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# The determination and distribution of nucleotides in dairy products using HPLC and diode array detection

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**Abstract**

A method for the determination of adenosine, cytidine, uridine, guanosine and inosine 5′-monophosphates in milk and dairy products was optimized and its performance evaluated. The technique was based on ion-pair reversed-phase HPLC separation of the nucleotides and diode array detection. The chromatographic separation was achieved using a C18 column and a gradient elution with a mixture of two solvents: solvent A, water/glacial acetic acid/tetrabutilammonium hydrogensulphate (TBAHS) and solvent B, methanol/glacial acetic acid/TBAHS. The effluent was monitored using a Diode Array detector set at 260 nm. Validation of the proposed method was carried out by standard additions method, with recoveries of 98.3%. The precision of the method was also evaluated and reported a coefficient of variation (CV) as less than 3.2. Upon development the technique was applied on different dairy products in order to study the distribution of nucleotides therein. The samples included bovine, ovine and caprine milks, the corresponding manually manufactured cheeses, whey cheese and whey. Three commercial cheeses made from bovine, ovine and caprine milks, respectively, and 20 infant formulae were also analysed. In contrast to their absence in cheese upon preparation nucleotides were present in cheese at the end of ripening; this observation led to the extraction of DNA in order to evaluate whether nucleotides were released from degradation of nucleoproteins. 

*Keywords:* Nucleotides; Dairy products; HPLC; Diode array

## 1. Introduction

Nucleotides are present in food mainly as nucleoproteins from which they are released and very efficiently absorbed (Wilson & Wilson, 1962). The reutilisation of these precursors of nucleotides spares the cost of de novo synthesis; in fact, nucleoproteins inhibit this metabolic pathway by feedback mechanisms (Palelle & Fox, 1989). Some authors hypothesised, therefore, that dietary nucleotides are energetically advantageous to fulfil the needs of the liver for nucleotides (López-Navarro, Bueno, Gil, & Sanchez-Pozo, 1996).

Evidence has shown that exogenous nucleotides can influence liver function. For example, supplementation of a parental nutritional solution with nucleotides facilitates liver recovery from ischaemia or partial hepatectomy (Palombo, Bowers, Clouse, McCullough, Forse, & Bistrian, 1993). Whether or not the liver requires dietary nucleotides under normal physiological conditions is an open question. Some authors believe that most of the ingested nucleotides are degraded within the intestine, but it has also been demonstrated that dietary nucleotides are incorporated into liver nucleic acids (Lopez-Navarro et al., 1996).

Several other functions are recognised for nucleotides, viz. modulation of lipoprotein metabolism, modification of the composition of the intestinal microflora, improvement of gastrointestinal tract repair after damage and participation in the immunitary response mediated by T cells. Moreover, rapidly growing tissues such as the intestinal epithelium and lymphoid cells lack significant capacity for de novo synthesis of nucleotides and require exogenous sources of these compounds (Sanchez-Pozo, Morrillas, Moltó, Robles, & Gil, 1994). Increased attention has been paid to the role of dietary nucleotides in infant nutrition. Recent studies suggested that dietary nucleotides are important in order to maintain normal growth and development in infants.

The content of free nucleotides in milk varies with the associated mammal species (Gil & Sanchez-Medina,

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1981). Hence, the aim of this work was to investigate, on the one hand, the nucleotide content of bovine, ovine and caprine milks, and, on the other, their distribution in different dairy products. As far as we know, there are no studies available on profiles of nucleotides in cheese and on whether the nucleotide content may be used as an objective parameter to indicate the type of milk employed.

Furthermore, human milk is the best source of nucleotides for young infants (Sugawara, Sato, Nakano, Idota, & Nkajima, 1995; Thorell, Sjoberg, & Hernell, 1996). Supplementation of adapted-milk formulae, with 5′-monophosphate adenosine (AMP), 5′-monophosphate cytidine (CMP), 5′-monophosphate uridine (UMP), 5′-monophosphate guanosine (GMP) and 5′-monophosphate inosine (IMP), using concentrations within the ranges found in human milk, is recommended by several organisations, namely, the Scientific Commitee for Food of the European Community (SCF, 1993). Thus, a third objective of this research effort was the monitorisation of the nucleotide content of infant formulae and follow-up milks available on the retail market.

In attempts to achieve the proposed objectives extraction procedures and a methodology using HPLC/ coupled to diode array detection were optimised and validated for application to the different dairy products analysed.

## 2. Materials

### 2.1. Apparatus

The chromatographic analysis was carried out in an analytical HPLC unit (Jasco) equipped with two pumps model PU-980, a diode array detector model MD-910 and an auto-sampler model AS-950. The loop volume selected was 20 μl.

The chromatographic separation was achieved with a Spherisorb $C_{18}$, (S10 ODS2) chromatographic column 10 μm (250×4.6 mm) from Waters (USA).

### 2.2. Reagents and standards

Nucleotides were obtained from Sigma Chemical Co (USA). Guanidinium isothiocyanate, ethylenediaminetetracetic acid, 2-mercaptoethanol, ethanol, tetrabutylammonium hydrogensulphate, silica, glacial acetic acid and methanol (gradient grade) were obtained from Merck (Darmstadt, Germany).

### 2.3. Samples

Samples of fresh raw bovine, ovine and caprine mature milks (i.e. with more than two months of lactation) were obtained directly from local farmers in the Trás-os–Montes region. Cheeses were manufactured manually using ovine and caprine milks. The drained whey was also analysed per si and further used as a substrate for the production of whey cheeses via the precipitation of whey proteins upon heat treatment. These whey cheeses were sampled, as well as the whey permeate.

Three commercial cheese types, i.e. bovine, ovine and caprine raw milk cheeses were randomly purchased from the market and analysed.

Twenty samples of commercially available infant foods manufactured by well-known companies, leaders in the market, were assayed. These included, 10 infant formulae based on cow's milk proteins, eight follow-up milks and two lactose-free infant formulae.

### 2.4. Sample preparation

The principal steps of sample preparation were: (1) extraction of the nucleotides followed by (2) acid precipitation of any macromolecules in the extract with recovery of the free nucleotides.

#### 2.4.1. Preparation of milk and whey extracts

A sample (3 ml) was added to 2 ml of perchloric acid solution 0.33M and homogenized. After centrifugation (5×1000 rpm) for 10 min, 3 ml of supernatant were neutralised with 200 μl of potassium carbonate (1.2M). This solution was then centrifuged (5×1000 rpm) for 10 min. If necessary, samples were filtered through Whatman W42 paper and subsequently, through 0.2 μm filter paper.

#### 2.4.2. Preparation of infant formulae and follow-up milk extracts

After homogenisation, 5.0 g of sample were dissolved in 20 ml of distilled water; 3 ml of the adapted milk formula solution followed the procedure of sample preparation described above.

#### 2.4.3. Preparation of cheese and whey cheese extracts

Each sample (25.0 g) was thoroughly homogenized and dispersed with 15 ml of perchloric acid solution 0.33M in a high-speed homogenizer (Ultraturrax, IKA-Werk, Jakob & Kunkel). After centrifugation (5×1000 rpm) for 20 min, 3 ml of supernatant were treated as described for milk and whey extracts. Samples were filtered through W42 paper and subsequently, through 0.2 μm filter paper.

### 2.5. Statistical analysis

Data are represented as the mean±standard deviation. Analysis of variance (ANOVA) was used to determine the effects of type of milk on nucleotide content. All statistical analyses were done with the Statview™

4.0 statistical package (Abacus Concepts, Berkeley CA, USA).

### 2.6. DNA extraction

Fresh milk was centrifuged (400 ***g*** at 4°C for 30 min) and the resulting cell pellet was used for DNA extraction. Otherwise, solid cheese (1.5 g) was subjected to DNA extraction in the following manner: samples were homogenised with 13.8 ml guanidinium isothiocyanate buffer [4 M guanidinium isothiocyanate, 50 mM Tris-HCl, 25 mM ethylenediaminetetracetic acid (EDTA) pH 7.5] and 1.2 ml of 2-mercaptoethanol on ice for less than 5 min, 5 ml of chilled ethanol were added thereafter and the samples were briefly mixed; the pellets that resulted from centrifugation (14,500 ***g***, at 4°C for 5 min) of the above mixture were dissolved in 300 μl guanidinium-HCl buffer (6 M guanidinium HCl, 25 mM EDTA pH 7.5); silica was subsequently added and the mixture was left for 15 min (tubes were gently mixed every 2 min) at room temperature to permit DNA adsorption to the silica particles; after centrifugation the buffer was removed and the particles were resuspended in 500 μl guanidinium-HCl buffer; upon renewed removal of the buffer the particles were washed twice with 70% ethanol, 50 mM Tris-HCl pH 7.2, 1 mM EDTA; finally, the DNA-coated particles were air-dried for 20 min, resuspended in 40 μl sterile $H_2O$ and upon dissolution at 50°C for 5 min the DNA-containing supernatant was stored at 4°C until further use.

The characteristics of extracted DNA were evaluated after performance of electrophoresis in 2% agarose gel and use of a photographic system equipped with software appropriate for image analyses, (System EDAS DC 120 Life Technologies).

### 2.7. Chromatographic conditions

The HPLC elution required a mixture of two solvents. Solvent A consisted of water/glacial acetic acid/5 mM tetrabutyl ammonium hydrogensulphate (TBAHS) in a 97.5:1.5:1.0 (v/v/v) ratio and solvent B consisted of methanol/glacial acetic acid/5 mM TBAHS) in a 97.5:1.5:1.0 (v/v/v) ratio. The two solvents were filtered and degassed before use. Gradient elution was carried out as follows: 0–5 min 100% A, 5–20 min linear gradient to 10% B, 20–23 min 10% B, 23–29 min linear gradient to 40% B, 29–30 min 40% B, 30–34 linear gradient back to 100% A (initial conditions), 2 min re-equilibration wash with 100% A. The flow rate was 1 ml/min.

### 2.8. Calculation of detection limits

The detection limits for each nucleotide under study were calculated as the concentration corresponding to three times the background noise, obtained with 10 determinations.

## 3. Results and discussion

### 3.1. Validity of the method

Under the assay conditions described, a linear relationship was obtained between the concentration of

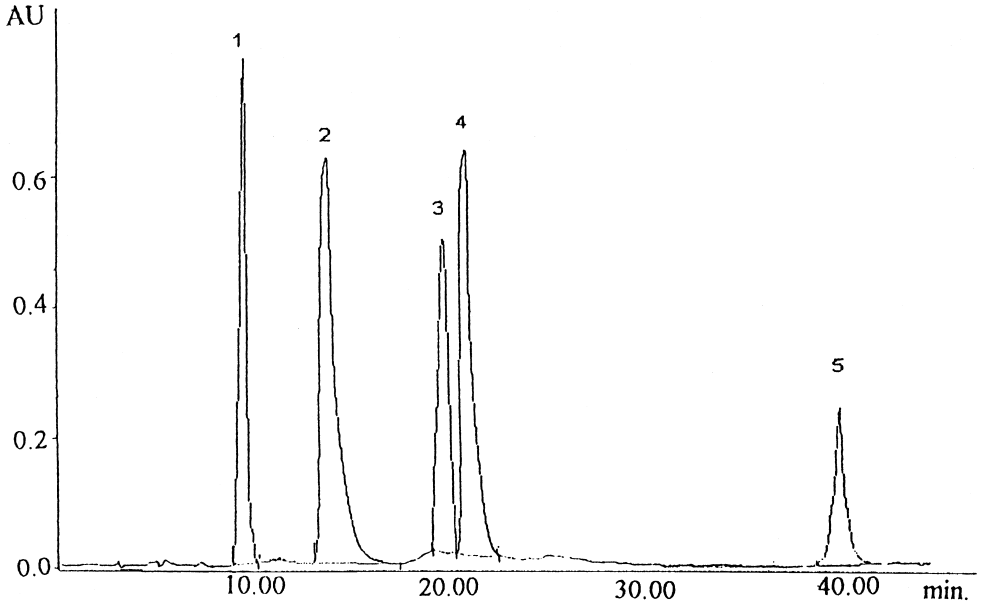


Fig. 1. Typical chromatogram for separation of five nucleotides (chromatographic conditions described in the text): 1, 5′-monophosphate cytidine; 2, 5′-monophosphate uridine; 3, 5′-monophosphate guanosine; 4, 5′-monophosphate inosine; 5, 5′-monophosphate adenosine. The concentration of each nucleotide injected onto the column was 15 mg/l.

different types of nucleotides and the UV absorvance at 260 nm. This linearity was maintained over the concentration range of 0.2–30 mg/l for CMP and UMP and of 0.4–30 mg/l for GMP, AMP and IMP. Fig. 1 shows a typical chromatogram of separation of the five nucleotides. Each nucleotide peak was identified by comparison of its retention time and by its spectrum.

The reliability of the method in terms of precision, recovery and sensitivity, was studied. The precision and accuracy of the extraction procedure was evaluated on three different matrices, including ovine milk, ovine cheese and an infant formula. Samples were analysed before and after the addition of known amounts of CMP, UMP, GMP, IMP and AMP. The results obtained from the recovery studies are listed in Table 1. These results confirmed that although the matrix composition is complex, it does not cause interference effects.

Six different extractions, as described above, were made for each sample and subsequently injected in duplicate. The CV reported was less than 3% ($n=6$). The detection limits, were 0.1 mg/l for CMP, UMP, IMP and GMP and 0.2 mg/l for AMP.

### 3.2. Raw bovine, ovine and caprine milks

Table 2 shows the contents of nucleotides assessed in the three types of milk, corresponding cheeses and subproducts.

Bovine milk yielded significantly different nucleotide patterns from those of ovine and caprine milks in both qualitative and quantitative terms, as apparent from inspection of Table 2. The total concentration of all nucleotides was lowest in bovine milk (ca 1.0 mg/100 ml), followed by ovine milk (8.0 mg/100 ml) and highest in caprine milk (14.4 mg/100 ml).

The ANOVA also indicate that the type of milk had a significant effect on the qualitative nucleotide profiles; CMP and AMP were present in all three types of milk but at very different concentrations (AMP was the more abundant nucleotide in caprine and ovine milks, but the less abundant in bovine milk, as observed from inspection of Table 2), whereas GMP and IMP were not detected in any of the three milk types analysed. The major nucleotide present in ovine and caprine milks but not detected in bovine milk was UMP; UMP represented per se more than 50% of the total content of nucleotides and was present in caprine milk at concentrations two-fold those in the ovine milk counterpart. Our results on the nucleotide contents of bovine, ovine and caprine milks are in good agreement with those reported by several research workers (Gil & Sanchez-Medina, 1981).

### 3.3. Ovine and caprine cheeses and corresponding drained whey, whey cheeses and whey upon heat treatment

The distribution of nucleotides when in a solid matrix such as cheese made from small ruminants' milks and the "fate" of nucleotides in secondary dairy products resulting from cheese manufacture, namely, whey cheese and whey upon heat treatment, was also analysed.

From inspection of the results listed in Table 2 we may conclude that during cheese manufacture the free nucleotides in milk are lost during whey syneresis; nucleotide contents in whey and whey cheeses derived therefrom parallel those found in the original plain milk.

Table 1
Statistical results for the recoveries obtained by the standard additions method

| Matrix | Nucleotide | Conc.[a] (mg/ml; $n=2$) | Recovery ± S.D. (%) |
|---|---|---|---|
| Ovine milk | 5′-monophosphate cytidine | 3.0 | 96.2 |
| | 5′-monophosphate adenosine | 3.0 | 97.4 |
| | 5′-monophosphate guanosine | 3.0 | 96.8 |
| | 5′-monophosphate uridine | 3.0 | 98.2 |
| | 5′-monophosphate inosine | 3.0 | 97.2 |
| Ovine cheese | 5′-monophosphate cytidine | 3.0 | 97.8 |
| | 5′-monophosphate adenosine | 3.0 | 98.8 |
| | 5′-monophosphate guanosine | 3.0 | 96.9 |
| | 5′-monophosphate uridine | 3.0 | 97.7 |
| | 5′-monophosphate inosine | 3.0 | 96.7 |
| Infant formulae | 5′-monophosphate cytidine | 3.0 | 101.0 |
| | 5′-monophosphate adenosine | 3.0 | 99.1 |
| | 5′-monophosphate guanosine | 3.0 | 97.6 |
| | 5′-monophosphate uridine | 3.0 | 97.9 |
| | 5′-monophosphate inosine | 3.0 | 97.2 |

[a] Concentration of nucleotide solution added to the samples before analysis.

Table 2
Nucleotides found in bovine, ovine and caprine mature milks, respective cheeses and subproducts[a]

| | 5′-Monophosphate cytidine | 5′-Monophosphate adenosine | 5′-Monophosphate guanosine | 5′-Monophosphate uridine | 5′-Monophosphate inosine |
|---|---|---|---|---|---|
| *Milk (mg/100 ml)* | | | | | |
| Bovine | 0.98±0.06 | Traces | <LOD | <LOD | <LOD |
| Ovine | 1.79±0.04 | 2.11±0.08 | <LOD | 4.09±0.23 | <LOD |
| Caprine | 2.67±0.05 | 3.34±0.07 | <LOD | 8.39±0.21 | <LOD |
| *Ovine cheese (mg/kg)*[b] | | | | | |
| 0 days ripening | <LOD | <LOD | <LOD | <LOD | <LOD |
| 30 days ripening | 0.27±0.01 | Traces | <LOD | 0.31±0.02 | <LOD |
| *Caprine cheese (mg/kg)*[b] | | | | | |
| 0 days ripening | <LOD | <LOD | <LOD | <LOD | <LOD |
| 30 daysripening | 0.29±0.02 | 0.19±0.02 | <LOD | 0.42±0.03 | <LOD |
| *Whey cheese (mg/kg)*[b] | | | | | |
| Ovine | <LOD | <LOD | <LOD | <LOD | <LOD |
| Caprine | <LOD | <LOD | <LOD | <LOD | <LOD |
| Whey (mg/100 ml) | | | | | |
| Ovine | 2.13±0.06 | 2.61±0.04 | <LOD | 4.89±0.10 | <LOD |
| Caprine | 2.97±0.09 | 3.54±0.11 | <LOD | 8.53±0.16 | <LOD |
| *Whey after heat treatment (mg/100 ml)* | | | | | |
| Ovine | 2.39±0.06 | 2.84±0.03 | <LOD | 5.61±0.07 | <LOD |
| Caprine | 3.56±0.07 | 4.20±0.06 | <LOD | 9.14±0.14 | <LOD |

[a] Values are expressed as mean±standard deviation of three samples analysed in duplicate ($n=6$). LOD, Limit of detection.
[b] Results are expressed for dry product.

Ovine and caprine cheeses ripened at room temperature (ca. 17°C) for 30 days, presented small amounts of CMP, AMP, and UMP by the end of the ripening period as shown in Table 2 total concentration of nucleotides was higher in caprine cheese than in ovine counterpart.

The analysis of commercially purchased cheeses showed that the bovine milk cheese contained traces of CMP and AMP, whereas small amounts of CMP, AMP and UMP were found in the ovine and caprine cheeses (Table 3), in the same order of magnitude as those reported for the experimental cheeses.

In the present study, DNA could be extracted from bovine, ovine and caprine milks and also from dairy products such as cheese and heat-denatured whey proteins (whey cheese). Ovine and caprine cheeses contained more DNA than bovine cheese.

Table 3
Nucleotides found in bovine, ovine and caprine commercial cheeses given as mg/1000 g of dry product[a]

| | Bovine | Ovine | Caprine |
|---|---|---|---|
| 5′-Monophosphate cytidine | Traces | 0.29±0.01 | 0.32±0.03 |
| 5′-Monophosphate adenosine | Traces | 0.21±0.00 | 0.28±0.01 |
| 5′-Monophosphate guanosine | <LOD | <LOD | <LOD |
| 5′-Monophosphate uridine | <LOD | 0.23±0.23 | 0.35±0.02 |
| 5′-Monophosphate inosine | <LOD | <LOD | <LOD |

[a] Values are expressed as mean±standard deviation of three samples analysed in duplicate ($n=6$). <LOD — <Limit of detection.

Analyses of the results suggests that during ripening some nucleotides may be released from degradation of nucleoproteins; enzymes present in cheese may hydrolyse molecules of nucleoproteins into nucleotides, nucleosides and free bases. Besides the therapeutic effects nucleotides may also be used as flavour enhancers in foods (Belem & Lee, 1997); thus, the nucleotides released during ripening may play an important role as precursors of several cheese flavour components. Such fact could lead to the use of nucleotide profile in a cheese as an objective parameter to indicate the type of milk used or predict the ripening time of the cheese. Nevertheless, further studies are warranted in order to determine this possibility. These studies include the need to establish kinetics of nucleoprotein degradation and concomitant release of nucleotides as well as to point out the major nucleotide present at each sampling point.

### 3.4. Analysis of infant formulae and follow-up milks

The results obtained on the analyses of 10 infant formulae based on cow's milk proteins, eight follow-up milks and two lactose-free infant formulae showed that only two (one infant formulae and one follow-up milk) of the 20 samples analysed presented the five nucleotides under study, an observation which may suggest a

supplementation step with those compounds in the production process. Among the five nucleotides analysed, CMP and UMP were the most abundant whereas GMP, AMP and IMP were in trace amounts. The concentrations of the dominating nucleotide, CMP, were 8.1 and 12.19 mg/100 g, in infant formulae and follow-up milk, respectively, whereas levels of UMP were 83% below those of CMP, i.e. 1.4 mg/100 g and 2.14 mg/100 g for infant formulae and follow-up milk, respectively. Neverthess, the levels of nucleotides were below the maximum limits allowed for suplementation (SCF, 1993; Directive 96/4/CE).

Ten other samples (five infant formulae and five follow-up milks) showed traces of cytidine monophosphate, probably owing to bovine milk composition. No nucleotides were detected in the remaining infant formulae and follow-up milks.

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