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Determination of ammonium in milk and dairy products by ion chromatography

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Abstract

To control the quality and the biochemical evolution of milk and dairy products during their technological transformations, it is interesting to determine their ammonium concentrations. A chromatographic method for the determination of this compound is proposed. The method is based on the separation of ammonium by cation-exchange chromatography and its detection by suppressed conductivity. With an appropriate sample preparation, this method enabled identification and quantification of ammonium with good repeatability (relative standard deviation of about 5%). Moreover, good sensitivity (less than 0.5 mg/kg) and no interference between ammonium and other matrix components were determined. It was also shown that this method offers a very promising alternative for studying changes in ammonium concentration of milk or caseinate after their heat treatments and in different dairy products such as yoghurt and cheeses (hard cooked and mould ripened cheeses).

Keywords: Deamidation; Dairy products; Ammonium

1. Introduction

Ammonium exists in milk and dairy products. Its origin and concentration depends on the nature of products considered. In raw milk and in the absence of microbial contamination, the concentration of ammonium is less than 5 mg/kg [1–3]. Then, after microbial contamination or different technological treatments of milk (sterilisation, acidification, ripening), the different milk compounds are modified. Globally, ammonium can be produced in three ways. The first are deamidation reactions of asparagine and glutamine residues present in the sequence of the milk proteins. These deamidations can be induced by severe heat treatments of milk or by enzymes during transformation of milk in yoghurt or different cheeses [1–3]. The second is a degradation of urea. As observed with the deamidation reactions, the urea degradation can be induced by severe heat treatments or by enzymes. The last reaction, which occurs especially in ripened cheeses, is an enzymatic degradation of the protein, peptides and amino acids with release of ammonium. Thus, to control the quality and the biochemical evolution of these products during their transformations, it is interesting to determine ammonium concentrations in these products. In this case, the ammonium ion can be considered as an indicator of the food quality. Different methods to determine this compound in milk and dairy products are described in the literature. Usual-

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ly, this cation, in dairy laboratories, is determined by alkaline distillation followed by titration by the Kjeldahl method [4,5], potentiometric method using an ammonia ion-selective electrode [6–8], enzymatic method [9,10] or flow injection analysis using a coloured indicator [11]. Each method has its own merits, but they are time-consuming and/or require much material; occasionally they are also susceptible to interference. For the determination of ammonium, an alternative method is ion chromatography. At the present time, the applications of ion chromatography are in the following areas: electroplating industry, environmental analysis (waste water), clinical chemistry and food and beverage industry [12–16].

This article describes the use of ion chromatography to determine ammonium concentrations in sodium caseinate and milk after different heat treatments and in different dairy products such as yoghurt, hard cooked and mould ripened cheeses.

2. Experimental

2.1. Sodium caseinate and skim milk – heat treatments

Sodium caseinate was prepared from raw skim milk. Briefly, milk was acidified to pH 4.6 with 1 *M* HCl (Carlo Erba, Val de Reuil, France) at 20°C. Curd was washed two times with water, redissolved at neutral pH, reprecipitated at pH 4.6 and the curd again washed two times. The casein was finally dispersed in 18 MΩ water, dissolved at pH 7.2 by addition of 1 *M* NaOH (Carlo Erba), and freeze–dried. Lyophilisat was reconstituted in 18 MΩ water at 20°C to obtain a final casein concentration of 24.5 g/kg.

Skim milk was obtained from Triballat (Noyal-sur-Vilaine, France).

In all cases, 0.1 g/kg of thimerosal (Sigma, St. Louis, MO, USA) was added to the suspensions to prevent bacterial and fungal growth.

A 12-g amount of sodium caseinate or skim milk, in sealed Pyrex tubes (external dimensions: 10×1.6 cm; volume: 15 ml), was submerged in an oil-bath. The temperatures were thermostatically regulated (±0.1°C) at 90, 100, 110, 120 and 130°C. The heating time was 30 min. This time included approximately a 5-min heating-up period. After heating, samples were immediately cooled to 20°C.

Before determination of ammonium by ion chromatography, sodium caseinate and skim milk samples not heated and heated for 30 min at different temperatures, were acidified to pH 4.6 using 1 *M* HCl. This acidification led to a precipitation of casein molecules. Casein precipitates were eliminated by filtration on a 0.42-μm filter. Filtrates were diluted two-fold with 18 MΩ water before injection (25 μl volume).

2.2. Yoghurt

Yoghurt was purchased from the market. The aqueous phase of yoghurt was recovered by yoghurt centrifugation (300 *g* during 15 min). Then, the aqueous phase was filtered on a 0.42-μm filter and diluted two-fold with 18 MΩ water before injection into the chromatographic system (25 μl volume).

2.3. Hard cooked and mould ripened cheeses

Hard cooked cheese (Emmental) and mould-ripened cheese (Camembert) were purchased from the market. Ammonium contents were determined in three types of samples: (i) Emmental cheese; (ii) surface of Camembert cheese and (iii) centre of Camembert cheese.

Sample preparation was the same in all cases. About 1 g of cheese sample was homogenised with 100 g of 18 MΩ water. Then, 1 *M* HCl was added to homogenate until the pH was 4.6. The insoluble part was separated by filtration through a 0.42-μm filter. The ammonium content was determined by ion chromatography of the filtrate (25 μl volume) without supplementary dilution.

2.4. Ammonium determination by ion chromatography

A Dionex DX-500 high-performance liquid chromatographic system (Jouy-en-Josas, France) was used.

Ammonium ion was separated on a cation-exchange column (CS15 IonPac column, 250×4 mm) fitted with a CG15 guard column. The CS15 IonPac analytical column stationary phase was composed of

a resin that is functionalised with carboxylate and phosphonate cation-exchange sites and crown ether groups, which provide excellent retention of ammonium and potassium. The IonPac CS15 packing is an 8.5 μm diameter solvent-compatible particle consisting of ethylvinylbenzene crosslinked with 55% divinylbenzene. The CS15 resin construct and properties are described in two previously published works [17,18]. With these stationary phase characteristics, cations can be separated with acidic eluents. H_2SO_4 (Carlo Erba) concentrations between 2.5 and 7.5 m*M* were tested (result not shown) and a good separation was obtained with 5 m*M* H_2SO_4. Moreover, adding acetonitrile (Carlo Erba) to the eluent modifies column selectivity (personal communication, Dionex). A range between 3 and 15% (v/v) of acetonitrile in the presence of 5m*M* H_2SO_4 was studied (result not shown) and a good separation was obtained with 9% (v/v) acetonitrile. Thus, from these preliminary experiments, separation under isocratic elution conditions using 5 m*M* H_2SO_4 and 9% (v/v) acetonitrile in 18 MΩ water was retained.

Separations were carried out at 20°C with a flow-rate of 1.2 ml/min. After elution and before detection by the conductivity detector, an autosuppression external water mode was used by a cation self-regenerating suppressor (CSRS-I, 4 mm). This system provided high capacity suppression of eluent and simplified the detection of ion chromatography because it maximised signal-to-noise ratio for high-sensitivity analysis [12].

Standard solutions of cations were prepared from 1000 mg/kg commercial solutions (Merck, Darmstadt, Germany). Before injection with an auto-sampling device (25 μl volume), suitable concentrations (0, 1, 2, 5, 10, 20 and 40 mg/kg of ammonium ion) were obtained by dilution with 18 MΩ water.

The detection limit was found by preparing dilute solutions of ammonium ion and by identifying the concentration that gave a signal calculated as twice the baseline noise.

In order to estimate the repeatability (or precision) of this method, relative standard deviation (RSD) was calculated as follows: RSD %=(σ100/m; σ and m being the standard deviation and the mean of series of determination, respectively). All samples were analysed in triplicate.

Additions of ammonium standard (+5, +10 and +20 mg/kg) to sodium caseinate, milk, aqueous phase of yoghurt and cheese extracts indicated that for these samples, there was no substantial matrix interference because the added concentrations were totally recovered (recovery>97%) and quantitative (Table 1).

Table 1
Recovery after addition of known ammonium concentration in different samples

	Recovery (%)		
	[Ammonium] added (mg/kg)		
	5	10	20
Sodium caseinate	100	100	99
Milk	100	100	98
Yoghurt	100	99	100
Cheeses	97	98	98

3. Results and discussion

3.1. General aspects

A typical chromatogram of standard cations (sodium, ammonium, magnesium, calcium and potassium) is presented in Fig. 1. The chromatographic peaks were identified by injecting each ion separately (chromatographic profiles not shown). As the pH value of eluent was about 1.0, ammonium ion was eluted in its fully protonated form. The chromatographic peaks were well resolved and consequently the quantification steps were easy. In this work, sodium, magnesium, calcium and potassium were not quantified. It is noteworthy that a step gradient after elution of ammonium could shorten the run time. However separation with isocratic elution condition was used because no reequilibration of the system was necessary between injections. Fast elution can be obtained using 15 m*M* methanesulfonic acid, 7.5 m*M* hydroxylamine and 5% acetonitrile [17].

Different types of relation between injected concentrations (up to 40 mg/kg) and chromatographic areas (conductivity signals) were tested (Fig. 2). The linear relation gave the following equation: $[NH_4^+]$ (mg/kg)=($2.57\cdot10^{-5}\times$conductivity signal) with a correlation coefficient (r^2) of 0.995. The cubic relation gave the following equation: $[NH_4^+]$ (mg/

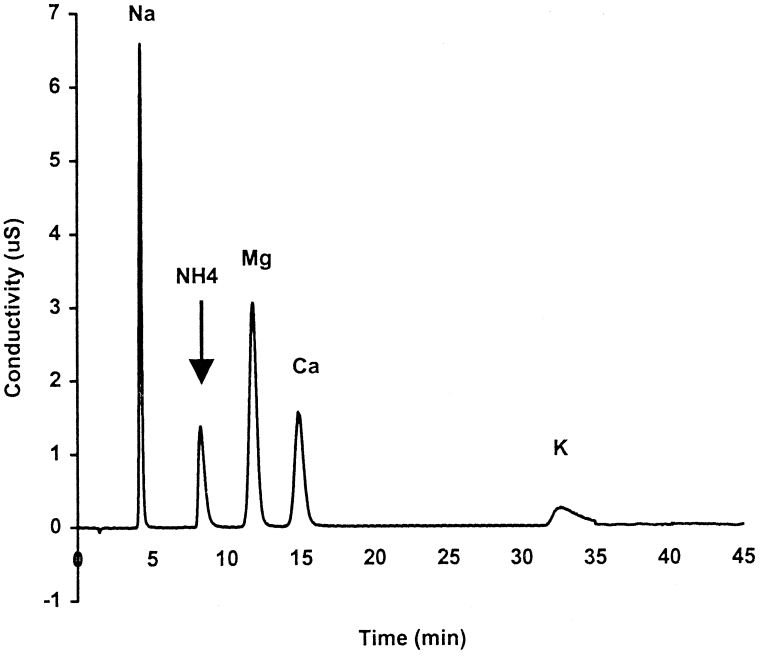

Fig. 1. Chromatogram of cations (sodium, ammonium, magnesium, calcium and potassium) of a standard mixture containing 5 mg/kg of each ion. The arrow on the chromatogram indicates the chromatographic peak of ammonium. Chromatographic conditions for analysis were as follows: eluent, 5 m*M* H_2SO_4 and 9% (v/v) acetonitrile; flow-rate, 1.2 ml/min; CS15 column with a CG15 as a guard column; injection volume, 25 μl; suppressed conductivity detection.

kg) = $(1.09 \cdot 10^{-18} \times \text{conductivity signal}^3) + (1.19 \cdot 10^{-12} \times \text{conductivity signal}^2) + (2.22 \cdot 10^{-5} \times \text{conductivity signal})$ with an r^2 of 0.9998. The quadratic relation gave the following equation: $[NH_4^+]$ (mg/kg) = $(3.46 \cdot 10^{-12} \times \text{conductivity signal}^2) + (2.13 \cdot 10^{-5} \times \text{conductivity signal})$ with an r^2 of 0.9998. Thus, cubic and quadratic relations have similar r^2 values. However, in this work, we used the quadratic calibration because its efficacy has been noted previously [19].

The detection limit was lower than 0.5 mg/kg.

3.2. Determination of ammonium in sodium caseinate: influence of heat treatment

Sodium caseinate consists of the casein fraction of milk which has been precipitated by acid at pH 4.6, collected and redissolved to neutral pH by addition of NaOH. In the food industry, casein molecules are used for their nutritional and functional properties (viscosity, water binding capacity, fat emulsification properties, whipping ability) [1,2]. As such molecules can undergo heat treatments, it was important to test their susceptibility to heat-induced deamidation. The increase in ammonium concentration in the aqueous phase after heat treatment can be taken as a measure for deamidation of casein molecules.

Sodium caseinate was heated at 90, 100, 110, 120 and 130 during 30 min. Chromatographic profiles of sodium caseinate not heated and heated at 120°C during 30 min are shown in Fig. 3A and B, respectively. In the absence of heat treatment, three chromatographic peaks were observed. The first chromatographic peak corresponded to sodium, the second to ammonium and the last to calcium. After the heat treatment, the second chromatographic peak, corresponding to ammonium, increased in intensity. It is noteworthy that in spite of important concentration of sodium, quantification of ammonium was possible. Quantitative results are reported in Fig. 4. After the various heat treatments, the free ammonium content in the pH 4.6-filtrates increased with the temperature of heat treatment. At 90 and 100°C,

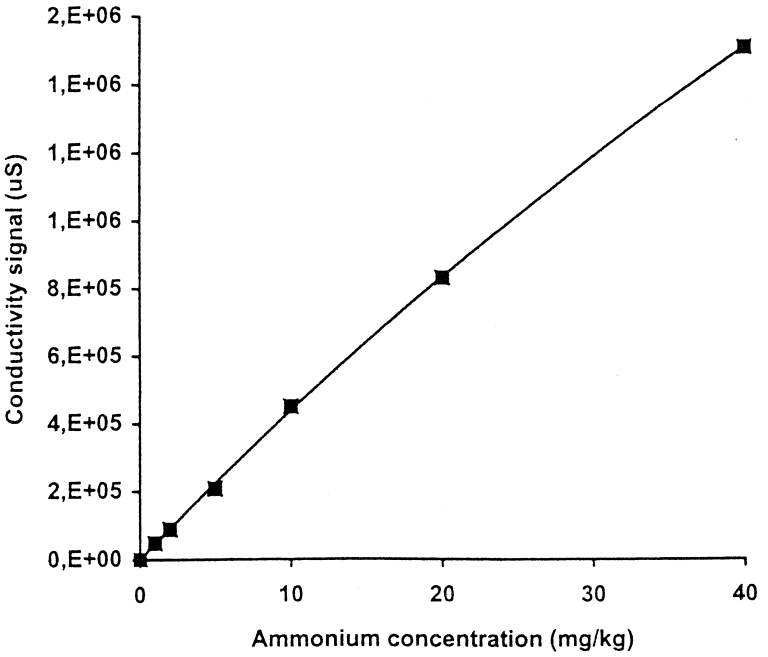

Fig. 2. Standard calibration curve for quantification of ammonium by ion chromatography. Chromatographic conditions are reported in the Experimental section and in the legend of Fig. 1.

the releases of ammonium were relatively low compared to those observed at 110, 120 and 130°C. During heat treatment of sodium caseinate, a nonenzymatic deamidation of casein molecules occurred ([7–10], and personal results). This heat-induced deamidation is a hydrolytic reaction that consists of the loss of ammonium from the asparagine and glutamine residues. Through this loss, these amino acids are converted into their acidic forms, aspartic acid and glutamic acid [20,21]. Results can be expressed in percentage of deamidation. 100% corresponds to the total amide content (24.2 m*M*) in the sodium caseinate suspension used. Theoretical glutamine and asparagine contents in sodium caseinate suspensions containing 24.5 g/kg in casein were indicated in Table 2. These contents were calculated from (i) the molar ratio of each casein in casein micelles: 4–1–4–1.6 for α_{s1}-, α_{s2}-, β- and κ-caseins and (ii) from the asparagine and glutamine content in these different casein molecules [1]. Thus, for a heating time of 30 min, the percentage of deamidation (0.5, 1.5, 3.1, 6.2 and 9.8%) increased with temperature (90, 100, 110, 120 and 130°C, respectively). Several authors ([7–10], and personal results) who studied the kinetics of heat-induced deamidation of sodium caseinate as a function of temperature (between 85 and 145°C), indicated a similar extent of casein deamidation to that determined in this study. In all cases, ammonium contents were not determined by ion chromatography. Thus, the similarity of results obtained by these authors and those obtained in this work, showed that this method to quantify ammonium is correct.

3.3. Determination of ammonium in milk: influence of heat treatment

As described for sodium caseinate, milk, in the dairy industry, can be heated. During heat treatment, nonenzymatic deamidation of proteins and degradation of urea occur. So, it was important to determine the increase in ammonium concentration in the milk aqueous phase.

Skim milk samples were heated at 90, 100, 110, 120 and 130°C during 30 min. Chromatographic profiles were more complex (Fig. 3C and D for milk not heated and heated at 120°C during 30 min,

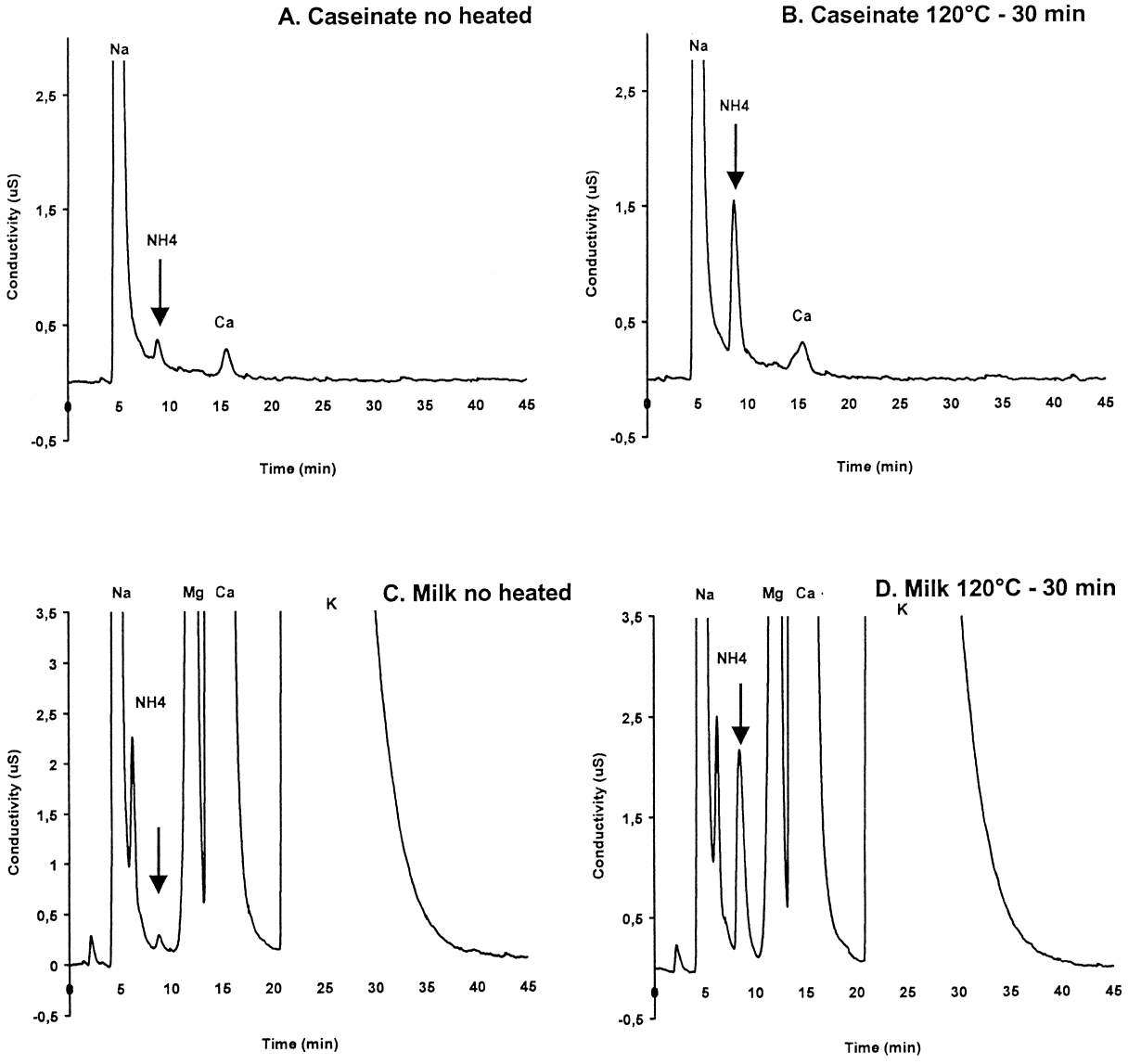

Fig. 3. Chromatograms of cations (sodium, ammonium, magnesium, calcium and potassium) present in the pH 4.6-filtrates of sodium caseinate and milk not heated (A and C, respectively) and heated at 120°C during 30 min (B and D, respectively). The arrows on the chromatograms indicate the chromatographic peak of ammonium. Chromatographic conditions are reported in the Experimental section and in the legend of Fig. 1. Ammonium contents are reported in Fig. 4.

respectively) than those observed with sodium caseinate (Fig. 3A and B). This complexity was due to the presence, in the aqueous phase of milk acidified to pH 4.6, of sodium, magnesium, calcium and potassium in important quantities. Chromatographic peak at 6.5 min was unidentified. At pH 4.6, the concentrations of sodium, magnesium, calcium and potassium in the milk aqueous phase were about 400, 120, 1200 and 1550 mg/kg, respectively [22]. However, in spite of high concentrations of these cations, no major change in retention time and in chromatographic peak resolution of ammonium was observed and its quantification was possible. Quantitative results are reported in Fig. 4. Thus, after the

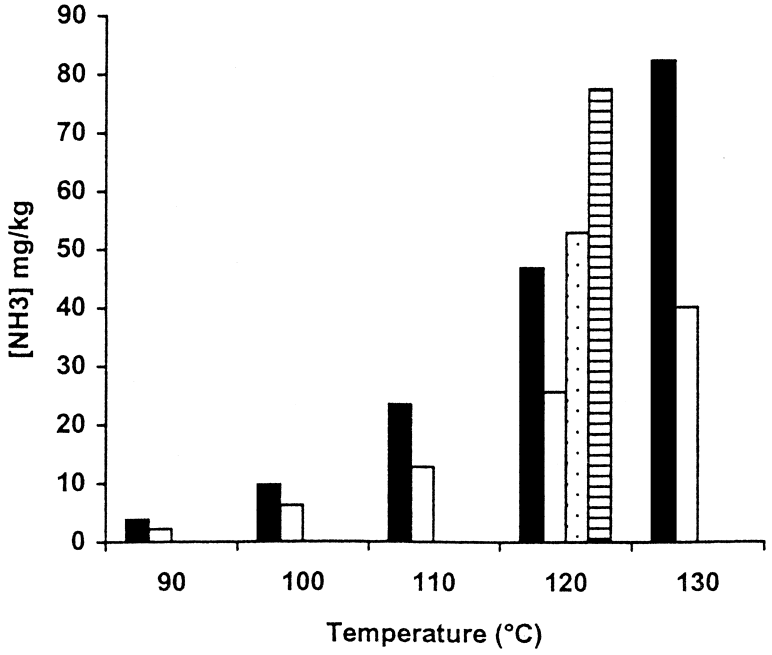

Fig. 4. Influence of the heat treatment on ammonia content in the aqueous phase of sodium caseinate (white histogram); sodium caseinate containing 5 m*M* urea (white histogram with point); sodium caseinate containing 10 m*M* urea (white histogram with draws) and skim milk (black histogram). Ammonia contents were determined by ion chromatography as reported in the Experimental section. Heat treatments were 90, 100, 110, 120 and 130°C during 30 min for sodium caseinate and skim milk and only 120°C during 30 min for sodium caseinate containing 5 and 10 m*M* urea. Relative standard deviation was about 5%.

various heat treatments, the free ammonium content in the pH 4.6-filtrates increased with heat treatments. At 90 and 100°C, the releases of ammonium were relatively low compared to those observed at 110, 120 and 130°C.

It is noteworthy that, for the same heat treatment, the ammonium contents were more important for milk than those determined for sodium caseinate (Fig. 4). Indeed, after sterilisation of milk, the origin and the evolution of the ammonium released are more complex than those observed with sodium caseinate. During severe heat treatments of milk, ammonium is formed consequently to casein deamidation, as shown in this work, but also consequently to deamidation of whey proteins and to urea decomposition [1,23]. To illustrate this phenomenon of decomposition of urea by heat treatment, sodium caseinate was heated at 120°C during 30 min in the presence of 0, 5 and 10 m*M* urea. Results are presented Fig. 4. In comparison with sodium casein-

Table 2
Theoretical repartition and content[a] of asparagine and glutamine residues in sodium caseinate suspension containing 24.5 g/kg in casein

	Casein concentration (g/kg) and m*M**	Asn residue (mol^{-1}) and m*M**	Gln residue (mol^{-1}) and m*M**	Amide (mol^{-1}) and m*M**
α_{s1}-CN	(9.26) 0.40*	(7) 2.80*	(13) 5.2*	(20) 8.0*
α_{s2}-CN	(2.30) 0.09*	(13) 1.17*	(17) 1.53*	(30) 2.7*
β-CN	(9.26) 0.38*	(5) 1.90*	(20) 7.60*	(25) 9.50*
κ-CN	(3.69) 0.20*	(8) 1.60*	(12) 2.40*	(20) 4.0*

[a] These contents were calculated from (i) the % of each casein in casein micelles: 37.8, 9.4, 37.8 and 15.1% for α_{s1}-, α_{s2}-, β- and κ-caseins, respectively and (ii) from the asparagine and glutamine content in these different casein molecules [13].

ate heated at 120°C in absence of urea, supplementary ammonium releases of 27.4 mg/kg (1.6 m*M*) and 52 mg/kg (3.05 m*M*) were determined for addition of 5 and 10 m*M* urea, respectively. These increases corresponded to a urea degradation of about 15%.

3.4. Determination of ammonium in yoghurt

Ammonium was determined in the aqueous phase of commercial yoghurt. The chromatographic profile, presented in Fig. 5A, was very similar to that observed with milk (Fig. 3C). Sodium, ammonium, magnesium, calcium and potassium were well separated. As observed with milk, the chromatographic peak between sodium and ammonium (retention time=6.5 min) was not determined. The ammonium content determined from this chromatographic profile is reported in Table 3.

In this work, the origin of ammonium was not

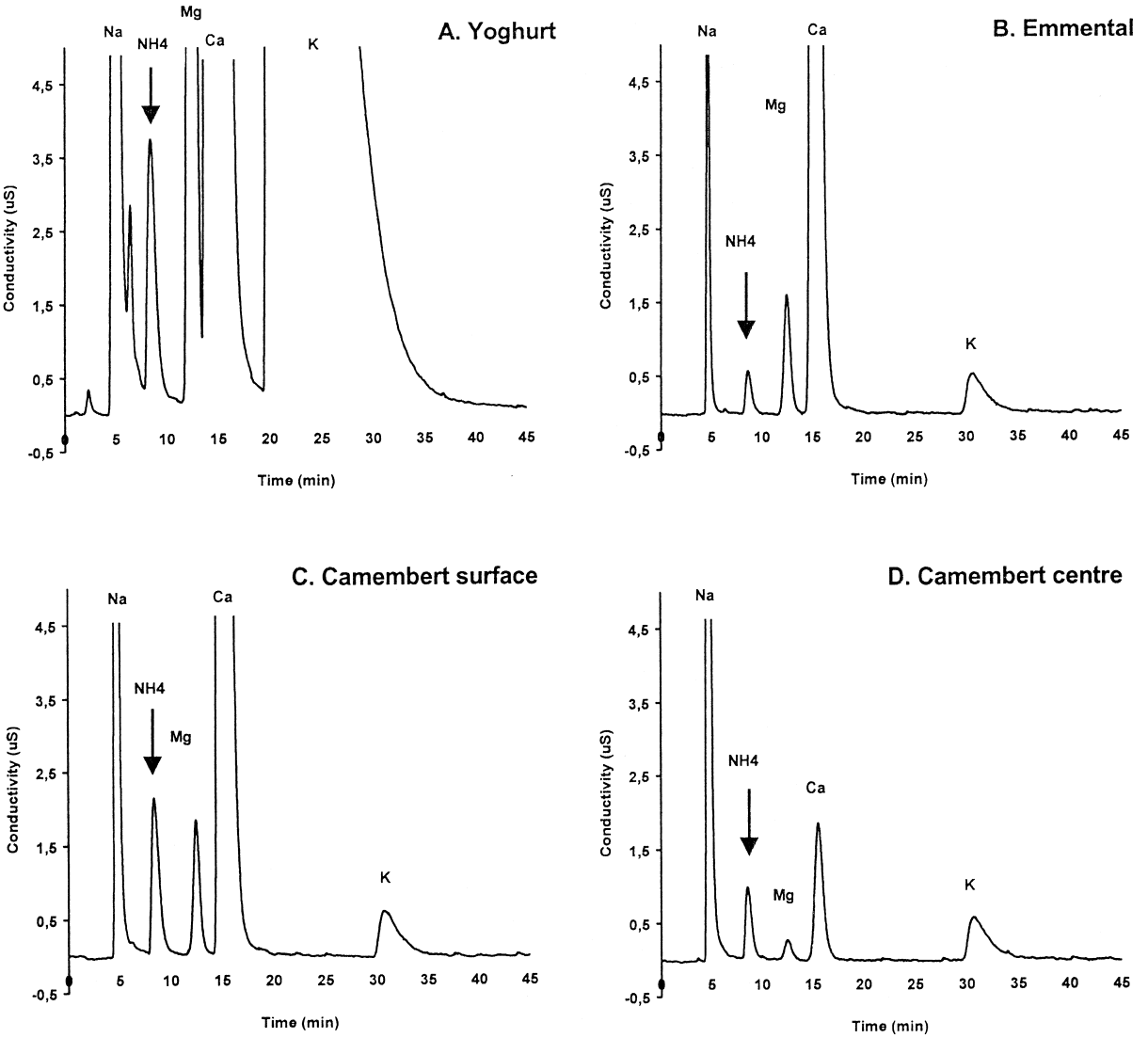

Fig. 5. Chromatograms of cations present in the aqueous phase of yoghurt (A), and cheese extracts of Emmental (B), surface of Camembert cheese (C) and centre of Camembert cheese (D). The arrows on the chromatograms indicate the chromatographic peak of ammonium. Chromatographic conditions are reported in the Experimental section and in the legend of Fig. 1. Ammonia contents are reported in Table 3.

Table 3
Ammonium content in different dairy products[a]

Dairy product	[Ammonium] (mg/kg)
Yoghurt	117
Emmental	550
Surface of Camembert cheese	2360
Centre of Camembert cheese	970

[a] The ammonium concentrations were determined by ion chromatography as indicated in the Experimental section. Chromatograms, corresponding to these determinations, are presented in Fig. 5A–D for yoghurt, Emmental, surface and centre of Camembert cheese, respectively. Relative standard deviation was about 5%.

determined precisely. The two main origins are described in the literature. In one part, it is reported that the level of ammonia nitrogen, in cultured milks, is due to the ability of the lactic bacteria to split urea [24,25]. On the other hand, although in the yoghurt, casein molecules are considered to be only weakly degraded, *Steptococcus thermophilus* and *Lactobacillus bulgaricus* may, during the fermentation, cause a significant degree of proteolysis. Indeed, both these organisms possess different exopeptidases and peptidases able to hydrolysate casein molecules with production of ammonium ion [24,25].

3.5. Determination of ammonium in hard cooked and mould ripened cheeses

Ammonium contents were determined in three types of samples: (i) Emmental cheese, (ii) surface of Camembert cheese and (iii) centre of Camembert cheese. Chromatographic profiles are presented in Fig. 5B–D, respectively.

As observed with milk and yoghurt, sodium, magnesium, calcium and potassium were identified qualitatively. In spite of high concentrations of these cations, no major change in retention time and in chromatographic peak resolution of ammonium was observed and its quantification was possible. Ammonium contents of these three samples, determined from these chromatographic profiles, are reported in Table 3.

Casein in unripened cheese is odourless, tasteless and water-insoluble and has a tough and rubbery consistency. During ripening of cheeses, the proteolytic enzymes of micro-organisms in cheese split this casein network into short-chain water-soluble compounds, e.g., polypeptides, peptides and amino acids. Then, amino acids may be further decomposed enzymatically by decarboxylation, transamination and deamination activities of the microbial flora on amino acids. This last biochemical reaction contributes to the production of NH_3 in the cheese and especially in mould ripened cheeses. Thus, ammonia is an important element in the aroma of these cheeses. In traditional Camembert cheeses, ammonium represents 7–9% of total nitrogen [3]. Concerning Camembert cheese studied in this work, we observed a low concentration of ammonium in the centre of the cheese and a high concentration at the cheese surface (Table 3). Similar results were observed by several authors [3,5,26]. This concentration difference between the centre and surface corresponds to a preferential proteolysis of casein by surface microflora. Theoretically, proteolytic enzymes responsible for the breakdown of casein molecules in cheese are plasmin of milk, proteases of *Penicillium* moulds and rennet (chymosin) necessary to coagulate milk. However, most of the proteolytic breakdown of casein can be attributed to proteases elaborated by *Penicillium* moulds [3,27]. Because the diffusion of these enzymes into the body of the cheese is limited [28], it is generally admitted that ammonium migrated into the interior of cheese. Thus, diffusion of ammonium from the surface toward the centre of the cheese and casein proteolysis with production of ammonium of casein in the centre of the cheese explain the important content of ammonium in this zone.

4. Conclusions

Several methods have been developed for determination of ammonium ion (alkaline distillation followed by titration, potentiometric or enzymatic methods) [4–11]. In this study, ion chromatography in combination with an adequate sample preparation has been successfully used for the qualitative and quantitative determination of ammonium. This new method is very specific for ammonium. Potential interfering substances such as other ions or matrix, do not affect ammonium determination.

In conclusion, the potential of this method appears very interesting because it seems possible to characterise specifically:

(1) Ammonium of different dairy products (milks, caseinates, whey products, purified milk proteins, yoghurts, cheeses).

(2) Ammonium in the aqueous phase of milk or derived products during different technological processes such as thermal treatment, acidification, membrane separation.

(3) The progress of biochemical reactions (especially proteolysis of casein molecules) by microorganisms during yoghurt and cheese manufactures.

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