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Characterisation of the casein variants in goat bulk milks using on-line RP-HPLC/ESI-MS

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Abstract — A HPLC/ESI-MS method was used to separate and analyse casein variants in goat bulk milks. From mass determination, about 25 casein components were characterised in each milk casein sample with up to 7 compounds obtained in the same HPLC fraction. The different caseins were each detected at various phosphorylation levels. Only casein compounds present at levels higher than 1% of total casein were obtained, as confirmed by the analysis of individual goat milks of known composition. The discrimination threshold obtained for the differentiation of molecular masses was quantified by the variation coefficient (CV = 0.02%). Assignment of masses to known compounds was made by reference to theoretical masses. Some compounds, in κCN, α_{s1}CN and βCN fractions could not be assigned. It is possible that they were non-described variants. A limitation of the method was revealed when variants present in the same RP-HPLC fraction had the same molecular mass (as was the case for α_{s1}CNA and α_{s1}CNB$_1$) or when their masses differed by ~80 g·mol^{-1} (as was the case for α_{s2}CNA and α_{s2}CNB). In this latter case, the 2 variants could not be differentiated from the different phosphorylation levels of the same, indeed the addition of a phosphoseryl residue increased the molecular mass by 80 g·mol^{-1}. To overcome these limitations, an improvement of the method was proposed. In the 3 bulk milks analysed the same main variants, κCN-Ile 2P, α_{s2}CN A11P, α_{s1}CN A and/or E were found, and the same non-identified component in βCN fraction. However, differences between the 3 bulk milks analysed were observed. A variation in the relative proportions of variants in κCN and βCN fractions was observed in the different milks and it is proposed, if confirmed, that it could be related to the selection stage of the producing goats. Thus, the HPLC/ESI-MS method applied to bulk milks collected throughout a country allowed for an accurate determination of the casein variants with a higher frequency. The analysis of individual milks would allow a fast and accurate characterisation of the casein phenotype of goats.

goat milk / casein variant / RP-HPLC/ESI-MS

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Résumé — Caractérisation des variants de la caséine contenue dans des laits de chèvre de grand mélange par RP-HPLC/ESI-MS. Une méthode RP-HPLC/ESI-MS a été utilisée pour la mise en évidence et la caractérisation des différents variants de la caséine présents dans des laits de chèvre de très grand mélange. L'identification des composés à partir des masses expérimentales était réalisée par référence aux masses théoriques des composés connus. Environ 25 composants ont été mis en évidence dans chaque lait. Dans une même fraction, jusqu'à 7 composés ont pu être séparés par leur différence de masse. Les différents niveaux de phosphorylation des caséines étaient individualisés. Les composants de la caséine présents à des teneurs supérieures à 1 % de la caséine totale ont été mis en évidence. Le seuil de discrimination entre 2 masses moléculaires, pour des constituants se trouvant en mélange dans un même pic chromatographique, était évalué par le coefficient de variation de la détermination (CV = 0,02 %). Certains des composés présents dans les fractions κCN, α_{s1}CN et βCN n'ont pu être identifiés. Il est possible que ce soient des variants non décrits. Une limitation à la caractérisation des composés est apparue concernant les variants ayant une même masse moléculaire ou ceux ayant des masses différant par 80 g·mol^{-1}. Dans ce cas, les 2 variants ne peuvent plus être distingués de 2 niveaux de phosphorylation différents d'un même variant puisque la masse ajoutée par l'addition du résidu phosphoryle est de 80 g·mol^{-1}. Pour résoudre ce problème, une amélioration de la méthode est proposée. Dans les 3 laits analysés ont été trouvés les mêmes variants comme constituants les plus abondants: κCN-Ile 2P, α_{s2}CN A 11P, α_{s1}CN A et/ou E, et le même composé non identifié dans la fraction βCN. Cependant, des différences entre les laits sont apparues concernant les proportions relatives des variants dans les fractions κCN et βCN. Il est probable, mais ce serait à confirmer, que ces variations soient en relation avec le niveau de sélection des chèvres ayant produit les laits. La méthode appliquée à des laits de grand mélange collectés sur différentes zones de ramassage a permis d'apprécier avec précision les variants principaux de la caséine et donc d'estimer le niveau de sélection du cheptel caprin dans ce secteur. Son application à des laits individuels permettrait une caractérisation rapide et précise du phénotype des animaux.

lait de chèvre / variant de la caséine / HPLC/ESI-MS

1. INTRODUCTION

Casein (CN) in goat milk shows a great heterogeneity with more than 16 variants described for the main κ, α_{s2}, α_{s1} and β caseins, amongst which 9 variants for the α_{s1}casein only [17]. The different α_{s1}casein variants (Fig. 1) differ not only by their amino acid sequences, resulting from amino acid deletions or substitutions, but also by their secretion levels in milk [11] which confers an agronomic importance on some of the α_{s1}CN variants. The trend in genetic selection promotes goats which possess high producing genotypes. Therefore, the genotypic characterictics of males used for insemination have been thoroughly studied [19]. Nevertheless, it has since remained difficult to quantify the progress of the selection directly of the entire lactating goat livestock for analytical reasons.

Casein (CN) variants were studied with conventional methods such as electrophoresis [1] or with high performance liquid chromatography (RP-HPLC) [13], in association with a compound identification through comparison to standards. With such a methodology, the precise characterisation of components requires their complete separation and isolation. So, the separation methods could only be successfully applied to milks with a simple variant pattern, as in individual milks or milks preferentially derived from homozygous goats. Casein variants in bulk milk could not be characterised. The identification of isolated compounds involved the determination of their complete amino acid sequence. It would normally be obtained by chemical sequencing of the protein, but can now be reached directly from the goat genoma [16]. This allows for the exact calculation of molecular mass of the protein which

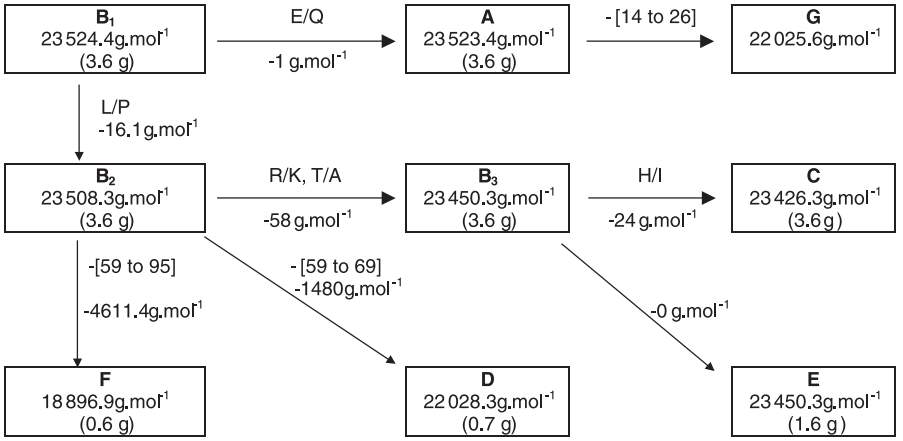

Figure 1. Variants of goat α_{s1} casein according to Grosclaude et al. [11]. For each variant are given: substitution or deletion of amino acids; molecular mass ($g \cdot mol^{-1}$) corresponding to the 10 P molecule, except for D (5 P) and F (4 P); secretion level in milk between brackets ($g \cdot L^{-1}$ for one allele).

substantially serves as the more usual and the more convenient reference. The molecular mass of a protein can now be obtained rapidly and with high accuracy using mass spectrometry [6, 10]. It can be used to characterise compounds with direct reference to their known theoretical masses. In this way Chianese et al. [7] characterised in individual goat milks 2 different βCNs with various phosphorylation levels. Coupling on-line RP-HPLC and electrospray mass spectrometry (RP-HPLC/ESI-MS), Léonil et al. [14] show the ability of this method to separate and characterise the caseins in cow milk.

In the work described here, we applied RP-HPLC/ESI-MS to some goat bulk milks collected in factories in order to evaluate the ability of this method to obtain separation and identification of the numerous casein variants contained in these milks even with unsatisfactory HPLC separation. In addition, some individual goat milks homozygous for known genotypes for α_{s1} casein were studied separately and after mixing in order to determine the discriminating limits of the method.

2. MATERIALS AND METHODS

2.1. Milks

The bulk milks were chosen from highly selected or more traditional herds. They were provided by 2 different factories. Milk I was collected in the Ille-et-Vilaine area (Triballat, Noyal-sur-Vilaine, France) from Alpine breed goats. The sample was taken out of a 10 000 L vat of milk. Milks II and III were collected in the Deux-Sèvres area (Sèvre et Belle Company, Celles sur Belle, France), and samples taken respectively from 7 000 L and 13 000 L vats. Goat breeds were mainly Alpine and Saanen. Storage of milk samples between milking and analysis was at 2 °C for 24 h for milks I and II and 48 h for milk III.

The individual milks of selected composition for α_{s1}CN were supplied by INRA Station d'Amélioration Génétique des Animaux (Castanet-Tolosan, France) where they have been genetically typed.

Composition of the milks, particularly total nitrogen matter (TNM), was obtained from an IR analysis (Dairy Lab, Multispec,

York, UK). Total casein in the milks was calculated as 0.7 × TNM.

2.2. Casein extraction

Skimmed milk was obtained by centrifugation of whole milk (500 *g*, 15 min, 35 °C). Caseins were obtained by precipitation at their isoelectric point. Briefly, an aliquot of 10 mL of skimmed milk (30 °C) was diluted 1 to 3 with water and 10% (v/v) acetic acid added up to pH 4.2. After standing for 10 min, a centrifugation step (1 000 *g*, 15 min, 20 °C) allowed the separation of a pellet and a supernatant which was discarded. The pellet was twice washed by dispersion in 25-30 mL of water with a Turrax blender and then recovered by centrifugation. The washed pellet was dispersed in 10 mL of a pH 7 buffer (0.1 mol·L^{-1} trishydroxymethyl-amino-methane/HCl, pH 7, containing 8 mol·L^{-1} urea, 1.3% Na_2 citrate and 10 mmol·L^{-1} dithiotreitol). The casein solution was kept for 30 min for equilibration before use.

2.3. RP-HPLC

Casein separation by RP-HPLC proceeded as previously described [13, 18]. A sample for injection was prepared from the casein solution by a × 0.2 dilution in the pH 7 buffer, then a × 0.17 dilution in 0.1% trifluoroacetic acid (TFA) in water in order to obtain a one g·L^{-1} casein solution. The pH was adjusted to 2.2 with 10% TFA. Injection of 100 µL of this solution on the column was performed. The amount of the different caseins in milk was calculated from the profile, as described elsewhere [18]. Accuracy on casein quantification was about 5% on a single determination, meaning ~10% on the relative proportions in milk.

2.4. RP-HPLC/ESI-MS

Molecular masses were determined by coupling between reversed phase high pressure liquid chromatography (RP-HPLC) and electrospray ionisation mass spectrometry (ESI-MS).

2.4.1. RP-HPLC separation

Samples were separated by RP-HPLC on a C18 column (2.1 mm in ∅, 15 cm length; Vydac 218 TP 5215, Hesperia, USA), at 40 °C and with a 0.25 mL·min^{-1} flow rate. Eluents were A: 0.1% TFA in water; B: 0.1% TFA in 20/80 water/acetonitrile (v/v). A linear gradient elution (1.33% B eluent·min^{-1}) was performed from 33% to 53% of B eluent. The sample for injection was the casein solution diluted × 0.5 in the pH 7 buffer, then × 0.4 in A eluent, the pH being adjusted to 2.2 with 10% TFA. Filtration on 0.45 µm cellulose membrane was achieved. Injection volume was 25 µL with 100 µg casein loaded on the column.

2.4.2. ESI-MS analysis

Electrospray mass spectrometer API III plus (Sciex, Thornhill, Ontario, Canada) was a triple quadrupole equipped with an atmospheric-pressure ionisation ion source. Samples delivered to the sprayer by splitter (1/8) of RP-HPLC flux, were sprayed at 55 °C, through a stainless-steel capillary held at a high voltage between 4.5 and 5.2 kV. The liquid nebulisation was aided by a coaxial air flow along the sprayer, adjusted between 0.3 and 0.4 MPa. The interface between sprayer and mass analyser consisted of a conical orifice of 100 µm ID (internal diameter). The potential at the orifice was 80 V. Conditions of potential apparatus equilibration and mass determination were those used by Léonil et al. [14]. After extraction of the TIC (total ionic current), protein mass spectra were obtained by averaging the signals from multiple scans. The charge number of the multicharged ions and the protein molecular mass determination were automatically obtained using a Power Macintosh Computer and a Sciex Version Mac. Spec. 3.3. Software.

2.5. Molecular mass

Compounds separated from casein and characterised by their experimental masses were assigned to known caseins using the following references for the theoretical masses: κCN [12]; α_{s2}CN [2-4]; α_{s1}CN [5, 8-9, 11, 15]; βCN [12, 20].

Casein is a phosphorylated protein with phosphoryl residues branched on seryl moieties. Each casein exists in milk at several phosphorylation levels. A phosphoryl residue has a mass ~80 g·mol^{-1}. So, all the components in a fraction differing by ~80 g·mol^{-1} can possibly be considered as the same casein variant at different phosphorylation levels.

Assignment was made when the difference between theoretical and experimental masses did not exceed 0.03%.

3. RESULTS AND DISCUSSION

3.1. RP-HPLC fractionation of casein

Caseins from the 3 bulk goat milks were separated by RP-HPLC into 5 main fractions using the method of Jaubert et al. [13], as shown for milk I in Figure 2. Caseins in the fractions were identified as κCN (fraction 1), α_{s2}CN (fraction 2), α_{s1}CN (fraction 4) and βCN (fraction 5) [13]. No reference was found for the identification of fraction 3. As shown on the figure, the method allowed partial separation of some of the different α_{s1}CN. Individual caseins in milks were quantified from their RP-HPLC profile (Tab. I). Significant differences between the 3 bulk milks were observed, with a decrease in α_{s1}CN and α_{s2}CN from milk I to III, balanced by an inverse variation of the κCN levels. Milk I with the highest α_{s1}CN level thus would correspond to herds with the highest selection progress. However few differences in TNM and total CN levels were observed between milks.

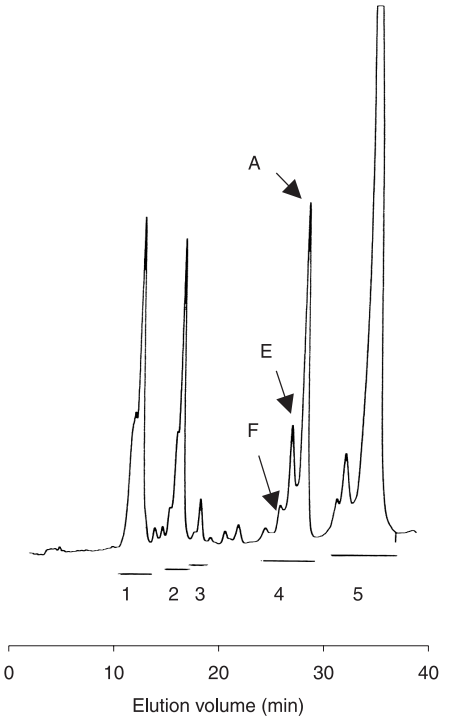

Figure 2. Casein fractionation by RP-HPLC according to Jaubert and Martin [13].
1. κCN; 2. α_{s2}CN; 3. non-identified; 4. α_{s1}CN, variants F, E, A; 5. βCN. Absorptivity at 214 nm.

3.2. Mass analysis

Mass determination in RP-HPLC fractions was performed on the 3 bulk milk caseins. The online RP-HPLC method used allowed us to also obtain the 5 main peaks, but did not allow separation of the α_{s1}CN into different peaks. Complete experimental results obtained for milk I and their interpretation are given in Table II as an example. As for the fractions, there was only one compound detected in fraction 3, but more in fraction 4 and 5. The ability of the method to discriminate between 2

Table I. Levels of total casein and casein fractions in the 3 goat bulk milks (from RP-HPLC data). g·kg^{-1} milk (% total CN).

Milk	TNM	Total CN	κCN	α_{s2}CN	Peak 3	α_{s1}CN	βCN
I	34.6	24.2	3.3 (13)	3.6 (15)	0.2 (1)	4.2 (17)	12.9 (53)
II	35.2	24.6	4.5 (18)	3.1 (13)	0.2 (1)	3.5 (14)	13.2 (54)
III	33.9	23.7	4.4 (18)	3.1 (13)	0.2 (1)	2.9 (12)	13.1 (55)

Table II. Experimental masses of casein constituents obtained by ESI/MS analysis of the RP-HPLC fractions from milk I and their assignment by comparison to theoretical data.

RP-HPLC fractions	Experimental masses (SD)	Relative proportion (a) in fraction %	Identification by comparison to known casein variants				
			Assigned		More than one assignment		Unknown
			Compound	Reference mass	Compound	Reference mass	
1 (κCN)	19 494.4(4.0)	8	. . .	. . .	. . .	. . .	UNK 1
	19 305.3(1.6)	50	κCN Ile 2P	19 304.4			
	19 295. (4.5)	42	κCN Val 2P	19 290.4			
2 (α_{s2}CN)	25 602.5(3.2)	55	α_{s2}CNA 11P	25 599.7			
	25 522.3(3.1)	18	. . . .	. . .	α_{s2} CNA 10P	25 519.7	
	25 448	14			or " B 10P	25 518.8	
	25 362	10					
	25 294	3					
3 (?)	25 588.4	100	α_{s2}CNC 11P	25 584.7			
					α_{s1} CN A 9P	23 443.4	
4 (α_{s1}CN)	23 447	8	. . . .	. . . .	or " B_1 9P	23 444.4	
	23 365.8(1.4)	33			or " E 10P	23 450.3	
	23 286.5(2.6)	33			or B_3 10P	23 450.3	
	23 316	4	. . .	. . .	. . . .	. . . .	UNK 4.1
	23 238.4(2.4)	12					
	23 160.1(4.1)	10					
5 (βCN)	23 853.0(2.3)	32	. . .	. . .	. . . .	. . . .	UNK 5
	23 773.1(2.6)	29					
	23 692	4					
	23 614	2					
	23 823.3(2.2)	15	βCN 6P	23 822.0			
	23 744.0(2.4)	14	" 5P				
	23 669	4	" 4P				

(a) calculated from the relative intensity of ionisation current in the fraction.

compounds with a low mass difference was shown from the results in fraction 1; 3 compounds were detected having the experimental masses 19 494.4, 19 305.3 and 19 295.1 g·mol^{-1}. Standard deviations of analyses were in the range 1.4 – 3.2 g·mol^{-1} for mass ranges about 20 000 g·mol^{-1}, meaning 0.01 to 0.02%.

3.3. Relative quantification

An attempt at a rough estimation of the proportion of compounds in each fraction was made. It was obtained from the relative intensity of ionisation current of molecules through the reconstructed spectrum. Indeed, the ionisation level is dependent on the nature of the compound. With the different casein variants being very close in composition and structure, we have inferred that the comparison of ionisation levels could give a valuable estimation of their proportions in one fraction. They are reported in Table II for milk I.

From these results, the lower amount of a compound can also be estimated thus allowing detection. In the single compound fraction 3, the compound detected amounted to ~1% of the total casein (Tabs. I and II). In a fraction having a complex composition, such as fraction 5, the compound detected in the lowest proportion amounted to 2% of the detected βCN. This amount also corresponded to about 1% of total casein. Thus only casein compounds present at a level higher than 1% have been detected.

3.4. Compound identification

In order to simplify the scheme of data presented in Table II and their interpretation, the masses were listed in decreasing order. Some of the compounds present had masses differing by ~80 g·mol^{-1}. Assignment of experimental masses to known compounds was made by comparison to theoretical masses. Different figures occurred:

3.4.1 Only one theoretical mass corresponded to the experimental compound, as it was in fraction 1 for κCN-Ile and κCN-Val, or in fraction 2 for α_{s2}CN 11P. Assignment was unambiguous.

3.4.2 More than one theoretical mass corresponded to the experimental one. For fraction 2, the mass 25 522.3 g·mol^{-1} could as well correspond to α_{s2}CN A 10P or to α_{s2}CN B 10P, as the theoretical mass of α_{s2}CN B is 1 g·mol^{-1} lower than α_{s2}CN A (Tab. I). In this case, the difference of one mass unit can be attributed to the substitution of glutamic acid (A variant) by lysine (B variant).

3.4.3 No theoretical mass was found corresponding to the experimental one. The compound was unknown (UNK) and remained non-identified, as it was for some of the compounds in each of the fractions 1, 2, 4 and 5.

3.4.4 Assignment in α_{s1}CN, fraction 4 was somewhat complex as α_{s1}CN exists as 9 or more different variants each at different phosphorylation levels [11]. Considering the theoretical molecular mass of variants (Fig. 1) on one side, and the accuracy of the method (established at about 0.01%) on the other side, it was observed: i) A and B_1 differ by only one mass unit that is lower than the discriminating power of the method; ii) E and B_3 have both the same sequence and molecular mass, but differ by their secretion level only; iii) A (or B_1) and E (or B_3) differ by 73 g·mol^{-1}. This value is close to the mass of a phosphoryl residue (80 g·mol^{-1}) however the accuracy of the method could possibly allow differentiation between these variants and the different phosphorylation level of one or the other variant.

As a consequence of these quantitative relations between α_{s1}CN theoretical masses, the A, E, B_1 and B_3 variants would remain indistinguishable by the method when they are eluted in the same RP-HPLC fraction. For B_1, the bias is low as it is a variant with low frequency in breeds (~0.03). For A and E, it is of importance since they are the main variants in Alpine and Saanen breeds respectively. Their allelic frequencies are 0.8 for the A variant in the Alpine breed and 0.7 for the E variant in the Saanen breed, as determined on males used for insemination

[19]. In effect, compounds were detected in fraction 4 with masses corresponding to either (or both) α_{s1}CN variant A and/or E (Tab. II), which could not be separated. Identification would involve obtaining these 2 compounds in 2 different RP-HPLC peaks, which could be possible using the RP-HPLC method of Jaubert and Martin [13], as shown in Figure 1, in which the different α_{s1}CN variants were separated in the increasing order F, B, E, A, having slightly different elution volumes. The RP-HPLC method used before ESI-MS analysis was not discriminant enough and had to be improved to obtain the separation of α_{s2}CN A and B on one side and of α_{s1}CN A and E on the other side into 2 different peaks. This would allow them to be separately analysed by ESI/MS. These conditions for the improvement of separation and to obtain the complete characterisation of the caseins were not assayed in the current work.

On the other hand, it is noteworthy that the F variant of α_{s1}CN was not identified in the milks, which could mean a level < 2% of total CN. The frequency of this variant has been reported in the range ~0.05 to 0.10. However, the secretion level of α_{s1}CN in F milk is far lower than that in A and E variants. Thus, it is likely that the compound was present in bulk milks at a concentration below the detection threshold of the method. The presence of a small peak only at the elution volume of F on the RP-HPLC profile of milk I in Figure 2 confirmed this hypothesis.

3.5. Comparison of caseins in the 3 bulk milks

The whole casein compounds characterised in the 3 milks are listed in Table III. The same constituents were present in the casein of the 3 milks with an even better

Table III. Full report of the different casein variants found in the 3 bulk milks.

Casein in RP-HPLC fraction	Variants and their phosphorylation levels		Proportion of the different variants in the fractions of milks % in a fraction		
			Milk I	Milk II	Milk III
1 κCN	UNK 1		8	17	2
	Ile	1-2P	50	53	61
	Val	1-2P	42	30	37
2 α_{s2}CN	A	11P	55	50	53
	(A	7-10P)*			
	(B	8-11P)*	45	50	47
3 α_{s2}CN	C	9-11P	100	100	100
4 α_{s1}CN	(A	7-9P)*			
	(B1	7-9P)*	74	71	76
	(E	8-10)*			
	UNK 4.1	3 levels	26	29	24
5 βCN	UNK 5	4 levels	67	59	55
	βCN	3-6P	33	41	45

* and/or.

discrimination of some compounds in one or the other milk granting the possibility to calculate one more phosphorylation level. The relative levels of compounds in the corresponding fraction are reported as well. Comparing the 3 milks, the main component in each fraction was the same casein variant at the same phosphorylation level: κCN-Ile 2P, α_{s2}CN A 11P, UNK 5. Nevertheless in fraction 4, the most abundant α_{s1}CN variant could not be determined as it was impossible to separately differentiate and quantify the main variants, A and/or E, which represent 75% of the total. A quantitative difference in the variant levels seemed to exist between milks, concerning κCN-Ile, with increasing proportions from milk I to III, and concerning UNK5, with decreasing proportions. It is likely that a variation in the respective A and E α_{s1}CN variants also occurred. These variations could be correlated to the selection level of goats, as the α_{s1}CN level in the milks was found increasing in the order III, II, I (Tab. I).

3.6. Analysis of individual milks

Analysis of the casein in individual milks from goats homozygous for α_{s1}CN, variant A, E and F, was performed to confirm the results obtained on bulk milks. Results of casein characterisation are summarised in Table IV. κCN variants were the same as in bulk milks. In FF milk fraction 2, an unknown compound, UNK 2, was characterised in addition to the A and/or B α_{s2}CN variants. In AA and EE milks, the characterised α_{s1}CN variants corresponded well with the genotype. However, two additional compounds corresponding to none of the known caseins were present in the

Table IV. Different casein variants characterised in 3 individual milks homozygous for α_{s1} casein and in the mix of the 3 at equal volumes.

Casein in RP-HPLC fraction	Variants and their phosphorylation levels		Different variants detected in the fractions of milks % in a fraction			
			AA	EE	FF	mixed AEF
1 κCN	Ile	1-2P	+	+		+
	Val	1-2P			+	+
2 α_{s2}CN	UNK 2				+	+
	A	11P		+		+
3 α_{s2}CN	C	9-11P	+	+	+	+
4 α_{s1}CN	A	7-9P	73			+
	E	8-10P		71		+
	F				ND	ND
	UNK 4.1	3 levels	27			+
	UNK 4.2	3 levels		29		+
5 βCN	UNK 5	4 levels	+		+	+
	βCN	3-6P		+	+	+

+, detected and characterised
ND, not detected.

milks, respectively UNK 4.1 (also present in bulk milks) and UNK 4.2. In FF milk, the characterisation of α_{s1}CN in peak 4 was not possible, as the amount of proteic material in the peak was too low. Thus α_{s1}CN variant F could not be characterised by the method even in individual FF milks, which corresponded to the highest concentration obtainable. The secretion level of α_{s1}CN in F milk is reported to be in the 1.2 g·L^{-1} range (Fig. 1), which corresponds to about 4% of the total casein. However, we have not quantified the F α_{s1}CN; it is possible that the α_{s1}CN content in the individual milk analysed was lower, due to the individual variability. Fraction 5 contained βCN 3–6P caseins and the already described UNK 5.

3.7. Non-identified compounds occurring in goat milks

In the milks were found 3 groups of non-identified compounds. The true characterisation of the compounds would require biochemical analyses and protein sequencing. Now, only some hypotheses can be proposed.

3.7.1. UNK 1 compound could be a new variant of κCN having a mass higher than that of the 2 known variants. If it were, it would represent a more primitive form of the protein. Or UNK 1 could originate from a glycosylated form of κCN-Val 2P, partially deglycosylated during the ionisation process [14].

3.7.2. UNK 2 compound was observed in individual milk F. It eluted in peak 2, which generally contained α_{s2}CN A and B. A series of 3 compounds differing by about 80 was obtained, meaning different phosphorylation levels. Compared to α_{s2}CN A 11P, it showed a molecular mass higher by about 15 g·mol^{-1}. It could prove to be a new variant of α_{s2}CN.

3.7.3. UNK 4.1 compound might be a variant of α_{s1}CN, its possible origin can be searched by derivation from a known higher molecular mass variant (Fig. 1).

3.7.4. UNK 4.2 compound was characterised in peak 4 of EE milk at 3 phosphorylation levels. Compared to UNK 4.1, the series were close, however the differences between homologous masses reached 2 to 6 units, a value which was slightly higher than the error of the method. This could mean that a different compound was present. As a matter of fact there was no probability of obtaining an adduct in the acidic conditions existing during analysis.

3.7.5. UNK 5. In the fraction 5, the βCN variant corresponding to the reference mass (23 822.0 g·mol^{-1}) was found in lesser amounts than a non-identified variant (23 853.0 g·mol^{-1}), UNK 5, having a mass lower by –26.3 g·mol^{-1}. This last had already been observed in individual milks by Chianese et al. [7] who also reported the phosphorylation level, i.e. 6P, for the compound with a mass ~23 853 g·mol^{-1}. Its biochemical characterisation, however, was not made. If the derivation is from the (CN used as reference and involves a monosubstitution, only a few possibilities arise from the difference in masses. They would be: Pro/Ala (–25.9 g·mol^{-1}), Tyr/His (–26.04 g·mol^{-1}) or Leu (Ile)/Ser (–26.08 g·mol^{-1}).

4. CONCLUSION

The RP-HPLC/ESI-MS method showed a highly increased resolutive capacity compared to RP-HPLC associated with a conventional detection method. The method applied to goat bulk milk allowed us to separate and identify the casein compounds present at levels higher than 1% of total casein with a 0.01 to 0.02% variation coefficient for mass determination. In a single bulk milk sample, up to 25 different compounds were separated. After characterisation by comparison to reference masses,

4 groups of compounds remained non-identified, eluting respectively in the same peaks as κ, α_{s1}, α_{s2} and β. They could correspond to non-described variants of goat casein. The main α_{s1}CN variants A and E, having the highest frequency in the milks and the α_{s2}CN A and B are not currently differentiated by the method. However it seems possible to overcome this point by an improvement of the RP–HPLC separation, in order to obtain a separation of α_{s1}CN variants F, E and A into 3 different peaks, as was obtained using the method of Jaubert and Martin [13]. In the 3 individual milks, the same casein variants were found as the main constituents. The proportion of constituents in a RP-HPLC fraction were generally close from one milk to the other, however for some of the caseins, κCN and βCN, proportions seem to vary between samples. The 3 bulk milks had consistently different levels of total α_{s1}CN (determined by RP-HPLC), 4.2, 3.5 and 2.9 $g \cdot kg^{-1}$ respectively in milks I, II and III, which confirms that they really originated from herds differing by their selection stage. It would thus be interesting to determine if the variation in the proportion of κCN and βCN variants was also in relation to selection.

The RP-HPLC/ESI-MS method appears to be a valuable tool for determining the different casein variants in an individual milk. When applied to bulk milks, it gives the main variant of each casein. If applied to individual milks, it allows the determination of the phenotype of each of the caseins in the milk and consequently the acquisition of data on the genotype of the lactating goats.

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