	5.85 ^b	5.54 ^c	6.02 ^a	***
1-Butanol	2.95 ^b	5.53 ^a	5.55 ^a	5.64 ^a	***
1-Pentanol	2.69 ^d	3.71 ^b	4.27 ^a	3.41 ^c	***
1-Hexanol	2.49 ^c	3.65 ^b	3.70 ^b	4.45 ^a	***
1-Heptanol	ND ^c	2.19 ^{ab}	2.55 ^a	1.88 ^b	***
1-Octanol	ND ^b	2.35 ^a	1.93 ^a	2.05 ^a	**
2-Propen-1-ol	ND ^d	4.29 ^b	5.15 ^a	3.51 ^c	***
1-Penten-3-ol	2.41 ^{ab}	1.99 ^b	2.43 ^{ab}	3.13 ^a	NS
3-Hexen-1-ol	ND ^c	3.22 ^a	3.02 ^a	2.48 ^b	***
1-Octen-3-ol	0.75 ^c	4.62 ^a	4.33 ^a	2.34 ^b	***
2-Propanol	4.21 ^d	6.20 ^b	6.99 ^a	5.88 ^c	***
2-Butanol	3.25 ^c	6.11 ^a	5.55 ^b	5.89 ^a	***
2-Pentanol	3.09 ^d	5.77 ^b	6.72 ^a	5.47 ^c	***
2-Heptanol	0.32 ^c	4.55 ^b	5.32 ^a	4.58 ^b	***
3-Octanol	ND ^b	2.90 ^a	ND ^b	ND ^b	***
2-Nonanol	ND ^c	2.28 ^b	2.50 ^b	3.15 ^a	***
2-Methyl-propanol	2.30 ^b	4.28 ^a	4.27 ^a	4.60 ^a	***
3-Methyl-1-butanol	3.00 ^c	5.12 ^b	5.07 ^b	5.49 ^a	***
2-Methyl-1-butanol	1.83 ^b	4.31 ^a	4.51 ^a	4.79 ^a	***
2-Ethyl-1-hexanol	0.11 ^b	3.23 ^a	3.23 ^a	3.00 ^a	***
3-Methyl-2-butanol	2.71 ^d	3.72 ^c	4.58 ^a	4.12 ^b	***
3-Methyl-3-buten-1-ol	2.49 ^b	3.20 ^a	3.25 ^a	3.12 ^a	***
3-Methyl-2-buten-1-ol	0.88 ^b	2.43 ^a	2.31 ^a	2.06 ^a	**
2,3-Butanediol	ND ^b	3.41 ^a	ND ^b	3.80 ^a	***
Phenol	1.29 ^b	2.38 ^a	2.54 ^a	2.09 ^{ab}	*
Total alcohols	45.21	104.46	101.98	99.81	
Aldehydes					
Acetaldehyde	3.57 ^c	4.40 ^a	4.05 ^b	4.38 ^a	***
Propenal	2.29	2.72	2.39	2.47	NS
Butanal	2.66 ^a	ND ^b	2.63 ^a	2.61 ^a	***
Pentanal	2.98 ^b	2.89 ^b	3.29 ^a	2.81 ^b	**
Hexanal	2.74 ^a	ND ^b	ND ^b	ND ^b	***
Heptanal	2.00	1.94	1.94	1.85	NS
Octanal	2.39	2.57	2.55	2.48	NS
Nonanal	2.71 ^b	3.19 ^a	3.12 ^a	3.04 ^a	**
Decanal	ND ^b	2.35 ^a	2.17 ^a	1.86 ^a	**
2-Methyl-propanal	2.62 ^c	4.04 ^a	4.14 ^a	3.41 ^b	***
3-Methyl-butanal	2.00 ^c	4.28 ^a	4.27 ^a	3.70 ^b	***
2-Methyl-butanal	0.11 ^c	4.16 ^a	4.18 ^a	3.25 ^b	***
2-Methyl-2-butenal	ND ^b	2.15 ^a	1.84 ^a	1.44 ^a	***
Benzaldehyde	2.51 ^c	3.00 ^a	2.91 ^{ab}	2.77 ^b	**
Benzeneacetaldehyde	0.22 ^b	3.18 ^a	3.00 ^a	2.57 ^a	***
Total aldehydes	28.79	40.87	42.48	38.65	
Sulfur compounds					
Methanethiol	ND ^b	1.31 ^{ab}	1.97 ^a	1.06 ^{ab}	*
Dimethyl sulfide	3.70 ^c	4.39 ^a	4.16 ^b	4.36 ^a	***
Carbon disulfide	2.71 ^c	3.74 ^b	3.77 ^b	4.42 ^a	***
Dimethyl disulfide	3.19 ^c	4.38 ^a	4.66 ^a	3.89 ^b	***
Dimethyl trisulfide	ND ^c	2.59 ^b	3.31 ^a	2.19 ^b	***
Methylthioethane	1.25 ^a	ND ^b	ND ^b	ND ^b	***
2,4-Dithiapentane	0.87 ^c	2.28 ^b	2.81 ^{ab}	3.29 ^a	***
S-Methyl-thioacetate	ND ^d	3.37 ^a	2.14 ^c	2.43 ^b	***
Total sulfur compounds	11.72	22.06	22.82	21.64	

Continued

Table 3 (Continued). Volatile components¹ [log(arbitrary units of area)] found by purge-and-trap extraction in the 4 Italian cheeses ripened under nonconventional conditions

Chemical class	Casciotta di Urbino	Barricato San Martino	Vento d'Estate	Ubriaco di Raboso	Statistical significance ²
Terpenes					
Tricyclene	ND ^b	ND ^b	2.74 ^a	0.51 ^b	***
2,6-Dimethyl-1,7-octadiene	ND ^b	2.12 ^a	2.29 ^a	2.40 ^a	***
α -Pinene	2.07 ^c	3.08 ^b	4.41 ^a	3.03 ^b	***
3,7-Dimethyl-1,6-octadiene	ND ^b	1.93 ^a	2.02 ^a	2.19 ^a	***
α -Fenchene	ND ^b	ND ^b	2.43 ^a	0.56 ^b	**
Camphene	ND ^c	2.15 ^b	3.77 ^a	2.10 ^b	***
3,7-Dimethyl-2-octene	ND ^b	0.44 ^b	1.76 ^a	1.97 ^a	***
β -Pinene	ND ^b	0.66 ^b	3.19 ^a	0.87 ^b	*
β -Myrcene	ND ^c	1.19 ^{bc}	3.45 ^a	2.06 ^{ab}	**
δ -2-Carene	ND	ND	1.41	ND	NS
1-Phellandrene	ND ^b	0.42 ^b	2.96 ^a	1.29 ^b	**
δ -3-Carene	ND ^c	1.90 ^b	3.97 ^a	0.91 ^c	***
α -Terpinene	ND ^b	ND ^b	2.62 ^a	0.71 ^b	**
<i>m</i> -Cymene	ND ^b	ND ^b	2.05 ^a	ND ^b	***
<i>p</i> -Cymene	0.10 ^c	2.51 ^b	4.00 ^a	2.65 ^b	***
DL-Limonene	1.94 ^b	2.61 ^{ab}	3.66 ^a	3.53 ^a	**
<i>Cis</i> -ocimene	ND ^b	0.32 ^b	2.92 ^a	0.76 ^b	**
1,8-Cineole	ND ^c	2.47 ^b	3.73 ^a	ND ^c	***
β -Terpinene	ND ^b	ND ^b	2.38 ^a	ND ^b	***
γ -Terpinene	ND ^b	1.45 ^{ab}	3.23 ^a	1.64 ^{ab}	*
7-Octen-2-ol-2,6-dimethyl	ND	0.61	0.62	1.58	NS
α -Terpinolene	ND ^b	0.99 ^{ab}	2.82 ^a	1.78 ^{ab}	*
1(1-methyl-1-ethenyl)4-methyl-benzene	ND ^b	0.85 ^{ab}	2.39 ^a	0.93 ^{ab}	*
Camphor	ND ^b	0.77 ^b	2.75 ^a	0.11 ^b	***
Menthone	ND ^b	1.93 ^{ab}	2.53 ^a	0.28 ^b	*
α -Isomenthone	ND	1.63	1.87	ND	NS
Menthol	ND	1.00	ND	0.84	NS
α -Terpineol	ND	ND	1.03	ND	NS
Total terpenes	4.12	31.05	72.99	32.71	

^{a-d}Means within a row with different superscript letters are different ($P < 0.05$).

¹Average value for 3 batches of each type of cheese, analyzed in duplicate. ND = not detected.

²The variability of the volatile components among the 3 batches of each type was not significant ($P > 0.05$).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

benzene derivatives were also identified, but they slightly distinguished the 4 cheeses (data not shown).

Twenty-eight terpenes were identified (Table 3). The highest number (23) was found in Vento d'Estate. Fourteen terpenes (e.g., tricyclene, camphene, δ -3-carene, α -terpinene, and *p*-cymene) statistically ($P < 0.001$ or $P < 0.01$) differentiated Vento d'Estate from the other cheeses. Only 3 terpenes were identified in Casciotta di Urbino cheese.

Thirty-one volatile compounds were identified by SPME extraction (data not shown). High molecular mass esters were also absent from Casciotta d'Urbino but were present (e.g., ethyl-decanoate, ethyl-dodecanoate) in the other 3 cheeses. Almost the same was found for ketones from 2-decanone to 2-tridecanone. 2(3*H*)-Furanone (5-ethylidihydro and δ -caprolactone) were the only lactones detected in the nonconventionally ripened cheeses.

Volatile FFA

Table 4 shows the composition of volatile FFA (C_2 to C_6) in the 4 Italian cheeses ripened under nonconventional conditions. The total amount varied markedly from Vento d'Estate (589.6 mg/100 g) to Barricato San Martino (210.6 mg/100 g), Ubriaco di Raboso (155.8 mg/100 g), and Casciotta di Urbino (32.5 mg/100 g). Although the concentration of acetic acid was higher in Ubriaco di Raboso and Barricato San Martino than in Vento d'Estate, this cheese was characterized mainly by elevated amounts of butyric (389.5 mg/100 g) and caproic (115.0 mg/100 g) acids. The concentrations of propionic and butyric acids statistically ($P < 0.001$) differentiated the 4 cheeses.

DISCUSSION

Although made with raw milk and without primary starters, each type of cheese had very similar microbial,

Table 4. Volatile FFA¹ (VFFA, mg/100 g) found in the 4 Italian cheeses ripened under nonconventional conditions

VFFA	Casciotta di Urbino	Barricato San Martino	Vento d'Estate	Ubriaco di Raboso	Statistical significance ²
Acetic acid	20.6 ^c	96.4 ^a	72.0 ^b	115.8 ^a	***
Propionic acid	0.2 ^d	20.7 ^a	11.6 ^b	6.7 ^c	***
Isobutyric acid	0.07 ^b	0.25 ^a	0.14 ^b	0.11 ^b	***
Butyric acid	7.7 ^d	85.4 ^b	389.5 ^a	25.8 ^c	***
Isovaleric acid	0.35 ^b	1.03 ^a	1.08 ^a	0.19 ^c	***
Isocaproic acid	0.02 ^b	0.04 ^a	0.05 ^a	0.02 ^b	**
Caproic acid	3.6 ^c	6.9 ^b	115.0 ^a	7.3 ^b	***
Total VFFA	32.57	210.62	589.61	155.81	

^{a-d}Means within a row with different superscript letters are different ($P < 0.05$).

¹Average value for 3 batches of each type of cheese, analyzed in duplicate.

²The variability of the volatile components among the 3 batches of each type was not significant ($P > 0.05$).

** $P < 0.01$; *** $P < 0.001$.

biochemical, and chemical features during 3 batches of manufacture, thus showing the reproducibility of each manufacturing protocol. Gross compositions and microbiological standards of the semihard cheeses were in agreement with those recommended in the guidelines for manufacture. Because of their marked contribution to ripening, especially when primary starters are not used (Fox et al., 1998), mesophilic lactic acid bacteria were mainly characterized. They varied from 7.0 to 9.0 log₁₀ cfu/g. Overall, endogenous lactic acid bacteria, which are present at very low numbers during the early phase of manufacture, become the dominant microbial flora (~8.0 log₁₀ cfu/g) during late ripening (Berthier et al., 2001). Both cell numbers and the number of identified species were the highest in Barricato San Martino and Vento d'Estate cheeses. These 2 cheeses were subjected to curd held at ~20 to 25°C for 20 h during manufacture, and especially to the longest time (4 mo) of ripening. Other environmental factors, which were strictly related to the manufacture of each cheese and not considered in this study (e.g., microbial composition of the raw milk, microbial flora from the manufacturing equipment), may theoretically have influenced the resulting endogenous microbiota. Nevertheless, *L. paracasei* and *Leuc. mesenteroides* were found in 3 of the nonconventionally ripened cheeses. Other reports (De Angelis et al. 2001; Di Cagno et al., 2003; Macedo et al., 2004) also found *L. paracasei* as the dominant species within the cheese endogenous microbiota, and species of *Leuconostoc* are also included in this group (Fox et al., 1998). Although usually not present at high cell densities within the milk endogenous microbiota at the end of ripening (Fox et al., 1998), species belonging to the *Enterococcus* (in Casciotta d'Urbino and Barricato San Martino cheeses), *Weissella* (in Vento d'Estate cheese), and *Lactococcus* (in Casciotta d'Urbino cheese) genera were found at 7.0 to 9.0 log₁₀ cfu/g. As shown

by other authors (Rossetti and Giraffa, 2005), genotypic typing of the identified lactobacilli was conclusive in showing differences between isolates, and the species of lactobacilli were in part grouped according to their origin.

Primary proteolysis was rather similar in the 4 Italian cheeses. High chymosin activity toward α_{s1} -CN and partial degradation of β -CN, especially by plasmin, was shown. Secondary proteolysis differed in part between the cheeses. As shown by urea-PAGE and PCA analyses of the pH 4.6-soluble nitrogen fraction and by FAA, Barricato San Martino and Vento d'Estate cheeses behaved similarly. These 2 cheeses were characterized by 1) an almost similar manufacturing protocol, which included the use of rennet paste and the longest ripening time, and 2) the most complex microbiota, which also included 2 species of mesophilic lactobacilli. An intense peptidase activity is generally attributed to the main species of mesophilic lactobacilli such as *L. paracasei* (Wouters et al., 2002; Poveda et al., 2003). Casciotta di Urbino cheese, which showed the lowest level of secondary proteolysis, was manufactured by using 20% of ewes' milk and, especially, liquid calf rennet. Compared with other Italian semihard and hard varieties manufactured with almost similar protocols but ripened under conventional conditions, the concentrations of FAA were approximately 100- to 200-fold lower, and the profile of the main FAA also differed markedly from those commonly found (Asp, Glu, Pro, Ile, Leu, Phe, and Lys; Resmini et al., 1988; Gobetti et al., 1999; Di Cagno et al., 2003). Several concomitant causes might be responsible for the low extent of secondary proteolysis compared with other Italian semihard varieties: 1) the absence of primary starters; 2) in part, the unusual composition of the cheese microbiota; and 3) the nonconventional conditions of ripening.

For most of the volatile components, Vento d'Estate and Casciotta di Urbino were the richest and poorest cheeses, respectively. These 2 cheeses differed mainly in technology (type of milk, rennet, curd held before salting, and time of ripening), endogenous microbiota, and plant materials (walnut leaves or hay) used during ripening. Esters were the most numerous chemical class. Other Italian, Spanish, and Portuguese (Moio and Addeo, 1998; Di Cagno et al., 2003; Fernández-García et al., 2004; Pinho et al., 2004) semihard and hard cheese varieties also contained esters as the main volatile compounds. Although comparison with these cheeses might be difficult because of the use of different extraction techniques, the ester profiles of Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso cheeses seemed to be more complex. Esters containing few carbon atoms contribute in a synergistic way to the fruity aroma of cheese because they have a perception threshold concentration 10-fold lower than their alcohol precursors (Preininger and Grosch, 1994). In particular, ethyl-hexanoate, which has a distinct odor of pineapple, and ethyl-butanoate were identified in Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso. Such compounds typically increase during ripening, mainly as a consequence of the microbial activity (Mariaca et al., 2001; Di Cagno et al., 2003). Methyl-hexanoate, which significantly ($P < 0.001$) differentiated Vento d'Estate, was also found in the vegetable species present in hay (Klesk et al., 2004). As usually found for "Grana" (Barbieri et al., 1994; Moio and Addeo, 1998) and semihard ewes' milk cheeses (Villasenor et al., 2000; Di Cagno et al., 2003), 2-alkanones with an odd number of carbon atoms were the main ketones in the 4 Italian cheeses. 3-Hydroxy-2-butanone, which is considered very important because of its low perception threshold (~ 0.12 mg/kg), was found in Casciotta di Urbino only. It is usually produced by reduction of diacetyl (2,3-butanedione) synthesized from pyruvate, lactose, or citrate by lactococci (Crow, 1990), which were the main microbial group found in Casciotta di Urbino. 2,3-Butanedione, also found at the highest level in this cheese, is characteristic of less matured cheeses (Buchin et al., 1998). 5-Hepten-2-one, which was found only in Vento d'Estate cheese, is typically identified in hay species (*Aphidius avenae*; Liu et al., 2001). The low redox potential in cheese, which was probably strengthened under the nonconventional conditions of ripening, favors the reduction of aldehydes and ketones to primary and secondary alcohols (Molimard and Spinnler, 1996). Primary, secondary, and branched-chain alcohols were found at high levels in Barricato San Martino, Vento d'Estate, and Ubriaco di Raboso cheeses. Secondary alcohols may be derived by the reduction of methyl ketones through the reductase activities of cheese en-

dogenous lactic acid bacteria (Molimard and Spinnler, 1996). Methyl-branched alcohols may derive through reduction of aldehydes formed from AA via Strecker degradation (e.g., 3-methyl-1-butanol from Leu; Jollivet et al., 1994). Nevertheless, alcohols such as propanol, 1-hexanol, 2-butanol, and 3-methyl-1-butanol, present especially in Ubriaco di Raboso cheese, were identified in wine by-products (Silva et al., 1996). 3-Octanol, which was found only in Barricato San Martino cheese, is one of the major alcohol components of thyme and rosemary (Ntezurubanza et al., 1985; Díaz-Maroto et al., 2005). The level of aldehydes in Barricato San Martino and Vento d'Estate cheeses (4 months of ripening) was lower than the levels of other chemical classes. This was in agreement with the profile of several other Italian cows' and ewes' semihard and hard cheese varieties (Moio and Addeo, 1998; Di Cagno et al., 2003). Usually, the low level of aldehydes, as unstable compounds that are reduced to alcohols or oxidized to acids, indicate an optimal maturation (Moio and Addeo, 1998; Carbonell et al., 2002). In contrast, the level of aldehydes in Casciotta di Urbino was rather high; in particular, hexanal, which is released from walnut leaves (Buttery et al., 2000), distinguished this cheese. 3-Methyl-butanol and 2-methyl-butanol, which are mainly found in Barricato San Martino cheese, are typically released from rosemary (Estevez et al., 2005) present in the herbal mixture used to ripen it. Dimethyl sulfide, carbon sulfide, dimethyl disulfide, and dimethyl trisulfide were the principal sulfur compounds found. These compounds were probably derived from the oxidation of metanethiol synthesized from breakdown of the sulfur-containing AA by microbial enzymes. Those compounds are considered essential for the characteristic aroma of cheeses such as Cheddar and Emmental, but some authors (Izco and Torre 2000) have speculated that they are not particularly important for the aroma of other cheeses. Overall, the presence of terpenes in cheese is appreciated, and these are not related to the ripening process but to the cows' diet. Vento d'Estate was particularly rich in terpenes. Tricyclene, camphene, β -myrcene, and 1,8-cineole identified from *Tanacetum vulgare* (Keskitalo et al., 2001), δ -2-carene and δ -3-carene from *Pimpinella anisum* (Lee, 2004), α - and β -terpinene from an alfalfa grass (Estell et al., 2005), and *p*-cymene and *cis*-ocimene from *Sambucus nigra* (Jorgensen et al., 2000) were all terpenes that might have been released from the hay used to ripen the cheese.

Lipolysis of the 4 Italian cheeses mainly depended on the use of rennet paste. Except for Casciotta di Urbino, all the cheeses were manufactured using rennet paste. This contains the pregastric esterase that preferentially hydrolyzes fatty acids esterified at the *sn*-3

position of glycerol (Woo and Lindsay, 1984), where most of the short-chain fatty acids are located. The concentration of C₂ to C₆ volatile FFA, and especially of butyric and caproic acids, was the highest in Vento d'Estate. These 2 FFA are mainly the end-products of esterase-lipase activities, which agreed with the highest level of esters found in this cheese. Acetic acid, found at the highest concentration in Casciotta di Urbino cheese, was shown to be present in walnut leaves (Buttery et al., 2000).

To the best of our knowledge, this is the first study on the characterization of semihard cheeses ripened under nonconventional conditions. Both technology traits and the use of different plant materials during ripening determined the individual characteristics of the cheeses. In part, ripening under plant materials seemed to influence the composition of the cheese endogenous microbiota, and especially the release of several volatile compounds during ripening.

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