# THE ANALYSIS OF 5'-MONONUCLEOTIDES IN PEDIATRIC FORMULAS BY HPLC

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## ABSTRACT

A reversed-phase HPLC method for the routine determination of supplemented 5'-mononucleotides (uridine 5' monophosphate, inosine 5' monophosphate, adenosine 5' monophosphate, guanosine 5' monophosphate, and cytidine 5' monophosphate) in pediatric formulas and milk products is described. Following sample dissolution, potential interferences were removed by strong anion-exchange solid-phase extraction. Chromatographic analyses were performed using a C18 stationary phase with gradient elution, UV detection and with quantitation achieved by an internal standard technique. A single laboratory validation was performed with recoveries of 92 – 101 % and repeatability RSD's of 1.0 – 2.3 %. The method was optimised for the rapid, routine analysis of nucleotide-supplemented bovine milk-based infant and follow-on formulas.

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## INTRODUCTION

Nucleotides are compounds of critical importance to cellular function. They operate as precursors to nucleic acids, as mediators of chemical energy transfer and cell signalling, and as integral components of coenzymes in the metabolism of carbohydrates, lipids and protein.

Nucleotide supplemented infant formulas have been reported to enhance immune response, influence metabolism of fatty acids and improve gastrointestinal tract repair after damage. With the proliferation of nucleotide-supplemented pediatric formulas, robust methods that incorporate minimal sample preparation and rapid chromatographic separations are required for routine product compliance analysis.

This method below describes a simple SPE sample clean-up that avoids the prior need to remove protein, coupled with a binary gradient reversed-phase liquid chromatographic system for the purpose of routine analysis of nucleotide supplemented infant formula. Analytical security is enhanced with an internal standard based quantitation.

## METHODOLOGY

### SAMPLE PREPARATION

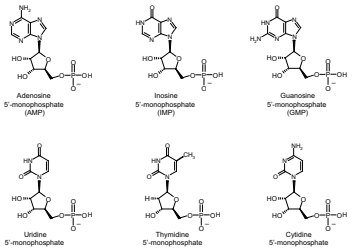
- Dissolve 1.0g powder in 30mL of NaCl/EDTA solution.
- Add 1mL of TMP intermediate standard (~80 µg mL$^{-1}$), mix well.
- Stand for 10 minutes to ensure complete hydration.
- Dilute to final volume of 50mL with water.

### SOLID PHASE EXTRACTION

- For each sample, place a SPE cartridge (Chromabond SB, 6mL x 1000mg) on SPE vacuum manifold.
- Condition cartridge with 4mL MeOH, followed by 2 x 5mL of $H_2O$.
- Load cartridge with 4mL of sample solution.
- Wash cartridge with 4mL of 0.3M KBr.
- Elute nucleotides with 4mL of 0.5M $KH_2PO_4$, pH 3.0 into a test tube.
- Filter an aliquot of the eluent through a 0.2 µm syringe filter into an autosampler vial ready for HPLC analysis.

### HPLC ANALYSIS

| | |
|---|---|
| Column: | Gemini $C_{18}$ 5µm 110Å 4.6 x 250mm (Phenomenex) |
| Mobile Phase A: | $KH_2PO_4$ (0.1M), pH=5.6 |
| Mobile Phase B: | MeOH 100% |
| Gradient: | Low pressure gradient |
| Detection: | Photo-diode array, quantify at 250nm (IMP), 260nm (GMP, TMP, AMP) and 270nm (CMP, UMP) |
| Quanititation: | Internal standard technique (TMP) |
| Injection: | 50 µL |




## RESULTS AND DISCUSSION

**CHROMATOGRAPHIC PERFORMANCE**

| Parameters[a] | CMP[b] | UMP | GMP | IMP | TMP | AMP |
|---|---|---|---|---|---|---|
| Retention time | 8.8 (0.22%)[c] | 11.8 (0.17 %) | 19.8 (0.15%) | 20.6 (0.10%) | 25.0 (0.04%) | 25.8 (0.04%) |
| Capacity factor | 0.6 (0%) | 1.2 (0.83%) | 2.7 (0%) | 2.8(0%) | 3.6 (0%) | 3.8 (0%) |
| Resolution | – | 6.3 (0.07) | 16.9 (1.11%) | 2.2 (0.45%) | 15.6 (0.19%) | 3.5 (0.57%) |
| Tailing | 1.3 (3.84%) | 1.2 (3.33%) | 1.0 (0%) | 1.0 (5.00%) | 1.1 (0%) | 1.1 (3.6%) |
| Theoretical Plates | 6810 (0.87%) | 8527 (5.51%) | 33692 (3.28%) | 60448 (1.22%) | 194363 (0.81%) | 241749 (0.22%) |
| Peak Area | 142255 (0.51%) | 200488 (1.39%) | 225242 (0.23%) | 122536 (0.75%)w | 488585 (0.11%) | 308754 (0.05%) |

[a] Calculations as defined by US Pharmacopeia.
[b] AMP = adenosine 5'-monophosphate, CMP = cytidine 5'-monophosphate, GMP = guanosine 5'-monophosphate, IMP = inosine 5'-monophosphate, UMP = uridine 5'-monophosphate, TMP = thymidine 5'-monophosphate.
[c] Mean (percent relative standard deviation) of six replicates of a mixed nucleotide standard.

**RESULTS OBTAINED FROM COMMERCIALLY AVAILABLE MILK PRODUC**

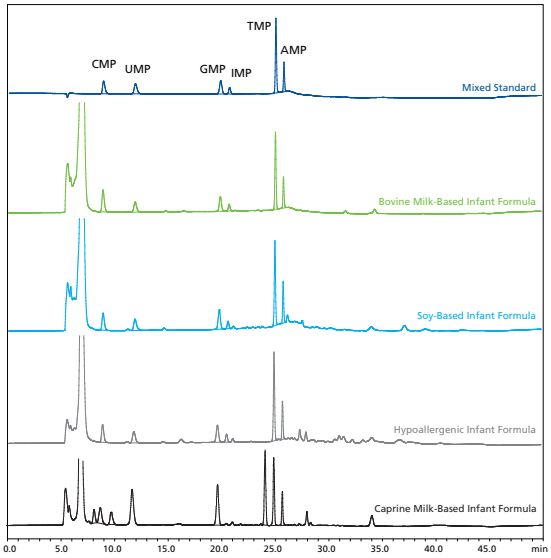
| Sample Type[a] | Nucleotide Supplemented | Results (mg/100g$^{-1}$) CMP[b] | UMP | GMP | IMP | AMP |
|---|---|---|---|---|---|---|
| Bovine milk-based IF | Yes | 11.6 (116%)[c] | 3.7 (95%) | 1.7 (106%) | 2.0 (125%) | 4.5 (145%) |
| Bovine milk-based FO | Yes | 6.0 (107%) | 2.4 (87%) | 0.9 (89%) | 1.0 (91%) | 2.1 (103%) |
| Bovine milk-based FO | No | 1.0 | 0 | 0 | 0.1 | 0 |
| Bovine milk-based FO | Yes | 8.5 (172%) | 2.4 (89%) | 1.0 (100%) | 1.0 (92%) | 2.3 (115%) |
| Bovine milk-based AN | Yes | 17.4 (120%) | 4.7 (75%) | 8.0 (107%) | 0 – | 7.2 (130%) |
| Soy-based IF | No | 0.1 | 0.3 | 0.3 | 0 | 0.5 |
| Caprine milk-based IF | No | 4.0 | 8.2 | 6.4 | 0.3 | 2.3 |
| Bovine milk-based WMP | No | 4.0 | 0 | 0 | 0 | 0 |
| Hypoallergenic IF | No[d] | 2.6 (101%) | 2.6 (92%) | 2.7 (85%) | 2.6 (96%) | 3.1 (100%) |
| Hypoallergenic IF | No | 0.0 | 0 | 0 | 0 | 0 |

[a] IF = infant formula, FO = follow-on formula, AN = adult nutritional product, WMP = whole milk powder.
[b] AMP = adenosine 5'-monophosphate, CMP = cytidine 5'-monophosphate, GMP = guanosine 5'-monophosphate, IMP = inosine 5' monophosphate, UMP = uridine 5'-monophosphate.
[c] Recovery as percentage of label claim.
[d] Hypoallergenic sample spiked with nucleotide mixed standard prior to analysis.
[e] – IMP not added

**METHOD PERFORMANCE**

| Analytea | Range (mg mL$^{-1}$) | Linear regression | $r^2$ | MDL (mg 100 g$^{-1}$)[b] | RSDr (%)[c] | HorRat[d] | $RSD_R$ (%)[e] | Recovery (%)[f] |
|---|---|---|---|---|---|---|---|---|
| AMP | 1.25 - 17.49 | y = 255805x + 11862 | 1.0000 | 0.19 | 2.0 | 0.4 | 4.5 | 99.6 (2.4%) |
| CMP | 0.61 - 8.55 | y = 287762x - 2493 | 0.9999 | 0.08 | 1.0 | 0.3 | 6.0 | 99.7 (1.9%) |
| GMP | 1.11 - 15.55 | y = 200342x - 1807 | 1.0000 | 0.06 | 2.1 | 0.4 | 5.2 | 100.5 (1.7%) |
| IMP | 1.09 - 15.27 | y = 198519x + 3879 | 1.0000 | 0.10 | 1.4 | 0.3 | 3.8 | 97.8 (2.4%) |
| UMP | 1.12 - 15.68 | y = 146931x - 1839 | 0.9999 | 0.08 | 2.3 | 0.5 | 8.6 | 96.5 (3.6%) |
| TMP | 1.61 - 22.54 | y = 150494x - 455 | 1.0000 | – | – | – | – | 100.1 (3.1%) |

[a] AMP = adenosine 5'-monophosphate, CMP = cytidine 5'-monophosphate, GMP = guanosine 5'-monophosphate, IMP = inosine 5'-monophos phate, UMP = uridine 5'-monophosphate, TMP = thymidine 5'-monophosphate
[b] Determined from $n$ replicates at or near the expected detection limit, MDL = $t_{n-1,\alpha=0.99} \times sd$, where $n$ = 10 and $\alpha$ = 0.01.
[c] RSD repeatability = sd/mean x 100 (n = 6)
[d] Horwitz ratio = RSD$_r$ / pRSD$_{eq}$ where pRSD$_{eq}$ = $C^{-0.15}$ at 10 ppm concentration level
[e] RSD intermediate reproducibility = sd/mean x 100 (n = 24)
[f] Mean recovery (percent relative standard deviation) at three levels each for bovine milk-based, soy-based, and hypoallergenic infant formulas"




Chromatographic performance was assessed by replicate analyses (n = 6) of a mixed nucleotide standard. Performance within recommended guidelines was achieved with the exception of capacity factor for CMP and UMP, however, this was deemed acceptable due to uncompromised peak integrity of these two compounds in all samples analysed.

Linearity of dose response was confirmed by least-squares regression analysis with acceptable values obtained for correlation coefficient. Plots of standard residuals showed no structure with only a small amount of random noise further demonstrating linearity. Method detection limits determined are well below levels typically supplemented to pediatric formulas. A Plackett-Burman robustness trial demonstrated that the method was found to be robust for seven factors which may have affected final results at levels typically expected during normal use.

Acceptable precision was achieved with HorRat (repeatability) calculated between 0.3 and 0.5. Method accuracy was demonstrated by measurement of recovery well within limits of 80 – 115 % at the 10 µg/g level suggested by AOAC. No evidence of method bias was found between the method described presently and one published previously (Gill and Indyk, 2007, *Int. Dairy J.* 17, 596-605).

The method was applied to a number of commercially available pediatric and nutritional powders. Products included for testing were infant formulas, follow-on formulas and an adult nutritional product. These products included a range of different sources; bovine milk, hydrolysed milk protein, caprine milk, and soy protein.

## CONCLUSIONS

A rapid robust analytical method for the analysis of 5'-mononucleotides supplemented to pediatric formulas is described

- Simple sample preparation without need for prior protein removal
- Rapid and thorough removal of interferences via SPE clean-up.
- Increased security of results through analysis by use of internal standard.
- Method applicable to a wide range of pediatric formulas

## ACKNOWLEDGEMENTS

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