ELSEVIER

PII: S0308-8146(96)00327-5

Food Chemistry, Vol. 59, No. 3, pp. 325–332, 1997

0308-8146/97 $17.00 + 0.00

RAPID COMMUNICATION

Enzymatic determination of carnitine in milk and infant formula

David C. Woollard,[a] Harvey E. Indyk[b] & Gerald A. Woollard[c]

[a]*Lynfield Food Services Centre, Ministry of Agriculture, PO Box 41, Auckland, New Zealand*
[b]*Anchor Products, PO Box 7, Waitoa, New Zealand*
[c]*Dept. Clinical Biochemistry, Auckland Hospital, Park Road, Auckland, New Zealand*

(Received 20 August 1996; revised version received 10 October 1996; accepted 10 October 1996)

A method has been developed for the routine estimation of carnitine in milk, milk powder and infant formula. Carnitine and its esters were measured following a selective alkaline hydrolysis scheme, with use of the coupled carnitine acetyl transferase–Ellman reaction to form the nitrophenolate anion chromophore. The method was automated using a centrifugal analyser to facilitate high sample capacity and improved precision. Endogenous total carnitine in whole milks varied between 20·2 and 28·1 mg/100 g solids, with free carnitine contributing 55–60% of the carnitine pool. A reliable estimate of the total carnitine content in milk is achieved from the sum of the acid soluble free and short-chain acylcarnitines, with the acid insoluble long-chain esters present at levels below the uncertainty of the methodology. Infant formulas demonstrated more diverse carnitine contents as a consequence of their complex and varied compositions and the increasingly common practice of supplementation.

INTRODUCTION

Current knowledge of the biochemistry of L(−)-carnitine, β-hydroxy-(γ-N-trimethylamino) butyric acid, has been reviewed by various authors over the past decade (Borum, 1983; Rebouche & Paulson, 1986; Bieber, 1988; Bremer, 1983). Carnitine participates in cellular membrane translocation processes, actively facilitating both the passage of long-chain fatty acids into the mitochondria prior to β-oxidation, and the removal of potentially toxic short- and medium-chain acyl groups. These, as well as other suspected functions involve the substrate specific L-carnitine acyl transferases (Borum, 1983, 1991; Lowes & Rose, 1989; Marzo *et al.*, 1990).

Carnitine is available to humans both through *in vivo* biogenesis involving lysine and methionine, and from a variety of dietary sources, notably meat and dairy products. The relative importance of the latter to human health is still under investigation (Rebouche, 1988; Feller & Rudman, 1988). In adults, absorption from food is both rapid and quantitative, with tissue accretion and conservation well controlled. Deficiency states are, however, recognised and may result from congenital blockage of the biosynthetic pathway, interaction with certain antagonist therapeutics or a dietary inadequacy of either carnitine, a precursor, or cofactor (Rebouche & Paulson, 1986). In infants, immature hepatic production of γ-butyrobetaine hydroxylase increases the importance of exogenous carnitine supply (Borum, 1991).

Human milk is reported to contain 35–70 nmol/mL (5–11 mg/L) total carnitine, which while sufficient to maintain normal plasma levels, is significantly lower than bovine milk 160–270 nmol/mL (25–43 mg/L) (Sandor *et al.*, 1982; Hamamoto *et al.*, 1988*a*). It is therefore evident that milk-based formula should generally meet the needs of infant nutriture unless the carnitine concentration is altered during manufacture. Thus, certain milk-based products assembled from depleted raw materials and all soy-based formulas are potentially carnitine deficient and may require supplementation, despite their high lysine and methionine content (Feller & Rudman, 1988).

Traditionally, radioenzymatic techniques utilising carnitine acetyltransferase (CAT) have supported clinical studies of carnitine metabolism and have been successful in determining carnitine and acylcarnitines based upon class solubility and retention on ion-exchange (Cederblad & Lindstedt, 1972; McGarry & Foster, 1976; Pace *et al.*, 1978; Rossle *et al.*, 1985;

Borum, 1990; De Sousa *et al.*, 1990), although it has been reported that long-chain acylcarnitines (>C-12) undergo incomplete radioisotope exchange (Minkler & Hoppel, 1993). An alternative enzymatic method based upon carnitine dehydrogenase has also been reported for serum samples (Takahashi *et al.*, 1994) and a microbiological assay based on the growth of *T. bovina* described for a range of clinical tissues (Baker *et al.*, 1992). These techniques have been refined and extended for diagnostic investigations and have provided a limited description of ester distributions (Bieber & Lewin, 1981; Bieber & Kerner, 1986). More recently, HPLC techniques incorporating pre-column derivatisation or isotopic exchange strategies, have become increasingly significant in view of inherent selectivity advantages allowing the discrimination of individual esters (Takeyama *et al.*, 1986; Arakawa *et al.*, 1989; Van Kempen & Odle, 1992; Minkler & Hoppel, 1993; Kamimori *et al.*, 1994; Matsumoto *et al.*, 1994; Schmidt-Sommerfeld *et al.*, 1995), while GLC methods have also been reported (Kumps *et al.*, 1994) and have the advantage of simpler interfacing with MS detection. Available techniques have been comprehensively reviewed previously (Lowes & Rose, 1989; Marzo *et al.*, 1990). HPLC has also been used for monitoring the carnitine chloride content of pharmaceutical preparations (Kamata *et al.*, 1994) and for D, L-isomer separation in drugs (Hirota *et al.*, 1994; De Witt *et al.*, 1994).

Validation of a CAT-based enzymatic procedure coupled to the colourimetric Ellman reaction, has been recently reported for application to milk in both manual and automated formats (Indyk & Woollard, 1995). This technique, though somewhat less sensitive than the radioenzymatic procedure, has proven reliability for the detection of free carnitine, and with incorporation of an ester hydrolysis scheme, was a potential candidate for a routine quality control procedure for total carnitine, thereby avoiding the requirement to engage the complexities of LC separation techniques. In this study, the automated procedure was selected in view of its high sample throughput and convenience. Related colourimetric procedures using centrifugal analysers have been reported for the determination of free and total carnitine in plasma (Roe *et al.*, 1992) and milk (Roos *et al.*, 1992). While plasma analysis may be achieved almost exclusively within the instrument, milk requires additional sample preparation prior to the enzymatic determination.

Despite its relevance to human health, there is limited information about the carnitine content and distribution in foods, with the exception of a few meat products (Mitchell, 1978; Borum, 1991). Information about bovine milk and derived dairy products is incomplete, although some data have been reported (Erfle *et al.*, 1970; Sandor *et al.*, 1982; Bosi & Refrigeri, 1983; Hamamoto *et al.*, 1988*a*; Indyk & Woollard, 1995). Such information will be increasingly required, particularly for products destined for infant nutrition and medical diets (Borum *et al.*, 1979; Ohtani *et al.*, 1985) and in view of the proliferation of carnitine within the health food industry.

The method presently described for free and acylcarnitines was utilised to generate information concerning the levels and distribution in milks, milk powders and a variety of infant formulas of differing composition.

EXPERIMENTAL

Apparatus

(a) Enzyme assays were performed in automated mode using a Cobas Fara II centrifugal analyser (Roche Diagnostics, Montclair, NJ).
(b) Circulating water bath with lid. For routine assays the temperature was held at 40±1°C, although temperatures up to 80°C were also evaluated.
(c) Graduated volumetric glassware (25, 50 and 100 mL) and auto-dispensers (0·5–10·0 mL) with disposable tips.
(d) pH meter (Orion Model 230 A, Boston, MA), calibrated with Volusol buffers (Rhone Poulenc, Clayton, Australia).
(e) Medium speed filter paper (Whatman No. 2) and 0·45 μm nylon disposable filters (Alltech, Deerfield, IL, USA).

Reagents

All solutions were prepared in water with resistivity >18 MΩ.

(a) *Chromogenic buffer reagent*: 30 mg DTNB (5,5′-dithiobis-2-nitrobenzoic acid, Sigma, St. Louis, MO), 9·0 g of Tris (trihydroxymethyl aminomethane) and 9·2 g of EDTA (Na salt) were dissolved in ca. 400 mL water. The pH was adjusted to 7·5 with HCl (5 M) and made to volume (500 mL). The reagent was stored frozen in 6 mL aliquots. Stable for up to 6 months.

(b) *Carnitine acetyl transferase (EC 2·3·1·7)*: 100 μl of enzyme suspension (5 mg protein/mL, 80 units/mg; Boehringer Mannheim, Auckland, NZ) was added to 1·0 mL water. Prepared fresh each day.

(c) *Acetyl CoA solution*: 8·3 mg of acetyl coenzyme A (Boehringer Mannheim) was dissolved in 25·0 mL water, divided into 3 mL aliquots and stored frozen for up to 3 months.

(d) *Carnitine standards*: (1) Stock solution (0·91 mM): 14·7 mg L-carnitine HCl (Aldrich, Milwaukee, USA) was dissolved in 100 mL water and frozen in 1·0 mL aliquots. These solutions were discarded after storage beyond 6 months. (2) Working solutions (91·0, 45·0 and 22·5 μmol/L): An aliquot of stock solution was thawed and 0·5 mL diluted with 4·5 mL water (91·0 μmol/L). Further dilutions (1:1 and 1:3, respectively) were made with water to prepare standards of 45·0 and 22·5 μmol/L. Discarded after use.

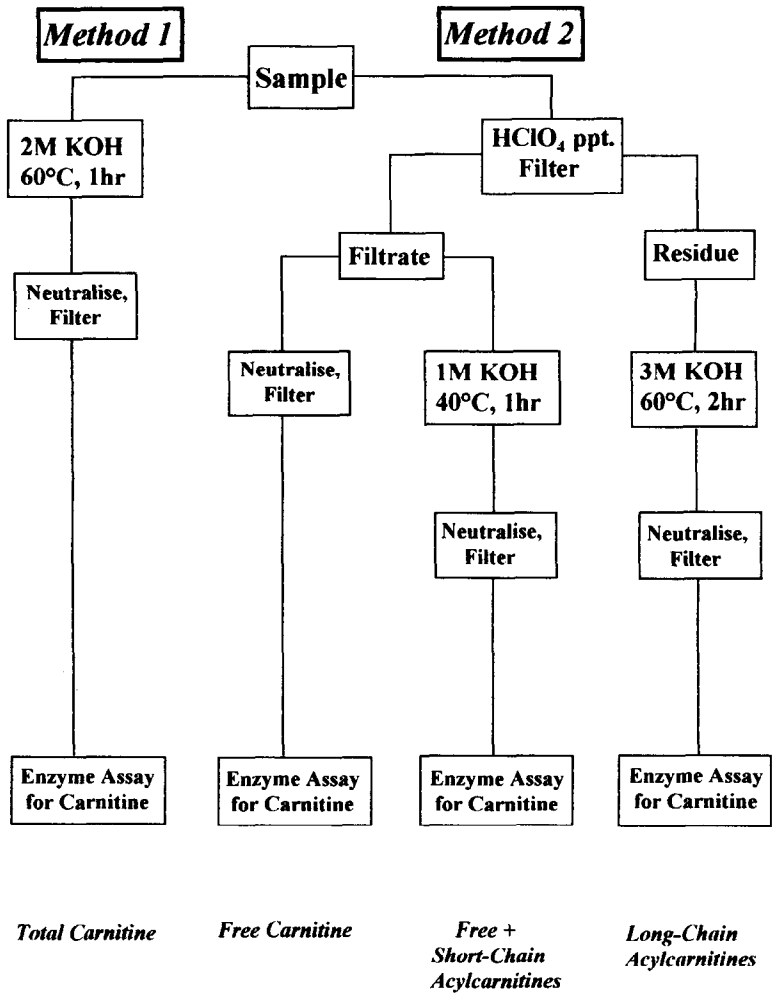

Fig. 1. Summary of described sample preparation scheme.

(e) *Working DTNB solution*: Frozen aliquots of chromogenic buffer reagent (6 mL) and acetyl CoA (3 mL) were thawed and mixed thoroughly. Discarded after use.

(f) *Perchloric acid (0·25 M)*: 36 mL of perchloric acid (70% w/v) was diluted to 1 L with water.

(g) *Potassium hydroxide (6 M)*: Potassium hydroxide pellets (340 g) were dissolved in 1 L water. Other molarities were prepared by dilution.

(h) L-Acetylcarnitine: ca. 20 mg of acetylcarnitine (P-L Biochemicals, Milwaukee, WI) was weighed accurately and dissolved in water (100 mL). This solution was used for recovery trials.

Sample collection

Samples of commercial milks, canned milk powders and infant formulas were collected from retail outlets and carnitine contents determined by the described procedures (Fig. 1).

Sample preparation

Method 1. Direct saponification

Powdered milk or infant formula (ca. 5·0 g) was accurately weighed into a conical flask, 40 mL of water added and dissolution achieved by warming. Liquid milk or ready-to-drink formula (40 mL) was sampled directly. The solution was made alkaline by addition of 40 mL of potassium hydroxide (4 M) and heated with stirring at 60°C for 60 min. After cooling, the extract was neutralised to pH 6·5 ± 0·5 with perchloric acid (70% w/v), transferred to a volumetric flask (100 mL) and made to volume with water. The precipitate was removed by filtration, discarding the first portion and collecting 10–20 mL. Approximately 5 mL was clarified by passage through a 0·45 μm membrane and kept under refrigeration (4°C) until ready for assay. During preliminary investigations, other alkali concentration (0·1–3·0 M), saponification time (1–3 hr) and temperature (20–80°C) combinations were evaluated.

Method 2. Acid precipitation

Samples, as for Method 1, were weighed and dissolved into tared volumetric flasks (100 mL). Perchloric acid (0·25 M, 40 mL) was added, mixed gently and allowed to stand for 60 min. The solution, at room temperature, was made to volume with water (vigorous shaking was avoided to prevent foam formation). The sample was filtered into a conical flask and 50–60 mL collected after

discarding the first filtrate. Two 25 mL aliquots were pipetted into individual 50 mL beakers for the separate sequential analyses of free and short-chain acyl carnitines, while the precipitate was retained for the estimation of long-chain carnitine ester contribution.

1) Free carnitine

The extract was adjusted to pH 6·5 ± 0·5 with potassium hydroxide (1·0 M). Thorough stirring is necessary to avoid localised alkaline conditions and thereby minimise acylcarnitine hydrolysis.

The solution was transferred quantitatively to a volumetric flask (50 mL), and made to volume with water. An aliquot was transferred directly to a vial (5 mL), although final clarification from residual suspended material occasionally required filtration through a 0·45 μm membrane. Carnitine extracts are stable for several weeks at 4°C and may be held until required for assay. Alternatively, prevention of possible spoilage through microbial growth was achieved by indefinite storage at −20°C.

2) Free + acid-soluble (short-chain) acylcarnitine

The extract was adjusted to pH 13 with potassium hydroxide (6 M), generally producing some turbidity. The beaker was placed in a covered water bath (40 ± 1°C) for 60 min, during which time the solution generally developed a yellow hue. Alternative time–temperature–pH combinations were also evaluated during preliminary development studies.

The extract was cooled to ambient temperature and pH reduced to 6·5 ± 0·5 with perchloric acid (70% w/v). During this procedure, the intensity of yellow coloration decreased, with concurrent volatilisation of sulphide and precipitation of potassium perchlorate. The extract was quantitatively transferred to a volumetric flask (50 mL) and made to volume with water. A clear aliquot was decanted into a vial (5 mL), with membrane clarification if necessary and held at 4°C until assay, or 20°C for long term storage.

3) Acid-insoluble (long-chain) acylcarnitine

The retained precipitate was collected by centrifugation (3000 rpm), washed (3×) with perchloric acid (0·25 M) to remove any residual acid-soluble carnitines and allowed to dry under vacuum desiccation. The dried residue (ca. 1–2 g) was mixed with water (5 mL) and following addition of an equal volume of potassium hydroxide (6 M), hydrolysed at 60°C for 120 min. The pH was returned to 6·5 ± 0·5 with perchloric acid (70% w/v), made to 20 mL and an aliquot refrigerated until required for assay. Alternative time–temperature combinations were evaluated during preliminary trials.

Analyte determination

The automated enzymatic determination protocol has been described previously (Indyk & Woollard, 1995). Briefly, extracts (20 μL) and reagent blank (20 μL water) were sampled (in duplicate) by the instrument probe and deposited into the appropriate positions of a 30-place cuvette rotor and diluted with water (50 μL). An infant formula control sample was included during each sample batch in order to monitor method precision. Calibration was performed concurrently against carnitine working standards (3-level), with each measurement determined in triplicate.

Working DTNB solution (165 μL) was added and cuvettes incubated under centrifugation at 37°C for 300 sec. Background absorbances {A1} were acquired at 405 nm, followed immediately by the addition of CAT reagent (5 μL). The enzymatic reaction proceeded under centrifugation for 600 sec, at which time final end-point absorbances were determined {A2}.

Instrument calculations ({A2}−{A1}×F) were performed automatically and reported in μmol/L, after subtraction of the reagent blank and linear interpolation. Sample extracts > 100 μmol/L carnitine were identified by the instrument and re-analysed following dilution with water. Final data was routinely expressed as mg/100 g of original sample.

RESULTS

Enzymatic assay

The described reagent compositions achieved optimum reaction kinetics, minimised the potential inhibitory effect of DTNB, satisfied volume limitations and maintained detector linearity. The pre-reaction incubation period was sufficient to account for any potential background interference from endogenous thiol-active compounds. Following addition of CAT, incubation at 37°C for 10 min was sufficient to achieve complete reaction, with stable absorbance values consistent with an end-point determination.

Instrumental figures of merit were equivalent to those reported previously, with linear regression exceeding 0·9993 (slope: 985·6 ± 83·4; reagent blank: 0·0199 ± 0·0028 au) and a detection limit of 3·0 nmol/mL (equivalent to 1·6 mg/100 g sample). These parameters were diagnostic of acceptable instrument performance, and assays repeated with fresh reagents if outside the expected range. Duplicate injections of each extract mitigated against the occasional intermittent air bubble, particulate or other transient event. Within-run instrumental relative standard deviation was estimated from duplicate injections to be 1·67% (n:62) and between-run relative standard deviation 2·81% (n:19), computed from duplicate values across a concentration range.

Sample preparation

Saponification of intact milk powders (Method 1) resulted in significant blank values {A1} which correlated with

Table 1. Carnitine in liquid and anhydrous milks (mg/100 g)[a]

Sample	Total carnitine[b]		Free carnitine	Free: Total
	Method 1	Method 2		
Skim milk	3.01 (2.88–3.54)	2.99 (2.85–3.33)	1.73 (1.43–1.93)	0.57
Whole milk	2.85 (2.65–3.39)	2.92 (2.60–3.40)	1.65 (1.34–2.02)	0.58
Skim milk powder	29.88 (26.36–34.76)	30.61 (25.83–33.73)	17.64 (15.67–19.60)	0.59
Whole milk powder	24.33 (20.22–28.05)	24.53 (21.58–27.67)	13.48 (11.64–16.89)	0.55

[a]Mean (range), $n = 5$.
[b]Total Carnitine = [Free + Acid-soluble + Acid-insoluble acylcarnitines] (Method 1) or [Free + Acid-soluble acylcarnitines] (Method 2).

hydrolysis temperature (0·2–1·0 au at 20–80°C resp.). This persistent Maillard background occasionally resulted in spectral saturation upon addition of the CAT reagent, precluding a consistently reliable assay at the higher temperatures. Carnitine esters are known, however, to be relatively resistant to saponification in intact samples, with higher temperatures and more forcing conditions generally desirable to achieve complete hydrolysis. The present study has demonstrated that optimum recovery with acceptable background was achievable at 60°C and this temperature was therefore chosen for general use.

To discriminate between free carnitine and acid-soluble short-chain acylcarnitine contributions, it is necessary to make selective use of alkaline hydrolysis. The preliminary removal of protein and fat by coprecipitation with perchloric acid (Method 2) facilitates milder saponification conditions under which the acylcarnitines are hydrolysed. Such conditions yield essentially colourless extracts subsequent to neutralisation, with blank values typically less than 0·08 au. It has been further demonstrated that carnitine is stable for up to 3 h during alkaline digestion under these conditions.

To facilitate the estimation of the acid-insoluble long-chain carnitine esters, the protein coprecipitate was recovered and separately hydrolysed, with recovery evaluated under several time–temperature–pH combinations. All such measured data for liberated carnitine (0 ± 2 mg/100 g sample) were within the experimental error of the technique and at or below the limit of detection and were therefore considered unreliable.

Analytical results

Replicate analysis of the infant formula control sample by method 2 indicated an overall assay repeatability (RSD_r) of 4·12% (n:5) and reproducibility (RSD_R) of 6·41% (mean:13·16 mg/100 g; n:18). Recovery of added carnitine and acetylcarnitine was quantitative ($99·7 \pm 0·8$% and $99·9 \pm 0·9$% resp.), with the latter ester indicative of the recovery of short-chain acylcarnitines present endogenously in milk.

Table 1 reports carnitine levels in domestic milks and milk powders, together with free to total carnitine ratios. The equivalence of total carnitine content as estimated by both Methods 1 and 2 reflects the experimentally negligible long-chain carnitine ester contribution.

Infant formulas are more complex materials and subject to wide variations in composition. This, in addition to supplementation protocols will often be revealed in altered carnitine distribution and content. A selection of formulas were assayed, and the free and total carnitine contents compiled in Table 2, together with declared label claims when available.

DISCUSSION

The current application of the centrifugal analyser to the enzymatic determination of carnitine in milk was an extension of early manual (Marquis & Fritz, 1964) and automated (Seccombe *et al.*, 1976) procedures, and has previously been validated both by comparison with a manual procedure and against independent radioenzymatic assay and HPLC methods (Indyk & Woollard, 1995). Performance parameters obtained in the present study further established the reliability of the test method.

Table 2. Carnitine content in commercial infant formulas (mg/100 g)[a]

Sample[b]	Total carnitine[c]	Free carnitine	Label claim
1	30.92 (5.32)	17.56 (3.69)	nd
2	25.23 (5.44)	13.86 (3.87)	nd
3	18.60 (3.78)	9.62 (3.02)	nd
4	23.83 (4.88)	16.89 (5.19)	nd
5	11.22 (3.71)	6.87 (4.19)	nd
6	21.73 (4.96)	15.02 (4.80)	nd
7	17.10 (4.00)	13.03 (2.87)	nd
8	12.20 (4.10)	5.98 (4.09)	5.0
9	20.16 (5.25)	12.68 (3.76)	6.5
10	11.03 (4.07)	11.04 (3.99)	8.0
11	9.78 (4.93)	9.69 (4.08)	5.0
12	4.14 (3.03)	4.11 (4.29)	4.0

[a]Mean (RSD%), $n = 3$.
[b]1–10 milk-protein based; 11–12 soy-protein based. 1–2 are Follow-On formula (1 year +). 3–10 are partially or fully vegetable oil filled. 8–12 contain added carnitine on ingredient list.
[c]Total carnitine determined by Method 2.
nd: not declared.

The colourimetric detection mode places some constraints on the sample preparation strategy selected for total carnitine estimation. Two protocols were evaluated, involving either hydrolysis of the intact sample, or removal of fat and protein prior to alkaline hydrolysis. The former approach requires relatively rigorous conditions in order to yield quantitative release of carnitine, thereby resulting in significant spectral background. Similar techniques involving direct saponification of intact biological tissues such as muscle (Borum *et al.*, 1977) and liver (Parvin & Pande, 1977) have avoided these constraints through use of the radioenzymatic detection technique commonly utilised in most clinical applications. However, such radioisotopic methods are inappropriate for use in routine food testing environments. Caramelisation has been previously noted in milk samples during alkaline hydrolysis and minimised with lower temperatures and longer digestion periods (Sandor *et al.*, 1982). Conversely, it has been reported that some acylcarnitines are resistant to cleavage and more rigorous conditions are necessary to achieve complete hydrolysis (Borum, 1990), while the importance of elevated alkali concentration in the release of acylcarnitines in dairy products has been emphasised elsewhere (Hamamoto *et al.*, 1988*b*). The present study has confirmed that optimal conditions for the estimation of total carnitine by intact sample hydrolysis were achieved at 60°C and 2 M alkali.

Removal of proximates by acid precipitation prior to saponification, minimises spectral complications resulting from photometric background. This approach has the added advantage of allowing a facile free carnitine determination by omission of the saponification step, but raises the question as to whether total carnitine is being determined subsequent to alkaline hydrolysis. The acid-soluble fraction has been reported to recover carnitine esters up to acyl-C8 in biological fluids (Minkler & Hoppel, 1993) and acyl-C10 in milk (Hamamoto *et al.*, 1988*b*). Ideally, a quality control method should recover all acylcarnitines present, but in practice, total carnitine is realistically represented by the acid-soluble fraction, since long-chain esters (> acyl-C12) reportedly contribute only 2–3% in milk (Hamamoto *et al.*, 1988*a*; Roos *et al.*, 1992; La Count *et al.*, 1995). Indeed, normal clinical tissues and fluids are generally dominated by both free- and acetylcarnitine, with higher homologues indicative of metabolic pathology (Erfle *et al.*, 1970; Arakawa *et al.*, 1989; Kerner *et al.*, 1984; Bieber & Kerner, 1986; Minkler & Hoppel, 1993; Kamimori *et al.*, 1994).

In this study, all samples were confirmed to contain long-chain carnitine esters at or below the method detection limit. Further, levels of acid-soluble carnitines were consistent with data obtained by direct saponification, indicating the essential equivalence of the free and short-chain fraction to total carnitine content in milks and infant formula. In practice, the low background inherent to Method 2 dictates that it is the preferred technique for routine analysis of total carnitine. Recovery of carnitine and acetylcarnitine has been shown here to be quantitative, while homologues up to octanoylcarnitine (< C-10) have previously been confirmed to be quantitatively recovered by the acid extraction technique (Bieber & Lewin, 1981; Kerner *et al.*, 1984; Hamamoto *et al.*, 1988*b*; Borum, 1990) and indeed palmitoylcarnitine at the physiological pH of plasma (De Sousa *et al.*, 1990).

Total carnitine contents of bovine whole and skim milk are in general agreement with published values (Sandor *et al.*, 1982; Bosi & Refrigeri, 1983; Hamamoto *et al.*, 1988*a*; Roos *et al.*, 1992; La Count *et al.*, 1995), while the range of carnitine levels found reflect both variability in composition and possible seasonal or lactational changes in commercial milk and milk powders. The ratio of free to total carnitine is also consistent with literature data, confirming the dominance of non-acyl carnitine (Erfle *et al.*, 1970; Hamamoto *et al.*, 1988*a*; Roos *et al.*, 1992).

Infant formulas are complex products based upon either milk or soy protein fractions in combination with various lipids, macro- and micronutrients (Borum *et al.*, 1979). These compositional variables impact on the wide range of endogenous carnitine found in commercially available products, with soy-protein contributing no carnitine and necessitating mandatory supplementation. In addition, milk-protein fractions can be partially or totally depleted of carnitine during processing, requiring final product fortification to achieve target nutritional levels. Nevertheless, milk-based formulas generally contain higher carnitine contents compared to human milk (Ohtani *et al.*, 1985; Sandor *et al.*, 1982), although bioavailabilty in formulas may be compromised with respect to breast milk (Borum, 1983). Carnitine supplementation and declaration may not reveal the contribution of total nutrient, since such information is commonly restricted to the free acid. With the continued interest in infant nutritional biochemistry, it is therefore of increasing importance that such dietary sources of carnitine are monitored more realistically.

CONCLUSION

A reliable analytical method for the routine determination of total carnitine has been developed for milk and infant formulas. The recommended extraction procedure removes proximates prior to alkaline hydrolysis, thereby minimising photometric problems associated with background Maillard reactions. The long-chain acylcarnitines are not routinely recovered, but their contribution to the carnitine pool has been demonstrated to be experimentally insignificant. The coupled enzyme–spectrophotometric reaction is performed by automated centrifugal analysis facilitating rapid and accurate quantitation and high sample capacity.

ACKNOWLEDGEMENT

The authors acknowledge with thanks the support of their respective affiliations and the facilities provided.

REFERENCES

Arakawa, N., Ha, T. Y. & Otsuka, M. (1989). An improved high-performance liquid chromatographic assay for the determination of free and esterified carnitine in animal tissues. *J. Nutr. Sci. Vitaminol.*, **35**, 475–479.

Baker, H., DeAngelis, B., Baker, E. R., Reddi, A. S., Khalil, M. & Frank, O. (1992). Routine microbiological assay for carnitine activity in biological fluids and tissues. *Food Chem.*, **43**, 141–146.

Bieber, L. L. & Lewin, L. M. (1981). Measurement of carnitine and O-acylcarnitines. *Methods Enzymol.*, **72**, 276–287.

Bieber, L. L. & Kerner, J. (1986). Short-chain acylcarnitines: identification and quantitation. *Methods Enzymol.*, **123**, 264–276.

Bieber, L. L. (1988). Carnitine. *Ann. Rev. Biochem.*, **57**, 261–283.

Borum, P. R., Broquist, H. P. & Roelofs, R. I. (1977). Muscle carnitine levels in neuromuscular disease. *J. Neurol. Sci.*, **34**, 279–286.

Borum, P. R., York, C. M. & Broquist, H. P. (1979). Carnitine content of liquid formulas and special diets. *Am. J. Clin. Nutr.*, **32**, 2272–2276.

Borum, P. R. (1983). Carnitine. *Ann. Rev. Nutr.*, **3**, 233–259.

Borum, P. R. (1990). Carnitine: determination of total carnitine using a radioenzymatic assay. *J. Nutr. Biochem.*, **1**, 111–114.

Borum, P. R. (1991). Carnitine. In *Handbook of Vitamins*, 2nd Edn., L. Machlin (Ed). Marcel Dekker Inc., NY., pp. 557–563.

Bosi, G. & Refrigeri, M. S. (1983). Determinazione gascromatografica della carnitina nel latte. *Rassegna Chim.*, **35**, 421–426.

Bremer, J. (1983). Carnitine metabolism and function. *Physiol. Rev.*, **63**, 1420–1468.

Cederblad, G. & Lindstedt, S. (1972). A method for the determination of carnitine in the picomole range. *Clin. Chim. Acta.*, **37**, 235–243.

De Sousa, C., English, N. R., Stacey, T. E. & Chalmers, R. A. (1990). Measurement of L-carnitine and acylcarnitines in body fluids and tissues in children and in adults. *Clin. Chim. Acta.*, **187**, 317–328.

De Witt, P., Deias, R., Muck, S., Galletti, B., Meloni, D., Celletti, P. & Marzo, A. (1994). High-performance liquid chromatography and capillary electrophoresis of L- and D-carnitine by precolumn diastereomeric derivatisation. *J. Chromatogr. B*, **657**, 67–73.

Erfle, J. D., Fisher, L. J. & Sauer, F. (1970). Carnitine and acetylcarnitine in the milk of normal and ketotic cows. *J. Dairy Sci.*, **53**, 486–489.

Feller, A. G. & Rudman, D. (1988). Role of carnitine in human nutrition. *J. Nutr.*, **118**, 541–547.

Hamamoto, M., Shimoda, K., Matsuura, N. & Matsuura, H. (1988*a*). Determination of free-, short-chain acyl and long-chain acyl carnitines in milk and milk products. *J. Jap. Soc. Nutr. Food Sci.*, **41**, 397–404.

Hamamoto, M., Shimoda, K., Matsuura, N. & Matsuura, H. (1988*b*). A method for the fractionation and determination of carnitines in milk and milk products. *J. Jap. Soc. Nutr. Food Sci.*, **41**, 389–395.

Hirota, T., Minato, K., Ishii, K., Nishimura, N. & Sato, T. (1994). High-performance liquid chromatographic determination of enantiomers of carnitine and acetylcarnitine on a chiral stationary phase. *J. Chromotogr. A*, **673**, 37–43.

Indyk, H. E. & Woollard, D. C. (1995). Enzymatic determination of free carnitine in milk and infant formula. *J. AOAC International*, **78**, 69–74.

Kamata, K., Takahashi, M., Terasima, K. & Nishijima, M. (1994). Liquid chromatographic determination of carnitine by precolumn derivatisation with pyrene-1-carbonyl cyanide. *J. Chromatogr. A*, **667**, 113–118.

Kamimori, H., Hamashima, Y. & Konishi, M. (1994). Determination of carnitine and saturated-acyl group carnitines in human urine by high-performance liquid chromatography with fluorescence detection. *Anal. Biochem.*, **218**, 417–424.

Kerner, J., Froseth, J. A., Miller, E. R. & Bieber, L. L. (1984). A study of the acylcarnitine content of sows colostrum, milk and newborn piglet tissues: demonstration of high amounts of isovalerylcarnitine in colostrum and milk. *J. Nutr.*, **114**, 854–861.

Kumps, A., Duez, P. & Mardens, Y. (1994). Gas chromatographic profiling and determination of urinary acylcarnitines. *J. Chromatogr. B.*, **658**, 241–248.

La Count, D. W., Drackley, J. K. & Weigel, D. J. (1995). Responses of dairy cows during early lactation to ruminal or abomasal administration of L-carnitine. *J. Dairy Sci.*, **78**, 1824–1836.

Lowes, S. & Rose, S. (1989). Analytical procedures for determining acylcarnitines in biological fluids. *Tr. Anal. Chem.*, **8**(5), 184–188.

Marquis, N. R. & Fritz, I. B. (1964). Enzymological determination of free carnitine concentrations in rat tissues. *J. Lipid Res.*, **5**, 184–187.

Marzo, A., Cardace, G., Monti, N., Muck, S. & Martelli, E. A. (1990). Chromatographic and non-chromatographic assay of L-carnitine family components. *J. Chromatogr. Biomed. Appns.*, **527**, 247–258.

Matsumoto, K., Ichitani, Y., Ogasawara, N., Yuki, H. & Imai, K. (1994). Precolumn fluorescence derivatisation of carnitine and acylcarnitine with 4-(2-aminoethylamino)-7-nitro-2,1,3-benzoxadiazole prior to high-performance liquid chromatography. *J. Chromatogr. A*, **678**, 241–247.

McGarry, J. D. & Foster, D. W. (1976). An improved and simplified radioisotopic assay for the determination of free and esterified carnitine. *J. Lipid Res.*, **17**, 277–281.

Minkler, P. E. & Hoppel, C. L. (1993). Quantification of carnitine and specific acylcarnitines by high performance liquid chromatography: application to normal human urine and urine from patients with methylmalonic aciduria, isovaleric acidemia or medium-chain acyl-CoA dehydrogenase deficiency. *J. Chromatogr. Biomed. Appns.*, **613**, 203–221.

Mitchell, M. E. (1978). Carnitine metabolism in human subjects. I. Normal metabolism. *Am. J. Clin. Nutr.*, **31**, 293–306.

Ohtani, Y., Higashi, A. & Matsuda, I. (1985). Carnitine concentration of formulas. *J. Pediatr. Gastroenterol Nutr.*, **4**, 845–846.

Pace, J. A., Wannemacher, R. W. & Neufeld, H. A. (1978). Improved radiochemical assay for carnitine and its derivatives in plasma and tissue extracts. *Clin. Chem.*, **24**, 32–35.

Parvin, R. & Pande, S. V. (1977). Microdetermination of (−)carnitine and carnitine acetyltransferase activity. *Anal. Biochem.*, **79**, 190–201.

Rebouche, C. J. & Paulson, D. J. (1986). Carnitine metabolism and function in humans. *Ann. Rev. Nutr.*, **6**, 41–66.

Rebouche, C. J. (1988). Carnitine metabolism and human nutrition. *J. Appl. Nutr.*, **40**, 99–111.

Roe, D. S., Terada, N. & Millington, D. S. (1992). Automated analysis for free and short-chain acylcarnitine in plasma with a centrifugal analyzer. *Clin. Chem.*, **38**, 2215–2220.

Roos, von N., de Vrese, M., Schulte-Coerne, H. & Barth, C. A. (1990). L-Carnitin in Milch von Monozygoten Zwillingskuhen. *Kieler Milchwirtschaftliche Forschungsberichte*, **44**(4), 363–370.

Rossle, C., Kohse, K. P., Franz, H.-E. & Furst, P. (1985). An improved method for the determination of free and esterified carnitine. *Clin. Chim. Acta*, **149**, 263–268.

Seccombe, D. W., Dodek, P., Frohlich, J., Hahn, P., Skala, J. P. & Campbell, D. J. (1976). Automated method for L-carnitine determination. *Clin. Chem.*, **22**, 1589–1592.

Sandor, A., Pecsuvac, K., Kerner, J. & Alkonyi, I. (1982). On carnitine content of the human breast milk. *Pediatr. Res.*, **16**, 89–91.

Schmidt-Sommerfeld, E., Zhang, L., Bobrowski, P. J. & Penn, D. (1995). Quantitation of short- and medium-chain acylcarnitines in plasma by radioisotopic exchange/high-performance liquid chromatography. *Anal. Biochem.*, **231**, 27–33.

Takahashi, M., Ueda, S., Misaki, H., Sugiyama, N., Matsumoto, K., Matsuo, N. & Murao, S. (1994). Carnitine determination by an enzymatic cycling method with carnitine dehydrogenase. *Clin. Chem.*, **40**, 817–821.

Takeyama, N., Takagi, D., Adachi, K. & Tanaka, T. (1986). Measurement of free and esterified carnitine in tissue extracts by high-performance liquid chromatography. *Anal. Biochem.*, **158**, 346–354.

Van Kempen, T. A. T. G. & Odle, J. (1992). Quantitation of carnitine esters by high-performance liquid chromatography. Effect of medium-chain triglycerides on the plasma carnitine ester profile. *J. Chromatogr. Biomed. Appns.*, **584**, 157–165.