ELSEVIER

Journal of Chromatography A, 881 (2000) 69–79

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Effect of the activity levels of the added proteolytic enzyme mixture on free amino acids in ripening Ossau-Iraty cheese

Jesús María Izco*, Aurora Irigoyen, Paloma Torre, Yolanda Barcina

Area de Nutrición y Bromatología, Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, Campus Arrosadía s/n, 31006 Pamplona, Spain

Abstract

A proteolytic enzymatic preparation (using one of three enzyme concentrations and, hence, one of three different enzymatic activity levels) was added (before clotting) to the milk used to manufacture Ossau-Iraty ewes'-milk cheese. The free amino acids were analysed by reversed-phase high-performance liquid chromatography and the sulphosalicylic acid-soluble N fraction was quantified by the trinitrobenzenesulphonic acid method for use as an index of proteolysis during ripening. Sensory analysis of the cheeses began after two months of ripening. Use of the enzymatic preparation increased the rate of release of amino acids in an amount proportional to the enzyme concentration employed. The effect of the preparation was more pronounced in the early months of ripening, with the differences in the free amino acid contents of the various batches decreasing as ripening progressed. Levels of certain free amino acids, such as taurine, tyrosine and valine, were virtually unaffected by the addition of the enzymatic preparation, whereas levels of such amino acids as serine, glycine, arginine and proline were reduced. Texture defects in the cheeses were observed, namely, reduced elasticity and creaminess and increased brittleness. Similarly, enzymatic treatment also gave rise to bitter flavours that were not characteristic of the normal taste and aftertaste of Ossau-Iraty cheese and these changes were proportional to the quantity of enzyme added.

Keywords: Cheese; Food Analysis; Amino Acids; Enzymes

1. Introduction

Ossau-Iraty cheese is manufactured from raw or pasteurized ewes'-milk in southwestern France and is one of the 33 cheeses that have been awarded an Appellation of Origin (A.O.) in France. It is an uncooked, pressed cheese that must be aged for a minimum of 90 days (60 days in the case of a related variety, Petit Ossau-Iraty cheese).

Few experiments in which accelerated ripening has been applied to ewes' milk cheeses are to be found [1]. Some experimental work on ewes' milk cheeses has been carried out on Manchego cheese in Spain (regulated by an A.O.) [2–4] and on Feta cheese in Greece [5]. Various methods can be used to accelerate cheese ripening [6–10], but, to a greater or lesser extent, proteolysis is a key process during ripening in all cheeses and, hence, research on accelerated ripening has mainly focused on the use of proteinases and peptidases [11,12]. The addition of enzymatic preparations directly to the milk before coagulation yields the best distribution of the enzymes, thereby enhancing contact between the enzymes and the particles in the coagulum [6,13] and avoiding the risk of overripening [4].

Amino acids and peptides contributing directly to

*Corresponding author. Tel.: +34-948-169-141; fax: +34-948-169-187.

E-mail address: jesus.izco@unavarra.es (J.M. Izco)

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00285-5

cheese flavour and aroma are released as the caseins are broken down during cheese ripening [14]. Quantification of the free amino acids (FAAs) can be quite useful for cheeses undergoing accelerated ripening, as a means of evaluating their effect on flavour and aroma intensity [15]. FAAs have for some time been known to play a major role in aroma formation in Cheddar cheese [16], and certain FAAs are extremely important factors in aroma development, e.g., proline in a Swiss-type cheese [17].

Sensory analysis is an essential tool in evaluating the results obtained when introducing technical innovations into the manufacturing processes employed for food products. Evaluation of cheese texture and flavour is indispensable in studying possible alterations caused by adding proteolytic enzymes, chiefly because of the activity of such enzymes on the proteins in the cheese [1,2].

The object of the present study was to establish a preliminary approximation of the effect of an added enzymatic mixture on proteolysis during ripening of Ossau-Iraty cheese, by analyzing the evolution of free amino acids as proteolytic indices. Finally, the aim of this work was to test a commercial proteolytic mixture by adding it to the milk, in order to obtain a ewes' milk cheese, Ossau-Iraty type, with accelerated ripening.

2. Experimental

2.1. Cheese manufacture

Three experimental batches E1, E2 and E3, each with an added enzymatic preparation containing one of three different concentrations of Peptidase R+ Prozyme 6 (Biocatalysts, Treforest Industrial Estate, Pontypridd, UK) and, hence, one of three different enzymatic activity levels (see Table 1), and one control batch (C) of type Ossau-Iraty cheese were prepared. For each batch, 200 l of pasteurized milk (20 s at 72°C) were incubated with starter culture (Flora Danica 50 U, Sochal, 92300 Levallois-Perret, France) at 11°C for 18 h, after which, it was stored at 4°C to halt bacterial activity until use. The milk was then heated to 32°C and starter culture (Flora Danica) was added (2 g/100 l) and allowed to act at that temperature for at least 15 min. An enzyme preparation (10 g/100 l) with one of three enzyme concentrations was added to the experimental cheese batches before the rennet. Next, 18 ml/100 l of rennet were added. After 20 min, the curd was comminuted to a particle size smaller than 5 mm at a temperature of 36°C, which was completed in less than 15 min. Part of the whey (20%) was then drawn off, and the curd was washed by replacing the volume removed with water heated to batch temperature. After preliminary pressing for 20 min to remove the whey, the curd was placed in moulds and pressed for 2.5 h. The cheeses were brined at 10°C in a saturated salt solution for 24 h and ripened at a temperature of less than 12°C and a relative humidity higher than 80%. Cheese mass ranged between 2 and 3 kg. Duplicate cheese samples were collected for physicochemical analysis on days 1, 15, 30, 60, 90 and 120 of the ripening process. Starting on day 60, a third sample was also collected for sensory analysis.

2.2. Physicochemical analyses

Dry matter (DM) was determined according to IDF-FIL standard 4 [18].

Table 1
Enzyme preparations used for each batch[a]

Batch designation	Type	Composition
C	Control	–
E1	Protease	Peptidase R (4UE)+Prozyme 6 (2UE)
E2	Protease	Peptidase R (10UE)+Prozyme 6 (5UE)
E3	Protease	Peptidase R (15UE)+Prozyme 6 (7.5UE)

[a] UE: Arbitrary unit of enzyme activity tested. Enzymatic mixtures were supplied by Biocatalysts (Treforest Industrial Estate, Pontypridd, UK).

The sulphosalicylic acid-soluble N (SSAN) fraction was quantified using the trinitrobenzenesulphonic acid (TNBS) method previously described [20].

2.2.1. Analysis of free amino acids

RP-HPLC analysis of the FAAs was performed according to the method of Barcina et al. [19]. A 1-g amount of comminuted cheese was weighed out, and 10 ml of 0.1 *M* HCl containing 0.4 m*M* methionine sulfone was added as the internal standard. The mixture was homogenized using an Ultra-Turrax blender, sonicated in a water bath for 20 min and centrifuged at 3000 *g* for 10 min. A 500-μl volume of supernatant was diluted (1:1, v/v) with trichloroacetic acid (40%). The mixture was left to react at 0–4°C for 10 min and centrifuged at 20 000 *g* for 10 min. A 25-μl volume of deproteinized supernatant was vacuum-dried. A 20-μl volume of redry solution (triethylamine–methanol–1 *M* sodium acetate; 1:2:2, v/v/v) was added to the dried samples, which were vacuum-dried again. A 20-μl volume of derivatizing solution (methanol–water–triethylamine–phenylisothiocyanate, 7:1:1:1, v/v) was added to the dried samples, which were then incubated for 10 min at room temperature before being vacuum-dried. Samples were resuspended in 100 μl of Pico-tag sample diluent (Waters, Milford, MA, USA) and were filtered through a type-HV hydrophilic filter (Millipore) with a pore size of 0.45 μm. Samples were analysed on a Waters HPLC system consisting of two 510 pumps, an ULTRA WISP 715 injector, a temperature control module and a 996 photodiode array detector set at 254 nm, operated using Millennium 2010 software. The column used was a Waters Pico-Tag C_{18} reversed-phase column maintained at 46°C. For identification and quantification of FAAs, a master solution of amino acids (Sigma, St. Louis, MO, USA) was used, to which methionine sulfone (Sigma) was added as an internal standard.

A gradient with two solvents was used to run the samples: solution A comprised 70 m*M* sodium acetate adjusted to pH 6.55 with acetic acid and added containing 2.5% acetonitrile and solution B was 45% acetonitrile, 40% water and 15% methanol. Before each injection, the column was equilibrated with solvent A for 20 min.

2.3. Sensory analysis

All samples were evaluated by at least eight trained assessors. A sensory analysis scoring sheet was developed specifically for this cheese, taking the following attributes into account: characteristic odour, sweet odour and bitter odour (odour analysis); elasticity, firmness, adherence, creaminess, brittleness, deformability and grittiness (texture analysis); characteristic taste, pungent taste, sweet taste, sour taste, salty taste and bitter taste (taste analysis); and characteristic aftertaste, persistence, sour aftertaste, salty aftertaste and bitter aftertaste (aftertaste analysis). The intensity of each attribute was scored according to an increasing scale from one to seven. In all, a total of 21 sensory attributes were evaluated.

2.4. Statistical treatment

The SPSS computer program (version 6.1, SPSS, Chicago, IL, USA) was used for statistical processing. Analysis of variance with 95% confidence intervals was run on each of the physicochemical parameters analysed to ascertain whether or not the differences between batches on each sampling date were significant. Stepwise discriminant analysis was also performed on the FAAs analysed to ascertain which of the different attributes were most useful in differentiating between batches and in classifying the cheeses according to batch. Wilk's lambda (λ) was used as the selection criterion for the attributes. When the discriminant functions had been derived, the standardized coefficients in the functions were calculated. The standardized coefficient values were indicative of the relative importance of each of the original attributes in each of the discriminant functions. The confusion matrix was also calculated to determine the percentage of cases that were correctly classified using only the first discriminant function.

3. Results

Fig. 1 shows the chromatograms of the analysis of FAAs from Control (C) and enzyme-treated cheeses (E1, E2 and E3) at 120 days of ripening. Extraction of FAAs and separation conditions were as given in the Experimental section. For quantification, a master

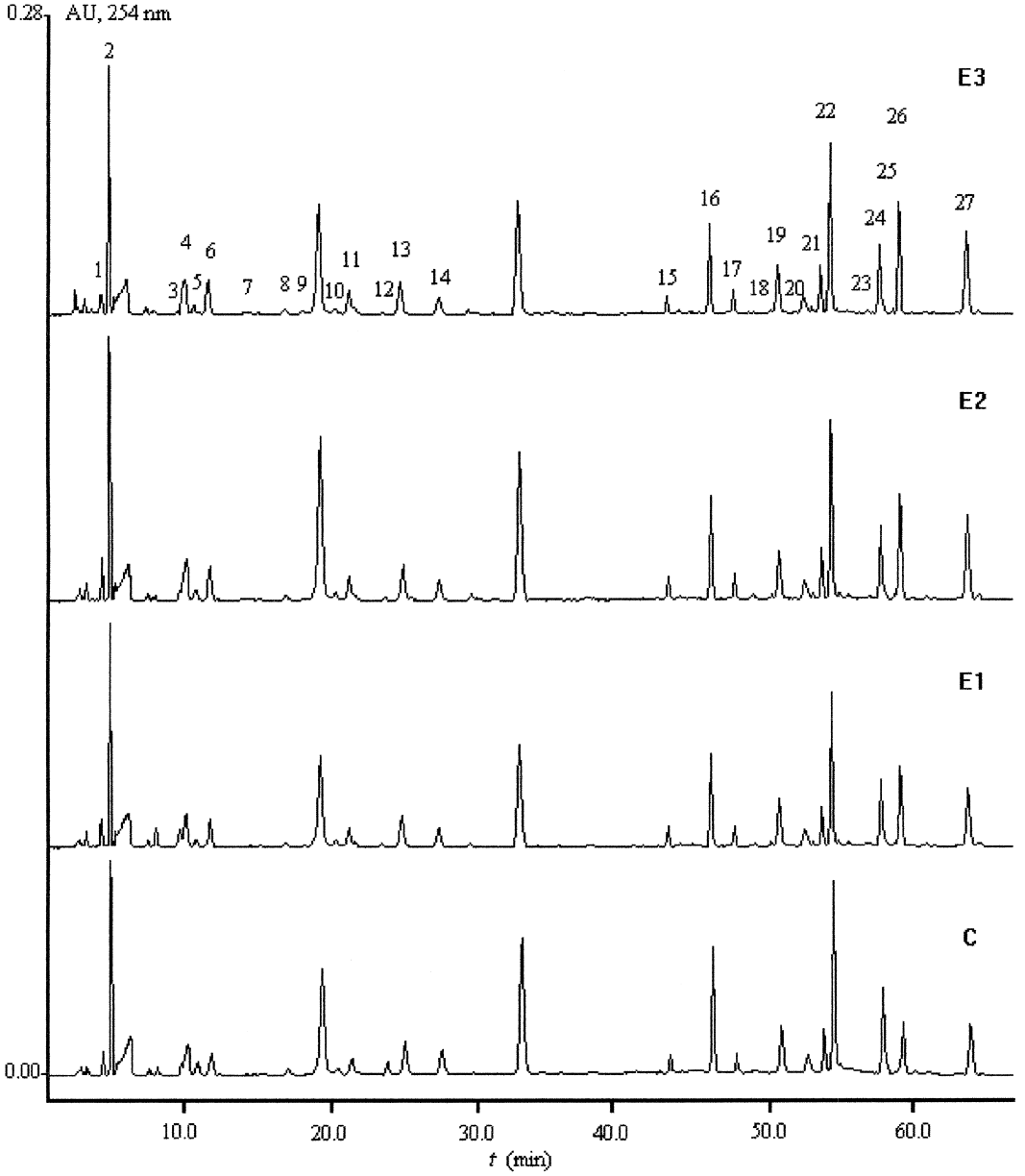

Fig. 1. Chromatograms of the analysis of free amino acids for control (C) and enzyme-treated (E1, E2 and E3) cheeses at day 120 of ripening. Conditions are given in the Experimental section. Peaks: 1=ASP, 2=GLU, 3=SER, 4=ASN, 5=GLY, 6=ASN, 7=TAU, 8=HIS, 9=GABA, 10=THR, 11=ALA, 12=ARG, 13=I.S. (methionine sulfone), 14=PRO, 15=TYR, 16=VAL, 17=MET, 18=CYST, 19=derivatizing agent (Ph-isothiocyanate), 20=CYS, 21=ILE, 22=LEU, 23=HYLYS, 24=PHE, 25=TRP, 26=ORN and 27=LYS.

solution of amino acids was used, to which methionine sulfone was added as an I.S. The cheese extraction and derivatizing process yield, together with intact FAAs, a number of unidentified peaks, although these rarely interfered with the identified peaks.

3.1. Physicochemical analyses

Tables 2 and 3 set out the results obtained for the cheeses on days 1, 15, 30, 60, 90 and 120 of the ripening period. On day 60 of ripening, batches E2 and E1, which were quite similar and became increasingly more similar to the control batch, presented the same levels of some of the main amino acids that, up to that point in ripening, had previously been higher than in the control batch, namely, glutamic acid, asparagine and glutamine. Levels of many of the amino acids were higher in batch E3 than in the other batches, however, as the ripening period progressed, the initial differences between this batch and the other cheese batches decreased.

Batch E3 had the highest total free amino acid (TFAA) values until day 60, but, after three months, the values were the same as in the other batches to which enzymes had been added. On day 30, the TFAA level in batch E1 was no longer significantly different from the level in the control batch, and this remained the case until the end of the ripening process.

The results show that, for SSAN as a proteolytic index, the differences between batches also diminished in the later stages of ripening, as has been

Table 2
Free amino acid (FAA*) and sulphosalicylic acid-soluble N (SSAN**) contents in the control (C) and enzyme-treated (E1, E2 and E3) cheeses on days 1, 15 and 30 of ripening[1]

Amino acid	Day 1				Day 15				Day 30			
	C	E1	E2	E3	C	E1	E2	E3	C	E1	E2	E3
ASP*	4.0^{b}	6.2^{a}	6.2^{a}	5.6^{a}	3.8^{b}	6.3^{a}	5.9^{a}	6.3^{a}	6.3^{b}	11.2^{a}	11.1^{a}	10.7^{a}
GLU	21.7^{c}	30.5^{b}	33.2^{a}	33.8^{a}	25.1^{c}	36.0^{b}	36.2^{b}	42.7^{a}	46.1^{c}	61.6^{b}	62.1^{b}	71.3^{a}
SER	2.6^{c}	4.0^{a}	3.7^{b}	4.3^{a}	4.5^{b}	7.3^{a}	7.0^{a}	8.3^{a}	7.9^{b}	12.8^{a}	11.0^{a}	8.6^{b}
ASN	11.6^{c}	15.1^{b}	16.4^{a}	16.4^{a}	18.9^{b}	30.7^{a}	32.5^{a}	36.5^{a}	43.6^{b}	58.5^{a}	60.7^{a}	67.0^{a}
GLY	0.8^{a}	0.5^{b}	0.5^{b}	0.4^{b}	1.2^{a}	1.1^{a}	1.1^{a}	1.1^{a}	5.2^{a}	2.8^{b}	2.2^{b}	2.0^{b}
GLN	4.8^{c}	7.7^{ab}	7.4^{b}	8.7^{a}	10.1^{b}	16.8^{a}	18.2^{a}	20.4^{a}	18.3^{c}	26.6^{b}	27.6^{b}	34.6^{a}
TAU	4.1^{a}	3.8^{a}	4.0^{a}	3.9^{a}	4.2^{a}	4.0^{a}	3.9^{a}	3.8^{a}	3.7^{a}	3.8^{a}	3.6^{a}	3.2^{a}
HIS	2.7^{d}	5.2^{c}	6.1^{b}	7.0^{a}	2.9^{d}	6.1^{c}	7.3^{b}	8.3^{a}	3.6^{d}	5.8^{c}	6.7^{b}	7.6^{a}
GABA	0.3^{b}	1.1^{a}	1.4^{a}	1.1^{a}	0.4^{c}	2.3^{ab}	2.8^{a}	2.0^{b}	0.6^{b}	2.7^{a}	3.3^{a}	2.6^{a}
THR	0.6^{b}	1.1^{a}	1.3^{a}	1.0^{a}	3.2^{b}	4.3^{a}	4.4^{a}	5.0^{a}	3.9^{b}	5.2^{ab}	5.6^{a}	5.9^{a}
ALA	3.1^{b}	5.1^{a}	4.4^{a}	4.6^{a}	5.7^{b}	8.1^{a}	8.2^{a}	8.9^{a}	8.8^{b}	10.3^{ab}	11.2^{ab}	13.0^{a}
ARG	nq	0.8^{a}	0.7^{b}	0.9^{ab}	2.7^{a}	2.5^{a}	2.3^{a}	2.8^{a}	6.2^{a}	4.8^{a}	4.9^{a}	5.4^{a}
PRO	22.7^{a}	22.9^{a}	21.5^{ab}	20.9^{b}	23.9^{a}	24.3^{a}	22.5^{a}	22.9^{a}	24.3^{a}	25.7^{a}	21.1^{b}	21.4^{b}
TYR	4.6^{a}	5.2^{a}	4.5^{a}	4.7^{a}	8.8^{a}	8.9^{a}	8.7^{a}	8.8^{a}	12.0^{a}	10.3^{a}	11.8^{a}	12.2^{a}
VAL	7.7^{a}	8.2^{a}	7.7^{a}	7.6^{a}	17.9^{a}	17.2^{a}	17.0^{a}	19.9^{a}	35.1^{a}	29.8^{a}	29.3^{a}	30.8^{a}
MET	1.1^{b}	1.7^{a}	1.3^{b}	1.9^{a}	3.2^{b}	4.2^{ab}	4.2^{ab}	5.5^{a}	6.2^{b}	8.3^{a}	7.6^{ab}	9.1^{a}
CYST	0.5^{d}	1.4^{c}	2.3^{b}	2.7^{a}	1.0^{d}	2.5^{c}	3.7^{b}	4.8^{a}	1.2^{d}	4.6^{c}	6.1^{b}	7.0^{a}
CYS	1.0^{c}	2.5^{b}	4.1^{a}	3.8^{a}	2.1^{c}	5.6^{b}	8.9^{a}	8.0^{a}	1.4^{c}	3.1^{b}	4.6^{a}	5.2^{a}
ILE	2.7^{b}	3.0^{b}	2.8^{b}	3.6^{a}	6.4^{b}	7.2^{b}	7.6^{b}	9.9^{a}	10.3^{b}	10.6^{b}	11.3^{ab}	14.5^{a}
LEU	10.5^{a}	11.0^{a}	9.1^{b}	11.4^{a}	34.3^{a}	30.9^{a}	29.1^{a}	34.6^{a}	40.4^{b}	31.3^{b}	44.1^{ab}	54.1^{a}
HYLYS	0.6^{c}	0.8^{b}	1.0^{ab}	1.0^{a}	0.4^{d}	2.0^{c}	2.4^{b}	3.0^{a}	1.9^{c}	3.9^{b}	4.6^{a}	3.8^{b}
PHE	8.3^{d}	9.3^{c}	10.5^{b}	11.9^{a}	21.9^{b}	24.6^{b}	28.0^{ab}	31.9^{a}	51.2^{b}	49.6^{b}	53.5^{ab}	60.1^{a}
TRP	0.3^{c}	0.6^{b}	1.0^{a}	0.9^{a}	0.5^{c}	1.3^{b}	1.7^{ab}	1.9^{a}	1.4^{d}	4.0^{c}	7.4^{b}	9.3^{a}
ORN	5.9^{b}	6.7^{a}	6.7^{a}	7.4^{a}	10.8^{b}	13.3^{ab}	14.8^{a}	16.2^{a}	17.3^{b}	22.2^{a}	23.7^{a}	25.5^{a}
LYS	3.6^{d}	8.0^{c}	9.8^{b}	12.5^{a}	6.6^{c}	13.5^{b}	16.5^{b}	21.6^{a}	9.5^{d}	16.2^{c}	22.1^{b}	26.7^{a}
TOTAL	125.8^{d}	162.2^{c}	166.9^{b}	177.7^{a}	221.7^{c}	283.2^{b}	296.3^{ab}	336.8^{a}	368.5^{c}	427.7^{bc}	459.3^{ab}	514.3^{a}
SSAN**	1.84^{c}	2.52^{b}	3.08^{a}	3.49^{a}	2.28^{c}	3.5^{b}	3.99^{ab}	4.51^{a}	4.76^{c}	6.75^{b}	7.56^{b}	8.61^{a}

[1] Different superscripts in the same row on the same sampling date indicate significant differences between the mean values ($p<0.05$, n=4). nq, not quantified.

* FAA contents are expressed as mg FFA/100 g dry matter.

** SSAN contents are expressed as mmol L-LEU/100 g dry matter.

Table 3
Free amino acid (FAA*) and sulphosalicylic acid-soluble N (SSAN**) contents in the control (C) and enzyme-treated (E1, E2 and E3) cheeses on days 60, 90 and 120 of ripening[1]

Amino acid	Day 60				Day 90				Day 120			
	C	E1	E2	E3	C	E1	E2	E3	C	E1	E2	E3
ASP*	8.2^{b}	13.6^{a}	13.1^{a}	15.5^{a}	12.7^{c}	15.6^{bc}	20.2^{a}	16.9^{ab}	15.5^{c}	21.4^{b}	26.4^{a}	22.2^{b}
GLU	86.2^{b}	99.8^{b}	96.2^{b}	123.5^{a}	127.7^{b}	144.9^{ab}	186.8^{a}	157.3^{ab}	165.5^{b}	197.3^{ab}	229.1^{a}	209.8^{a}
SER	10.7^{b}	16.9^{a}	6.6^{c}	3.9^{d}	14.2^{b}	22.2^{a}	12.1^{b}	5.1^{c}	15.4^{b}	26.5^{a}	13.3^{b}	6.1^{c}
ASN	99.0^{b}	115.7^{b}	115.9^{b}	146.9^{a}	142.2^{b}	175.6^{ab}	225.8^{a}	181.9^{ab}	192.2^{b}	235.7^{ab}	288.2^{a}	254.8^{a}
GLY	6.5^{a}	2.9^{b}	2.7^{b}	3.1^{b}	9.8^{a}	5.4^{c}	7.9^{b}	6.9^{c}	15.0^{a}	9.9^{c}	11.7^{b}	10.6^{bc}
GLN	34.5^{b}	43.5^{b}	42.2^{b}	59.0^{a}	46.0^{b}	64.4^{a}	78.2^{a}	74.9^{a}	69.0^{b}	96.6^{a}	110.0^{a}	111.0^{a}
TAU	3.5^{a}	3.6^{a}	3.2^{a}	3.1^{a}	3.4^{ab}	3.8^{a}	3.7^{a}	2.8^{b}	3.5^{a}	3.2^{a}	2.9^{a}	2.7^{a}
HIS	9.1^{ab}	7.8^{c}	8.0^{bc}	10.1^{a}	11.8^{ab}	10.4^{b}	14.4^{a}	14.3^{a}	19.6^{a}	15.6^{b}	17.9^{ab}	17.5^{ab}
GABA	0.5^{b}	3.3^{a}	2.9^{a}	2.8^{a}	2.1^{b}	2.7^{ab}	2.9^{a}	0.8^{c}	nq	3.2^{b}	3.9^{b}	7.8^{a}
THR	5.4^{b}	6.7^{ab}	5.6^{b}	7.8^{a}	12.2^{b}	15.6^{ab}	19.6^{a}	16.1^{ab}	15.3^{c}	21.6^{ab}	24.7^{a}	17.7^{bc}
ALA	11.6^{c}	15.8^{b}	14.5^{bc}	20.8^{a}	17.2^{c}	22.3^{b}	27.7^{a}	26.6^{ab}	20.7^{c}	28.5^{b}	34.8^{a}	38.9^{a}
ARG	26.9^{a}	10.0^{c}	12.1^{bc}	13.6^{b}	31.0^{a}	7.6^{c}	10.8^{bc}	13.2^{b}	30.8^{a}	8.7^{b}	9.3^{b}	5.2^{c}
PRO	34.3^{a}	28.7^{b}	20.4^{c}	27.1^{b}	40.8^{a}	33.5^{a}	36.6^{a}	33.5^{a}	58.3^{a}	46.5^{b}	45.9^{b}	41.9^{b}
TYR	19.0^{ab}	17.0^{b}	21.2^{ab}	23.5^{a}	25.8^{b}	28.5^{b}	35.2^{a}	36.3^{a}	30.8^{b}	40.1^{a}	44.8^{a}	32.8^{b}
VAL	79.6^{a}	62.7^{bc}	58.2^{c}	71.2^{ab}	109.9^{a}	94.4^{a}	99.8^{a}	93.0^{a}	127.4^{a}	115.3^{a}	135.2^{a}	116.2^{a}
MET	14.2^{b}	16.4^{b}	16.4^{b}	21.8^{a}	19.0^{b}	23.7^{b}	29.8^{a}	30.2^{a}	30.9^{b}	39.1^{a}	43.0^{a}	40.8^{a}
CYST	1.6^{c}	7.0^{b}	7.8^{ab}	8.1^{a}	2.0^{c}	8.5^{ab}	8.2^{b}	10.1^{a}	1.3^{c}	7.7^{ab}	8.1^{a}	7.1^{b}
CYS	2.2^{c}	3.8^{b}	4.2^{b}	6.4^{a}	1.5^{c}	6.5^{ab}	3.7^{bc}	9.0^{a}	nq	nq	nq	nq
ILE	21.8^{b}	22.6^{b}	24.3^{b}	33.1^{a}	33.2^{c}	39.6^{b}	47.0^{a}	51.1^{a}	35.0^{b}	41.0^{b}	53.3^{a}	53.6^{a}
LEU	123.5^{a}	96.0^{b}	98.5^{b}	124.5^{a}	114.6^{ab}	101.5^{b}	110.9^{ab}	122.3^{a}	204.8^{a}	196.5^{a}	206.3^{a}	206.8^{a}
HYLYS	2.5^{c}	5.2^{b}	6.2^{ab}	6.8^{a}	4.9^{b}	8.5^{a}	8.6^{a}	9.4^{a}	2.2^{c}	6.4^{a}	5.9^{a}	4.6^{b}
PHE	93.1^{ab}	75.8^{c}	82.3^{bc}	94.3^{a}	113.5^{a}	107.9^{a}	104.7^{a}	106.7^{a}	138.5^{a}	128.1^{ab}	123.8^{ab}	120.2^{b}
TRP	4.9^{d}	6.4^{c}	10.7^{b}	13.6^{a}	9.8^{c}	11.0^{c}	14.8^{b}	18.9^{a}	11.7^{a}	6.6^{c}	8.3^{bc}	10.2^{ab}
ORN	21.0^{c}	37.3^{b}	40.9^{b}	55.8^{a}	27.5^{b}	64.8^{a}	62.0^{a}	69.0^{a}	42.3^{b}	87.0^{a}	95.0^{a}	104.7^{a}
LYS	25.1^{c}	37.1^{b}	43.7^{b}	58.2^{a}	37.3^{c}	57.3^{b}	85.0^{a}	91.2^{a}	60.2^{c}	85.4^{b}	114.1^{a}	114.7^{a}
TOTAL	744.6^{b}	755.3^{b}	757.5^{b}	954.0^{a}	969.8^{b}	1076.0^{ab}	1256.3^{a}	1197.0^{a}	1305.7^{b}	1467.2^{ab}	1655.8^{a}	1557.5^{ab}
SSAN**	8.59^{c}	10.51^{b}	11.69^{b}	13.85^{a}	10.30^{c}	13.06^{b}	14.78^{a}	15.18^{a}	10.41^{b}	13.24^{a}	13.97^{a}	14.38^{a}

[1] Different superscripts in the same row on the same sampling date indicate significant differences between the mean values ($p<0.05$, $n=4$). nq: not quantified.

* FAA contents are expressed as mg FFA/100 g dry matter.

** SSAN contents are expressed as mmol L-LEU/100 g dry matter.

reported previously [3]. SSAN values were highest in batch E3 throughout the entire ripening period. However, the values did not differ significantly from those for batch E2 after three months or from those for batch E1 after four months. SSAN values were higher than in the control batch in all of the cheese batches to which enzymes were added during manufacture, suggesting an increase in proteolysis brought about by use of the enzyme.

The discriminant analysis selected the parameters shown in Table 4. Proline and glycine can be observed to have more weight in function 1. In fact, concentrations of those amino acids were higher in batch C than in the other batches. This is also reflected in Fig. 2, in which function 1 clearly discriminated the control cheeses from the experimental cheese batches. Cheese batches E2 and E3 exhibited some overlap, which is in agreement with the fact that, in the later stages of ripening, the values for many of the amino acids were similar in these two batches. Plotting of discriminant functions 1 and 2 yielded good classification of the cheeses (with 97.92% of the ''grouped'' cases being correctly classified), as shown in Table 5.

3.2. Sensory analysis

Fig. 3 presents the profiles obtained from sensory

Table 4
Discriminant analysis: summary of parameter (FAA) selection and standardized canonical discriminant function coefficients

Step	Parameter entered	Wilks' λ	Significance	Function 1	Function 2	Function 3
1	CYSTA	0.51477	0.0000	0.97480	−1.65332	0.08799
2	SER	0.25132	0.0000	0.35886	1.23351	0.12085
3	HYS	0.06372	0.0000	8.26155	−0.06074	−1.33507
4	PRO	0.02999	0.0000	−4.26813	2.16509	2.29884
5	ASP	0.02404	0.0000	2.00274	4.17922	−2.03000
6	GLY	0.01829	0.0000	−3.91279	−3.02098	−1.83401
7	HILYS	0.01546	0.0000	−1.05057	1.22070	0.65532
8	LEU	0.01401	0.0000	−1.51397	−0.59692	2.15961
9	ORN	0.01274	0.0000	1.17743	1.73851	−0.01878
10	ASN	0.00942	0.0000	−7.30156	0.28179	−15.06109
11	ALA	0.00796	0.0000	1.97620	−0.10787	4.38243
12	GLU	0.00654	0.0000	3.47573	−4.95659	10.89540

analysis of the four cheese batches after 90 days of ripening. Differences between the batches with respect to odour were especially noticeable at two months of ripening, with batch E3 earning the lowest scores for sweet odour and for characteristic odour. After four months of ripening, the aroma profiles for the different cheese batches had become very similar. The largest differences recorded between the cheese batches were found in the texture profiles. Although the behaviour of the control batch was quite uniform between two and four months of ripening, that was not the case for the cheese batches made using added enzyme. The three experimental batches generally earned lower scores for elasticity and creaminess and higher scores for brittleness than the control batch. The taste and aftertaste profiles for

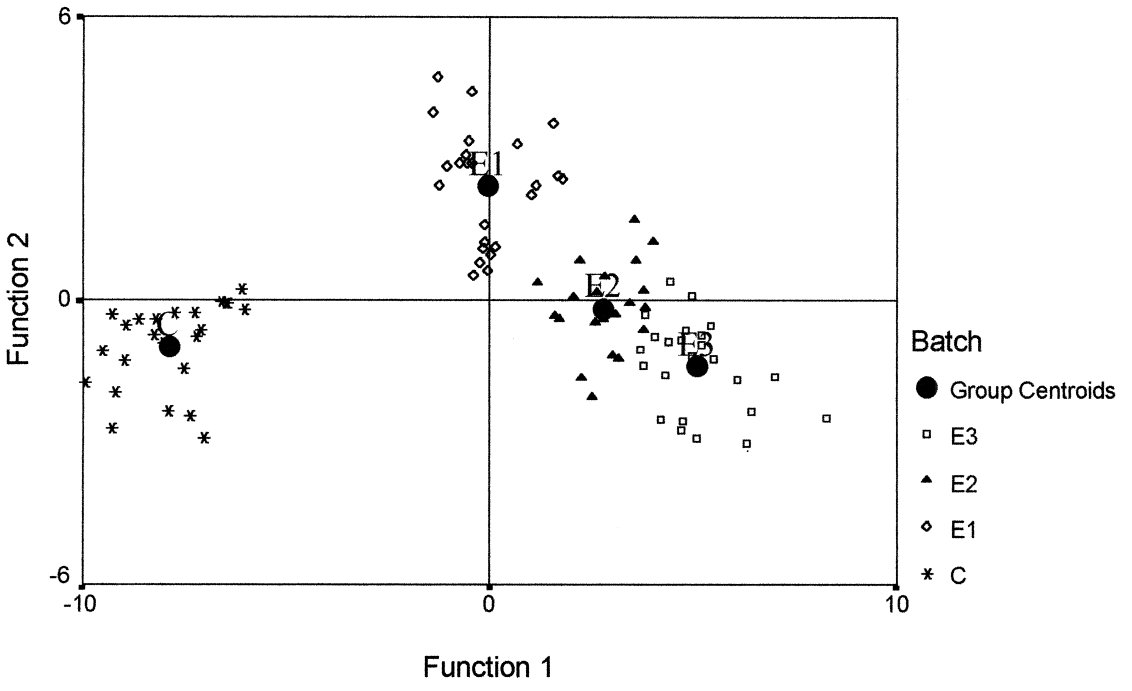

Fig. 2. Plot of the canonical discriminant functions obtained using the FAA analysis results, classing control cheese (C) and enzyme-treated cheeses (E1, E2 and E3).

Table 5
Classification results based on discriminant analysis[a]

Actual group	No. of cases	Predicted group membership			
		1	2	3	4
Group 1 C	24	24 100.0%	0 0.0%	0 0.0%	0 0.0%
Group 2 E1	24	0 0.0%	24 100.0%	0 0.0%	0 0.0%
Group 3 E2	24	0 0.0%	0 0.0%	24 100.0%	0 0.0%
Group 4 E3	24	0 0.0%	0 0.0%	2 8.3%	22 91.7%

[a] Percent of ''grouped'' cases correctly classified: 97.92%.

batches E1 and E2 were quite similar to the profiles for the control batch, with some slight differences at 90 days of ripening. Batch E3 presented the largest discrepancies in these parameter values, and that batch earned higher bitterness scores than the other batches, particularly for aftertaste, throughout the ripening period spanned by the sensory analysis tests.

4. Discussion

The behaviour of certain amino acids over the ripening period deserves mention. For instance, except for day 60 in the case of valine, concentrations of taurine and valine did not vary significantly between the cheese batches on the different sampling dates. In addition, the glycine concentration in batch C was higher than in the rest of the batches over the entire ripening period, except on day 15, on which values in all the batches were about the same. The situation for arginine, an amino acid related to bitter flavours in cheese [19], was similar. The concentration of that amino acid increased at a higher rate in the control batch than in the other batches. Fernández-García et al. [2] added Neutrase to a blend of bovine and ovine milks (30:70, v/v) and found increases of 100% for tyrosine and valine on day 45 and even greater increases for arginine. However, on combining Neutrase and Palatase, Fernández-García et al. [4] recorded decreases in the concentrations of glutamic acid, asparagine, arginine,

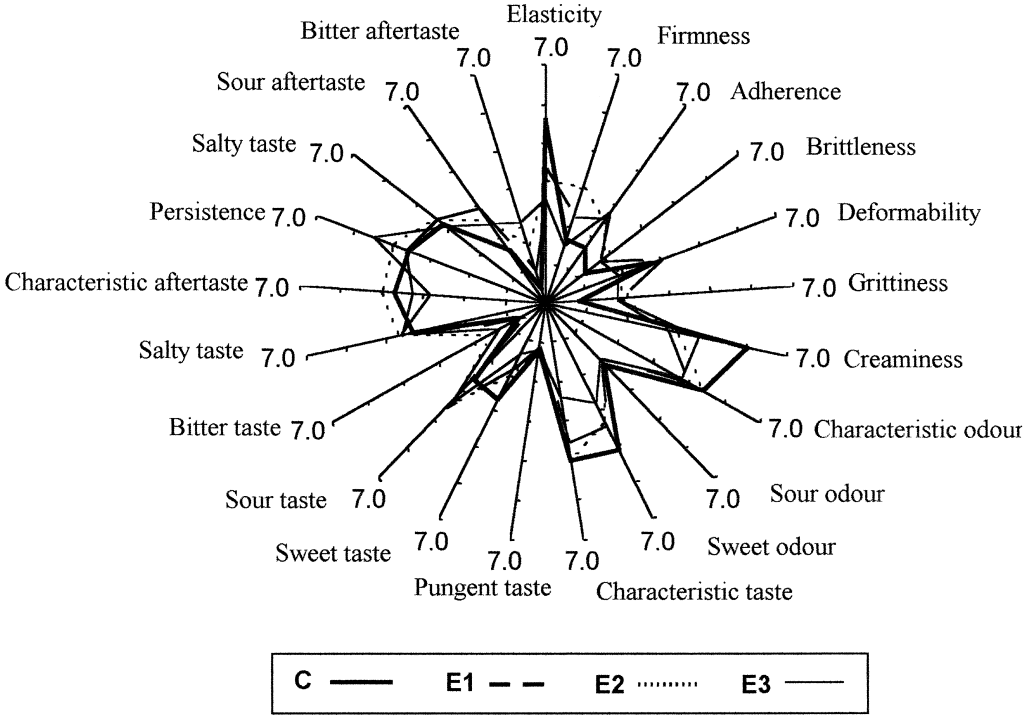

Fig. 3. Sensory scores for the control cheese (C) and enzyme-treated cheeses (E1, E2 and E3) on day 90 of the ripening period.

valine and tyrosine. Conversely, using Flavorage (a lipase plus proteinases and peptidases,) Fernández-García et al. [3] had earlier recorded increases in all of the amino acids, although the differences with respect to the control batch grew smaller as ripening advanced.

Levels of proline, an amino acid related to sweet flavours in cheese [21], also increased at a higher rate in the control batch than in the experimental batches. Other researchers, using proteinases in cheeses made from cows' milk, have also reported the same or very much lower levels of this amino acid in cheeses manufactured using added enzymes [21,22]. The behaviour of the amino acid serine was also special, in that the concentration of that amino acid decreased with increasing concentrations of the enzymes employed in this study. Thus, in batch E3, the concentration of this amino acid was the same as in the control batch on day 30 and then fell to lower levels until the end of ripening. Similarly, in batch E2, the serine concentration was lower than or equal to that in the control batch from day 60. Interestingly, in batch E1, the concentration of this amino acid was higher than that in the control batch throughout the ripening period. Law and Wigmore [22] observed a slight decrease in the amount of serine in Cheddar cheese treated with Neutrase.

The differences in TFAA levels at the start of the ripening period decreased progressively as ripening advanced. Vafopoulou et al. [5] found that differences between Feta cheese batches made from ovine milk to which enzymes had been added tended to diminish with cheese aging, indicating that the contribution of proteinases to proteolysis was limited to the early stages of ripening. Differences with respect to the control batch at the end of ripening were also observed to decrease by Fernández-García et al. [3] using Flavorage in a Manchego-type cheese made from milk blends.

The effect of the activity of the added enzyme was discernible in that the increased activity resulted in higher SSAN values, the highest values being recorded for batch E3, followed by batch E2 and then batch E1. This finding agrees with the results obtained by some other workers, who have reported that increasing the concentration of the enzyme used to accelerate cheese ripening in a cows' milk Cheddar cheese [25–27] and in a ewes' milk Manchego-type cheese [28] yielded an increase in the amino acid content. Using different encapsulated proteases added to the milk in a Manchego-type cheese, Picón et al. [23,24] reported an increase in proteolysis, measured as the phosphotungstic acid-soluble N (PTAN) content. Conversely, using the same enzyme as one of the preceding research teams, Ardö and Pettersson [29] failed to record any increase in the PTAN levels in a Swedish cheese, whereas Pahkala et al. [30] observed different levels of increase in an Edam-type cheese, depending on the enzyme employed.

Clearer differences between the control batch and the cheese batches made with added enzyme were observed on the basis of the SSAN contents than on the basis of the TFAA contents. In fact, the correlation coefficient between the TFAA and SSAN values over the ripening period were higher for the control batch (0.957) than for batches E1, E2 and E3 (0.946, 0.916 and 0.927, respectively). This may be due to the fact that SSAN levels were quantified using the TNBS method, which may react with low-molecular-mass peptides as well as with the FAAs [20]. The enzymatic preparation probably brought about an increase in the release of low-molecular-mass peptides, which reacted with the TNBS, thereby heightening the differences between the cheese batches containing added enzyme and the control batch.

Bitter aromas and texture defects have sometimes been recorded for cheeses to which proteolytic enzymes were added to accelerate ripening [6,9,10]. However, in this experiment, bitter aromas were only clearly detectable in batch E3, and were probably attributable to the higher activity levels of the enzymatic preparation added to that batch. Using a neutral protease from *Aspergillus oryzae* in Cheddar cheese, Fedrick et al. [31] reported that bitter flavours became more pronounced at higher concentrations of added enzyme. Similar results were obtained by Alkhalaf et al. [32] using Rulactine in a Saint-Paulin cheese. Conversely, using differing concentrations of two neutral proteases from *Bacillus subtilis* in a Manchego-type cheese made from ewe's milk, Núñez et al. [28] did not record any significant differences in bitter aromas between any of the experimental batches and the control batch. The differing results reported by these workers may be due to the fact that ovine caseins are less likely to

release bitter peptides in response to the action of the proteinases than are bovine caseins [1]. Using encapsulated chymosin and a neutral protease from *Bacillus subtilis* in a Manchego-type cheese made from ewe's milk, Picón et al. [23,24] likewise failed to record bitter flavours. However, bitter flavours were reported in Manchego cheese made from a blend of bovine and ovine milk with added proteolytic enzymes [4,6] and in a Feta-type cheese made from ewe's milk [5].

5. Conclusions

This paper contributes new data on the effect of adding enzymatic preparations to the milk used in the manufacture of French Ossau-Iraty cheese to help complete existing knowledge regarding the application of accelerated ripening methods to ewes' milk cheeses.

The enzymatic preparation used in batches E1, E2 and E3 promoted release of amino acids proportional to the enzyme concentration employed. In any event, the action of the enzyme preparation was more marked in the early months of ripening and, as the ripening period progressed, the differences in the FAA contents decreased. This fact is noticeable for some major amino acids, such as glutamic acid and asparagine. Taurine, tyrosine and valine levels were virtually unaffected by the enzymatic treatments, whereas serine, glycine, arginine and proline levels were lowered.

Overall, the enzymatic preparation considered here gave rise to texture defects in this cheese, resulting in decreased elasticity and creaminess and increased brittleness. Similarly, use of the preparation also gave rise to bitter overtones proportional to the concentration of added enzyme. Those overtones are not intrinsic components of the characteristic taste and aftertaste of Ossau-Iraty cheese. The defects were more pronounced in batch E3, because of the higher level of activity of the enzymatic preparation added to that batch. The sensory results obtained in the present experiment may be related to the levels of certain amino acids. For instance, levels of proline, an amino acid related to sweet flavours in cheese, were higher in the control batch than in the other cheese batches. Fig. 3 shows that the enzyme-treated cheese batches earned lower scores for sweet odour than did the control batch.

As expected, the enzymatic mixture increased the proteolysis that occurred during ripening and, therefore, promoted the acceleration of ripening of Ossau-Iraty-type cheese. However, the action of the enzyme preparation was more marked in the early months of ripening. Also, the enzyme had an adverse impact on the organoleptic characteristics of the Ossau-Iraty cheese, especially at higher concentrations.

It is necessary to indicate that other trials, in which this enzymatic mixture was tested again, were carried out and similar results were obtained. Regarding to the SSAN values (not shown), the preparation increased the proteolysis that occurred during ripening and also generated defects in the sensory characteristics of the cheese. This fact confirms the conclusions obtained in this study.

Acknowledgements

This research work is part of the project ''PL-921298'' of the European AAIR Program. The author is grateful to the Government of Navarra for the financial support provided for this study.

References

[1] E. Fernández-García, R. López-Fandiño, Rev. Esp. Cienc. Tecnol. Aliment. 34 (1994) 353.
[2] E. Fernández-García, M. Ramos, C. Polo, M. Juárez, A. Olano, Food Chem. 28 (1988) 63.
[3] E. Fernández-García, A. Olano, D. Cabezudo, P.J. Martín-Alvarez, M. Ramos, Enzyme Microb. Technol. 15 (1993) 519.
[4] E. Fernández-García, R. López-Fandiño, L. Alonso, M. Ramos, J. Dairy Sci. 77 (1994) 2139.
[5] A. Vafopoulou, E. Alichanidis, G. Zerfiridis, J. Dairy Res. 56 (1989) 285.
[6] E. Fernández-García, Aliment. Equip. Tecnol., July–August (1986) 53.
[7] P.F. Fox, Food Biotechnol. 2 (1988) 133.
[8] M. El Soda, M. Saada, Egyptian J. Dairy Sci. 14 (1986) 115.
[9] M. El Soda, Int. Dairy J. 3 (1993) 531.
[10] P.F. Fox, J.M. Wallace, S. Morgan, C.M. Lync, E.J. Niland, J. Tobin, Antonie van Leeuwenhoek 70 (1996) 271.
[11] P.F. Fox, M.B. Grufferty, in: P.F. Fox (Ed.), Food Enzymology, Elsevier, London, 1991, p. 219.
[12] P.F. Fox, J. Law, Food Biotechnol. 5 (1991) 239.

[13] B.A. Law, Dairy Ind. Int. 45 (1980) 15.
[14] S. Visser, G. Hup, F.A. Exterkate, J. Stadhouders, Neth. Milk Dairy J. 37 (1983) 169.
[15] F.V. Kosikowski, J. Dairy Sci. 71 (1988) 557.
[16] B.K. Dwivedi, CRC Crit. Rev. Food Technol. 3 (1973) 457.
[17] G.J. Moskowitz, S.S. Noelck, J. Dairy Sci. 70 (1987) 1761.
[18] International Dairy Federation, Standard No. 4, Determination of Dry Matter in Cheese, in: Official Methods of Analysis, Vol. 1, Ministry of Agriculture Fisheries and Food, Madrid, 1958.
[19] Y. Barcina, F.C. Ibáñez, A.I. Ordóñez, Food Control 6 (1995) 161.
[20] J.M. Izco, P. Torre, Y. Barcina, Food Control 1 (2000) 7.
[21] N. Zaki, S.A. Salem, Indian J. Dairy Sci. 45 (1992) 303.
[22] B.A. Law, A. Wigmore, J. Dairy Sci. 50 (1983) 519.
[23] A. Picón, P. Gaya, M. Medina, M. Núñez, J. Dairy Sci. 77 (1994) 16.
[24] A. Picón, P. Gaya, M. Medina, M. Núñez, J. Dairy Sci. 78 (1995) 1238.
[25] B.A. Law, A. Wigmore, J. Dairy Res. 49 (1982) 137.
[26] I.A. Fedrick, J.W. Aston, S.M. Nottingham, J.R. Dulley, N.Z. J. Dairy Sci. Technol. 21 (1986) 9.
[27] J-C.C. Lin, I.J. Jeon, H.A. Roberts, G.A. Milliken, J. Food Sci. 52 (1987) 620.
[28] M. Núñez, A.M. Guillén, M.A. Rodríguez-Marín, A.M. Marcilla, P. Gaya, M. Medina, J. Dairy Sci. 74 (1991) 4108.
[29] Y. Ardö, H.E. Pettersson, J. Dairy Res. 55 (1988) 239.
[30] E. Pahkala, V. Antila, M. Laukkanen, Meijeritiet Aikakkirja 43 (1985) 33.
[31] I.A. Fedrick, J.S. Cromie, J.R. Dulley, N.Z. J. Dairy Sci. Technol. 21 (1986) 191.
[32] W. Alkhalaf, L. Vassal, M.J. Desmazeaud, J.C. Gripon, Lait 67 (1987) 173.