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The analysis of pantothenic acid in milk and infant formulas by HPLC

David C. Woollard[a], Harvey E. Indyk[b,*], Scott K. Christiansen[c]

[a]*Lynfield Food Laboratory, AgriQuality Ltd, PO Box 41, Auckland, New Zealand*
[b]*Anchor Products, PO Box 7, Waitoa, New Zealand*
[c]*Wyeth Nutritionals, PO Box 2109, Georgia, Vermont, USA*

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Abstract

A method is described for the estimation of pantothenic acid in milk and infant formula based on deproteination of sample and direct analysis by reversed-phase HPLC with a low pH phosphate:acetonitrile eluent and multiwavelength UV detection. Several LC columns were evaluated and a C_8 chemistry selected for routine application. Comparative data are provided against compendial microbial and GLC procedures. Based on the low content of bound, endogenous pantothenate in milk, the method provides a reliable measure of total vitamin B_5 for compliance control during infant formula production.

Keywords: Pantothenic acid; HPLC; Milk; Infant formulas

1. Introduction

Pantothenic acid (vitamin B_5) occurs primarily bound as part of coenzyme A (CoA) and acyl carrier protein (ACP) and may also be found in the free acid form in various tissues. These coenzymes are essential in mediation of the many critical reactions involved in intermediary metabolism and though acting primarily through an active sulphydryl group, are controlled by specific steric characteristics of the central pantothenate moeity (Fox, 1990; Wyse, Song, Walsh & Hansen, 1985).

Adult human intake of this vitamin has generally been considered adequate in view of the absence of deficiency in normal populations, the ubiquitous distribution of pantothenic acid in the human diet and limited synthesis by intestinal microflora (Fox, 1990; Song, 1993). There is limited information concerning absorption and bioavailability of dietary pantothenate, which has made a definitive recommended daily allowance elusive (van den Berg, 1997). In contrast to most foods and tissues where pantothenate is generally present in bound coenzyme form, free pantothenic acid dominates in human and bovine milk (Guilarte, 1989; Hartman & Dryden, 1974; Renner, 1989; Song, Chan, Wyse & Hansen, 1984). Based on the relative pantothenate levels in blood and milk of human subjects, the mammary gland effectively concentrates this vitamin from the maternal circulation for delivery to the infant (Song et al., 1984). Infant formulas are generally supplemented with pantothenic acid as the calcium salt, in accordance with regulatory requirements to ensure adequate vitamin intake to non-breastfed infants (Infant Formula Act, 1980).

Reported concentrations of pantothenate in foods and tissues have been quantitatively diverse due both to the various enzymatic strategies employed to release bound vitamin (Gonthier, Fayol, Viollet & Hartmann, 1998; Song et al., 1984) and the range of methods used to quantitate released pantothenate in the free form. Analysis of pantothenate in foods has most commonly been accomplished by either microbiological assay (MBA) (Angyal, 1996; AOAC, 1995; Bell, 1974; Skeggs & Wright, 1944) or radioimmunoassay (RIA) (Walsh, Wyse & Hansen, 1979). More recent developments applied to certain foods, milks or clinical tissues include a radiometric-microbiological assay (RMA) (Guilarte, 1989), indirect enzyme immunoassay (ELISA) (Finglas, Faulks, Morris, Scott & Morgan, 1988; Gonthier, Boullanger, Fayol & Hartmann, 1998), gas liquid chromatography (GLC)

* Corresponding author. Tel.: +64-7-889-3989; fax: +64-7-887-1502.
E-mail address: harvey.indyk@nzdairy.co.nz (H.E. Indyk).

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(Davidek, Velisek, Cerna & Davidek, 1985; Tesmer, Leinert & Hotzel, 1980), GLC–MS (Banno, 1997) and capillary electrophoresis applied to enantioseparation of pantothenate DL-stereoisomers (Kodama, Yamamoto & Matsunaga, 1998). These approaches, despite successful analytical attributes, have distinct disadvantages in the routine food quality control laboratory. MBA is tedious, manipulative and often exhibits relatively poor precision, RIA and RMA require the use of radioisotopes and scintillation counting, both difficult in the food environment, ELISA requires the aquisition of non-commercially available antisera and GLC requires extensive sample preparation and derivatisation procedures. More recently, high performance liquid chromatography (HPLC) has been utilised for the estimation of free pantothenate in infant formulas, relying on single channel, low-wavelength UV (<200 nm) for detection and quantitation (Endo, Yao, Saotome & Kamei, 1991; Romera, Ramirez & Gil, 1996; Zuo & Zhang, 1988). HPLC is potentially the most facile technique for this analysis, although poor spectral specificity in the low UV suggests uncertainty in establishing unambiguous peak identity and purity.

This study reports a similar, simplified HPLC protocol for the estimation of free pantothenic acid in milk and infant formulas appropriate for routine compliance monitoring and provides a reasonable estimate of total vitamin content in view of the minor contribution of coenzyme bound pantothenate. Expedient column selection combined with photodiode array detection and on-line spectral analysis has provided confidence in the accurate quantitation of putative analyte.

2. Materials and methods

2.1. Equipment

Three HPLC instruments in different laboratories were used in the study: a manual system consisting of two 510 pumps, a rheodyne 7125 injector, 680 controller, 8×10 radial compression module (Waters, Milford, MA, USA) and an SPD-M10A diode array detector controlled with Class software (Shimadzu, Tokyo, Japan). The other systems were integrated HP1050 and HP1100 instruments respectively, incorporating autosampler, quaternary pump, on-line degasser and diode array detector, controlled with ChemStation software (Hewlett Packard, Waldbronn, Germany).

Initial studies involved the use of the Resolve C_{18}, 5 μm, 100×8 mm cartridge column, non-endcapped, 10% carbon content (Waters). Several other C_{18} columns were evaluated and the following selected for further investigation: Nomura Develosil ODS-MG-5, 5 μm, 250×4.6 mm, dense end-capped, 15% carbon content, low ligand density (Phenomenex, Torrance, CA, USA); YMC AQ312, 5 μm, 150×4.6 mm, hydrophilic endcapped, 16% carbon load (YMC Inc., Wilmington, NC, USA); Platinum EPS, 3 μm, 53×7 mm, non-endcapped, reduced ligand density (Alltech, Deerfield, IL, USA), and a Luna Phenyl-C_6 column, 5 μm, 250×4.6 mm, 18% carbon (Phenomenex) evaluated for its combined aliphatic/aromatic selectivity. A Luna C_8 column, 5 μm, 250×4.6 mm, hydrophobic shielded endcapped, 13.5% carbon (Phenomenex), was also selected for further study.

2.2. Reagents

Water (>18 MΩ), *d*-pantothenic acid, hemicalcium salt (Sigma, St Louis, MO, USA), acetonitrile (HPLC grade, BDH, Merck, Poole, UK), potassium dihydrogen orthophosphate, orthophosphoric acid and glacial acetic acid (AR grade, BDH).

Phosphate buffer (0.1 M, pH 2.25) was prepared by dissolving 13.61 g KH_2PO_4 in water (1.0 l) and adjusting pH with H_3PO_4. Acetic acid (3.0% v/v) was prepared by diluting 15 ml to 0.5 l with water.

Mobile phase was prepared by mixing, subsequent to separate filtration and degassing, phosphate buffer (970 ml) and acetonitrile (30 ml). Alternative buffer:acetonitrile eluent proportions were prepared as required.

2.3. Standard preparation

Stock pantothenic acid standard (500 μg/ml): Approximately 140 mg calcium pantothenate was dried at 105°C for 2 h over silica gel in a vacuum oven. After cooling in dessicator, 108.8 mg was accurately weighed into a small beaker. It was dissolved in a small volume of water and transferred to a volumetric flask (200 ml), rinsing beaker with ethanol:water (1:1 v/v) and made to volume, stoppered and mixed thoroughly. 4 ml aliquots were dispensed into screw-capped vials and stored at −18°C. Stock standard was stable for 6 months.

Working standards (5.0 and 10.0 μg/ml): Prepared by dilution of stock 1:100 and 1:50 respectively with water in separate volumetric flasks (50 ml). These standards were prepared fresh daily and discarded after use.

2.4. Sample preparation

Sample powder (0.5 g) was accurately weighed into a disposable, graduated centrifuge tube (10 ml). A sample blank and control sample were included in each analytical schedule. Approximately 5 ml warm water (<40°C) was added with vortex mixing and allowed to stand for 20 min. 0.5 ml acetic acid (3% v/v) was added to each sample, vortexed and allowed to stand for 20 min. The samples were then made to volume (10.0 ml) and centrifuged at 3500 rpm (1000 *g*) for 15 min. An aliquot (1–2 ml) was withdrawn from the aqueous serum phase (avoiding lipid and sediment), clarified through a 0.45

μm membrane filter (Sartorius, Goettingen, Germany) attached to a 10 ml luer-tipped disposable syringe and filtrate collected in a 1.8 ml screw-capped autosampler vial. The extract was then ready for injection.

For samples of poor homogeneity, a 5 g sample was weighed into a 100 ml flask and the analysis scaled accordingly.

2.5. HPLC analysis

Preliminary optimisation studies were performed with the Resolve column. Following purging of the analytical column with at least 15 volumes of acetonitrile (100%) and acetonitrile:water (50:50 v/v), the system was equilibrated with mobile phase (phosphate buffer, pH 2.25:acetonitrile, 97:3 v/v). Isocratic flow rate was adjusted to between 1 and 2 ml/min to elute pantothenic acid standard within ca. 10–20 min and to maintain suitable operating pressure. Working standards (10–20 μl) were injected until a reproducible response was achieved, followed by sample extracts. Chromatography was continued until a major, late eluting unknown peak was removed (ca 35 min) before the next sample injection. Following completion of the sample schedule, the column was purged sequentially with acetonitrile:water (50:50 v/v), water (100%), acetonitrile:water (50:50 v/v) and finally acetonitrile (100%) for column storage between runs.

Multichannel UV detection at 200, 205 and 240 nm was routinely used during analysis (dual channel detection at 200 and 205 nm was a minimum requirement to allow spectral ratioing, with monitoring at 240 nm considered expedient in order to further verify the absence of potential interferences). During optimisation studies, spectral scans were acquired for all peaks of interest, both for purity evaluation of target analyte and to monitor potential artefacts.

Based on information acquired from the Resolve studies, alternative columns were evaluated. Analytical conditions were comparable for the YMC AQ312, Luna Phenyl-C_6 and Luna C_8 columns, except for minor modifications in flow rate. The Develosil ODS-MG-5 column was operated at higher acetonitrile content (5% v/v), while the Platinum EPS was optimised with phosphate buffer (100%) alone. For these columns, it was expedient to remove retained components with acetonitrile (100%) for 5 min, following elution of pantothenate, prior to re-equilibration under initial conditions.

2.6. Calculation

For routine analysis, quantitation was achieved with a single-level external standard: Pantothenic acid (mg/100 g) $= A_{sa}/A_{std} \times C_{std} \times 10 \times 100/wt \times 1/1000$ where: A_{sa} is area (or height) of pantothenic acid in sample, A_{std} is area (or height) of pantothenic acid in standard, C_{std} is concentration of pantothenic acid in standard (μg/ml), wt is sample weight (g).

Alternatively, on-line quantitation may be achieved against a multilevel standard calibration and linear regression.

2.7. Comparative methods

Samples were subjected to analysis for free pantothenic acid (no enzymatic hydrolysis) by the reference microbiological assay utilising *Lactobacillus plantarum* and turbidometric estimation at 620 nm (Angyal, 1996; AOAC, 1995; Bell, 1974).

Several samples were also analysed by a modified GLC procedure (Davidek et al., 1985). Briefly, sample powder (2 g) was digested in 40 ml HCl (25% v/v) at 95°C for 5 h to yield pantoyl lactone and alanine. The extract was transferred to a 100 ml beaker containing 0.5 g KH_2PO_4 and neutralised to pH 5.0 with NaOH (40%). Ethyl laurate internal standard was added and the solution extracted (4×) with dichloromethane (20 ml) in a separatory funnel. The pooled extracts were evaporated and the analysis completed by a 1 μl cold injection onto a BPX70 capillary column (30 m×0.25 mm) with flame ionisation detection. The carrier gas was hydrogen with an oven temperature gradient of 80–210°C (5°C/min), 210–250°C (30°C/min) and held at 250°C (3 min).

3. Results

3.1. Chromatography

Fig. 1 illustrates single wavelength (205 nm) chromatography achieved for a standard, sample blank and both milk and formula sample extracts with the Resolve C_{18} system.

Fig. 2 illustrates multiwavelength (200, 205 and 240 nm) chromatograms of a typical infant formula extract analysed with both Platinum and Develosil columns. The preferred column, based on superior selectivity, was the Luna C_8, and a multiwavelength chromatogram of the same sample is depicted in Fig. 3.

All analytical columns selected for evaluation demonstrated acceptable resolution of pantothenic acid in sample extracts, with multiwavelength photodiode array detection providing confidence in peak purity (200:205 ratio of 1.67 ± 0.03). As apparent from the above figures, there were unique selectivities associated with each column chemistry. Several other hydrophobic columns failed to perform during preliminary trials due to phase collapse and were not subjected to further study.

Potential artefact peaks which were variably resolved from pantothenate remain unidentified, although their spectral properties indicate free organic or amino acids.

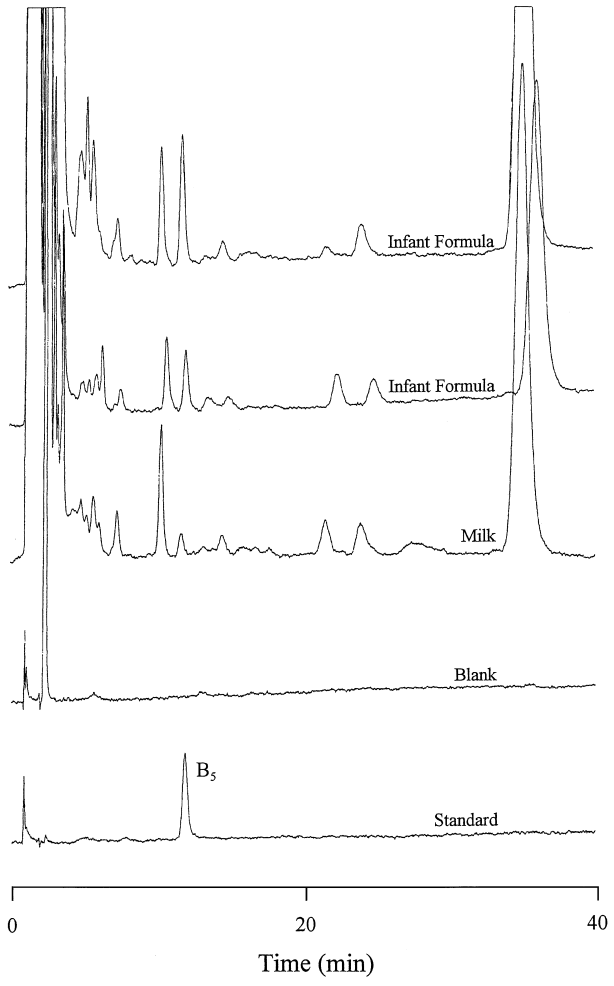

Fig. 1. Single channel UV (205 nm) chromatograms obtained with Resolve C_{18} column. Eluent: phosphate (0.1 M, pH 2.25):acetonitrile (97:3 v/v); flow: 1.8 ml/min; inj. vol: 10 μl.

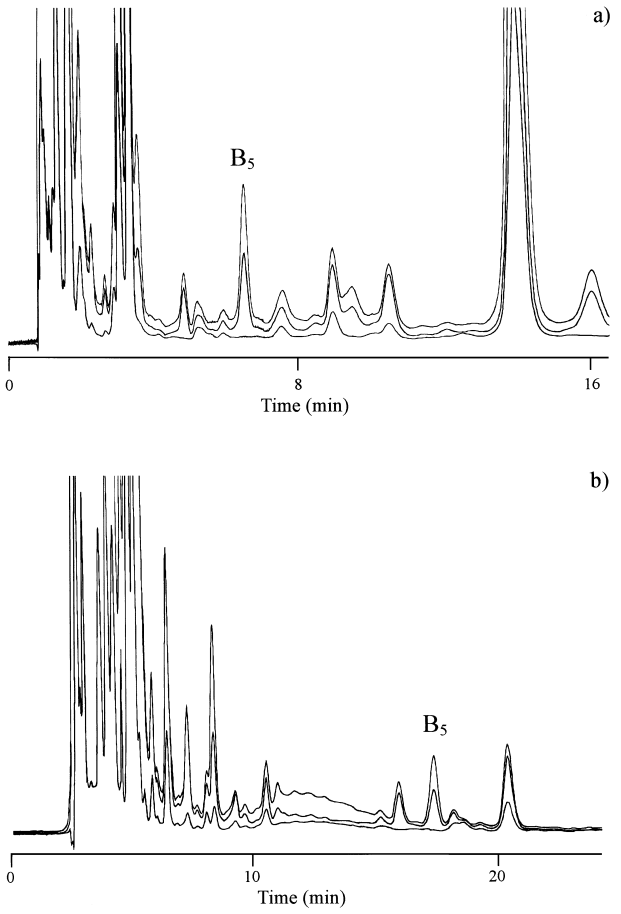

Fig. 2. Multiwavelength UV chromatograms (upper: 200; middle: 205; lower: 240 nm) of a typical infant formula extract obtained with either Platinum (a) or Develosil (b) C_{18} columns. Eluent: phosphate (0.1 M, pH 2.25); flow: 1.5 ml/min; inj. vol: 50 μl (Platinum); Eluent: phosphate (0.1 M, pH 2.25):acetonitrile (95:5 v/v); flow: 1.0 ml/min; inj. vol: 50 μl (Develosil). For both columns, eluent was step-changed to acetonitrile following elution of pantothenic acid, prior to reequilibration under initial conditions.

3.2. Method performance

Detector response for LC system 1 was evaluated with multilevel standards (0.50 to 25.0 μg/ml) and found to be linear (r^2: 0.99998) with a regression $y = 29504x - 342$. The overall method detection limit (MDL) was estimated following replicate analysis of unfortified whole milk powder containing endogenous levels of free pantothenic acid (EPA, 1984) and found to be equivalent to 0.30 mg/100 g anhydrous sample (*n*: 11). Comparable performance was observed with both the HP1050 and HP1100 LC systems.

Recovery was evaluated, in duplicate, with a two-level pantothenic acid augmented whole milk powder and estimated to be 98.0 and 99.8% (100 and 300% addition level). Replicate analysis of the NIST SRM 1846 (Gaithersburg, MD, USA) provided a further indication of quantitative recovery by comparison against the reference value (Sharpless et al., 1997).

Method precision was estimated by calculating between-run reproducibility for both an unfortified whole milk powder and an in-house control infant formula powder. Replicate analyses yielded RSD_R of 4.90% (mean: 2.07 mg/100 g; *n*: 12) and 2.68% (mean: 6.94 mg/100 g; *n*: 22) respectively, while a value of 4.55% was estimated for the NIST SRM 1846 (mean: 4.73 mg/100 g; *n*: 5).

Method accuracy was initially assessed using the Resolve column, by comparison of pantothenic acid levels estimated against both the microbiological assay (MBA) and GLC techniques for a range of infant formulas and unfortified milk and the data presented in Table 1.

Both HPLC and MBA methods estimate free analyte and demonstrate comparability, with an analysis of mean pantothenate by the paired, two sample *t*-test (2-tail) confirming no significant difference (t_{meas}: 1.18 vs t_{crit}: 2.13, df: 15, *P*: 0.05), with *r* of 0.971 and linear regression $y = 1.070x - 0.237$. The limited number of samples assayed initially by GLC precluded a reliable evaluation of its equivalence to HPLC, although the

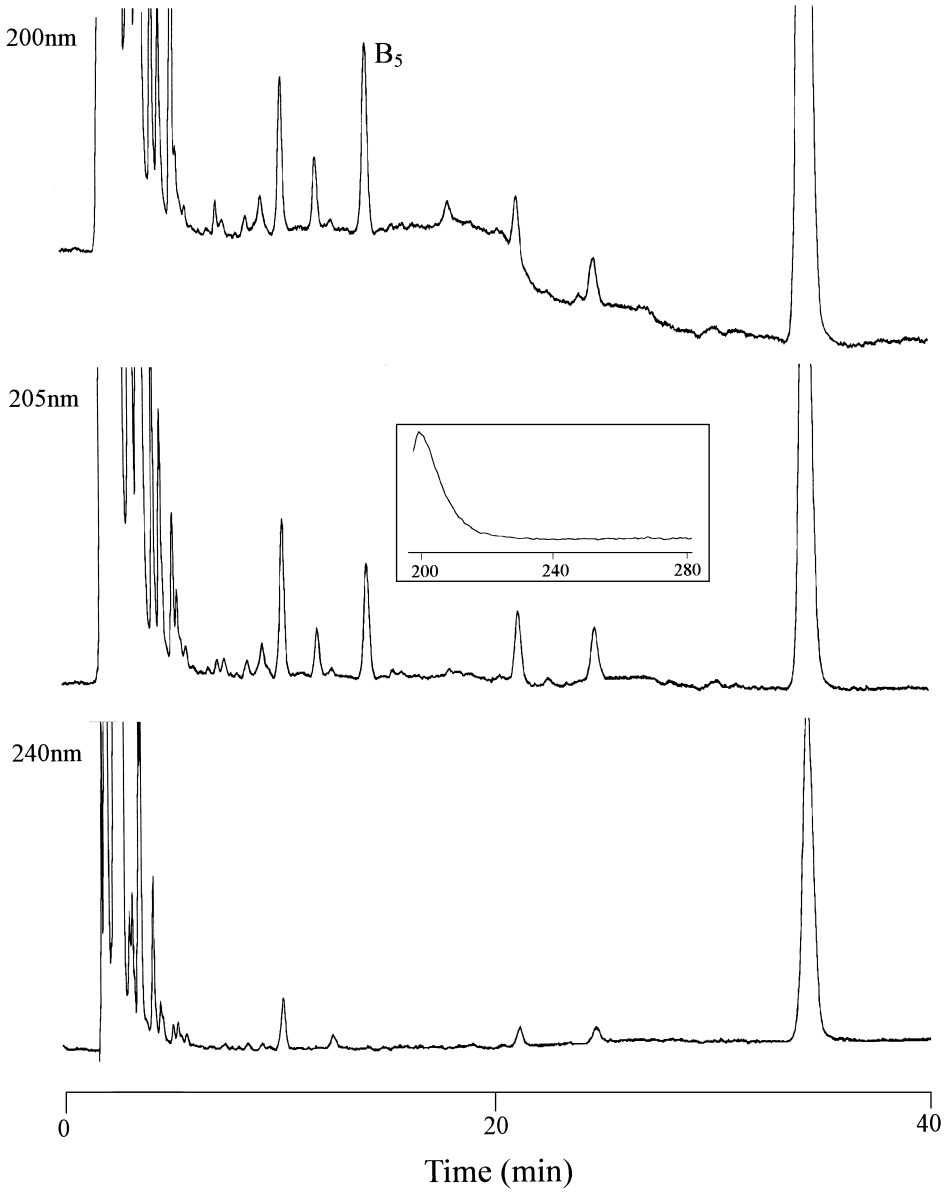

Fig. 3. Multiwavelength UV chromatogram (200, 205, 240 nm) of a typical infant formula extract obtained with Luna C_8 column. Insert illustrates UV spectral scan of pantothenic acid. Eluent: phosphate (0.1 M, pH 2.25):acetonitrile (97:3 v/v); flow: 1.4 ml/min (increased to 1.8 ml/min at 18 min); inj. vol: 10 μl.

GLC technique has been demonstrated to be less precise than HPLC following replicate analysis of a typical milk powder (mean: 5.35 mg/100 g; RSD_R: 17.01%; n: 30).

A range of samples sourced from both New Zealand and the USA, and including the NIST SRM 1846 were subjected to analysis with alternative LC columns. Comparative data are presented in Table 2, along with MBA and GLC results.

The above data demonstrate that each column was capable of reliable quantitation of pantothenate in milk powder and infant formula, confirming the suitability of the proposed HPLC method for application to the routine analysis of infant formulas. It was also apparent during these later studies and further comparisons with the Luna column, that there was no significant difference between HPLC and GLC (t_{meas}: 0.62 vs t_{crit}: 2.06, df: 25, P: 0.05), with r of 0.933 and linear regression $y = 1.026x - 0.004$.

None of the columns was capable of unambiguously identifying pantothenate in infant formulas based on protein hydrolysate, due to the abundance of free peptides and amino acids present in such products.

4. Discussion

Organic acids frequently represent unique chromatographic separation challenges, with strategies for increased

resolution limited to optimising column length, temperature, organic modifier content, ionic strength or pH adjustment of eluent in order to suppress both analyte dissociation and silanol ionisation. Retention under reversed-phase conditions is generally the result of several interaction mechanisms between polar solute, eluent, stationary phase and underlying silica, with the consequence that column selection and optimisation are rarely facile (Bruzzoniti, Mentasti & Sarzanini, 1998; Claessens, van Straten, Cramers, Jerzierska & Buszewski, 1998; Vailaya & Horvath, 1998).

Recent HPLC methods for the determination of pantothenic acid in infant formulas have advocated reversed-phase separation at both low pH and low organic modifier concentration (Endo et al., 1991; Romera et al., 1996; Zuo & Zhang, 1988). It is recognised that both of these requirements, while essential for successful retention of a polar and ionisable analyte, represent potential limitations to effective reversed-phase performance, especially with the older *type A* silica column chemistries. Thus, both conventional end-capping protection of residual silanols and bonded-phase integrity are vulnerable to low pH, with variable selectivity commonly reported under such conditions (Dolan, 1998; Kirkland, 1996). Further, the predominantly aqueous eluents (>95%) required to effect retention of such polar analytes, can initiate collapse of the hydrophobic bonded phase, with potential variability, or loss of retention. This problem has previously been described during pharmaceutical analysis (Woollard, 1986).

The six columns selected during column evaluation represent different solutions to the above considerations. The Resolve C_{18} cartridge column is representative of a first generation, non-endcapped *type A* silica chemistry,

Table 1
Pantothenic acid content in milk and infant formula powders (mg/100 g)[a]

Sample[b]	HPLC (Resolve C_{18})	MBA	GLC	CD[c]
1	2.41	2.55	2.60	–
2	6.50	6.50	7.25	3.0
3	5.11	4.10	nd[d]	2.5
4	3.43	3.65	nd	2.5
5	4.21	4.20	nd	2.5
6	3.95	4.00	nd	2.6
7	7.17	5.80	nd	4.0
8	6.24	6.25	7.56	3.0
9	6.32	6.20	nd	3.0
10	7.20	7.25	10.09	5.5
11	10.26	9.70	nd	4.0
12	5.08	5.12	3.56	2.7
13	4.92	4.90	nd	2.7
14	4.28	4.48	nd	2.7
15	4.78	5.05	nd	2.7
16	4.84	4.80	nd	2.7

[a] HPLC and MBA data reported as means of duplicate determinations; GLC as single determinations.
[b] 1 = skim milk; 2 = whole milk; 3–9 = whey-based formula, partially oil filled; 10,11 = milk-based formula, oil filled; 12–16 = goat milk-based formula, oil filled.
[c] CD = minimum can declaration.
[d] nd, not determined.

Table 2
Pantothenic acid content in milk and infant formula powders (mg/100 g)[a]

Sample[b]	HPLC						MBA	GLC	CD
	Luna C8	Resolve C18	YMC AQ 312	Develosil ODS-MG	Platinum EPS	Luna Phenyl-C6			
1[c]	4.76 (6)	4.73 (5)	4.20	4.48 (3)	4.63 (3)	4.69 (3)	4.79 (9)	5.19 (4)	4.87 ± 0.73
2	2.16 (4)	2.07 (12)	2.47	2.07 (3)	2.09 (3)	2.16	2.05 (3)	2.65	–
3	7.04 (4)	6.94 (22)	7.80	6.22 (3)	6.81 (3)	6.89	7.00 (48)	5.83	2.5
4	3.47 (3)	3.47 (8)	4.45	3.42 (4)	3.46 (3)	3.49	3.50 (33)	4.05	2.5
5	6.13 (3)	6.36	8.49	6.07 (3)	6.28 (3)	6.13	6.55 (4)	5.95	2.5
6	9.41 (5)	9.35 (6)	10.46	9.23 (3)	9.37 (3)	9.62	9.36 (3)	11.35	4.0
7	14.43 (5)	14.67 (3)	14.52	14.59 (3)	14.25 (3)	14.22	14.66 (3)	11.73	6.0
8	5.25 (3)	5.44 (3)	4.50	5.30 (4)	5.34 (3)	4.86	5.53	nd[d]	2.7
9	4.61 (3)	5.31	4.68	4.75	4.69	4.55	4.47	5.07	2.4
10	5.32 (3)	4.81	4.83	5.42	5.07	5.01	4.77	4.04	2.1
11	3.19 (3)	3.23	3.10	3.24	3.15	3.10	2.78 (3)	3.30	1.5
12	2.26 (6)	2.20	2.49	2.07 (4)	2.16 (4)	2.18	1.79 (3)	2.37 (3)	1.4
13	2.89 (3)	3.98	2.93	3.01	3.09	2.96	2.91 (3)	2.97	1.5
14	int[e]	int	int	int	int	int	3.54 (3)	3.69	2.1

[a] MBA values are means of data from two independent laboratories. All data are means of at least duplicate determinations, higher replications given in parentheses.
[b] 1 = NIST SRM 1846; 2 = whole milk; 3–5 = whey-based formula, partially oil filled; 6,7 = milk-based formula, oil filled; 8 = goat milk-based formula, oil filled; 9–12 = milk-based formula, oil filled; 13 = soy-based formula, oil filled; 14 = hydrolysed protein-based formula, oil filled.
[c] NIST SRM 1846, 'reference' 4.87 ± 0.73 mg/100 g.
[d] nd, not determined.
[e] int, chromatographic interference precludes quantitation.

while the 3 μm Platinum EPS represents a modern *type B* silica, non-endcapped equivalent offering enhanced silanophilic interaction with polar analytes. The Luna C_8 and high carbon density Phenyl-C_6 columns are based on low-acidity *type B* silica, reduced free silanol, exhaustive end-capped and shielded bonded phase ligand. The latter column represents a unique selectivity attributed to the combination of aliphatic and aromatic functionalities. The YMC AQ312 column incorporates an embedded hydrophilic endcapping reagent to facilitate bonded ligand activity in predominantly aqueous mobile phases, while the Develosil column exploits the potential of very low C_{18} ligand density to function in high aqueous eluent conditions. These columns also have reduced vulnerability to the low pH (<2.5) required for pantothenate retention (pK_a: 4.4). The tendency for conventional hydrophobic bonded phase columns to fail under conditions of low organic solvent content may be difficult to overcome and often requires a rigorous ligand activation protocol prior to analysis.

Pantothenic acid has no significant chromophore, necessitating LC detection at non-selective low wavelength (~200 nm) with increased risk of interference from most organic substances. The relatively weak retention reported in previous studies was observed to result in potential coelution of unknown milk components with pantothenic acid. Previous techniques (Endo et al., 1991; Romera et al., 1996; Zuo & Zhang, 1988) employed single channel UV detection, which in our experience was found to be incapable of distinguishing analyte from artefact. Several of the artefact peaks eluting under these conditions show UV spectra qualitatively similar to pantothenate, and may be organic acids or free amino acids present in milk as identified by others under similar chromatographic conditions (Akalin, Kinik & Gonc, 1997; Bevilacqua & Califano, 1989). The dominant late eluting peak in all milks and milk-based formulas exhibits a UV spectra similar to several compounds removed during column clean-up, although the equivalent peak present in the NIST SRM displays a qualitatively distinct spectrum. Multiwavelength UV detection has therefore been determined in this study to be mandatory for the unambiguous evaluation of putative pantothenate peak identity and integrity under such chromatographic conditions.

Previous HPLC procedures for estimating free vitamin B_5 in infant formulas have employed sample preparation schemes incorporating deproteination by pH adjustment (Romera et al., 1996; Zuo & Zhang, 1988) and further purification with solid phase extraction (Endo et al., 1991) prior to chromatographic analysis. Addition of acetic acid was found, in the present study, to effectively achieve a protein and fat free fraction suitable for direct injection.

Comparability of data based on independent analytical techniques is indicative of an unbiased estimate of analyte level. For a wide range of infant formulas, milk powders and the NIST SRM, the equivalence of several independent HPLC determinations with both non-enzymatic MBA and GLC data confirms the suitability of the liquid chromatographic method to routine compliance control of vitamin B_5 supplemented formulas. Further, equivalence with GLC data supports evidence for the low content of CoA-bound pantothenate in milk and formula, since the sample treatment implemented prior to GLC analysis releases both free and bound analyte (Davidek et al., 1985; Tesmer, et al., 1980).

Previous studies have confirmed that both human and bovine milk contain predominantly free pantothenate, with approximately 10% bound, largely as CoA. Levels previously reported for bovine milk are slightly higher than for human milk and range 2.5–4.0 mg/100 g anhydrous milk, measured as total B_5 (Fox, 1990; Gonthier et al., 1998; Renner, 1989; Scott, Bishop, Zechalko, Edwards-Webb, Jackson & Scuffam, 1984; Walsh et al., 1979). Our study reports a mean of 2.1 mg/100 g free vitamin for a control whole milk powder sample, consistent with reported values.

Vitamins are generally added to bovine milk during the production of infant formulas, and since the majority of endogenous pantothenate exists free in milk, an enzymatic pretreatment is not essential in estimating the level of this vitamin in formulas. This conclusion is further supported by a recent study which confirmed the equivalence of MBA estimation, both with and without enzymatic hydrolysis in such products (Endo et al., 1991). All infant formulas tested by the proposed HPLC method confirmed compliance with declared label claims based on recommendations of the Infant Formula Act (1980).

5. Conclusions

The HPLC protocol described, emphasises the importance of column selection and multiwavelength detection to achieve an unambiguous chromatographic estimation of pantothenic acid content in milk and infant formulas. Based on its superior selectivity and robustness under long-term, routine conditions, the Luna C_8 column was selected for future application. The method provides a result based on free pantothenic acid, which has been demonstrated to be a reliable estimation of vitamin B_5 content due to the low contribution of bound pantothenate in milk.

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