Sensory and Mass Spectrometric Analysis of the Peptidic Fraction Lower Than One Thousand Daltons in Manchego Cheese

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ABSTRACT

A total of 107 different peptides, all derived from α_{S1} -, α_{S2} -, and β -casein, were identified in different fractions of artisan or industrial Manchego cheese at 4 and 8 mo of ripening, and their sequences were examined. Most of these peptides are described for the first time in Manchego cheese. Taste characteristics (umami and bitter) were assigned based on their AA sequence and the position of these AA within the sequence. The umami taste was predominant in all fractions analyzed by the panelists, and the peptides EQEEL, QEEL, and EINEL, containing a high number of glutamic residues, were found within the fractions. However, in several fractions described as having umami characteristics, no peptides responsible for this taste were detected. Therefore, compounds other than peptides seem to be involved in the umami properties of water-soluble extracts lower than 1,000 Da of Manchego cheese.

Key words: cheese taste, mass spectrometry, sensory analysis, umami

INTRODUCTION

Manchego cheese is the main Spanish variety having a protected denomination of origin. It has a very complex and characteristic flavor. Compounds that contribute to cheese flavor are mainly produced as a consequence of the biochemical changes during ripening. Most research on Manchego cheese flavor has been focused on its volatile components (e.g., González-Viñas et al., 2001; Fernández-García et al., 2002; Gómez-Ruiz et al., 2002a). In addition, cheese taste is one of the most important organoleptic attributes, and the

correct balance of sapid compounds is essential to cheese quality (McSweeney, 1997). Engels and Visser (1994) and Taborda et al. (2003) have analyzed water-soluble extracts (WSE) of different types of cheeses and suggested that low molecular weight compounds such as small peptides and AA, short-chain fatty acids, volatile compounds, and organic acids and ions are responsible for the basic taste of cheeses. Molina et al. (1999) fractionated the WSE of cheese made from milk of different species, and differences in the taste were established depending on the origin of the milk.

Recently, studies on the relationship between the particular organoleptic characteristics of Manchego cheese and the presence of small peptides, AA, short-chain fatty acids, volatile compounds, other organic acids and ions in the different fractions of WSE lower than 1,000 Da (WSE <1,000 Da) have been carried out in our laboratory (Taborda, 2001). Volatile compounds have been related to the flavor and small peptides and AA to the taste. However, no peptides have been identified in the different fractions. Identification of peptides in cheeses is an intensive and difficult task; nevertheless, liquid chromatography-tandem mass spectrometry (MS/MS) allows the identification and sequencing of a large number of peptides in a relatively short analysis time. Several configurations of mass spectrometers now provide MS/MS data with sufficient mass accuracy to deduce peptide sequences of enzymatically digested proteins from low-energy collisionally induced MS/MS spectra (Careri and Mangia, 2003). High-performance liquid chromatography-MS/MS has been used by Gómez-Ruiz et al. (2002b, 2004, 2006) to identify the bioactive peptides with demonstrated angiotensin I-converting enzyme inhibitory activity in different types of cheeses.

Peptides have a wide range of tastes, and their importance to the sensory perception of foods has been recognized. However, not many peptides have been identified and related to the taste of cheese (Polo et al., 2000). Despite some claims, the real impact of small peptides on the taste of cheese has not been clearly demonstrated. The objectives of this study were to identify taste-active peptides by HPLC-MS/MS in dif-

Received May 8, 2007.

Accepted June 4, 2007.

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ferent fractions of WSE of raw and pasteurized Manchego cheeses at 2 different stages of ripening, and to relate their presence to the sensory characteristics of these fractions, as previously evaluated by expert panelists.

MATERIALS AND METHODS

Samples of Cheese

Protected denomination of origin Manchego cheese samples (artisan and industrial), at 4 and 8 mo of ripening, were purchased in the local supermarket. The samples were analyzed in duplicate.

Isolation and Fractionation of the WSE <1,000 Da

The WSE <1,000 Da was obtained following the method described by Salles et al. (1995). Briefly, 50 g of cheese was homogenized with 100 mL of water and held in a water bath at 40°C for 1 h. After centrifugation ($3,800 \times g$; 20°C, 30 min), the supernatant was filtered and ultracentrifuged ($100,000 \times g$; 20°C, 30 min). The resultant supernatant (WSE) was UF at 4°C in an ultrafiltration cell with a 1,000-Da cutoff cellulose membrane (YM1, Dia-Flo, Millipore, Bedford, MA) in an ultrafiltration cell (Diaflo, Millipore) to obtain the WSE <1,000 Da. The WSE <1,000 Da was then fractionated by gel-permeation chromatography (GPC) with a Superformance Sephadex G10 column (1.6×60 cm, Pharmacia, Uppsala, Sweden) with manual injection. Elution was done with Milli-Q water (Millipore) to allow sensory evaluation at a constant flow of 2 mL/min. The detection was carried out at 280 nm in a 2138 Uvicords detector (LKB, Uppsala, Sweden). Five fractions were automatically collected. Two milliliters of each fraction was lyophilized, redissolved in 200 μ L of water:trifluoroacetic acid (TFA) (1,000:0.37), and sonicated for 30 min before analysis by reversed-phase (RP) HPLC-MS/MS.

RP-HPLC-MS/MS Analysis

The 5 fractions obtained from the WSE <1,000 Da of the different cheeses were analyzed by analytical RP-HPLC-MS/MS with a Hi-Pore C₁₈ column (250×4.6 mm, 5 μ m particle size, Bio-Rad Laboratories, Richmond, CA) in an HP Agilent 1100 system (Agilent Technologies, Santa Clara, CA) connected to an Esquire-LC quadrupole ion trap (Bruker Daltonik GmbH, Bremen, Germany). The HPLC system was equipped with a quaternary pump (Agilent Series 1100) and a variable wavelength detector (Agilent Series 1100) in combination with an autosampler (Agilent Series 1000). Data were processed by using

ChemStation for LC 3D Systems (Agilent Technologies). The injection volume was 100 μ L and the samples were eluted at a flow rate of 0.8 mL/min. Solvent A was a mixture of water:TFA (1,000:0.37) and solvent B was a mixture of acetonitrile:water:TFA (800:200:0.27). A step gradient system of water and acetonitrile was used as the mobile phase: solvent A (100%) for 5 min, then a linear gradient to 40% solvent B in 60 min, and from 40 to 70% solvent B in 5 min. The absorbance was monitored at 214 nm. The flow (0.8 mL/min) was split postcolumn by placing a T-piece (Valco, Houston, TX) connected with a 75- μ m i.d. polyetheretherketone (PEEK) outlet tube of an adjusted length to give approximately 20 μ L/min of flow directed into the mass spectrometer via the electrospray interface. The mass spectrometer used nitrogen as the nebulizing and drying gas and operated with an estimated helium pressure of 5×10^{-3} bar. The capillary was held at 4 kV. By using electrospray ionization in the positive ion mode, mass spectra were acquired from m/z 100 to 900. Approximately 15 spectra were averaged in the MS analyses, and approximately 5 spectra were analyzed in the multiple MSⁿ analyses. The signal threshold to perform auto MSⁿ analyses was 5,000, and the precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage increase from 0.35 to 1.4 V. Automation of data processing was achieved by using DataAnalysis (version 3.0, Bruker Daltonik) and the software package Biotools (version 2.1, Bruker Daltonik).

Taste of the Identified Peptides

The taste characteristics of the identified peptides were assigned depending on the AA and their positions in the sequence. For the umami taste, all the sequences suggested by Roudot-Algaron (1996) and Maehashi et al. (1999) as capable of giving such a taste were searched for within our identified peptides in Manchego cheese. Ten peptides with umami taste were found: PSE, RKE, SAEQK, ENINEL, INEL, EQEEL, QEEL, NVVGET, VVGET, LEQL.

Bitter peptides were selected to look for the sequences capable of giving such a taste, as suggested Kim and Li-Chan (2006). Thirteen peptides with the sequences PPF and PFP (which showed high bitterness values in the work of the above-mentioned authors) were considered in this study: VVAPFP, VVAPFPE, VVAPFPEV, VAPFP, VAPFPE, APFPE, VPPFL, PFPKY, GPVRGPFP, VRGPFP, RGPFP, GPFP, GPFPI.

Sensory Analysis

A panel of 6 experienced panelists (5 women and 1 man, ages 25 to 50) were selected from 12 participating

Table 1. Identified peptides in the permeate <1,000 Da of gel-permeation chromatographic fractions of artisan and industrial Manchego cheeses at 4 and 8 mo of age

Item	Sequence	Protein fragment	Observed mass	Calculated mass	Fraction ¹
α_{S1} -CN					
1.	RPK	α_{S1} -CN (1–3)	399.2	399.267	4, 6, 7
2.	RPKHPIK	α_{S1} -CN (1–7)	874.4	874.557	6
3.	PKHP	α_{S1} -CN (2–5)	477.3	477.277	8
4.	HPIK	α_{S1} -CN (4–7)	493.2	493.309	2, 4, 6, 7, 8, 9
5.	IKHQ	α_{S1} -CN (6–9)	524.2	524.314	8
6.	HQGL	α_{S1} -CN (8–11)	453.2	453.241	4, 7, 8, 9, 10
7.	VLNEN	α_{S1} -CN (15–19)	587.2	587.299	3
8.	VLNENL	α_{S1} -CN (15–20)	700.3	700.383	1, 3
9.	LNEN	α_{S1} -CN (16–19)	488.2	488.233	1, 3
10.	LNENL	α_{S1} -CN (16–20)	601.3	601.314	1, 3, 8
11.	NLLR	α_{S1} -CN (19–22)	514.4	514.333	4, 7
12.	LLR	α_{S1} -CN (20–22); α_{S1} -CN (98–100)	400.3	400.287	4
13.	LLRF	α_{S1} -CN (20–23)	547.4	547.356	4
14.	VVAP	α_{S1} -CN (24–27)	384.2	384.245	3, 4, 6, 7, 8
15.	VVAPFP	α_{S1} -CN (24–29)	628.3	628.366	3, 4
16.	VVAPFPE	α_{S1} -CN (24–30)	757.3	757.408	1, 3, 6, 7, 8
17.	VVAPFPEV	α_{S1} -CN (24–31)	856.4	856.477	3
18.	VAPFP	α_{S1} -CN (25–29)	529.2	529.297	4
19.	VAPFPE	α_{S1} -CN (25–30)	658.3	658.343	1, 3
20.	APFPE	α_{S1} -CN (26–30)	559.3	559.271	1, 3, 4, 7, 8
21.	FRKE	α_{S1} -CN (32–35)	578.3	578.325	4
22.	RKE	α_{S1} -CN (33–35); α_{S2} -CN (32–34)	431.2	431.257	6
23.	RKEN	α_{S1} -CN (33–36)	545.3	545.299	6
24.	KENIN	α_{S1} -CN (34–38)	616.3	616.325	1, 3
25.	ENINEL	α_{S1} -CN (35–40)	730.3	730.357	1, 3
26.	NINE	α_{S1} -CN (36–39)	488.2	488.233	1, 3
27.	INEL/LNEI	α_{S1} -CN (37–40); α_{S2} -CN (83–86)	487.3	487.271	1, 3, 4, 6, 8
28.	SIEDQ	α_{S1} -CN (48–52)	590.2	590.262	8
29.	IEDQ	α_{S1} -CN (49–52)	503.2	503.233	6
30.	IEDQA	α_{S1} -CN (49–53)	574.3	574.267	8
31.	EDAK	α_{S1} -CN (55–58)	461.2	461.219	1
32.	EDAKQ	α_{S1} -CN (55–59)	589.3	589.278	1, 3, 6
33.	DAKQM	α_{S1} -CN (56–60)	591.2	591.276	3, 8
34.	AKQMK	α_{S1} -CN (57–61)	604.3	604.344	7
35.	SAEQK	α_{S1} -CN (75–79)	561.2	561.283	6
36.	PSE	α_{S1} -CN (87–89)	331.2	331.145	1, 3
37.	SERY	α_{S1} -CN (88–91)	553.2	553.257	9
38.	ERYL	α_{S1} -CN (89–92)	579.3	579.309	5, 7, 9, 10
39.	LEQL/NKKI	α_{S1} -CN (95–98); β -CN (27–30)	501.3	501.287	3, 6
40.	LLRL	α_{S1} -CN (98–101)	513.4	513.371	4, 7, 9
41.	LRL	α_{S1} -CN (99–101)	400.4	400.287	7
42.	LKKY	α_{S1} -CN (101–104)	550.4	550.355	4
43.	KKYN	α_{S1} -CN (102–105)	551.3	551.314	7
44.	NVPQL	α_{S1} -CN (105–109)	569.3	569.325	3
45.	VPQL	α_{S1} -CN (106–109)	455.3	455.282	3, 4
46.	PQL	α_{S1} -CN (107–109)	356.3	356.213	7
47.	EIVPK	α_{S1} -CN (110–114)	584.4	584.361	1, 3, 4, 6, 7, 8, 9
48.	IVPK	α_{S1} -CN (111–114)	455.3	455.318	4, 7, 8
49.	AHQ	α_{S1} -CN (129–131)	354.2	354.172	4, 8
50.	LPL	α_{S1} -CN (159–161); β -CN (75–77); β -CN (135–137); β -CN (137–139)	341.2	341.239	4
α_{S2} -CN					
51.	EKNM	α_{S2} -CN (24–27)	520.2	520.239	1, 3
52.	AIHP	α_{S2} -CN (28–31)	436.3	436.251	4
53.	AIHPR	α_{S2} -CN (28–32)	592.4	592.352	4, 7
54.	IHPR	α_{S2} -CN (29–32)	521.3	521.315	4
55.	PRKE	α_{S2} -CN (31–34)	528.3	528.309	3, 4, 7
56.	VVRN	α_{S2} -CN (44–47)	486.4	486.299	4, 7, 8
57.	VRN	α_{S2} -CN (45–47)	387.3	387.233	7
58.	PQY	α_{S2} -CN (94–96); α_{S2} -CN (178–180)	406.2	406.193	7
59.	LKKI	α_{S2} -CN (165–168)	500.2	500.376	3
60.	VRYL	α_{S2} -CN (205–208)	549.3	549.335	4, 5

Continued

Table 1 (Continued). Identified peptides in the permeate <1,000 Da of gel-permeation chromatographic fractions of artisan and industrial Manchego cheeses at 4 and 8 mo of age

Item	Sequence	Protein fragment	Observed mass	Calculated mass	Fraction ¹
<i>β</i> -CN					
61.	EQEE	<i>β</i> -CN (2–5)	533.2	533.204	1
62.	EQEEL	<i>β</i> -CN (2–6)	646.3	646.288	3
63.	QEEL	<i>β</i> -CN (3–6)	517.2	517.246	1, 3
64.	NVVGET	<i>β</i> -CN (7–12)	697.2	697.276	6
65.	VVGGET	<i>β</i> -CN (8–12)	503.2	583.233	3
66.	ITHINK	<i>β</i> -CN (23–28)	724.4	724.433	6
67.	HINK	<i>β</i> -CN (25–28)	510.3	510.299	7, 8
68.	HINKK	<i>β</i> -CN (25–29)	638.3	638.394	7
69.	KIEK	<i>β</i> -CN (29–32)	516.3	516.334	3, 4, 6
70.	IEKF	<i>β</i> -CN (30–33)	535.3	535.308	7
71.	LQDK	<i>β</i> -CN (45–48)	502.3	502.282	3
72.	DKIHP	<i>β</i> -CN (47–51)	608.3	608.335	2, 3, 4, 6, 7, 8, 9
73.	DKIHPP	<i>β</i> -CN (47–52)	755.4	755.404	4, 7, 8, 9, 10
74.	FTGPIPN	<i>β</i> -CN (62–68)	744.3	744.388	8
75.	TGPIP	<i>β</i> -CN (63–67)	483.3	483.277	3
76.	TGPIPN	<i>β</i> -CN (63–68)	597.3	597.32	1, 3, 4, 6, 7, 8, 9
77.	GPIPN	<i>β</i> -CN (64–68)	496.2	496.272	4
78.	PQN	<i>β</i> -CN (71–73)	357.1	357.172	6
79.	TPVVVPP	<i>β</i> -CN (80–86)	707.4	707.429	3, 6, 8
80.	PVVVPP	<i>β</i> -CN (81–86)	606.3	606.381	8
81.	VVVPP	<i>β</i> -CN (82–86)	509.3	509.329	3, 6, 8
82.	VVVPPF	<i>β</i> -CN (82–87)	656.3	656.397	8
83.	VPPF	<i>β</i> -CN (84–87)	458.3	458.263	2, 4, 7, 8, 9, 10
84.	VPPFL	<i>β</i> -CN (84–88)	571.8	571.344	4, 8, 9
85.	IMGVPK	<i>β</i> -CN (92–97)	643.3	643.384	4, 6
86.	GVPKV	<i>β</i> -CN (94–98)	498.3	498.324	7
87.	VPKVK	<i>β</i> -CN (95–99)	569.3	569.397	4
88.	KVKE	<i>β</i> -CN (97–100)	502.3	502.319	6, 8
89.	PFPKY	<i>β</i> -CN (110–114)	650.4	650.354	4
90.	LPPT	<i>β</i> -CN (151–154)	426.3	426.255	8
91.	PQSVL	<i>β</i> -CN (159–163)	542.3	542.314	3
92.	PIQA	<i>β</i> -CN (184–187)	427.3	427.254	3
93.	YQEP	<i>β</i> -CN (191–194)	535.2	535.235	3
94.	PVLGP	<i>β</i> -CN (194–198)	481.3	481.297	6, 7, 8
95.	VLGP	<i>β</i> -CN (195–198)	384.3	384.245	7
96.	GPVRGPPF	<i>β</i> -CN (197–204)	825.4	825.457	4, 7
97.	GPVR	<i>β</i> -CN (197–200)	427.3	427.262	4, 8
98.	VRGP	<i>β</i> -CN (199–202)	427.8	427.262	7
99.	VRGPFP	<i>β</i> -CN (199–204)	671.3	671.383	7
100.	RGPFP	<i>β</i> -CN (200–205)	687.4	685.398	7
101.	GPFP	<i>β</i> -CN (201–204)	416.2	416.213	9
102.	GPFP	<i>β</i> -CN (201–205)	529.3	529.297	8
Different CN					
103.	RP/PR	α_{S1} -CN (1–2); α_{S2} -CN (31–32); κ -CN (46–47)	271.1	271.172	7, 9
104.	QP/PQ/PK	Various fragments (α_{S1} -, α_{S2} -, β -, and κ -CN)	243.1	243.129	4, 6, 8
105.	RF	α_{S1} -CN (22–23); κ -CN (16–17)	321.1	321.187	5
106.	RL	α_{S1} -CN (100–101); α_{S2} -CN (161–162)	287.2	287.203	4
107.	YP	α_{S1} -CN (151–152); β -CN (60–61); β -CN (114–115); κ -CN (35–36)	278.1	278.134	6

¹Identification of fractions: 1 = fraction 1 artisan cheese, 4 mo old; 2 = fraction 2 artisan cheese, 4 mo old; 3 = fraction 1 artisan cheese, 8 mo old; 4 = fraction 2 artisan cheese, 8 mo old; 5 = fraction 3 artisan cheese, 8 mo old; 6 = fraction 1 industrial cheese, 4 mo old; 7 = fraction 2 industrial cheese, 4 mo old; 8 = fraction 1 industrial cheese, 8 mo old; 9 = fraction 2 industrial cheese, 8 mo old; 10 = fraction 3 industrial cheese, 8 mo old.

subjects to conduct sensory analyses. They were trained to recognize the basic tastes with standard solutions (salty, umami, astringent, sour, sweet, and bitter) prepared in mineral water according to Molina

et al. (1999). These solutions were presented to the panelists as such and in mixtures, and the results were discussed among them. Before each session, panelists tasted the reference solutions to memorize their tastes

and intensities. They were then requested to assess the taste of the gel-permeation fractions of the WSE <1,000 Da. The fractions were lyophilized before reconstitution with 2 mL of mineral water. Aliquots were placed directly onto panelists' tongues. The presence and intensity of each basic taste was evaluated on a scale ranging from 0 to 100. As a reference, the taste intensity scores of the standard solutions were taken as 80.

RESULTS AND DISCUSSION

Peptide Identification by RP-HPLC-MS/MS

The 5 GPC fractions obtained from the WSE <1,000 Da were analyzed by RP-HPLC coupled online to a mass spectrometer with the aim of identifying the peptides responsible for the taste of artisan and industrial Manchego cheeses. Identification of peptide components in complex protein hydrolysates (e.g., cheese) requires more information than just the masses of the peptides. However, unlike de novo peptide sequencing, a complete sequencing of the peptide components is not always necessary because the sequences of the precursor proteins are known. In our case, the identification approach involved searching for the masses and partial sequences (sequence tags) in a database of ovine milk proteins, including sequence modifications caused by genetic variants and posttranslational modifications (phosphorylation and glycosylation). In a second step, the MS/MS spectrum of the target peptide was compared with the MS/MS spectra of the peptides selected in the database having the same molecular weight. In most cases, the MS/MS spectrum unambiguously matched one sequence of the group of peptides selected by mass. This ideal situation would occur if the fragmentation process could be controlled so that each peptide was cleaved between every 2 consecutive AA and a single charge was retained on the N-terminal group. However, the fragmentation process in mass spectrometers is far from ideal, and some peptides exhibited fragmentation patterns that deviated significantly from the expected fragmentation pattern, and these had to be identified manually.

A total of 107 different peptides were identified in the different fractions of artisan or industrial Manchego cheese at 4 and 8 mo of ripening. Table 1 shows the sequences, protein fragments, CN of origin, and observed and calculated masses of the peptides identified by MS/MS in the different GPC fractions of Manchego cheeses. Peptides were mainly detected in fractions 1 and 2. In fraction 3 only a few peptides were identified, all of them found in cheeses at 8 mo of ripening. No peptides were detected in fractions 4 and 5. These results agree with those of Fernández et al.

(1998), who analyzed the WSE of Cheddar cheese by GPC. Those authors also found the peptides concentrated in the first eluting fractions, a full range of AA in the middle eluting fractions, and Phe, Tyr, and Trp in the last eluting fractions.

All peptides were unequivocally identified, except in the case of INEL/LNEI, for which it was not possible to differentiate between the 2 isomeric AA Leu and Ile. The presence of both residues in the studied peptide prevented us from using MS/MS spectra of the m/z 86 immonium ions of Leu or Ile residues. In addition, the w- and d-series daughter ions were not sufficiently strong to use as a tool to distinguish both residues. Peptides originating from α_{S1} -CN dominated the degradation products, with 50 peptides arising from this protein. Ten of the identified peptides corresponded to α_{S2} -CN fragments, and 42 to β -CN fragments. No peptides were found derived from p- κ -CN. Several dipeptides (103 to 107 in Table 1) were also identified. The AA sequence of these dipeptides was identified in several CN; therefore, they may have their origin in different CN fractions. The highest number of peptides was identified in artisan cheeses. These cheeses usually show a higher proteolysis because they are elaborated with raw milk. In fractions 1 and 2 of the artisan Manchego cheese at 8 mo of ripening, 66 peptides were identified, whereas 38 peptides were identified only in industrial cheeses with the same ripening time. Several peptides (EIVPK, DKIHP, TGPIPN) were identified in 7 of the 8 analyzed fractions. Most of these peptides are described for the first time in Manchego cheese. Among the 107 identified peptides, only 15 had previously been reported in this type of cheese, in a study on the identification and formation of angiotensin I-converting enzyme inhibitory peptides (Gómez-Ruiz et al., 2002b, 2004). Peptides 2, 4, 5, 7, 16, 19, 21, and 24 from α_{S1} -CN and 77 and 98 (Table 1) from β -CN have been identified in Emmental juice (Gagnaire et al., 2001). Additionally, peptides with the same AA sequence as peptides 16, 18, 20, and 33 derived from α_{S1} -CN have previously been reported in Cheddar cheese (Alli et al., 1998; Fernández et al., 1998).

Contribution of Peptides to the Umami and Bitter Tastes in Manchego Cheese

The sensory evaluation of GPC fractions 1 to 3 of artisan and industrial milk cheeses at 4 and 8 mo of ripening showed that in both types of cheeses, umami was the predominant taste in all fractions, followed by salty and bitter. The salty taste is usually attributed to the presence of greater amounts of salts rather than to the presence of specific peptides (Molina et al., 1999).

Table 2. Scores for umami and bitter tastes and possible peptides and AA responsible for such tastes in the Sephadex G-10 gel-permeation fractions 1 to 3 from the water-soluble extract with a molecular weight <1,000 Da of raw and pasteurized Manchego cheeses after 4 and 8 mo of ripening¹

Taste	Fraction	Raw milk cheese				Pasteurized milk cheese			
		4 mo old		8 mo old		4 mo old		8 mo old	
		Score	Peptide	Score	Peptide	Score	Peptide	Score	Peptide
Umami	1	42	ENINEL, INEL, PSE, QEEL	43	INEL, PSE, LEQL, EQEEL, QEEL	40	RKE, INEL, SAEQK, NVVGET	37	ENINEL, INEL, VVGET
	2	44	*	65	INEL	47	*	38	*
	3	40	*	30	*	29	*	20	*
Bitter	1	6	VVAPFP, VAPFP, APFPE	3	VVAPFP, VVAPFPE, VVAPFPFPEV, VAPFPE, APFPE	—	VVAPFPE	—	VVAPFPE, APFPE, VPPFL, GPFP
	2	48	●	ND	VVAPFP, VAPFP, APFPE, VPPFL, PFPKY, GPVRGPFP	—	VVAPFPE, APFPE, GPVRGPFP, VRGPFP, RGPFP	—	VPPFL, GPFP
	3	40	Tyr	3	Phe, Tyr	8	Phe, Tyr	—	Phe, Tyr

¹An asterisk (*) indicates no umami peptides were identified; a minus (—) indicates the taste was not detected; a bullet (●) indicates no bitter peptides were identified.

Astringent and sour tastes were detected slightly, and the sweet taste was absent. In general, the scores for the intensity of the tastes were higher for artisan cheeses than for industrial cheeses. Gómez-Ruiz et al. (2002a) found that Manchego cheeses made from raw milk had a more complex volatile profile and odor intensities than their pasteurized milk counterparts throughout ripening.

Consequently, only peptide sequences related to the umami and bitter tastes were investigated among the different peptides identified in the RP-HPLC-MS/MS analysis. Taste-active peptides and the scores for the umami and bitter tastes given by the panelists are shown in Table 2.

Different umami peptides were found in fractions 1 and 2 of all cheese samples (Table 2). The high scores for umami taste in these fractions may be due to the presence of such umami peptides. The pair Glu-Glu (EE) has previously been reported as an umami peptide (with a taste by itself) in synthetic form (Maehashi et al., 1999) and in fish hydrolysates (Noguchi et al., 1975). In our study, a peptide containing EE in its sequence (QEEL, β -CN) was found in fraction 1 of artisan cheeses at 4 and 8 mo of ripening. However the highest umami intensity was found in fraction 2 of 8-mo-old artisan cheeses, in which only the peptide INEL was identified. Hence, it is possible that some other peptides contribute to the umami taste described in this fraction, because the peptide INEL was also identified in fraction 1, in which the umami taste was perceived with less intensity. In addition, other compounds, such as free AA, are capable of giving an umami taste. Glutamic acid has been reported to be the predominant AA in fraction 1 of ovine milk cheeses (Molina et al., 1999), and Taborda (unpublished re-

sults) found that Glu and Asp were the main free AA in fraction 1 (accounting for 90 to 100% of the total free AA). Therefore, the umami taste of fractions 1 and 2 may be due to umami-active peptides or to free Glu and Asp residues. Moreover, Maehashi et al. (1999) showed that many umami peptides did not have an umami taste per se, although their combination with 0.02% 5'-inosine monophosphate produced a delicious, "full" umami taste.

No umami peptides were found in fraction 3. However, RP-HPLC-MS/MS analysis identified the ammonium ions of Phe (except in 4-mo-old artisan cheese) and Tyr (except in 8-mo-old industrial cheese). These AA are retarded in GPC because of hydrophobic interactions, so they were expected in fraction 4 or 5. However, it should be noted that pure water was used as the eluent to allow sensory analysis; therefore, the separation was not based on molecular size alone. Both aromatic AA showed a significant umami-enhancing effect on the umami taste of monosodium glutamate-NaCl mixtures. This is a novel phenomenon for the so-called bitter AA (Lioe et al., 2005).

Regarding bitterness, it is generally accepted as a consequence of CN degradation (Lemieux and Simard, 1992). Using omissions tests to determine taste-active compounds of Camembert cheese, Engel et al. (2001) showed that peptides were the major contributors to the bitter taste, although the bitterness of Camembert was due not only to its chemical properties but also to the chemical environment and pH at which it was tasted. Moreover, Kim and Li-Chan (2006) recently showed that bulky hydrophobic AA at the C terminus and bulky basic AA at the N terminus were highly correlated with bitterness. In our study, no relation between peptides and bitterness was established (Ta-

ble 2), although the selected peptides joined the characteristics of bitterness described previously. The peptide RGPFPPI, found in fraction 2 of industrial cheeses at 8 mo of ripening, did not lead to any bitter taste, as perceived by the judges, although the heptapeptide RGPFPPIV had a high bitterness intensity (Kim and Li-Chan, 2006). Probably, the independent sensory evaluation of the subfractions of the WSE did not take into account complex taste interactions that may exist among those subfractions in the crude WSE. Peptides are not systematically responsible for cheese bitterness, and some compounds involved in other taste characteristics may disturb the perception of bitter stimuli (Engel et al., 2000).

The taste of peptides by themselves is discussed controversially in the literature, including studies reporting that the “delicious” beefy meaty peptide does not have any umami or other taste (van Wassenaar et al., 1995; Hau et al., 1997). The individual taste of most peptides is probably weak or even undetectable, and their role in eliciting an intense umami taste remains unclear. In our study, the umami taste was predominant in all fractions, even in those in which umami-tasting peptides were not found. Therefore, it is difficult to understand the real impact of peptides on the umami properties of the WSE <1,000 Da of Manchego cheese. In a study on soy sauces, Lioe et al. (2006) concluded that peptides, especially those containing glutamyl residues, did not contribute significantly to the umami taste of koikuchi and tamari shoyu. In addition, those peptides with a taste similar to that of monosodium glutamate or umami peptides are known to have the property of masking the taste of foods (Ohyama et al., 1988), so other tastes of the fractions could be underestimated, and this could be the reason for the predominant umami taste in all fractions. These results are in agreement with those reported by Salles et al. (2000) in the evaluation of taste compounds in WSF of goat cheeses, in which the direct impact of the low molecular weight compounds on the taste of cheeses was minimized. However, the same group reported that the omission of peptides and AA in a model WSE led to a significant decrease in the umami taste (Engel et al., 2001).

In our study, the high scores for umami taste in fraction 1 of artisan cheeses at 4 and 8 mo of ripening could be attributed to the peptides EQEEL and QEEL, which contain a high number of glutamic residues. Furthermore, the presence of salts could be important in enhancing the flavor peptides, and other mentioned factors, such as interactions among peptides, the presence of new peptides still not evaluated sensorially, and free and conjugated AA, can have an influence on the overall taste.

ACKNOWLEDGMENTS

This work was financially supported by the projects AGL2005-03381 from the Spanish Ministry of Education and Science (MEC) and S-505/AGR/0153-01 from the Autonomous Community of Madrid. G. Taborda wishes to express his appreciation to the University of Caldas and Colciencias (Colombia) for the financial fellowship.

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