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Quantification of non-protein nitrogen components of infant formulae and follow-up milks: comparison with cows' and human milk

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The composition of fourteen infant formulae and six follow-up milks with regard to their free amino acids (including taurine), free nucleotides, orotic acid, and free and total L-carnitine content was studied. The levels found were compared with the limits established in European legislation and with the composition of human and cows' milk samples. HPLC methodologies, optimized and validated for the matrices under study, were used, except for free and total L-carnitine contents that were quantified using a flow-injection manifold, also optimized and validated for the matrices under study. Global statistical treatment of the results by cluster analysis indicated similarities between the contents of the N compounds under study of infant formulae, follow-up milks and cows' milk and differences with regard to human milk composition. The principal component analysis showed that 60·2 % of the variation in data was due to the first principal component, and the second component represented 23·8 % of the total information. Nucleotide profiles, orotic acid, and free and total L-carnitine contents explain the main differences observed between human milk and the other milks studied (cows' milk, infant formulae and follow-up milks). Cows' milk is distinguished from infant formulae and follow-up milks mainly owing to the different uric acid contents and free amino acids profiles.

Free amino acids: Nucleotides: Orotic acid: Uric acid: L-Carnitine: Infant formulae

Over the last 30 years, there has been an evolution in the appreciation of the functional roles that some minor non-protein-N compounds play in neonatal nutrition. Owing to the special biochemical needs of the developing neonate a group of compounds considered as semi-essential or conditionally essential, namely, taurine and other free amino acids, L-carnitine and, more recently, free nucleotides was identified (Anderson *et al.* 1988; Goedhart & Bindels, 1994; Rassin, 1994). Studies that have defined the role that the latter compounds play in early development have reflected on properties such as infant growth, maturity of enzymic synthesis in infants, and the presence of the compounds in human milk coupled with animal and human studies.

On the other hand, progressive attempts have been made by the infant nutrition industry to bring the composition of infant formulae closer to that of human milk, not only with regard to major components, but also with regard to minor compounds that may be involved in the development of the newborn. Follow-up milks are given to infants after 4–6 months of age in order to make the transition from human milk or infant formulae to cows' milk. However, the composition of these artificial milk formulae, relative to the above-mentioned N compounds, does not correspond to that of cows' milk, or to that of human milk.

European regulations, directives 91/321/CE and 96/4/CE (European Community, 1991*a*, 1996), establish the type and respective limits of compounds that can be added to meet the necessary nutritional requirements for infant formulae and follow-up milks. With regard to taurine, its content must be equal or greater than 10 μmol/100 kJ (42 μmol/100 kcal). Supplementation with other essential and semi-essential amino acids is also allowed to raise the nutritive value of proteins, but only in the proportions necessary for that purpose. Thus, the evaluation of free essential and semi-essential amino acids in infant formulae is pertinent. Another important aspect relating to free amino acids composition (essential or not) results from the observation that milks of each species have a distinctive free amino acid pattern, which may reflect the relative importance of the free amino acids during early postnatal development. However, information on free amino acids content in human milk is very limited (Pamblanco *et al.* 1989; Sarwar *et al.* 1998).

Abbreviations: AMP, adenosine 5′-monophosphate; C, cows' milk; CMP, cytidine 5′-monophosphate; FM, follow-up milks; GMP, guanosine 5′-monophosphate; H, human milk; IF, infant formulae; IMP, inosine 5′-monophosphate; PCA, principal component analysis; UMP, uridine 5′-monophosphate.

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European regulations also establish L-carnitine content; it must be superior to 1·79 μmol/100 kJ (7·5 μmol/100 kcal; European Community, 1996). Human milk contains lower levels of total carnitine when compared with cows' milk. Thus, milk-based formulae generally meet the needs of infant nutrition unless the carnitine concentration is altered during manufacture (Woollard *et al.* 1997).

The importance of dietary nucleotides in infant nutrition has been the subject of active research for the last decade (European Community, 1991*b*). Human milk is known to contain a significant amount of free nucleotides when compared with the composition of mature cows' milk (Gil & Sanchez-Medina, 1982). Nucleotide supplementation of infant formulae is recommended by European legislation up to the levels found in human milk (European Community, 1996). Supplementation is allowed to a maximum of 0·598 mg/100 kJ (2·50 mg/100 kcal) for cytidine 5′-monophosphate (CMP), 0·418 mg/100 kJ (1·75 mg/100 kcal) for uridine 5′-monophosphate (UMP), 0·359 mg/100 kJ (1·50 mg/100 kcal) for adenosine 5′-monophosphate (AMP), 0·120 mg/100 kJ (0·50 mg/100 kcal) for guanosine 5′-monophosphate (GMP) and 0·239 mg/100 kJ (1·00 mg/100 kcal) for inosine 5′-monophosphate (IMP). However, total concentration cannot exceed 1·2 mg/100 kJ (5 mg/100 kcal).

Other endogenous-N milk compounds such as uric and orotic acids appear naturally in these formulae and because their levels can be good indicators of the quality of cows' milk used their quantification is also important (Ferreira *et al.* 1998).

The objective of the present work was to evaluate the composition of the major free amino acids including taurine, free nucleotides, free and total L-carnitine, and uric and orotic acids in commercially available infant formulae and follow-up milks, and compare the levels found with European standards and with human milk and cows' milk samples. To this end, precise, reproducible, rapid and economic analytical procedures validated for these matrices were used, which included reversed-phase HPLC separations for amino acids, nucleotides, orotic acid and uric acid, and flow-injection analysis with spectrophotometric detection for quantification of free and total L-carnitine. The results obtained using these methodologies were analysed by statistical multivariate analysis in order to understand the differences between some compounds of the non-protein-N fraction of infant formulae, follow-up milks, human milk and cows' milk.

Materials and methods

Sampling

Twenty samples of powder from adapted milk formulae were analysed, including two different types of formulation: infant formulae (fourteen samples from different brands, numbered from IF1 to IF14); follow-up milks (six samples from different brands, numbered from FM15 to FM20). The number of analysed samples reflected market availability.

Samples of cows' and human milk were also analysed, including four samples of mature raw cows' milk (within 2 months of lactation, numbered from C21 to C24), and four samples of mature human milk (within 1 month of lactation, obtained by manual expression, numbered from H25 to H28). These samples were collected in sterile polypropylene containers, transported fresh or frozen and stored at −20°C until analysis.

Reagents and solutions

The L-amino acids kit, *o*-phthalaldehyde, AMP, CMP, UMP, GMP, IMP, 5,5′-dithiobis-2-nitrobenzoato, N′-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid, tri(hydroxymethyl)-aminomethane, $Na_2EDTA.2H_2O$, aminopropyl glass beads (200–400 mesh, 75 Å mean pore diameter), 25 % (v/v) glutaraldehyde solution, L-carnitine, acetyl-CoA and carnitine acetyltransferase (*EC* 2.3.1.7; 2·8 mg protein/ml, approximately 90 units/mg at pH 8·0) were obtained from Sigma Chemicals Co. (St Louis, MO, USA) 2-Mercaptoethanol, tetrabutylammonium hydrogensulfate, glacial acetic acid, acetonitrile and methanol were from Merck (Darmstradt, Germany). All other chemicals were of analytical reagent grade.

Water used possessed a resistance greater than 15 MΩ, was filtered through a membrane of 0·45 μm porosity and was, subsequently, degassed.

Reversed-phase high-performance liquid chromatography separations

Apparatus. The chromatographic analyses were carried out in a Jasco high-performance liquid chromatograph equipped with two type PU-980 pumps and a type AS 950 auto-sampler (Jasco Corporation, Tokyo, Japan). The loop volume selected was 20 μl. A Jasco multiwavelength diode array detector MD-910 and the Borwin PDA Controller Software (JMBS Developpements, Le Fontanil, France) were also used. Chromatographic column was selected according to the compounds under evaluation.

Taurine and free amino acids. For the analysis of free amino acids, including taurine, a method validated for the matrices under study, involving pre-column derivatization with a mixture of *o*-phthalaldehyde and 2-mercaptoethanol and separation by reversed-phase HPLC, on a C_{18} (S10ODS2) column and using gradient elution of 0·05 M-phosphate buffer pH 5·3 and methanol (Mendes *et al.* 1998), was used. The sample preparation was performed as described by Ferreira *et al.* (1997*b*).

Nucleotides. Determination of AMP, CMP, UMP, GMP and IMP in dairy products was based on ion-pair reversed-phase separation (C_{18} chromatographic column) and with diode array detection (Oliveira *et al.* 1999; Ferreira *et al.* 2001), set at 280 nm. Gradient elution was with water–glacial acetic acid–5 mM-tetrabutyl ammonium hydrogensulfate (97·5:1·5:1·0, by vol.) and methanol–glacial acetic acid–5 mM-tetrabutyl ammonium hydrogensulfate (97·5:1·5:1·0, by vol.). Preparation of milk, infant formula and follow-up milk extracts and chromatographic conditions were performed as described in Ferreira *et al.* (2001).

Orotic and uric acids. Orotic and uric acids were also determined by HPLC, using an amina-bonded silica

column (Spherisorb NH_2; Waters Corporation, Milford, MA, USA), isocratic elution with acetonitrile–HCl 0·01 M (84:16, v/v) and detection at 280 nm. Preparation of milk, infant formula and follow-up milk extracts and chromatographic conditions were performed as described previously (Ferreira & Ferreira, 1997; Ferreira *et al.* 1998).

Flow-injection analysis evaluation of free and total L-carnitine

L-Carnitine content was determined by using flow-injection analyses with an immobilized carnitine acetyltransferase bioreactor. The methodology developed and validated previously (Ferreira *et al.* 1997*a*) was based on the spectrophotometric determination through its reaction with carnitine acetyltransferase coupled with acetyl-CoA and dithiobenzoate. The merging zones technique was used to minimize acetyl-CoA consumption. The flow-injection manifold and sample preparation for free L-carnitine determination are described in Ferreira *et al.* (1997*a*). Total L-carnitine was determined after saponification according to the method described by Woollard *et al.* (1997); this saponification enables the estimation of acid-soluble free and short-chain acylcarnitines.

Statistical analysis

Data are represented as means and standard deviations. ANOVA was used to determine the effects of brand, on the one hand, and type of formulation on the other, on the compounds under study. Two separate sets of 'between-types' and 'within-type' analyses were carried out. Fisher's protected least significant difference *t* test at the 5 % significance level was applied to experimental results to assess intrapair differences. ANOVA statistical analyses were done with the Statview™ 4.0 statistical package (Abacus Concepts, Berkeley, CA, USA).

In order to make meaningful comparisons between powdered formulae and liquid milks possible, it was necessary to dilute according to label instructions. Therefore, 13 g (243 kJ; 58 kcal) adapted milk formula powder was reconstituted to 100 ml with water.

Global statistical analyses of results were carried out by cluster analysis and principal component analysis (PCA), which were conducted to determine similarities and differences between samples of infant formulae (IF), follow-up milks (FM), human milk (H) and cows' milk (C), considering all the variables under study. Data were standardized, with twenty-eight rows corresponding to twenty-eight N-compound profiles (14 IF, 6 FM, 4 C, 4 H), and the column vectors (X_1, X_2,...X_i,...X_{12}) representing the following compounds: glutamic acid, threonine, taurine, AMP, CMP, UMP, GMP, IMP, orotic acid, uric acid, free and total L-carnitine. The X matrix was used for cluster analysis and for PCA.

Another PCA was carried out, calculated without the human milk, to search any grouping of IF and FM types.

Cluster analysis of the X matrix was carried out using the single linkage method with Euclidean distances (Mardia *et al.* 1979), as implemented in the Statistics for Windows package (Microsoft Corporation, CA, USA), which involves the scaling of distance measures by observed variable ranges. PCA of X was carried out in the usual way, based on the sums of squares and products matrix $S=X'X$ (Mardia *et al.* 1979).

Results and discussion

Free amino acids

Free amino acids were separated and identified in adapted milk formulae (IF and FM), cows' milk and human milk samples, with the aim of identifying the major free amino acids that contributed to nearly 70 % of the total free amino acids. The total content of free amino acids was also evaluated.

As apparent from Tables 1 and 2, milk infant formulae and follow-up milks provided similar qualitative profiles of free amino acids, but quantitatively significant differences were sometimes observed. The prevailing amino acids in both formulae were glutamic acid, taurine, threonine and serine, but their concentrations were affected differently by the type of formulation. ANOVA showed that this variable was not significant for glutamic acid ($P=0·412$), serine ($P=0·206$) and threonine ($P=0·478$), in contrast to the taurine content, which was significantly affected ($P=0·0012$); follow-up milks presented lower concentrations of taurine (Tables 1 and 2), with the exception of two samples.

With regard to the influence of the type of brand, data pertaining to the major amino acids indicate significant differences ($P<0·001$) among the commercial brands of infant formulae, especially for threonine and taurine, which are confirmed by their high F values (F 121·49 and F 95·20 respectively). Dispersion among samples was lower for serine and glutamic acid contents, as observed by F values (F 19·68 and F 20·41 respectively).

With regard to the effect of brand of follow-up milks on the content of threonine the dispersion among samples was lower when compared with infant formulae (F 41·3) (but the sampling was also inferior). Lower dispersion among serine and glutamic acid contents was also noticed (F 9·56 and F 34·42 respectively). In contrast, a large variability among commercial brands for taurine (F 7142·9) was reported for the follow-up milks assayed. No significant statistical similarities were found between any of these samples ($P=0·0001$) as indicated in Table 2.

Separation and identification of free amino acids in cows' milk showed that the major free amino acids were glutamic acid (4·89 (SD 0·335) mg/100 ml), taurine (1·43 (SD 0·389) mg/100 ml), histidine (1·44 (SD 0·269) mg/100 ml) and threonine (0·292 (SD 0·085) mg/100 ml). The same study in human milk revealed that glutamic acid (29·7 (SD 2·54) mg/100 ml) and taurine (3·71 (SD 0·560) mg/100 ml) were also the more abundant, but the other major free amino acid was alanine (2·57 (SD 0·14) mg/100 ml) and not histidine (1·02 (SD 0·22) mg/100 ml). Total free amino acid content was five times higher in human milk (49·8 (SD 2·61) mg/100 ml) than in cows'

Table 1. Principal free amino acids and total free amino acid content of infant formulae (IF) (mg amino acid/100 g product)*

(Mean values and standard deviations of two determinations)

	Threonine		Taurine		Glutamic acid		Serine		Total free amino acids	
Samples	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
IF1	21·6[c]	2·2	37·9[c,f]	1·2	30·2[a,d]	2·1	10·3[a]	1·3	104·8	4·1
IF2	14·5[d]	0·1	30·8[d,h]	0·2	24·5[b,g]	1·9	11·5[a,d]	0·9	80·1	2·2
IF3	33·4[e]	3·5	38·0[c,f]	2·5	28·7[a,e]	2·3	5·65[b,e]	0·23	106·7	4·3
IF4	3·88[f]	0·0	38·0[f]	2·3	38·1[c]	1·8	8·11[c]	0·88	89·4	1·6
IF5	29·7[b,g]	1·3	44·7[g]	0·7	32·1[a,d,f]	1·1	12·1[d]	1·4	121·9	4·1
IF6	31·5[e,g]	1·0	33·2[d]	2·1	33·1[d,f]	1·7	10·1[a]	1·0	110·2	2·2
IF7	ND[a]		28·2[a,h]	2·6	24·4[b,g]	1·2	11·4[a,d]	1·0	67·1	4·2
IF8	ND[a]		17·8[i]	1·1	26·6[b,e]	1·0	4·77[b]	0·22	51·3	1·1
IF9	ND[a]		32·6[d]	1·3	29·0[a,e]	2·0	5·77[b,e]	0·75	70·2	2·1
IF10	1·41[a,f]	0·01	5·63[e]	0·24	21·4[g]	1·5	11·4[a,d]	1·0	42·5	1·1
IF11	Traces[a]		24·5[a]	0·4	34·0[f]	1·4	6·21[b,e]	0·42	67·2	1·2
IF12	1·60[a,f]	0·01	10·4[b]	0·01	33·1[d,f]	2·1	6·11[b,e]	0·33	54·1	2·8
IF13	26·6[b]	2·6	34·5[c,d]	2·4	23·1[b,g]	2·1	10·1[a]	0·1	94·3	4·1
IF14	14·3[d]	0·8	55·6[j]	0·6	38·2[c]	1·0	7·11[c,e]	1·0	127·2	3·2

ND, not detected.
[a,b,c,d,e,f,g,h,i,j]Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).
* For details of procedures, see p. 128.

milk (10·7 (SD 1·45) mg/100 ml) and taurine content was three times higher in human milk.

It was observed that reconstituted infant formulae contained a mean value of 3·8 mg glutamic acid and 4·0 mg taurine/100 ml. Reconstituted follow-up milks contained 3·7 mg glutamic acid and 1·8 mg taurine/100 ml. When compared with the composition of cows' milk and human milk, it was observed that these concentrations were significantly lower than those obtained for human milk, and slightly lower than those found in cows' milk. The mean content of taurine found in infant formulae was similar to that found in human milk, which suggests supplementation with this compound, within the levels allowed by European legislation.

Nucleotides

The results obtained on the analyses of the fourteen infant formulae and six follow-up milks showed that only two (IF8 and FM18) of the twenty samples analysed presented the five 5′ nucleotides under study. 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 8·10 and 12·19 mg/100 g in IF8 and FM18 respectively. The levels of UMP were 1·40 and 2·14 mg/100 g in IF8 and FM18 respectively. Nevertheless, the levels of nucleotides were below the maximum limits allowed for supplementation (European Community, 1996).

Ten other samples (IF1, IF2, IF3, IF4, IF9, IF13, IF14, FM16, FM17, FM19) showed traces of CMP. No nucleotides were detected in the remaining infant formulae and follow-up milks.

Cows' milk yielded significantly different nucleotide patterns from those of human milk. ANOVA indicated that the origin of milk had a significant effect on the qualitative nucleotide profiles. CMP was quantified in cows' milk and in human milk, with mean concentrations of 0·98 (SD 0·08) and 1·64 (SD 0·065) mg/100 ml respectively. UMP, GMP, AMP and IMP were not quantified in cows'

Table 2. Principal free amino acids and total free amino acid content of follow-up milks (FM) (mg amino acid/100 g product)*

(Mean values and standard deviations of two determinations)

	Threonine		Taurine		Glutamic acid		Serine		Total free amino acids	
Samples	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
FM15	11·2[a]	1·4	39·8[a]	0·02	22·1[a]	2·3	11·0[a,c]	0·1	83·9	3·3
FM16	21·5[b]	0·76	4·47[b]	0·31	30·0[b,c]	1·9	8·04[b]	0·42	84·9	2·9
FM17	21·6[b]	2·0	2·12[c]	0·06	31·4[b,c]	2·1	10·1[a]	1·0	65·1	2·9
FM18	2·88[c]	0·37	Traces[d]		28·2[b,e]	1·8	12·0[c]	0·9	64·2	1·9
FM19	17·1[d]	2·15	34·5[e]	0·33	24·2[a,e]	1·9	12·4[c]	0·9	88·2	0·9
FM20	19·6[b,d]	0·53	1·01[f]	0·08	34·2[c]	2·2	5·23[d]	0·21	61·0	3·2

[a,b,c,d,e,f]Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).
* For details of procedures, see p. 128.

milk, but they were present in human milk with mean concentrations of 1·64 (SD 0·065), 1·043 (SD 0·073), 0·323 (SD 0·022), and 0·908 (SD 0·022) mg/100 ml respectively. These results on the nucleotide contents of human milk are in good agreement with those reported by other research workers (Gil & Sanchez-Medina, 1982).

Supplementation of infant formulae and follow-up milks with nucleotides is not a common practice by the industry.

Orotic acid, uric acid, free and total L-carnitine

The analytical results for orotic acid, uric acid, and free and total L-carnitine contents of infant formulae and follow-up milks are reported in Tables 3 and 4 respectively.

Infant formulae and follow-up milks provided similar qualitative profiles of orotic acid, uric acid, and free and total L-carnitine. ANOVA showed that this variable (infant formulae/follow-up milk) was not significant ($P=2{\cdot}405$, $P=0{\cdot}9726$, $P=0{\cdot}513$, $P=0{\cdot}464$, for orotic acid, uric acid, and free and total L-carnitine respectively).

Within brands of infant formulae and within brands of follow-up milks dispersion was high ($P=0{\cdot}0001$). The great variability among commercial brands may reflect different modifications made to the cows' milk (by the manufacturer) used in the formulation of adapted milk formulae.

Significant differences between the levels of uric acid in cows' milk and in human milk were obtained ($P=0{\cdot}0001$). Cows' milk contained, in general, levels twice as high as those reported in human milk; 1·99 (SD 0·106) and 0·877 (SD 0·057) mg/ml respectively. No significant differences ($P>0{\cdot}05$) between the levels of uric acid in reconstituted infant formulae (0·96 (SD 0·26) mg/100 ml) and the levels obtained for human milk were reported.

With regard to orotic acid the mean content found in cows' milk was 4·67 (SD 0·594) mg/100 ml, similar to the average concentration of orotic acid in reconstituted adapted formulae (3·25 (SD 0·81) mg/100 ml). No orotic acid was detected in human milk.

Human milk presented 0·527 (SD 0·057) and 0·81 (SD 0·13) mg/100 ml of free and total L-carnitine respectively. Cows' milk contained, in general, levels six times higher; 3·13 (SD 0·28) and 4·21 (SD 0·69) mg/100 ml of free and total L-carnitine respectively. These contents are approximately the published values (Woollard *et al.* 1997). Reconstituted adapted milk formulae, generally, contained levels three times higher (a mean content of

Table 3. Orotic acid, uric acid, and free and total L-carnitine content of infant formulae (IF) (mg/100 g product)*

(Mean values and standard deviations of three determinations)

	Orotic acid		Uric acid		Free L-carnitine		Total L-carnitine	
Samples	Mean	SD	Mean	SD	Mean	SD	Mean	SD
IF1	$26{\cdot}7^{a,e,f,i}$	1·0	$6{\cdot}80^{a,e}$	0·11	$9{\cdot}55^{a}$	0·21	$18{\cdot}6^{a,g}$	0·5
IF2	$25{\cdot}1^{a,f}$	0·9	$8{\cdot}02^{b}$	0·07	$13{\cdot}6^{b}$	0·30	$21{\cdot}6^{b,f}$	1·1
IF3	$21{\cdot}8^{b}$	1·1	$6{\cdot}10^{a}$	0·09	$7{\cdot}79^{c}$	0·27	$10{\cdot}5^{c}$	0·7
IF4	$37{\cdot}6^{c}$	1·7	$10{\cdot}8^{c}$	0·10	$14{\cdot}1^{b,h}$	0·10	$23{\cdot}1^{b}$	2·1
IF5	$15{\cdot}1^{d}$	0·8	$5{\cdot}12^{d}$	0·90	$13{\cdot}0^{d}$	0·10	$13{\cdot}0^{d}$	0·1
IF6	$19{\cdot}7^{b}$	1·3	$4{\cdot}87^{d}$	0·21	$9{\cdot}49^{a}$	0·71	$10{\cdot}9^{c}$	0·6
IF7	$28{\cdot}1^{e,i}$	0·7	$5{\cdot}12^{d}$	0·09	$13{\cdot}8^{b,h}$	0·40	$15{\cdot}9^{e}$	0·6
IF8	$24{\cdot}8^{a}$	0·8	$7{\cdot}10^{e}$	0·07	$7{\cdot}79^{c}$	0·07	$11{\cdot}2^{c,d}$	0·9
IF9	$27{\cdot}3^{f,i}$	1·0	$10{\cdot}2^{c}$	0·60	$16{\cdot}8^{e}$	0·40	$19{\cdot}8^{a,f}$	0·6
IF10	$35{\cdot}1^{g}$	0·5	$7{\cdot}22^{e}$	0·19	$14{\cdot}2^{h}$	0·30	$17{\cdot}4^{e,g}$	0·8
IF11	$16{\cdot}9^{d}$	1·1	$8{\cdot}81^{f}$	0·19	$9{\cdot}62^{a}$	0·11	$9{\cdot}80^{c}$	0·17
IF12	$27{\cdot}1^{f,i}$	0·8	$9{\cdot}22^{f}$	0·27	$17{\cdot}5^{f}$	0·50	$26{\cdot}5^{h}$	1·2
IF13	$31{\cdot}7^{h}$	0·8	$6{\cdot}10^{a}$	0·29	$12{\cdot}2^{g}$	0·30	$18{\cdot}3^{a,g}$	0·7
IF14	$27{\cdot}6^{e,i}$	1·2	$11{\cdot}8^{g}$	0·18	$9{\cdot}69^{a}$	0·09	$11{\cdot}7^{c,d}$	0·9

^{a,b,c,d,e,f,g,h}Mean values within a column with unlike superscript letters were significantly different ($P<0{\cdot}05$).
* For details of procedures, see p. 128.

Table 4. Orotic acid, uric acid, and free and total L-carnitine content of follow-up milks (FM) (mg/100 g product)*

(Mean values and standard deviations of three determinations)

	Orotic acid		Uric acid		Free L-carnitine		Total L-carnitine	
Samples	Mean	SD	Mean	SD	Mean	SD	Mean	SD
FM15	$23{\cdot}1^{a}$	0·3	$8{\cdot}67^{a}$	0·13	$9{\cdot}89^{a}$	0·11	$17{\cdot}5^{a}$	0·8
FM16	$14{\cdot}2^{b}$	0·7	$4{\cdot}17^{b}$	0·09	$9{\cdot}69^{a}$	0·09	$15{\cdot}2^{b}$	1·0
FM17	$34{\cdot}1^{c}$	1·1	$10{\cdot}9^{c}$	0·20	$14{\cdot}0^{b}$	0·4	$17{\cdot}3^{a}$	0·5
FM18	$34{\cdot}6^{c}$	1·1	$11{\cdot}8^{d}$	0·19	$15{\cdot}2^{c}$	0·7	$19{\cdot}1^{a}$	0·8
FM19	$25{\cdot}1^{a}$	0·5	$5{\cdot}19^{e}$	0·19	$11{\cdot}9^{d}$	0·9	$26{\cdot}1^{c}$	1·0
FM20	$15{\cdot}7^{b}$	1·2	$4{\cdot}99^{e}$	0·20	$7{\cdot}69^{e}$	0·07	$9{\cdot}89^{d}$	0·09

^{a,b,c,d,e}Mean values within a column with unlike superscript letters were significantly different ($P<0{\cdot}05$).
* For details of procedures, see p. 128.

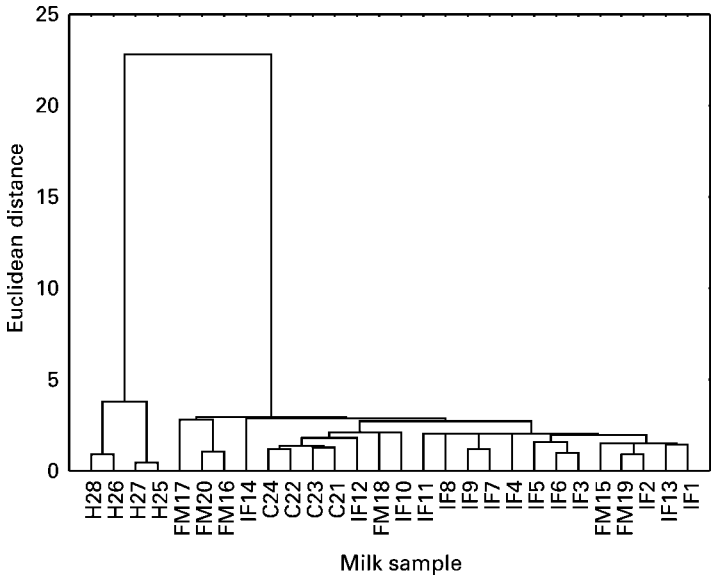

Fig. 1. Dendogram obtained from cluster analyses of infant formulae (IF1 to IP14), follow-up milks (FM15 to FM20), cows' milk (C21, C22, C23, C24) and human milk (H25, H26, H27, H28). Variables were adenosine 5′-monophosphate, cytidine 5′-monophosphate, uridine 5′-monophosphate, guanosine 5′-monophosphate, inosine 5′-monophosphate, orotic and uric acids, free and total L-carnitine, taurine, glutamic acid and threonine.

1·5 mg free L-carnitine and 2·2 mg total L-carnitine/100 ml) when compared with human milk, but half of the quantity present in cows' milk. All the samples of adapted milk formulae presented levels of L-carnitine above the minimum required by European legislation (European Community, 1996). It was observed that L-carnitine labels are commonly restricted to the free compound.

Global statistical analyses

Cluster analysis indicated similarities between the composition of infant formulae, follow-up milks and cows' milk with regard to the N compounds under study and differences with regard to human milk composition (Fig. 1). Although cluster analysis is useful as a general type of analysis, it does not relate clusters to variables because it only takes into consideration a distance matrix, and consequently does not provide an answer to which compounds are responsible for the observed differences between clusters (type of milk product). PCA, on the other hand, is based on a correlation matrix or on a sum of squares and products matrix between variables, which enables the reduction of dimensionality with no loss of significant amounts of information, and relates individual observations (i.e. type of milk) to N compounds under study.

The PCA results show that two principal components are enough to describe the main features of the data, losing no more then 17 % of the information, with the first component (component 1) by itself condensing slightly over 60 %, and the second (component 2) representing 23·8 % of the total information. The loadings and scores of all milks on the first two principal components are presented in Table 5 and plotted in Fig. 2 respectively.

PCA is more effective and more informative than cluster analysis with regard to the necessary simplification of the results. GMP, IMP, AMP, UMP, orotic acid, glutamic acid, and free and total L-carnitine are the main reasons human milk is different from the other milks studied (cows' milk and adapted milk formulae). Cows' milk is separated from infant formulae and follow-up milks mainly owing to the different threonine and uric acid contents. The exception was FM18 that may be considered part of the cows' milk group in the PCA; its composition is quite similar to cows' milk, only free L-carnitine, total L-carnitine and glutamic acid are slightly lower. A PCA was calculated without the human milk to see any grouping of FM and IF types (Table 6 and Fig. 3). GMP, AMP and IMP were not used as variables in this PCA because they were not detected in adapted milk formulae or in cows' milk.

IF8 and FM18 differ from others owing to their CMP and UMP contents. A group of samples including IF4, IF9, IF10, IF12 and FM17 could be considered in that these adapted milk formulae were more similar to cows'

Table 5. Principal component analysis loadings on principal components 1 and 2

Variables	Component 1	Component 2
CMP	−0·661589	−0·642444
UMP	−0·972589	−0·203239
GMP	−0·975322	−0·167663
AMP	−0·974159	−0·167986
IMP	−0·975254	−0·167452
Orotic acid	0·892438	−0·295279
Uric acid	0·387429	−0·798883
Free L-carnitine	0·720780	−0·619928
Total L-carnitine	0·693603	−0·570845
Glutamic acid	−0·965240	−0·195634
Taurine	−0·184637	0·530935
Threonine	0·281452	0·752582

CMP, cytidine 5′-monophosphate; UMP, uridine 5′-monophosphate; GMP, guanosine 5′-monophosphate; AMP, adenosine 5′-monophosphate; IMP, inosin 5′-monophosphate.

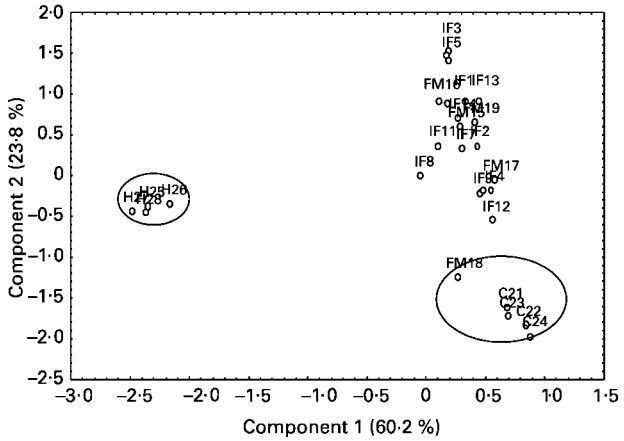

Fig. 2. Global plot of principal component analysis scores on principal components 1 and 2. IF, infant formulae; FM, follow-up milks; C, cows' milk; H, human milk samples.

Table 6. Principal component analysis loadings on principal components 1 and 2 (without human milk)

Variables	Component 1	Component 2
CMP	−0·776444	−0·491549
UMP	−0·217228	−0·912582
Orotic acid	−0·794462	0·065942
Uric acid	−0·900827	0·175735
Free L-carnitine	−0·902294	0·284962
Total L-carnitine	−0·819691	0·326351
Glutamic acid	−0·468076	0·413229
Taurine	0·503916	0·416569
Threonine	0·689721	0·201429

CMP, cytidine 5′-monophosphate; UMP, uridine 5′-monophosphate.

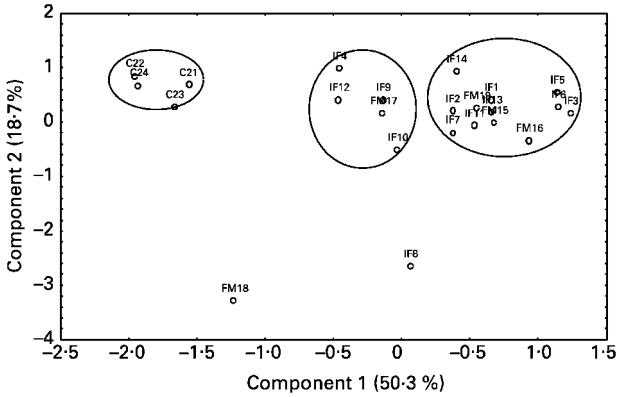

Fig. 3. Plot of principal component analysis scores calculated without the human milk. IF, infant formulae; FM, follow-up milks; C, cows' milk samples.

milk than the remainder; separation from cows' milk was due to slightly lower contents of uric acid and free and total L-carnitine. Another group of samples that includes the remaining thirteen samples was assigned; these samples contained more threonine and taurine and less uric and orotic acids, and free and total L-carnitine than cows' milk. Inside this last group IF5, IF6 and IF3 were those with higher threonine and lower orotic acid contents.

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