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Oligopeptides and free amino acids in Parma hams of known cathepsin B activity

Stefano Sforza[a], Alessandro Pigazzani[a], Maurizio Motti[a], Cecilia Porta[b], Roberta Virgili[c], Gianni Galaverna[a], Arnaldo Dossena[a], Rosangela Marchelli[a,*]

[a]*Dipartimento di Chimica Organica ed Industriale, Università di Parma, Parco Area delle Scienze 17/a, I-43100, Parma, Italy*
[b]*Consorzio del Prosciutto di Parma, Via M. dell'Arpa 8/b, I-43100, Parma, Italy*
[c]*Stazione Sperimentale per l'Industria delle Conserve Alimentari, viale Tanara 31/A, I-43100, Parma, Italy*

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Abstract

Sixteen Parma hams were manufactured following regular standard procedures up to the 13th month of processing and then divided into two groups according to established levels of cathepsin B activity in fresh hams. Each group was selected in order to include the same number of hams with low or high cathepsin B activity. The two groups were further split to be kept for an additional month at two different temperatures (18 and 26 °C) and then analysed for proximate composition, free amino acids and oligopeptides (MW < 500 Da). All hams were analysed by sensory analysis (panel test) to be scored for saltiness, bitterness and the flavour of "ageing". Fresh hams with higher cathepsin B activity were shown by means of two-way ANOVA to yield dry-cured hams more proteolysed ($P < 0.01$), while the higher temperature of the final ageing period seemed to play a major role in lowering ham dryness. Bitterness was particularly well perceived in most proteolysed hams and it was significantly related to the higher amounts of lipophilic amino acids and lipophilic oligopeptides (identified by mass spectrometry), in agreement with the literature. Thus, the endopeptidase activity in fresh hams, together with the presence of specific lipophilic amino acids and oligopeptides in dry-cured hams, can be regarded as a molecular marker of bitter taste development.

1. Introduction

Parma ham is a well known dry-cured meat product, obtained from heavy pig thighs (12–14 kg), mildly salted and long aged (at least 12 months). The manufacturing of Parma ham has not changed throughout the centuries, and it is mainly based on the reduction of muscle water activity (a_w) due to sodium chloride diffusion and to the dehydration which takes place during ageing. No other additive, beside salt, is added to the raw thighs during the entire process, yielding Parma ham (Parolari, 1996).

The sensory and functional properties of the product are closely related to both meat quality and manufacturing practices (Virgili & Parolari, 1991). One of the most important processes taking place during ham ageing is the proteolysis of muscle proteins. Endogenous exo- and endopeptidase activities, together with processing parameters (salt amount, ageing temperature and length of the ageing phase), greatly influence the proteolysis degree, yielding different amounts and patterns of free amino acids (FAAs) and peptides (Pearson, Wolzak, & Gray, 1983).

Indeed, partially proteolysed meat products are known to be an important dietary source of essential amino acids. The sensory properties of free amino acids are also very well established: except for glutamic acid (which has a distinct meat broth taste known as "umami"), L-amino acids may be sweet or bitter, according to their side-chain, being perceived as bitter when bearing a lipophilic side chain (Belitz & Grosch, 1985). In a recent paper (Virgili, Schivazappa, Parolari, Bordini, & Degni, 1998), a high endopeptidase activity has been associated with a high perception of bitterness in aged hams and correlated with a high content of methionine, asparagine and isoleucine.

* Corresponding author. Tel.: +39-0521-905410; fax: +39-0521-905472.
E-mail address: marchell@unipr.it (R. Marchelli).

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On the other hand, very little is known about the small peptides generated in meat products during technological treatments. In dairy products, the beneficial properties of peptides have been well demonstrated; these include their ability to act as metal ion carriers, antioxidant, opioid-like, antihypertensive, antimicrobial or immunoenhancing effects (Andrews & Varley, 1994). However, some peptides were shown to be able to trigger an allergic reaction in sensitive subjects.

Oligopeptides are generally bitter and, in agreement with the behaviour of free amino acids, lipophilic residues increase bitterness (Belitz & Grosch, 1985). Some small peptides carrying lipophilic side-chains were found to be responsible for the bitter taste (Akamatsu, Yoshida, Nakamura, Asao, Iwamura, & Fujita, 1989): in particular, the oligopeptide fraction with MW < 1800 Da (Aristoy & Toldra, 1995). Also, the development of the typical flavour in aged hams has been associated with the peptide fraction (HansenMoller, Hinrichsen, & Jacobsen, 1997; Rodriguez-Nunez, Aristoy, & Toldrà, 1995).

In this paper, the main proteolytic products (NPN, FAA and small peptides) have been investigated in order to find correlations with the cathepsin B activity in the fresh muscle, the final ageing temperature and the sensory traits of dry-cured hams. The amino acid composition and the sequence of several small peptides were identified by HPLC–MS analysis.

2. Materials and methods

2.1. Analysis of fresh muscle

Portions (about 50 g) of *Semimembranosus* muscle in the cuscion section of fresh hams were taken for the analysis of the proteolytic enzyme (two replicate samples were analysed for each assay). Cathepsin B was determined by fluorimetric assays, as reported in the literature (Parreno, Cussò, Gil, & Sarraga, 1994), with N-CBZ-L-Arg-L-Arg-7-amido-4-methyl coumarin (N-CBZ-Arg-Arg-AMC) as substrate. Cathepsin B activity is expressed as nmol 4-methyl-aminocoumarin (AMC) $\times$ g muscle^{-1} $\times$ min^{-1}.

The pH values were measured in fresh hams (1 h and 2 h post-mortem) to verify that no PSE (pale soft exudative) or DFD (dark firm dry) hams were included in the trial.

2.2. Analysis of dry-cured hams

Two adjacent 1-cm thick slices including *Semimembranosus*, *Semitendinosus* and *Biceps femoris* (BF) muscles were taken for chemical analyses. Samples were vacuum-packaged and stored at 2 °C until analysed.

2.3. Chemical analysis

Trimmed and minced lean meat from BF muscle was analysed for the following parameters:

1. Proximate composition according to AOAC methodologies (AOAC, 1990);
2. Non-protein nitrogen (NPN), as the percent ratio between nitrogen soluble in 5% trichloroacetic acid and total nitrogen (Careri, Mangia, Barbieri, Bolzoni, Virgili, & Parolari, 1993); and
3. Free amino acids (FAAs) following a given HPLC technique (Virgili, Parolari, Schivazappa, Soresi Bordini, & Borri, 1995), by means of a double derivatisation with orthophthaldialdehyde (OPA) for primary amino acids and fluorenylmethoxycarbonyl chloride (FMOC-Cl) for proline detection.

2.4. Oligopeptide analysis

Ten grams of BF were homogenised with 88.4 ml of 0.1 M hydrochloric acid and 1.6 ml of a 0.3 mM aqueous solution of *(L,L)-phenylalanyl-phenylalanine* (Phe-Phe) as internal standard. The resulting solution was centrifuged at 10000 rpm for 20 min, filtered and added to an equal volume of 10% TCA. The supernatant solution was left at 5 °C overnight, then centrifuged and filtered. An aliquot (7 ml) of the liquid phase was extracted three times with ethyl ether and evaporated under reduced pressure. The residue was dissolved in 3 ml of an aqueous solution of 0.1% TFA (pH$\approx$3) and the pH was adjusted to 4 with 1 M KOH. The solution was filtered through a 0.45-μm filter, then filtered again through Amicon YC05 Ultrafilters with a molecular weight cut-off of 500 Da, using an Amicon Micropartition system MPS-1. The filtrate was evaporated under nitrogen and dissolved in 200 μl of an aqueous solution containing 0.2% acetonitrile and 0.1% formic acid.

The final solution was analysed by HPLC–MS with a RP-C18 column (Jupiter Phenomenex, 300 Å, 250$\times$4.6 mm) using an elution gradient: eluent A: water, 0.2% acetonitrile, 0.1% formic acid; eluent B: water/acetonitrile (65/35, 0.1% formic acid). The gradient was: isocratic 99%A (0–15 min), from 99 to 0%A (15–60 min), isocratic 0%A (60–69 min), from 0 to 99%A (69–70 min). The detector was an API 150 EX mass spectrometer equipped with a turbo ion spray interface and a single quadrupole analyser. The detector was set in the 100–700 m/z range with the following conditions: ion spray voltage 5500 V, turbo temperature 400 °C, orifice voltage 20 V, ring voltage 200 V, step 0.1 Da, dwell time 0.5 ns, pause time 5.0 ns. The full-scan chromatograms were analysed in order to determine the molecular weights of the most intense peaks. Extract ion chromatograms (XIC) for the desired molecular weights were

obtained (±0.5 u.m.a) in order to better integrate the chromatographic peaks. The amounts of peptides were semi-quantified as peptide/internal standard peak area ratios.

2.5. Sensory analysis

A panel of 10 trained members evaluated 1-mm thick slices of the hams for the three following attributes: bitter, aged and salty. The attributes were rated by numeric scales ranging from 0 (devoid of the attribute) to 9 (maximum perception). At each session, four hams were analysed and the presentation order followed a full balanced design to minimise carry over effects.

2.6. Statistical analysis

Principal Component Analysis (PCA), one- and two-way analysis of variance (ANOVA) and correlation coefficients were computed by means of the SPSS 9.0 program, using standard statistical procedures.

3. Results and discussion

3.1. Ham grouping and treatments

Several pig thighs (16) were selected with a pre-established pH (no PSE or DFD were allowed) and cathepsin B activity, and grouped according to different levels of enzymatic activity: eight thighs with high cathepsin B values (mean value 1.8 (±0.1) nmol AMC×g muscle^{-1} × min^{-1}) and eight thighs with low cathepsin B activity (mean value 1.3 (±0.1) nmol AMC×g muscle^{-1} × min^{-1}). Both groups, hereinafter referred to as HC and LC, respectively, were manufactured following the standard procedures for the production of Parma ham up to 13 months of total ageing; then, for one additional month, four hams of the HC group and four hams of the LC group were maintained at a higher temperature (HT, $T = 26$ °C), while the remaining hams were kept at the lower temperature (LT, $T = 18$ °C), generating four classes of hams (HC–HT, HC-LT, LC-HT and LC-LT).

Dry-cured hams were analysed for salt, moisture, protein content, and additionally, NPN, FAA and oligopeptides, in order to find suitable proteolysis markers. Small peptides were studied by HPLC–MS (single quadrupole analyser) in the full scan (100–700 Da) mode and by the extract ion technique (XIC).

3.2. Oligopeptide identification

The molecular weights of the most intense peaks were determined by total scan chromatograms: 17 oligopeptides (subsequently labelled with capital alphabetic letters), giving peaks with retention times ranging from 22 to 41 min, were characterised on the basis of their MW (Table 1).

Table 1
Molecular weight (MW) and retention times (t_r)[a] of the oligopeptides identified in the aged hams

Peak identification	t_r (min)	MW (Da)
A	22	188
B	23	202
C	24	188
D	25	310
E	29	222
F	30	260
G	31	260
H	33	262
I	34	294
J	35	216
K	36	230
L	36	319
M	37	319
N	37	244
O	38	244
P	39	230
Q	41	216

[a] Chromatographic conditions: RP-C18 column (Jupiter Phenomenex, 300 Å, 250×4.6 mm) using an elution gradient: eluent A: water, 0.2% acetonitrile, 0.1% formic acid; eluent B: water/acetonitrile (65/35, 0.1% formic acid). The gradient was: isocratic 99%A (0–15 min), from 99% to 0%A (15–60 min), isocratic 0%A (60–69 min), from 0% to 99%A (69–70 min).

Low molecular weight dipeptides were found to be most abundant in 14-month-old Parma ham, whereas tri-, tetra- and pentapeptides, below 500 Da, seem to be present in minor amounts. This finding may support the hypothesis that the endogenous proteolytic process during ham ageing leads to an accumulation of products of low molecular weight, as already suggested (Hansen-Moller et al., 1997). However, higher molecular weight peptides eventually formed are, with the present extraction method (10% TCA), probably coprecipitated with the proteins or filtered off by ultrafiltration.

The semi-quantitative amounts of the above oligopeptides were calculated by measuring the ratio between the area of the peptide and that of a suitable internal Phe-Phe standard, by assuming an equal relative response factor per mole. Extract ion chromatograms (XICs) for the desired molecular weights were extracted in order to better-integrate the chromatographic peaks. A full scan chromatogram of (a) a ham sample, (b) the corresponding XIC of the peptides with molecular weight 260 Da (peaks F and G) and (**c**) the XIC of Phe-Phe are shown in Fig. 1.

To identify the probable compositions of the selected oligopeptides, the corresponding mass spectra were analysed by examining the small fragmentation peaks associated with the base molecular protonated peak. Since the fragmentation of a peptide often generates the free amino acids, it was possible to identify the amino

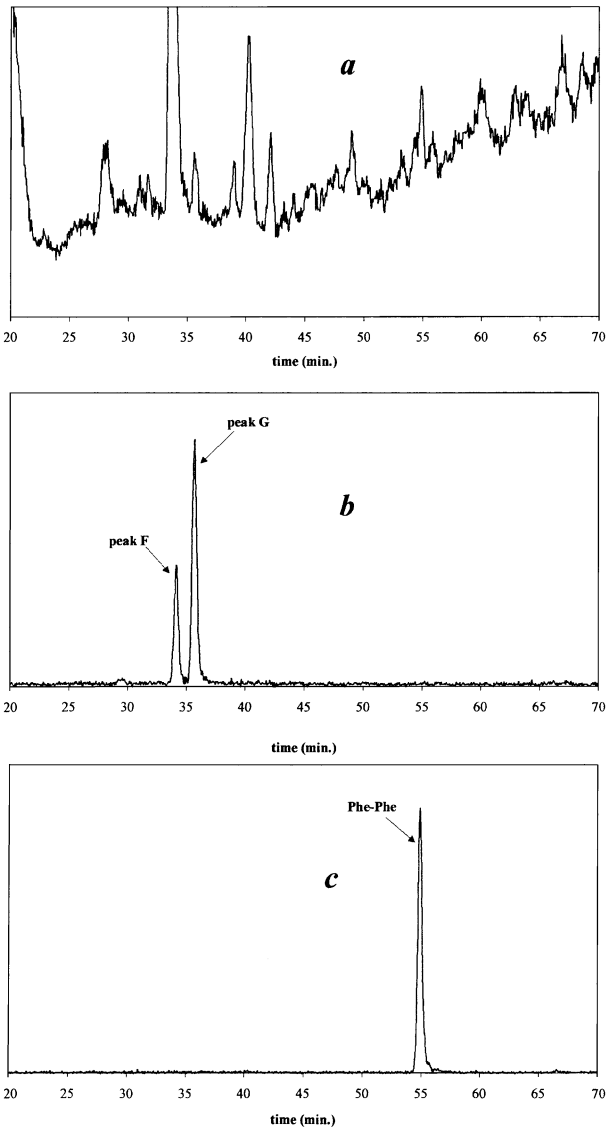

Fig. 1. Full scan chromatogram (a) of a ham sample and two extract ion chromatograms obtained from the same chromatogram corresponding to the peaks with a molecular weight of 260 Da (b, peak F and G) and to the standard Phe-Phe (c).

acid compositions of most dipeptides. Results are reported in Table 2.

3.3. Correlation of chemical parameters with cathepsin B activity and temperature treatment

Chemical parameters related to proteolysis (FAA, oligopeptides and NPN), together with the proximate compositions of hams belonging to the four groups (HC-HT, HC-LT, LC-HT and LC-LT), were compared by one-way analysis of variance. Parameters showing significant differences ($P \leqslant 0.5$) are reported in Table 3. Cathepsin B and sodium chloride content were included in Table 3 to show that ham groups differ significantly in the cathepsin activities (meeting the requirements of the experimental design), but are similar in their salt contents, allowing the inhibitory effect of sodium chloride on proteolysis to be disregarded.

All products were rather homogeneous in moisture and salt contents, as shown by the relative standard deviations, in agreement with the standard values usually found in Parma long-aged hams (Parolari, 1996; Virgili & Parolari, 1991). In contrast, the increased variability found for NPN, single FAAs and peptides could be ascribed to the different characteristics of the raw matter and to the different ageing treatments.

Significant differences between the HC-HT and the LC-LT groups were found for the amino acids alanine, leucine, lysine, phenylalanine and threonine and for a small peptide (peak A, MW = 188, aa composition Gly and Leu(Ile)). The former group was characterised by high values of NPN, single FAAs and dipeptide A.

On the other hand, the peptide M (MW = 319) increased significantly in the less-proteolysed class of hams (LC-LT), appearing to be further hydrolysed at a higher enzyme activity and ageing temperature.

No significant differences between the four classes of hams in the total amounts of peptides identified, or the total amino acid contents were observed. However, although the total FAA content decreased regularly following the order HC-HT > HC-LT > LC-HT > LC-LT (19.0 > 18.5 > 17.6 > 15.2 g/100 g protein, respectively), the amount of peptide identified showed a discontinuous trend (7.64 > 6.22 < 5.56 < 6.44 μmol/100 g muscle, respectively). Thus, the amount of oligopeptide cannot be regarded as an index of enhanced proteolytic activity, being, at the same time, products and substrates of the proteolytic activity, in contrast with the total NPN and FAAs, which are end-products.

Dry-cured ham parameters were further analysed by means of two-way analysis of variance to look for significant differences generated by interaction or addition of the main effects, cathepsin B activity and ageing temperature. In the case of NPN, the influence of cathepsin B activity was enhanced by ageing temperature (Table 3) suggesting an additive mechanism between the main effects ($F = 8.816$, significativity of $F = 0.004$), while a significant interaction between the two factors was found for the moisture content ($F = 4.683$, significativity of $F = 0.051$) of dry-cured hams. In particular, as shown by data reported in Table 3, raw hams with low cathepsin activity yielded dry-cured hams with a moisture content in agreement with the different ageing temperatures: higher temperatures gave drier hams; on the other hand, hams with high cathepsin activity were unaffected by temperature change. A tentative explanation may be the following: hams with high cathepsin activity could be more prone to fast liquid loss during the first curing steps, leading to the onset of an outer rind, preventing a regular dehydration in the following phases of processing.

Table 2
Identification of oligopeptides by HPLC–MS

Peptide	MW (Da)	Possible amino acid composition	Fragmentation peaks attributed to free amino acids	Assigned composition
A	188	AlaVal, GlyIle, GlyLeu	132 (Leu or Ile)	Gly + Leu(Ile)
B	202	AlaIle, AlaLeu, SerPro	132 (Leu or Ile)	Ala + Leu(Ile)
C	188	AlaVal, GlyIle, GlyLeu	132 (Leu or Ile)	Gly + Leu(Ile)
D	310	GluTyr	182 (Tyr)	Glu + Tyr
E	222	GlyPhe, ThrCys	166 (Phe)	Gly-Phe[a]
F	260	IleGlu, LeuGlu, AsnGln, AsnLys, GlyGlyGlnAsn, GlyGlyLys, GlyAlaAsn, GlyGlyGlyAla	132 (Leu or Ile)	Glu + Leu(Ile)
G	260	IleGlu, LeuGlu, AsnGln, AsnLys, GlyGlyGlnGln, GlyGlyLys, GlyAlaAsn, GlyGlyGlyAla	132 (Leu or Ile)	Glu + Leu(Ile)
H	262	ProPhe, IleMet, LeuMet, AspGlu	166 (Phe)	Pro-Phe[a]
I	294	IleTyr, LeuTyr, GluPhe	166 (Phe)	Glu + Phe
J	216	ProThr, ValVal	No identifiable aa peak	–
K	230	ProAsp, ValIle, ValLeu	118 (Val	Leu(Ile) + Val
L	319	AspTrp, GlyProPhe, GlyIleMet, GlyLeuMet, GlyAspGlu, AlaValMet, AlaThrGlu, SerValAsp, SerThrIle, SerThrLeu, ProThrCys, ValValCys, ValThrThr	No identifiable aa peak	–
M	319	AspTrp, GlyProPhe, GlyIleMet, GlyLeuMet, GlyAspGlu, AlaValMet, AlaThrGlu, SerValAsp, SerThrIle, SerThrLeu, ProThrCys, ValValCys, ValThrThr	No identifiable aa peak	–
N	244	ProGlu, IleIle, IleLeu, LeuLeu	132 (Leu or Ile)	Leu(Ile) + Leu(Ile)
O	244	ProGlu, IleIle, IleLeu, LeuLeu	132 (Leu or Ile)	Leu(Ile) + Leu(Ile)
P	230	ProAsp, ValIle, ValLeu	No identifiable aa peak	–
Q	216	ProThr, ValVal	No identifiable aa peak	–

[a] Assignments of Gly-Phe and Pro-Phe were made by comparison with authentic samples.

Table 3
Proximate composition data (g/100 g muscle), FAA (g AA/100 g protein) and peptides identified (μmol peptide/100 g muscle = (Area peptide)/(Area Phe-Phe) × (μmol Phe-Phe/100 g muscle) for the four groups of Parma hams[a]

	HC-HT	HC-LT	LC-HT	LC-LT	Overall	%RSV
Cathepsin B[b]	1.83a	1.78a	1.28b	1.34b	1.56±0.2	18.6
Sodium chloride	5.5	5.5	5.6	5.2	5.4±0.4	7.41
Moisture	63.0a,b	63.0a,b	61.8a	63.7b	62.9±1.0	1.58
Alanine	1.59a	1.52a	1.32a,b	1.20b	1.41±0.23	16.3
Leucine	1.39a	1.32a,b	1.21a,b	1.04b	1.24±0.22	17.7
Lysine	1.92a	1.83a,b	1.71a,b	1.53b	1.75±0.27	15.4
Phenylalanine	0.96a	0.93a,b	0.83a,b	0.73b	0.86±0.16	18.6
Threonine	0.94a	0.84a,b	0.81a,b	0.74b	0.83±0.11	13.2
Peptide A	4.09a	2.22b	2.82a,b	2.36a,b	2.87±1.32	46.0
Peptide M	0.51a	0.69a,b	0.56a	0.90b	0.66±0.24	36.4
NPN[c]	33.0a,b	31.7b	29.7b,c	28.1c	30.6±2.5	8.2

[a] Mean values for each group and for all hams (with relative standard deviations) are listed. Numbers regarding different groups with different letters are significantly different ($P<0.05$), as shown by LSD test of one-way analysis of variance.
[b] As nmol AMC × min^{-1} × g^{-1} muscle.
[c] NPN is expressed as percent ratio between nitrogen soluble in 5% trichloroacetic acid to total nitrogen.

3.4. Correlations of FAAs and oligopeptides with the sensory properties of the aged hams

Several small peptides have been reported to be bitter (Belitz & Grosch, 1985) and bitterness has often been related to the hydrophobic residues present in the peptide chain (Akamatsu et al., 1989; Belitz & Grosch, 1985; Ishibashi, Ono, Kato, Shigenaga, Shinoda, Okai, & Fukui, 1988). In the present work, a good correlation between the bitter taste and the proteolysis index ($r^2=0.49$, $P<0.01$) was observed. This finding confirms the role played by the proteolytic products in human taste.

The total amount of FAAs increased, along with the NPN value (linear regression: $r=0.86$), since amino acids are the final catabolites of the protein cleavage; however, by correlating every single FAA amount with bitterness, the highest correlation coefficients were obtained for the lipophilic ones (Table 4).

Table 4
Correlation coefficients and significance between amino acids (mg AA/100 g protein) and selected oligopeptides (μmol peptide/100 g muscle) with sensorial properties (panel test evaluation)

FAAs and peptides	Bitter	Salty	Aged
Gln	+ 0.74**		
Phe	+ 0.73**		
Ile	+ 0.71**		
Leu	+ 0.71**		
Asn	+ 0.71**		
Gly	+ 0.70**		
Glu	+ 0.68**		
Lys	+ 0.68**		
Val	+ 0.67**		
Ala	+ 0.64**		
Arg	+ 0.63**		
Ser	+ 0.58*		
Thr	+ 0.57*		
Tyr	+ 0.54*		
Pro		+ 0.60*	
Total F.A.A.	+ 0.62**		
E (Gly-Phe)	+0.76**		
C (Gly + Leu(Ile))	+0.55*		
N (Leu(Ile) + Leu(Ile))	+0.54*		
G (Glu + Leu(Ile))	+0.54*		−0.61*
K (Leu(Ile) + Val)	−0.51*		
D (Glu + Tyr)			+0.63**
J (MW = 216)		+0.63*	
NPN	+ 0.70**		

*$P < 0.05$.
**$P < 0.01$.

In particular, three lipophilic amino acids, i.e. phenylalanine, isoleucine and leucine, well known for their low bitterness threshold values (Belitz & Grosch, 1985), showed a good response. In addition, glutamine and asparagine, which are generally considered as carriers of neutral taste, appear in Table 4 with a highly positive correlation (Belitz & Grosch, 1985).

Apart from amino acids, several small peptides appeared to be able to impart, at least partially, a bitter taste to the aged hams. In particular, dipeptides carrying one lipophilic amino acid (Leu, Ile or Phe) are mostly related to bitterness, in agreement with the literature (Maehashi, Matsuzaki, Yamamoto, & Udaka, 1999). Accordingly, Gly-Phe (the most related in our samples) is one of the most bitter peptides, with a bitterness intensity of 0.83, relative to caffeine (Ishibashi, Sadamori, Yamamoto, Kanehisa, Kouge, Kikuchi, Okai, & Fukui, 1987). Surprisingly, the dipeptide K (Leu + Val or Ile + Val) appeared to be negatively correlated with the bitter perception.

The peptide D (Glu + Tyr) was found to be positively related to the aged taste, which is a major accepted trait in dry-cured ham. This finding is in agreement with the role postulated for short peptides containing at least one glutamic acid, reported to be responsible for the onset of the so-called "umami taste", associated with meat products (Maehashi et al., 1999). However, this property is still controversial (Van den Oord & Van Wassenar, 1997). Peptide G (Glu + Leu or Glu + Ile) is positively related to bitter and negatively to the aged taste in agreement with the negative relationship between aged and bitter (linear regression, $r = -0.69$; $P < 0.01$). Finally, a good correlation with saltiness was found for the amino acid proline and for peptide J (Val + Val or Pro + Thr).

4. Conclusions

In dry-cured Parma hams the sensory properties, and particularly bitterness, are related to a well defined proteolytic patterns. According to the present results, bitterness increased when a high level of endopeptidase activity was present in the raw pig thighs, yielding a high degree of proteolysis at the end of the ageing time. The high proteolysis generated a greater amount of lipophilic amino acids and oligopeptides, favouring the perception of the unpleasant bitter aftertaste in the final product. FAA analysis and the determination of small peptides, by HPLC–MS, permitted the identification of molecules involved in the development of bitterness in dry-cured hams. The lipophilic amino acids leucine, isoleucine and phenylalanine, and the dipeptides Gly-Phe, Gly + Leu(Ile) and Leu(Ile)-Leu(Ile), were found to be strongly related to the bitter taste. Therefore, they can be considered as "molecular markers" of this undesired taste.

Finally, the significant relationship between the sensory properties and the chemical composition provides new tools for assessing the nutritional and organoleptic qualities of the aged hams.

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