Evaluation of Peptides Generated in Italian-Style Dry-Cured Ham during Processing

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Low molecular weight nitrogenous compounds (M_w < 3000 Da) were measured from 29 Parma hams at different production stages (i.e., 3, 25, 125, 211, 365, and 485 days after slaughter). Precolumn derivatization with AQC and reversed phase HPLC of the samples revealed that the content of low molecular weight nitrogenous compounds increased during processing of the hams. Principal component analysis of the non-amino acid peaks showed five groupings of the samples corresponding to the different production stages. In the first 365 days of processing five different peptide peaks are formed. Post ripening lead to the formation of six additional peptide peaks. Partial least squares modeling of the major peptide peaks and sensory data show that the formation of peptides is highly correlated to the flavor formation of Parma ham.

Keywords: *HPLC; peptides; multivariate statistics; cured ham*

INTRODUCTION

Traditional dry-cured ham from southern Europe is considered a high-quality product regarding the flavor. The development of the desired mature flavor requires a long processing time and is costly (Toldra et al., 1993). Intense proteolysis has been observed during the dry-curing process (Molina and Toldra, 1992). Proteolytic enzymes of the meat are active during ripening (Toldra et al., 1993), and a decrease in peptide size is observed (Rodriguez-Nunez et al., 1995). The proteolytic enzymes are also responsible for an increase of free amino acids during processing (Aristoy and Toldra, 1991).

The aroma is formed by complex chemical reactions, and a microbial succession is observed during ripening, coinciding with changes in the profile of volatile compounds, indicating that microorganisms are involved in aroma formation (Hinrichsen and Pedersen, 1995).

In the field of flavor analysis of dry-cured hams the interest has in recent years been directed mainly toward volatile compounds (Berdagué et al., 1991; Barbieri et al. 1992; Careri et al., 1993; Hinrichsen and Pedersen, 1995). Berdagué et al. (1991) found that aliphatic aldehydes, alcohols, and methyl ketones were the dominant volatile compounds in French dry-cured hams. Barbieri et al. (1992) found that Parma hams contained more esters compared to French hams which contained higher amounts of aldehydes and alcohols. In a study including sensory properties and chemical data Careri et al. (1993) showed that hams with high levels of tyrosine and lysine, and of methyl-branched short-chain esters and alcohols had the highest acceptability scores. A correlation between mature flavor and methyl-branched aldehydes, secondary alcohols, methyl ketones, ethyl esters, and dimethyl trisulfide was shown by Hinrichsen and Pedersen (1995).

The flavor of the water soluble fraction of the dry-cured hams received less attention than the volatiles, but water soluble compounds could be very important for the flavor.

Amino acids as free acids or as hydrochlorides are known to have a great impact on the flavor of a variety of foods. In seafoods, glutamic acid and glycine are taste-active compounds, and in meats, glutamic acid,

and inosine 5′-monophosphate are important for taste (Fuke and Konosu, 1991).

Peptides generated by enzymatic action on proteins in foods can also be important for the flavor. In Swiss cheese, a number of peptides could be identified, and it was assumed that the bitter-tasting peptides contributed positively to the overall flavor (Mojarro-Guerra et al., 1991). Yamasaki and Maekawa (1978) found a peptide with delicious taste from beef treated with papain, and co-operative action of protease and peptidase was necessary to generate the flavor-related peptides in cocoa seeds (Voigt et al., 1994).

Knowledge about the composition of the mature flavor in traditional dry-cured ham could be of interest for use in other meat products.

The aim of the present study was to develop an HPLC method for determination of N-containing water soluble compounds using selective precolumn fluorescense derivatization and to find out whether these compounds are important for the development of the mature flavor in dry-cured ham.

MATERIALS AND METHODS

Products. Twenty-nine Parma hams were purchased at a factory in Langhirano, Italy. The hams were processed according to the traditional processing scheme based on 25 days of dry salting at 1−3 °C, resting for 90 days at 1−4 °C, drying and primary ripening for 90 days at 15−20 °C at RH 60−90%. Pork fat containing pepper was then smeared on cut surfaces followed by final ripening for 160 days at 17−18 °C and then post-ripening for additional 120 days as described by Virgili and Parolari (1991). Samples for analysis were taken at specific stages in the process: five hams prior to salting (3 days), five hams after salting (25 days), five hams after drying (125 days), five hams after first ripening (211 days), five hams after second ripening (365 days, fully matured product) and three hams after post-ripening (485 days).

Sampling. The hams were derinded leaving 1 cm of fat left on the hams. Representative samples for analysis were then taken from the cushion part, which included biceps femoris, semimembranosus, and semitendinosus. In this way only intra muscular fat was included in the samples. Samples were stored in sealed vacuum bags under vacuum at −20 °C for no longer than 2 months.

Chemicals. All amino acid standards were obtained from Sigma (St. Louis, MO). Triethylamine, di(*N*-succinimidyl)-
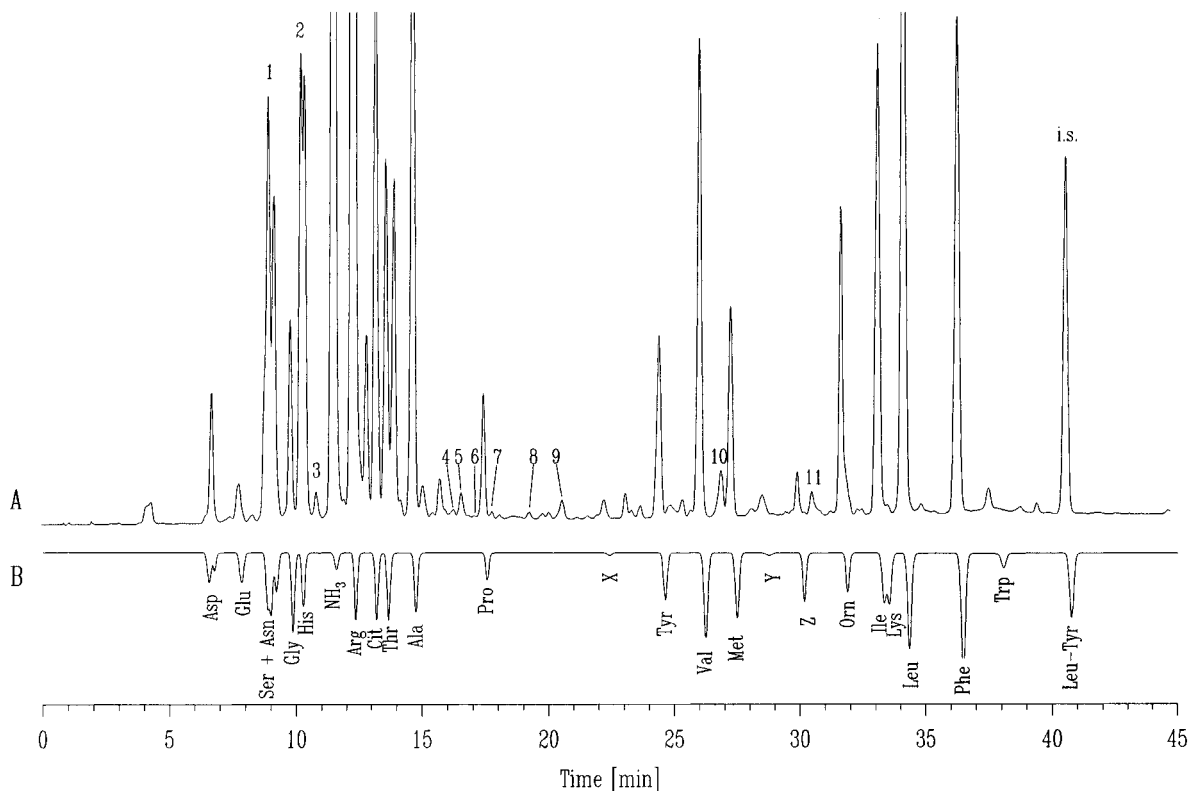
Figure 1. Selectivity of the chromatographic system: (A) Parma ham sample added internal standard; (B) standard solution containing amino acids at 25 μM. Peaks 1−11 were identified as marker compounds for the maturity of the Parma ham.

carbamate, and 6-aminoquinoline were obtained from Merck (Darmstadt, Germany). Demineralized water was treated in a Milli-Q Plus Water Purification System from Millipore (Bedford, MA). Methanol (MeOH), acetonitrile (MeCN), and tetrahydrofuran (THF) were of HPLC grade obtained from Romil (Loughborough, Leicestershire, UK). All other chemicals were of analytical grade. 6-Aminoquinoyl-*N*-hydroxysuccimidylcarbamate (AQC) were prepared according to the procedure described by Cohen and Michaud (1993).

High-Performance Liquid Chromatography Instrumentation. The HPLC system was a Hitachi system from Merck (Darmstadt, Germany) consisting of an AS-4000 autosampler, an L-6200 gradient pump, an F-1080 fluorescence detector, an L-5025 column oven, and a D-6000 HPLCmanager software installed on a Compaq Descpro 386s PC. An AS-4000 autosampler editor program was used for modifying the derivatization procedure.

Chromatographic System. The column was a Hypersil BDS (3 μm, 60 mm × 4.6 mm i.d. Hewlett Packard) operated at 40 °C.

The mobile phases consisted of A [0.2 M potassium phosphate buffer, pH 6.0/water/triethylamine (125:875:1; v/v/v)] and B [MeCN/water (800:200; v/v)].

Gradient profile: 0−52 min, 98%−70% A; 52.1−52.5 min, 70%−20% A; 52.6−55.6 min, 20% A; 55.7−56.7 min, 20%−98% A. Flow: 1.5 mL/min.

The detection principle was fluorescence with excitation at 245 nm and emission at 395 nm.

Ultrafiltration. Microcon 3 ultrafiltration units, with a cutoff value of 3000 daltons were obtained from Amicon (Beverly, MA). Prior to usage, the filters were purified by spinning 250 μL of 25 mM sodium phosphate buffer, pH 6.0, through the filter at 12000*g* for 20 min. Remaining liquid was carefully removed by tapping the unit on absorbent paper. The procedure was repeated once.

Sample Pretreatment. Three milliliters of 25 mM sodium phosphate buffer, pH 6.0, containing the internal standard (0.20 mM *d*-Leu-Tyr) was added to 0.50 g of meat. After homogenization using a Kinematica Polytron PT 3000 (Littau, Schwitzerland) fitted with a 12 mm tip, the homogenate was centrifuged for 5 min at 14000*g* at 5 °C, and 400 μL of extract

was transferred to a purified Microcon 3 ultrafiltration unit. The unit was centrifuged at 10000*g* for 1 h. Twenty-five microliters of the resulting filtrate was transferred to an autosampler vial, diluted with 225 μL of water, and analyzed. All samples were analyzed in duplicate.

Standard Solutions. Linearity of the standard curves for the amino acids based on peak height was investigated in the range 0.25−200 μM. Solutions were prepared in water containing 0.05% (w/v) sodium azide.

Derivatization. The AS-4000 autosampler was programmed by means of the AS-4000 program editor software (Merck, Darmstadt, Germany) to perform the derivatization.

Fifteen microliters of 10 mM AQC dissolved in MeCN and 50 μL of 0.2 M sodium borate buffer, pH 8.8, were mixed with 50 μL of sample extract. After 10.0 min at 60 °C, 1.5 μL of a mixture of acetonitrile/acetic acid (1:1; v/v) was added. Five microliters of the mixture was injected in the HPLC system.

Repeatability. For inter-assay variability, ten different samples were analyzed and one was selected on the basis of the content of amino acids and the compounds which were found in the principal component analysis of the grouping of the samples. The sample was analyzed ten times during one day.

Preparation of "In-House Reference Material". Extracts from the samples used for the repeatability test were pooled, divided into 500 μL portions, and stored at −25 °C. The material was analyzed in duplicate every day and served as an in-house reference material.

Statistical Analyses. Peptide peaks from HPLC were examined by principal component analysis (PCA) in the Unscrambler (CAMO A/S, 1994). Same data were correlated to sensory data by projection on latent structures (PLS) also in the Unscrambler.

RESULTS AND DISCUSSION

Selectivity of the Chromatographic System. In Figure 1 the selectivity of the chromatographic system is illustrated. The pH and the slope of the gradient were optimized in order to detect the highest number of peaks

Table 1[a]

ID	RRt (%)	RSD (%)	height (μV s)	RSD (%)	n
1	18.40	0.89	139 242	7.7	10
2	24.70	0.77	327 840	10.9	10
3	27.30	0.67	341 446	12.4	10
4	30.30	0.40	1025 047	4.6	10
5	40.00	0.22	13 245	11.0	10
6	41.10	0.21	44 910	9.0	10
7	42.10	0.21	66 420	12.8	9
8	43.70	0.05	71 626	9.7	7
9	46.70	0.17	21 335	24.0	10
10	66.60	0.44	53 546	13.1	10
11	74.90	0.10	11 285	11.9	10
d-Leu-Tyr	100	0.10	340 754	21.3	10

[a] Repeatability: One sample selected on basis of the content of amino acids and the 11 marker compounds was analyzed 10 times. The retention time is calculated relative to the internal standard.

in the chromatogram and simultaneously separate as many amino acids as possible. When a blank sample is run, water passed through a Microcon 3 filter, the peaks labeled X, Y, and Z as well as the ammonia peak are always seen. No attempts were made to identify the unknown peaks, but the heights of X, Y, and Z could be reduced by washing the Microcon 3 filter with buffer prior to usage. The dipeptides carnosine and anserine, characteristic for pork meat, appeared in the chromatograms after 45 min (after elution of the internal standard) as two peaks off scale (not shown). The peaks were identified by comparing the relative retention time to authentic standards. These two peptide peaks were excluded in the further processing of results.

Principle of Detection and Derivatization. Different principles for detection of the peptide peaks were considered including UV and fluorescence. UV detection was not used due to its low sensitivity and general unspecificity at the wavelengths where peptide peaks show maximum absorbance. Fluorescence labeling of the free N-terminal part of the peptides was chosen as

the best procedure due to the high sensitivity and the fact that it allows the method to distinguish compounds containing a free primary or secondary amino group from other compounds. This derivatization procedure does, however, mean that peaks observed in a chromatogram are not necessarily an amino acid or a peptide, but it only implies that the compounds contain a primary or secondary amino group.

The conditions for the derivatization, i.e., time, excess of reagent, and temperature were optimized using lysine and ham extracts. Lysine was selected because it forms an intermediate derivative which elutes faster than the final derivative which in the single product elutes after 5 min at 65 °C.

Derivatization using AQC is advantageous compared to *o*-phthalaldehyde or phenyl isothiocyanate, because it is not necessary to remove excess of the AQC reagent. AQC reagent is rapidly hydrolyzed to 6-aminoquinoline, which has different excitation and emission wavelengths compared to the derivatized compounds. This excess reagent cannot be seen in the chromatograms (Cohen and Michaud, 1993).

Repeatability. In Table 1 the repeatability of determination of the 11 marker peptide peaks are shown. Peak height was chosen for the calculations, as it gives less relative variation compared to using peak area. Comparing peak height and area for determination for the amino acids gives the same result.

Evaluation of Peptide Generation. The action of endogenous and microbial enzymes within meat leads to the formation of low molecular weight substances, which by themselves contribute to the flavor or serve as intermediates in further reactions. All such reactions will generate an alternating pattern of low molecular weight substances dependent on enzyme specificities and process conditions. Small peptides will be generated during processing, and they can act as flavor contributors or as substrate for further catalysis. In
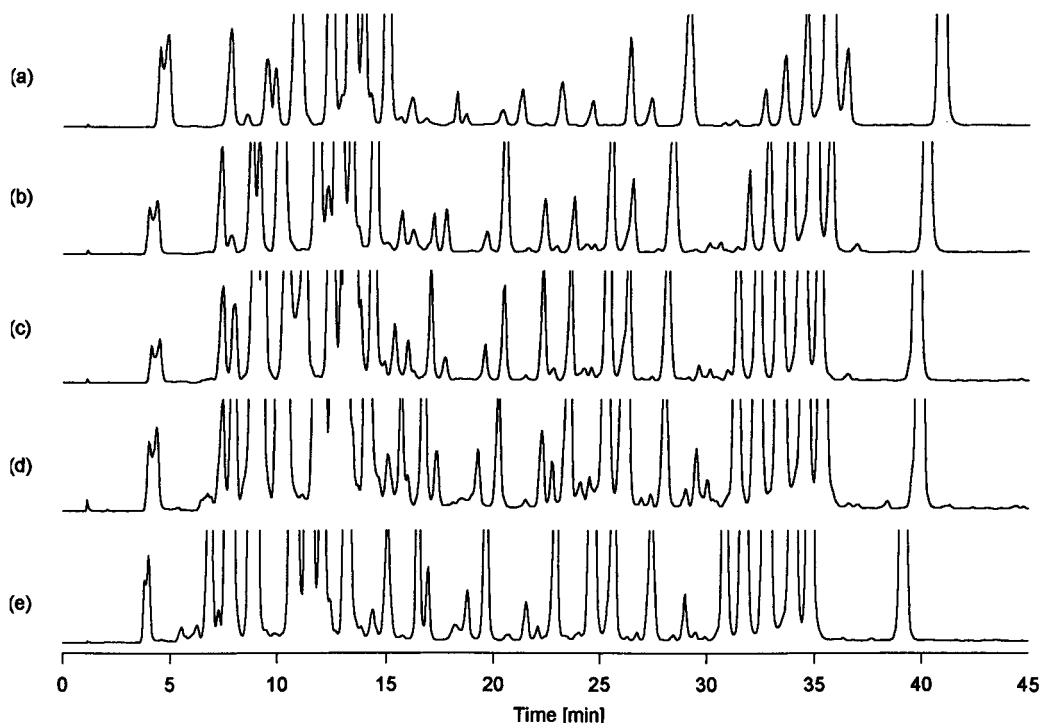

Figure 2. Typical chromatograms of extracts from Parma ham. Raw meat (a) and meat after post-salting (b), after first (c) and second ripenings (d), and finally after post-ripening (e) are shown. Evidently the amount and number of low molecular weight nitrogenous compounds increase during processing.
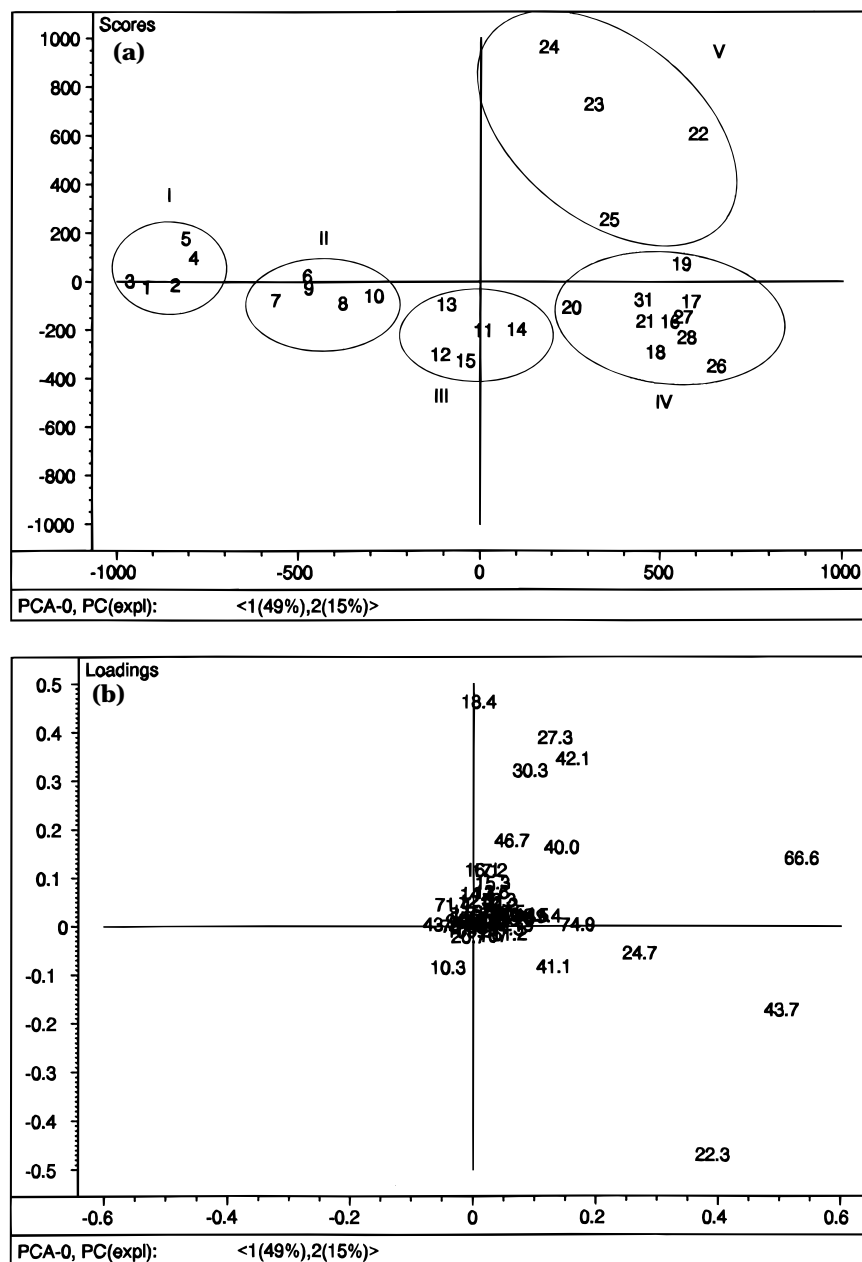
Figure 3. (a) Scores plot after principal component analysis of peptide peaks in all hams (1–28, 31). Peak areas resulting from free amino acids were excluded from the resulting data matrix. Then peak areas were scaled by the square root in order to get data within the same range. Arabic numerals designate hams of different storage times: 1–5 are after 3 days; 6–10, 25 days; 11–15, 125 days; 16–20, 211 days; 21–25, 365 days; and 26–28, 485 days. Ham 31 is spoiled. The Roman numerals designate the different groupings. (b) Loadings plot. The numbers assign relative retention times.

Figure 1 peaks originating from free amino acids are marked. As the free amino acids are the ultimate products of proteolysis, the amount of these compounds will be much higher compared to the peptides, which represent a dynamic state in which both formation and breakdown take place. Therefore, the peak hights of amino acids are much larger compared to the other peaks in the chromatograms. In Figure 2a–e are shown typical chromatograms of extracts from raw meat (Figure 2a), after post-salting (Figure 2b), after first (Figure 2c) and second ripening (Figure 2d), and finally after post-ripening (Figure 2e). Evidently the peak heights and variety of low molecular weight nitrogenous compounds increase during processing.

To recognize the latent patterns of peptide generation, the peak areas from the chromatograms were exposed to principal component analysis. Peak heights resulting from free amino acids were excluded from the resulting

data matrix. The peak heights were scaled by the square root in order to get data within the same range and then subsequently exposed to principal component analysis. Results from principal component analysis of peak heights of crude meat extracts are presented as scores and loadings plots in Figure 3a,b. The two first principal components describe 49% and 15%, respectively, of the total variation in the data (Figure 3).

In the scores plot in Figure 3a, five groupings are observed: group I with raw hams (3 days), group II with hams after salting (25 days), group III with hams after drying (125 days), group IV with hams after first (211 days) and second ripening (365 days), and finally group V with fully matured hams (485 days). Groups I–IV are essentially described by the first principal component, whereas group V is described by the second principal component.

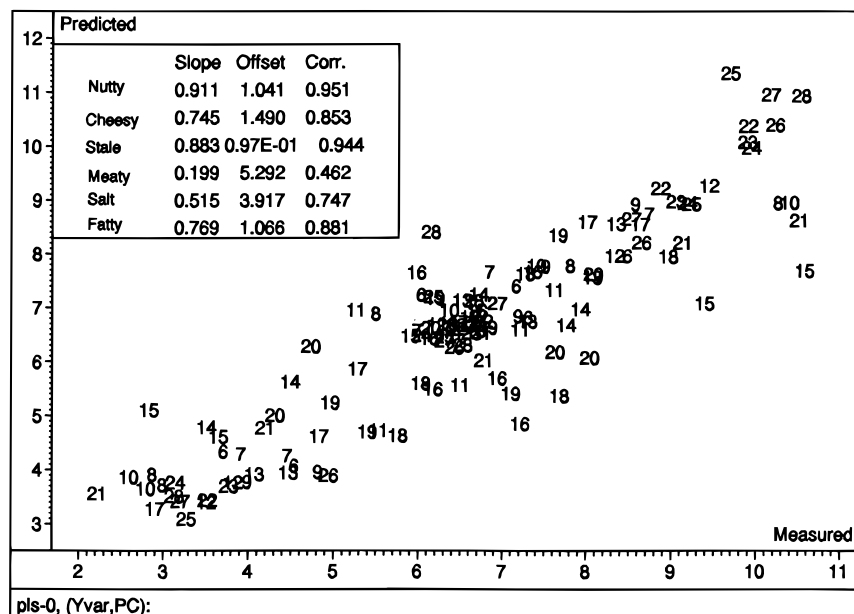The loadings plot in Figure 3b reveals which com-

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	Slope	Offset	Corr.
Nutty	0.911	1.041	0.951
Cheesy	0.745	1.490	0.853
Stale	0.883	0.97E-01	0.944
Meaty	0.199	5.292	0.462
Salt	0.515	3.917	0.747
Fatty	0.769	1.066	0.881

Figure 4. Intensities of flavor attributes (Hinrichsen and Pedersen, 1995) versus predicted values based on partial least squares modeling with two principal components to describe the relationship between peptide peaks and sensory profiles.

pounds are responsible for the observed groupings. On the first principal component are placed the compounds with relative retention times of 24.7, 41.1, 43.7, 66.6, and 74.9. On the second principal component are placed the compounds with relative retention times of 18.4, 27.3, 30.3, 40.0, 42.1, and 46.7. These compounds are mainly responsible for the groupings observed in Figure 3a.

By comparing the scores and loadings plots (Figure 3a and b), samples in groups I and II are characterized by a low content of the compounds with relative retention times of 24.7, 41.1, 43.7, 66.6, and 74.9, whereas group IV has a high content of these compounds. Group III has an average content. Group V is also characterized by a high content of these compounds and by a high content of compounds with relative retention times of 18.4, 27.3, 30.3, 40.0, 42.1, and 46.7.

There is obviously a relationship between processing and formation of low molecular weight nitrogenous substances. The first 365 days of processing the peptide peaks with relative retention times of 24.7, 41.1, 43.7, 66.6, and 74.9 are formed (groups I−IV) and the post-ripening leads to the formation of additional peptide peaks with relative retention times of 18.4, 27.3, 30.3, 40.0, 42.1, and 46.7. The peptide generation can in this way be divided into two phases, the first including raw meat, hams after salting, hams after drying, and hams after first and second ripening, and the second including the fully matured hams. This is in accordance with previous observations in which a similar phase division was observed on the basis of the volatile compound content (Hinrichsen and Pedersen, 1995). In a study on flavor peptides Aristoy and Toldrá (1995) compared raw pork and dry-cured ham. They found an increase in the levels of peptides and free amino acids due to the dry-curing process. Taste descriptions of gel filtration fractions showed that only samples from dry-cured ham had fractions with savory taste. These fractions were relatively rich in hydrophilic nitrogen compounds. Rodriguez-Nuñez et al. (1995) observed a more intense formation of peptides below 2700 Da during the first 3.5 months of processing of Spanish Serrano ham but were unable to detect a change in the peptide formation

late in the ripening. This is not in accordance with the presented results and may be due to different technologies as these authors applied a more crude separation technique which may not cover differences in the peptide patterns.

In order to demonstrate the relevance of the presented results the peaks of the peptides were correlated to earlier published sensory data (Hinrichsen and Pedersen, 1995). In Figure 4 the resulting model is shown as a plot of measured values versus predicted values. The model was generated by partial least squares modeling, where all data were autoscaled. All sensory attributes were described by the model except that *meaty* was less convincing. This model shows that the formation of peptide peaks is highly correlated to the flavor formation and that peptides are involved in the overall flavor of Parma ham.

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