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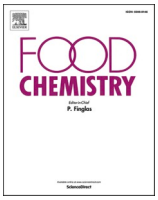

Volatile compounds, gamma-glutamyl-peptides and free amino acids as biomarkers of long-ripened protected designation of origin Coppa Piacentina

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ABSTRACT

Coppa Piacentina is an Italian protected designation of origin (PDO) dry-cured product obtained from the muscle of pork neck and ripened for at least six months. Metabolomics- and volatilomics-based strategies, combined with a chemical characterization of free amino acids were applied to identify biomarkers of long-ripened Coppa Piacentina PDO. Long ripening induced a significantly increase of total free amino acids, mainly represented by glutamic acid, involved in the umami taste perception. Untargeted metabolomics, performed using UHPLC-HRMS, allowed to identify 32 putative gamma-glutamyl-peptides, known as main contributors to the kokumi taste. Unsupervised and supervised multivariate statistics observed a clear modification of these peptides over the ripening, with gamma-glutamyl-peptides which significantly increased in long-ripened samples. A volatilomics-based strategy, conducted with GCxGC-MS, was then performed, and 93 different compounds were identified, with aldehyde and ketones deriving from the lipid auto-oxidation which increased according to ripening.

1. Introduction

Dry-cured meats are produced based on traditional practices and by applying a wide variety of processing conditions (Toldrá, 2006). In Europe, several PDO (protected designation of origin) dry-cured products obtained from pork have been established, with specific regulations which drive their production. Coppa Piacentina is a PDO dry-cured product obtained from the muscle of pork neck and can only be produced by factories within the Salami Piacentini PDO Consortium. This typical Italian product acquired the PDO status according to the specific regulation (EC) n. 1263/96, and its production must be located in the province of Piacenza (Italy). Briefly, based on the Coppa Piacentina PDO disciplinary, the raw muscle of neck was mechanically treated in order to remove residual blood, spiced up with salts and other flavoring compounds and then refrigerated. Then, the product is coated in a natural casing, securely tied, left to dry at room temperature, and ripened within a relative humidity of 70–90 % and a range temperature of 10–20 °C. The entire process, from salting to dry ripening, spans a minimum of six months.

Ripening represents a key step for the generation of aroma and taste attributes which characterized the final dry-cured products (Seong et al., 2015). During this step, several variable conditions, such as time, temperature and relative humidity, could change as a function of the final characteristics of the product. The flavor of dry-cured products, Coppa Piacentina PDO included, is based on a pool of different volatile and non-volatile compounds which are produced also during the ripening step (Sforza et al., 2006). Non-volatile molecules include peptides and free amino acids, which are released from the muscle proteins by the action of endopeptidases, mainly calpains and cathepsins, and exopeptidases (Pearson et al., 1983). The pool of these proteinaceous compounds directly contributes to the final taste, and indirectly to certain aroma compounds (Toldrá, 2006). Recently, Rutigliano et al. investigated the proteolysis which occurs in Coppa Piacentina PDO during 8 months of ripening, determining a significant enrichment in free amino acids in the first 5 months of ripening (Rutigliano et al., 2023). It is well known that an excessive amount of free amino acids

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provides unpleasant bitter and sour taste to dry-cured meat products (Virgili et al., 1998). In addition, free amino acids are also precursors of other compounds with volatile characteristics, such as aldehydes, ketones and alcohols, which are produced during the Strecker degradations via chemical modifications and hydrolysis reactions (Zhou et al., 2020). Volatile compounds could also be produced during lipolysis. Lipolysis occurs during the ripening of dry-cured meat products, inducing an increase in the free fatty acids amount due to the degradation of phospholipids (Toldrá, 2006). Then, unsaturated fatty acids are further oxidized to volatile compounds, leading to the formation of aliphatic hydrocarbons, alcohols, aldehydes and ketones (Ba et al., 2012).

In our previous work, several chemical changes induced by oxidation reactions have been already investigated and determined as relevant in the building of the final sensory profile of Coppa Piacentina PDO (Rocchetti et al., 2023b). Starting from this point, in the present work we deeply investigated for the first time volatile and non-volatile marker compounds of long-ripened Coppa Piacentina PDO. To this aim, foodomics approaches have been applied as powerful tool for understanding and depicting the changes in volatile compounds, glutathione and gamma-glutamyl-peptides. The application of untargeted foodomics, based on both metabolomics- and volatilomics-based strategies, combined with a chemical characterization of free amino acids was performed for identifying biomarkers of long-ripening samples and investigating the changes occurring during the ripening of dry-cured meat.

2. Materials and methods

2.1. Materials

AccQ-Fluor reagent kit and amino acid standard mix were obtained from Waters (Milford, MA, U.S.A). All the other solvents, standards, salts, acids and bases were of analytical grade and purchased from Sigma-Aldrich (Milan, Italy).

2.2. Samples collection

The samples were provided by a salami factory belonging to the "Salami Piacentini PDO Consortium" and were composed by 5 biological replicates of fresh Coppa Piacentina samples having undergone to 0 (raw meat), 60, 90, 180 and 240 days of ripening (total 25 samples). The samples were produced according to the guidelines reported in the European Union regulation of PDO foodstuffs as "Coppa Piacentina" (Commission Regulation EU, 1996). In particular, biological replicates were obtained from five distinct swine of the Duroc x (Landrace x Large White) breed. The swine were fed with a mixture of cereals and soybean meal in proportions complying with the Coppa Piacentina PDO disciplinary. The T0 samples (raw meat) weighted 3300 ± 25 g and presented the 38 % of dry matter (DM, determined based on ISO 1442:1997). The T60 (DM 52 %), T90 (DM 52 %), T180 (DM 57 %), and T240 (DM 66 %) samples weighted 2010 ± 14 g, on average. The samples were received under vacuum conditions at 4 °C, minced and stored at −20 °C before each analysis.

2.3. Free amino acids analysis

In this work, 2.5 g of sample were homogenised with 25 mL of HCl 0.01 N with a homogenizer (Ultra Turrax IK149 T50 digital, IKA, Staufen, Germany) for 2 min at a speed of 22000 rpm and maintained in agitation at room temperature for 2 h. The samples were centrifuged (6000 × g) at 4 °C for 20 min and the supernatant was brought to 50 mL with deionized water. Then, 900 μL of samples were mixed with 10 μL of norleucine (30 mg/mL in HCl 0.1 M), used as internal standard. A calibration curve for amino acid quantification was prepared by mixing 40 μL of Norleucine (2.5 mM in HCl 0.1 M), 40 μL of amino acids standard mix (2.5 mM) and 40 μL of hydroxyproline, asparagine, tryptophan and glutamine (2.5 mM in HCl 0.1 M). Derivatization of samples and standard solutions was performed with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) according to the method described by Luparelli et al. (2023). The derivatised samples were injected in the HPLC/ESI-MS system consisting in a Vanquish pump and autosampler, and a TSQ Fortis triple-quadrupole mass spectrometer (Thermo-Fisher Scientific, San Jose, CA, USA). The separation was performed with a XBridge BEH C18 column (2.5 μm, 3.0x75 mm; Waters). The mobile phase was composed by H_2O + 0.2 % CH_3CN + 0.1 % HCOOH (eluent A) and CH_3CN + 0.1 % HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100 % A for 1.8 min, from 100 % A to 50 % A by linear gradient in 11.4 min and 0.8 min at 50 % A plus washing step at 0 % A (100 % B) and reconditioning. Flow rate was set at 0.2 mL/min, injection volume 5 μL and sample temperature 19 °C. Detection was performed by using Waters SQ mass spectrometer with the following conditions: ESI source in positive ionisation mode, source voltage 4.5 kV, source temperature 270 °C, desolvation temperature 200 °C. The acquisition was performed in SIM mode.

2.4. Determination of glutathione and gamma-glutamyl-peptides by UHPLC-HRMS

The semi-quantitative analysis of glutathione and the gamma-glutamyl-peptides was done using a Q Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) coupled to a Vanquish ultra-high-pressure liquid chromatography (UHPLC) pump. A hydroalcoholic solution (80 % methanol) acidified with 0.1 % formic acid was used to extract 1 g of each Coppa Piacentina PDO sample. The extraction was promoted by ultrasounds, as reported previously (Rocchetti et al., 2023a). Following a centrifugation step (6000 × g) for 15 min at 4 °C, the extracts were filtered in UHPLC vials through 0.22 μm cellulose syringe filters and then analyzed by a hyphenated UHPLC-Orbitrap mass spectrometry system.

The mobile phases consisted in water and acetonitrile (both UPLC-MS grade, from Sigma–Aldrich, Milan, Italy), considering gradient elution (6–94 % acetonitrile in 35 min) and 0.1 % formic acid as phase modifier. The column selected for the chromatographic separation was an ACQUITY UPLC Waters BEH C18 (2.1 × 100 mm, 1.7 μm). More details regarding the HRMS conditions are reported elsewhere (Rocchetti et al., 2023a). The analysis was done combining a full scan analysis (*m/z* 80–1200) under positive ionization conditions (mass resolution of 70,000 at *m/z* 200) with a data-dependent (Top N ions = 3) MS/MS mode (mass resolution of 17,500 at *m/z* 200). A flow rate of 200 μL/min and an injection volume of 6 μL were considered. The fragmentation of the Top-N ions was achieved using typical collisional energies of 10, 20, 40 eV. The collected instrumental data were processed using the software MS-DIAL (version 4.90) for automatic peak finding, LOWESS normalization, and annotation that was achieved through a spectral matching against the comprehensive FooDB database (https://www.foodb.ca, access date: 15 June 2023). The identification step of the different metabolites was based on mass accuracy, isotopic pattern, and spectral matching, then calculating a total identification score (cut-off > 80 %) and considering a level 2 of confidence in annotation, according to the comprehensive guidelines described by Kodra et al. (2022). Finally, glutathione (GSH) together with all the other peptides identified were grouped and semi-quantified according to a hydroalcoholic standard solution of glutathione (Merck KGaA, Darmstadt, Germany) analyzed under the same instrumental conditions. The results were finally expressed as mg equivalents (Eq.)/100 g of samples for each ripening time (n = 5).

2.5. SPME bidimensional (GC X GC)-MS volatile analysis

Two grams of minced sample were transferred into a 20-mL

headspace vial. The analysis was performed with a GCxGC-MS system consisted of a Shimadzu GCMS-TQ8040 NX (Shimadzu, Kyoto, Japan) equipped with a flow modulator set at a modulation time of 4 s for all experiments. Samples were prepared for gas chromatography by solid-phase microextraction (SPME) using a carbon wide range/PDMS fibre (Shimadzu, Kyoto, Japan). Before extraction, the samples were equilibrated at 40 °C for 20 min under agitation. The headspace was sampled at 40 °C for 20 min and desorption performed into the GC injection port at 260 °C for 5 min. The following column set configuration was adopted: a 1D SLB-5 ms fused silica capillary column (Supelco, 20 m × 0.18 mm x 0.18 μm film thickness) coupled to a 2D SupelcoWAX (Supelco, 5 m × 0.32 mm dc, and df 0.25 μm film thickness). The oven temperature was set at 40 °C for 2 min. Then, the temperature was increased to 220 °C at 6 °C/min. Mass spectra were acquired using a *m/z* range of 50–650. The Shimadzu LabSolutions - Post Run software (Shimadzu, Kyoto, Japan) was used to process the two-dimensional chromatographic data, eliminate noise peaks and identify peaks with signal-to-noise ratios exceeding 100. Only those peaks identified in all the five biological replicates were considered for the further elaboration. Peak identification was performed by the mass spectra matching with the NIST20 database. In addition, the Linear Retention Index (LRI) was calculated after running a series of alkanes (C7-C20, Sigma Aldrich) on the same equipment and under the same chromatographic conditions of the sample and compared to the literature values (NIST Chemistry WebBook). The compounds were considered identified when their mass spectra matched at least 80 % those available in the computerized library (NIST20 database) and the LRI had less than 30 units of difference to those reported in the literature for columns of the same polarity (NIST Chemistry WebBook). The compounds were considered tentatively identified when the identification was based only in the mass spectrum match with computerized library (at least 80 % of similarity) (Dias et al., 2021). The relative abundance of compounds was expressed as the logarithm base 10 of the total integrated area of each compound divided by the fresh weight of the sample.

2.6. Statistical analysis

All the samples of Coppa Piacentina were singularly analysed (total 25 samples, 5 biological replicates per ripening time) for the free amino acids and volatilomics analyses. For the metabolomic analysis three technical replicates were performed for each biological sample (total 75 samples, 3 technical replicates for each set of 5 biological replicates per ripening time). Data were expressed as the mean ± standard deviation. Statistical analysis of the amino acid results was performed by using SPSS version 26.0 (SPSS Inc., Chicago, IL, USA). The data were subjected to ANOVA with Tukey's post hoc test to determine the differences between samples. The results from the volatilomics and Orbitrap-MS analysis were analysed by MetaboAnalyst 5.0 (Pang et al., 2021). Imputation was performed for missing value which were then substituted by LoDs (1/5 of the minimum positive value of each variable). Furthermore, the data were normalized by median, Log transformed and then auto-scaled. The transformed data were then subjected to principal component analysis (PCA) followed by k-means cluster analysis and orthogonal partial least squares - discriminant analysis (OPLS -DA), in order to identify similarities and differentiation among samples. A variables importance in projection (VIP) approach was used to rank the prediction ability of each marker peptide and volatile compound during the ripening time, selecting as minimum cut-off a value > 1.

3. Results and discussion

3.1. Changes of free amino acids during Coppa Piacentina PDO ripening process

The free amino acid concentration levels in raw pork neck and in the ripened samples have been evaluated and reported in Table S1 of supplementary material.

The content of total free amino acids ranged from 535.61 mg/100 g on DM in the raw pork neck (T0) to 1812.99 mg/100 g on DM after 240 days of ripening. In particular, a significant increase ($p < 0.01$) in the amount of total free amino acids was identified from T0 to T180, while no significant increase was identified from 180 days and 240 days. A similar trend was also determined for the majority of amino acids, except for threonine, asparagine, phenylalanine and arginine, where no significant differences were identified according to the ripening time. On the contrary, the amount of free glutamine decreased from the starting raw material to the final product after 240 days of ripening, moving from 79.63 mg/100 g to 31.80 mg/100 g on DM. Among the different amino acids, arginine was detected as the most abundant amino acid in the raw pork neck (105.11 mg/100 g on DM), followed by glutamine, phenylalanine (48.25 mg/100 g on DM) and tyrosine (47.12 mg/100 g on DM). After 240 days of ripening, the free amino acid profile changed. In fact, glutamic acid and lysine became the most abundant free amino acids, with concentration of about 257.46 mg/100 g and 235.50/100 g on DM, respectively. High concentrations also characterized alanine (168.47 mg/100 g on DM), leucine (122.14 mg/100 g on DM) and aspartic acid (10.11 mg/100 g on DM).

The variation in free amino acids is strictly related to the physico-chemical changes, microbial and enzymatic dynamics which occur during the ripening of dry-cured meat products. In particular, proteolysis performed by the action of endogenous muscle peptidases has already demonstrated responsible for the degradation of muscle proteins into peptides and free amino acids (Mora et al., 2013). The results here reported were in line with the ones recently published by Rutigliano et al. (Rutigliano et al., 2023), who analysed the profile in free amino acids of Coppa Piacentina samples collected before and after 200 and 320 days of ripening. In particular, a significant increase in free amino acids in both external and internal parts of Coppa Piacentina samples was identified according to the ripening period, reaching the maximum amount of 1200 mg/100 g of total free amino acids after 5 months of ripening (expressed on wet weight). Among the different amino acids, they determined glutamic acid, glutamine, lysine and alanine as the most abundant ones in the ripened samples, in agreement with the results presented here. In addition, the profile of free amino acids at the end of ripening was also similar to those characterised in other dry-cured products, as Chinese dry-cured lamb ham, Portuguese and Iberian dry-cured ham (Alfaia et al., 2004; Jurado et al., 2007; Luo et al., 2021).

It has been observed a different susceptibility of each amino acid to the proteolysis, with aspartic acid and glutamic acids which presented the highest increase percentages from the raw meat (T0) to the last day (T 240) of ripening. These two amino acids, which represented more than the 20 % of the total amount of free amino acids released in long-ripened Coppa Piacentina, are attributed to umami tastes of dry-cured meat products (Sforza et al., 2006). Furthermore, in the present work, the analysis of samples collected during the first stages of ripening (after 60 and 90 days) allowed to better understand and investigate if the proteolysis and the liberation of free amino acids mainly occurred in the first or last part of ripening. In Fig. 1, it was reported the kinetic changes of total free amino acids during the ripening of Coppa Piacentina samples.

From the day 90 to 180, it was detected the highest rate of total free amino acids increase (slope of 10.58), suggesting that proteases are mainly involved in the later stages of protein degradation, as also reported by Toldrà et al. in dry-cured ham (Toldrá et al., 2000). During the ripening period 180—240 days the rate decreased (slope 0.71), probably due to the reduced enzyme activity as well as to further degradation reactions to any other compounds such as volatile molecules.

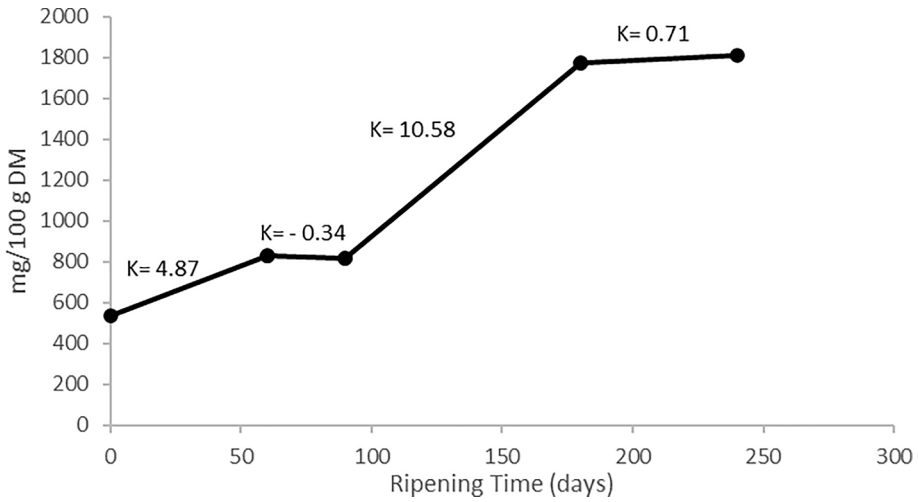

Fig. 1. Kinetic of total free amino acid released during the ripening of Coppa Piacentina PDO. Results are reported as the mean of the five different replicates (n = 5). "K value" represents the slope of the linear equation between two different time points.

3.2. Changes of glutathione and gamma-glutamyl-peptides during the ripening process

The UHPLC-HRMS analysis was used to provide more insights into the evaluation of gamma-glutamyl-peptides as biomarker compounds of Coppa Piacentina samples, as related with the progress of ripening. The analysis allowed the putative identification (according to a level 2 of confidence in annotation) of 32 peptides that are reported in supplementary material (Table S2). In particular, for each peptide identified, we have reported: the total Identification score (provided by the MS-Dial software), the reference *m/z*, the adduct type, the MS1 isotopic profile, the MS/MS spectra (where available), the cumulative abundance of each peptide in the pooled QC sample, and the raw abundance values of each peptide annotated in the different Coppa Piacentina samples. Therefore, the putative metabolite annotation has been reached by using a combination of HRMS, MS/MS (where available), isotopic profile and pattern of each metabolite detected, spectral library search (against the comprehensive database FooDB), and the global ID score resulting from the sum of each main parameter (such as spectral matching and RT alignment). Also, the level 2 of confidence in annotation allowed to confirm the structural identity of eight compounds, namely *N*-gamma-L-Glutamyl-L-valine, gamma-Glutamylarginine, gamma-Glutamylproline, gamma-Glutamyl-S-methylcysteine, gamma-Glutamylserine, gamma-Glutamyltyrosine, gamma-L-glutamyltaurine, and gamma-Glutamylglutamic acid. Overall, the interest towards gamma-glutamyl-peptides in meat science is recent (Rocchetti et al., 2021; Wang et al., 2022; Yang et al., 2019); these compounds are a series of small molecular peptides that contain a γ-carboxyl group of glutamic acid at the *N*-terminus in the molecule and have been described as main contributors to the kokumi taste. Accordingly, kokumi is a sensation that promotes the palatability of food, and it has been specifically defined as a sensation giving "continuity", "mouthfulness", "thickness", and "richness" to the meat product. Starting from these background conditions, we tested the prediction ability of gamma-glutamyl-peptides during ripening by using a combination of both unsupervised and supervised statistical approaches (Fig. 2).

Firstly, the PCA score plot reported in Fig. 2A allowed to observe a clear separation between fresh *vs* ripened meat samples, with two principal components able to explain a 58.6 % of the total variability thus allowing to postulate a great ability of these peptides to explain the chemical changes observed. This trend was confirmed by inspecting the OPLS-DA score plot (Fig. 2B) revealing a goodness-of-fitting (R^2Y) > 0.8 and a goodness of prediction (Q^2) > 0.6. Additionally, the permutation testing (N = 100 random permutations) and Hotelling's T^2 test allowed to exclude model overfitting and significant outliers on the prediction model. By checking the behaviour outlined by unsupervised and supervised models it was clear that these peptides were quite modified overtime. The best biomarkers of the trends detected in Fig. 2 were then

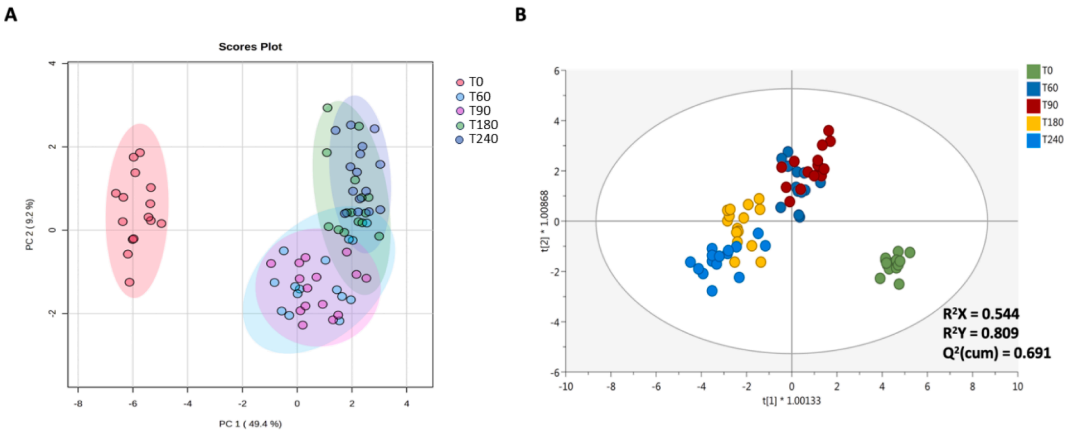

Fig. 2. A) Unsupervised PCA score plot resulting from the unsupervised k-means clustering of the different peptides identified in the Coppa Piacentina PDO samples during ripening, revealing three major groups. B) OPLS-DA score plot of the peptides identified in the long ripened (T-180 and T-240) and short ripened (T-0, T-60 and T-90) Coppa Piacentina PDO samples.

extrapolated trough a VIP selection method; overall, 17 peptides exhibited a VIP score > 1, thus resulting in a high prediction ability (Table S3). In particular, the highest VIP scores were detected for five compounds, namely gamma-Glutamyltryptophan (1.542), gamma-Glutamylthreonine (1.441), Glutathione (1.290), N,N'-Bis(gamma-glutamyl)cystine (1.264), and gamma-Glutamylglutamic acid (1.250). Overall, the kokumi threshold concentration has not yet reported in scientific literature on the discriminant peptides detected; the only available concentration, as reported by Wang et al. (2022) is reported for gamma-Glutamylglutamic acid, corresponding to 17.5 μmol/kg. Therefore, further studies combining sensory analysis and omics studies are necessary to better investigate the biochemical mechanisms.

According to scientific literature (Yang et al., 2019), gamma-glutamyl peptides are widely found in bacteria, plants and mammals. Indeed, these compounds can be considered as the products or by-products arising from the GSH cycle in organisms. Accordingly, the most important enzymes involved in these biochemical pathways are represented by γ-glutamyl cysteine synthetase (GCS), γ-glutamyl transpeptidase (GGT), and GSH synthetase. Microbes, including *Bacillus* spp. and lactic acid bacteria are reported to use the free amino acids in the raw material to synthesize the γ-glutamyl peptides via GGT and GCS catalysis (Yang et al., 2019; Wang et al., 2022). As well, the proteins in the raw materials are degraded to amino acids or such peptides by proteases. Besides, the free amino acids are used to provide γ-glutamyl acceptors for the synthesis under the action of GGT or GCS. Under our experimental conditions, we observed a significant increase of Glu during ripening followed by a significant reduction of Gln (Table S1 of supplementary material). A similar trend was noticed by Rutigliano et al. (2023), investigating the important role of endogenous proteolytic enzymes trough a proteomic approach. However, these authors did not evaluate the chemical changes of gamma-glutamyl-peptides and the quantitative changes of GSH, thus only postulating that the donor substrates of the glutamyl group could be preferentially provided by glutamine, glutathione and glutamic acid. In this regard, we reported in Table 1 the semi-quantitative changes of GSH and total gamma-glutamyl-peptides during the 240 days of ripening; it was interesting to notice that an inverse and significant ($p < 0.05$) correlation was found between, on one side, the reduction of GSH (from 53.93 to 0.63 mg/100 g on DM) and, on the other side, the increase of these kokumi-related peptides (from 8.46 up to 18.12 mg/100 g on DM).

Therefore, the HRMS findings demonstrated that, under our experimental conditions, the donor substrates of the glutamyl group were preferentially provided by glutathione (showing a sharp and significant decrease during ripening), followed by glutamic acid and glutamine. The proposed biosynthetic pathway of gamma-glutamyl-peptides in Coppa Piacentina PDO samples during ripening is provided as Fig. S1.

Overall, as also reviewed by Yang et al. (2019), we hypothesized a first reaction between glutamic acid and cysteine catalysed by the enzyme GCS to synthesize the peptide γ-glutamyl cysteine; thereafter, a reaction catalyzed by the enzyme GS (using the amino acid glycine) leads to the synthesis of the tripeptide glutathione. Finally, the last reaction is catalyzed by the enzyme GGT able to transfer an amino acid residue to the glutathione, thus leading to the synthesis of different γ-glutamyl-peptides. However, future *ad-hoc* studies are needed considering that no comprehensive microbiological and metagenomic data are available to date on Coppa Piacentina PDO samples during ripening and only few reports have been published (Busconi et al., 2014). Additionally, in this work, it's not fully possible to discern between the GSH involved in the biosynthesis of gamma-glutamyl-peptides and that directly involved in the redox changes of the meat matrix during ripening; in particular, it is known that the GSH:GSSG ratio is among the most important cellular systems to fight against the oxidative stress typical of ripened food products, as also reported in our previous work (Rocchetti et al., 2023a) when considering the modifications of the lipidic fractions.

Table 1
Semi-quantitative analysis of reduced glutathione (GSH) and total gamma-glutamyl peptides considering their modifications during ripening time (240 days) expressed on dry matter (DM). Different letters within one column identified the presence of a significative differences among samples or group of samples ($p < 0.05$). The results are expressed as mean value ± standard deviation ($n = 5$).

Ripening time (days)	Glutathione (GSH) (mg/100 g on DM)	Total gamma-glutamyl peptides (mg/100 g on DM)
T0	53.93 ± 3.88^{b}	8.46 ± 0.99^{a}
T60	1.58 ± 0.88^{a}	12.39 ± 2.08^{b}
T90	1.78 ± 0.78^{a}	12.90 ± 2.57^{b}
T180	0.42 ± 0.31^{a}	18.34 ± 2.10^{c}
T240	0.63 ± 0.42^{a}	18.12 ± 2.55^{c}

3.3. Volatilome profile of Coppa Piacentina PDO during ripening

A comprehensive list regarding the volatile compounds identified during the ripening of Coppa Piacentina PDO is reported in the supplementary material file (Table S4). In summary, the GCxGC-MS analysis allowed to identify 93 compounds, belonging to 8 different chemical families, being: alcohols, aldehydes, alkanes, alkenes, aromatic compounds, ketones, terpenes and others. Thereafter, ANOVA analysis allowed to identify significant differences ($p < 0.05$) in the abundance of these chemical families (Table 2).

Overall, aldehydes represented the chemical family which was mostly affected by the ripening process. Hexanal was the most abundant aldehyde detected after 240 days of ripening and its abundance significantly increased during the ripening process. This compound, at high concentration responsible for the rancid perception, is the main product of the oxidation of linolenic acid derivatives (Domínguez et al., 2019). This fitted with the findings obtained in our previous work, where the concentration of linolenic acid derivatives statistically decreased during the ripening of Coppa Piacentina PDO and a small rancid-like flavour was appreciated by sensory panellists in the samples ripened for 240 days (Rocchetti et al., 2023a). If linear aldehydes are known to originate from the auto-oxidation of unsaturated fatty acids, branched-chain aldehydes derive from the Strecker degradation of amino acids (Bermúdez et al., 2015). Overall, 2-metyl butanal was not identified in the raw meat, while started to be detectable from 60 days of ripening till the end. This compound is associated with the proteolysis and decarboxylation of amino acids occurred during the Streaker reaction (Virgili et al., 2007). A similar trend was observed for ketones, whose total abundance

Table 2
Changes of total abundance of volatile families identified in Coppa Piacentina PDO samples according to the ripening time. Different letters within one row identified the presence of a significative differences among samples or group of samples ($p < 0.05$). The results are expressed as the sum of the total area abundance after log10 data transformation.

Chemical family	T0	T60	T90	T180	T240
Alcohols	45.29 ± 0.99^{c}	40.61 ± 1.16^{b}	32.28 ± 0.79^{a}	46.84 ± 0.82^{c}	37.35 ± 1.05^{a}
Aldehydes	5.59 ± 0.22^{a}	41.38 ± 1.33^{b}	51.12 ± 1.83^{c}	77.06 ± 2.96^{d}	89.70 ± 3.08^{e}
Alkanes	94.86 ± 0.70^{c}	67.84 ± 1.82^{a}	68.02 ± 1.34^{a}	73.90 ± 0.68^{b}	73.95 ± 4.27^{b}
Alkenes	21.75 ± 0.62^{d}	21.47 ± 0.24^{a}	26.57 ± 0.24^{b}	26.59 ± 0.57^{c}	27.40 ± 0.37^{b}
Aromatic compounds	17.67 ± 0.48^{c}	16.72 ± 0.94^{c}	21.27 ± 0.98^{d}	10.74 ± 0.65^{b}	5.23 ± 0.13^{a}
Ketones	18.84 ± 0.44^{a}	23.56 ± 1.01^{c}	19.20 ± 0.71^{b}	29.88 ± 1.76^{d}	29.62 ± 0.82^{d}
Terpenes	9.95 ± 0.28^{a}	22.25 ± 0.79^{b}	22.56 ± 0.99^{b}	22.80 ± 1.69^{b}	22.00 ± 0.58^{b}
Other classes	40.65 ± 0.30^{c}	5.38 ± 0.31^{a}	10.77 ± 0.62^{b}	10.43 ± 0.31^{b}	5.13 ± 0.13^{a}

significantly increased from the initial to the end of ripening. Ketones can be produced during different pathways, with β-oxidation performed by microbial metabolism and the lipid oxidation which represented the mainly ones (Sirtori et al., 2020). Among the terpenes, only 3-carene and *o*-cymene were already detected in the raw meat, while the others started to become detectable during ripening. The presence of these compounds originated from the spices and herbs which are used during the production of dry-cured products (i.e., pepper, cloves, nutmeg and cinnamon) and are mixed on the surface of raw meat before the refrigerated resting step (Commission Regulation EU, 1996).

Other chemical families reported a different trend in their abundance during the ripening of Coppa Piacentina. In particular, the abundance of total alcohols significantly decreased during the ripening, reaching the lowest abundance after 240 days of ripening. A similar trend was observed also by Huan et al. in the volatile profile of Jinhua ham (Huan et al., 2005). Among the different alcohols, only 2-methyl-1-dodecanol, 2-propyl-1-heptanol, 1-hexadecanol, 1-hexanol, 2-butyl-1-octanol and 1-octen-3-ol started to appear during the ripening of samples. Their appearance could be related to the degradation of amino acids and autoxidation of fatty acids (Karpiński et al., 2020). In particular, the presence of linear alcohols such as 1-hexadecanol, 1-hexanol and 1-octen-3-ol could be from the oxidative degradation of fatty acids (Luo et al., 2021). Under the aromatic compound family are included ethylbenzene, 1,3-dimethyl-benzene, toluene and *p*-xylene. Their total abundance changed during the ripening period, reaching the maximum level after 90 days from the beginning of ripening, and then significantly decreased till the end. A similar trend was observed also by Huan et al. in the volatile profile of Jinhua ham (Huan et al., 2005). Finally, total alkanes and alkenes decreased their abundance during the ripening of samples. Unbranched alkanes and alkenes may come from the autoxidation of lipid, while, on the contrary, branched alkanes could be originated from the oxidation of branched fatty acids, which are naturally present in the animal tissue (Berdagué et al., 1991).

In order to better investigate the differences among Coppa Piacentina samples during its ripening, PCA analysis followed by k-means cluster analysis (score plot reported in Fig. 3A) was performed.

The interpreted value of PC1 and PC2 were 42.8 % and 17.1 %. The non-hierarchical K-means cluster analysis revealed that Coppa Piacentina samples were clearly divided in two distinct clusters, which differentiated the raw pork neck to the ripened samples. Furthermore, an additional separation was identified among ripened samples, which allowed the discrimination between the short-time (T-60 and T-90) and the long-time (T-180 and T-240) ripening period.

Subsequently, the supervised statistical analysis OPLS-DA was employed to quantitatively differentiate the volatile compounds which were mainly generated by a long period of ripening (Fig. 3B). The model highlighted a clear separation among the two different groups of samples, and the orthogonal latent vector was useful to better identify those compounds which mainly characterized the long-ripened Coppa Piacentina PDO samples (i.e., T-180 and T-240). The OPLS-DA model provided acceptable parameters of cross-validation, with the fit parameter (R^2Y (cum) = 0.98) and the predictive ability (Q^2 (cum) = 0.96) which clearly discriminated the two clusters. In order to gain a deeper insight into the volatilomic differences which characterized the long-ripened Coppa Piacentina samples, different volatile compounds were screened based on the VIP values that were generated by the OPLS-DA model. The cut-off criterium was set at VIP scores higher than 1, defining high prediction score. After data filtering, 23 different compounds were selected for their high discriminant potential when considering their role in the formation of volatile compounds after a long ripening period (Table 3).

These discriminating compounds included alcohols, aldehydes, alkanes and alkenes, ketones, terpenes, aromatic and other compounds. Among them, 12 compounds were up-regulated in the long-ripened samples, including 3,3-dimethyl- 2- butanone, 2-octenal, 2-decenal, 2-nonenal and pentanal which were exclusively representative of those samples ripened for 180 and 240 days. Overall, 2–3,3-dimethyl- butanone is a methyl ketone which is produced during the decarboxylation of β-keto acids or the β-oxidation of saturated fatty acids, and act as precursors in the formation of fatty flavor during the ripening of meats (Shi et al., 2019). Next, all the other compounds exclusively identified in the long-ripened samples were aldehydes, known as the major contributors to the overall flavor of fermented and ripened meats owing to their low perception thresholds (Zhang et al., 2023). These compounds originate from the auto-oxidation of unsaturated fatty acids (Bermúdez et al., 2015) and represent the third step of lipid autoxidation, after the formation of peroxides (Amaral et al., 2018), which were previously demonstrated to significantly increase during the ripening of Coppa Piacentina (Rocchetti et al., 2023b). Finally, among these VIP marker compounds, 10 were already reported in literature as key odorants responsible for the pork dry-cured meat flavour (Chen et al., 2023; Li et al., 2022; Segura-Borrego et al., 2022; Xu et al., 2022). By combining previous literature data with chemometrics, 2-heptanone, 2-heptenal, 2-nonenal, 2-octenal and octanal, up-accumulated after 180 and 240 days

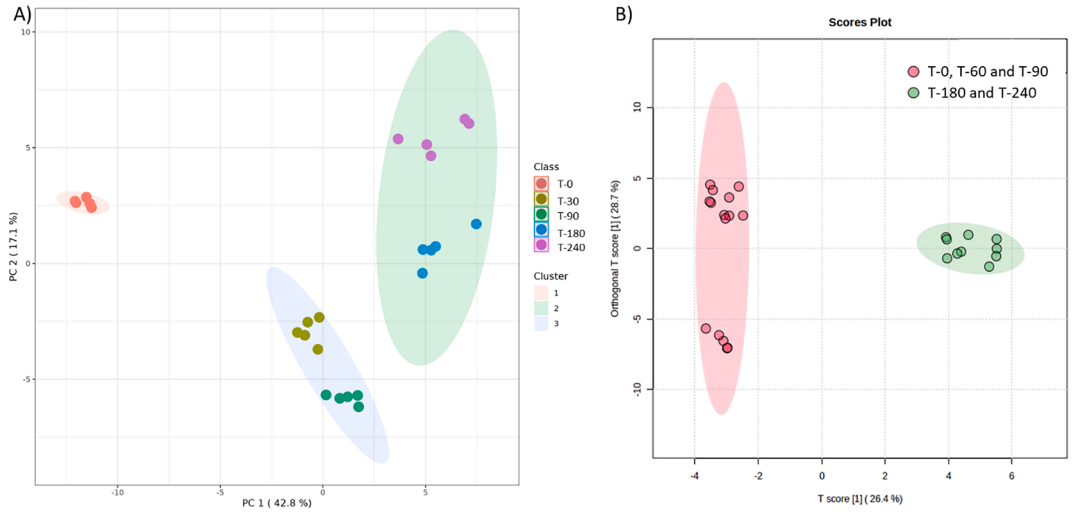

Fig. 3. A) PCA score plot resulting from the unsupervised k-means clustering of the different volatile compounds identified in the Coppa Piacentina PDO samples during ripening, revealing three major groups. B) OPLS-DA score plot of the volatile compounds identified in the long ripened (T-180 and T-240, green dots) and short ripened (T-0, T-60 and T-90, red dots) samples of Coppa Piacentina. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Significant VIP marker compounds which better discriminate the volatilomic profile of Coppa Piacentina samples subjected to a short (T0, T60, T90) and long ripening period (T180 and T240). Those compounds up-accumulated in the long-ripened samples (T180 and T240) are signed with a "X".

Volatile compound	VIP [t]	VIP [*ortho*-t]	Up-accumulation in long-ripened samples	Key odorants
1-Butanol, 3-methyl- *	1.468	0.063		Burnt, Cocoa, Floral, Malt
2-Decene, 5-methyl-, (Z)-	1.804	0.540		
2-Butanone, 3,3-dimethyl-	1.607	0.827	X	
2-Decenal, (E)-	1.688	0.717	X	
2-Heptanone *	1.134	0.958	X	Blue Cheese, Fruit, Green, Nut, Spice
2-Heptenal, (E)- *	1.461	0.578	X	Nutty
2-Nonenal, (E)- *	1.709	0.649	X	Potato peel
2-Octenal, (E)- *	1.685	0.719	X	Toasted corn
2-Octene, (E)-	1.251	0.604		
3-Carene	1.252	0.744	X	
Acetoin	1.563	0.708	X	
Allyl ethyl ether	1.100	0.863		
Benzaldehyde *	1.114	0.957		Meaty, floral
Benzene, 1,3-dimethyl-	1.314	0.794	X	
Benzeneacetaldehyde	1.060	0.700	X	
1-Tridecene	1.313	0.914	X	
Heptane, 2,2,4,6,6-pentamethyl-	1.093	0.650		
Heptanal *	1.508	0.764		Citrus, Fat, Green, Nut
Hexanal *	1.119	0.947		Green, grass
Octanal *	1.099	1.011	X	Waxy, fatty
Pentanal *	1.717	0.611		Nutty
p-Xylene	1.247	0.985		
Toluene	1.262	1.202		

*Key odorants of pork dry-cured meat products according to Chen et al. (2023), Segura-Borrego et al. (2022), Xu et al. (2022).

of ripening, can be identified as potential key odorants and volatile biomarkers of long-ripened Coppa Piacentina PDO.

4. Conclusions

In this work, the combination of untargeted and targeted analysis allowed to identify compounds may have potential as markers for depicting and discriminating long-ripened Coppa Piacentina PDO. The chemical analysis revealed the impact of ripening in the release of free amino acids, which significantly increased over time, due to the proteolytic activity of enzymes that mainly occurred from 180 days of ripening. Untargeted metabolomics provided new insight in the changes of gamma-glutamyl-peptides, non-volatile compounds contributors to the kokumi taste, whose concentrations significantly increased in the long-ripened Coppa Piacentina PDO. Finally, ripening was demonstrated to modify the volatilome profile of Coppa Piacentina PDO, with 2—3,3-dimethyl- butanone, 2-octenal, 2-decenal, 2-nonenal and pentanal which were representative of those samples ripened for 180 and 240 days. Taking all together these results, the application of foodomic strategies and the chemical characterization of free amino acids was demonstrated useful for identifying biomarkers of long-ripened Coppa Piacentina PDO. In addition, we provided new insight in the volatile and non-volatile compounds which are implied in the development of the final taste of Coppa Piacentina PDO after 6 months of ripening. Given these premises, furtherer analysis should investigate the contribution of humidity and temperature on these chemical modifications characterizing Coppa Piacentina PDO during the ripening process.

CRediT authorship contribution statement

Giulia Leni: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Data curation, Formal analysis. **Gabriele Rocchetti:** Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Data curation, Formal analysis. **Terenzio Bertuzzi:** Visualization, Methodology, Data curation. **Alessio Abate:** Formal analysis. **Alessandra Scansani:** Visualization, Investigation. **Federico Froldi:** Visualization, Investigation. **Aldo Prandini:** Visualization, Supervision, Project administration, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.138225.

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