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Liquid chromatographic method for the determination of lutein in milk and pediatric formulas

Brendon D. Gill*, Harvey E. Indyk

Fonterra Co-operative Group Ltd, P.O. Box 7, Waitoa, New Zealand

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ABSTRACT

A simple and rapid method has been developed for routine compliance testing of lutein in pediatric formulas. Following a mild saponification procedure (70 °C for 10 min) demonstrated to be benign to lutein, lipophilic components were partitioned by a single extraction into hexane:diisopropyl ether (75:25 v/v) and concentrated in mobile phase. Baseline chromatographic separation of lutein and zeaxanthin (internal standard) was achieved using a C_{30} column and isocratic elution with methanol:dichloromethane (70:30 v/v). Lutein concentration was quantitated against zeaxanthin, which corrected for incomplete extraction recovery. Performance parameters assessed included recovery (101–108%) and repeatability (2.2% RSD). The method was applied to the analysis of lutein-supplemented pediatric formulas, unsupplemented milk powders, and bovine and human milk.

1. Introduction

Carotenoids are a diverse class of polyisoprenoid hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls), of which approximately 600 have been isolated from natural sources, predominantly in their most thermodynamically stable all-*trans* configuration. Within this group, there is a wide range of polarity, solubility and stability, and these features are of significance in the development of suitable analytical methods for their quantitative estimation in foods (Craft & Soares, 1992).

Carotenoid structure confers remarkably diverse physiological functions in photosynthesis, pro-vitamin A activity, antioxidant status, immune function, cell differentiation and several pathologies (Oliver & Palou, 2000). Since lutein and zeaxanthin are the only dietary carotenoids in the macular pigment, they have received increasing attention for their dietary potential to reduce age-related macular degeneration (Lietz, Mulokozi, Henry, & Tomkins, 2006), although this view has recently been challenged (Trumbo & Ellwood, 2006). Further, lutein and zeaxanthin are both considered to be protective in neonatal retinal epithelium, and infant formulas are therefore increasingly supplemented with lutein to more closely resemble human breast milk (Jewell, Mayes, Tubman, Northrop-Clewes, & Thurnham, 2004).

Contemporary methods for the selective analysis of carotenoids in foods and clinical applications are based on high performance liquid chromatography (HPLC), and comprehensive recent reviews are available (Barua, Olson, Furr, & van Breemen, 2000; Blake, 2007; Eitenmiller & Landen, 1999; Oliver & Palou, 2000; Su, Rowley, & Balazs, 2002). Photodiode-array detection is considered to be essential, given the subtle differences in characteristic UV–vis spectra of the many individual carotenoids potentially present in natural foods, while mass spectrometric detection methods have also been advocated for confirmation of identity (Breithaupt, 2004; Dachtler, Glaser, Kohler, & Albert, 2001; Rodríguez-Bernaldo de Quirós & Costa, 2006; Schlatterer & Breithaupt, 2006). Reversed phase separation systems are most commonly described utilising monomeric or polymeric C_{18} chemistries, although C_{30} columns are increasingly applied in view of improved selectivity (Aman, Bayha, Carle & Schieber, 2004; Breithaupt, 2004; Dachtler et al., 2001; Li, Qin, Yin, & Tang, 2007; Mercadante, Steck, & Pfander, 1999; Rousell, Raley, & Hofsommer, 1996). Nevertheless, normal phase separations have also been reported in view of their enhanced retention of polar xanthophylls and resolution of isomeric carotenoids (Panfili, Fratianni, & Irano, 2004).

Saponification and direct solvent extraction methods are generally advocated for carotenoid analysis of foods, with the latter technique often preferred for low fat samples and when efficient extraction of target analytes can be demonstrated. However, for complex foods and those containing high fat, alkaline hydrolysis is frequently required to remove potentially interfering lipid and chlorophyll, improve recovery and convert xanthophyll esters (Ball, 2006; Barua et al., 2000; Eitenmiller & Landen, 1999; Hart & Scott, 1995; Konings & Roomans, 1997; Larsen & Christensen, 2005; Rodríguez-Bernaldo de Quirós & Costa, 2006). Supercritical fluid

* Corresponding author. Tel.: +64 7 887 1235; fax: +64 7 887 1502.
E-mail address: brendon.gill@fonterra.com (B.D. Gill).

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extraction and enzymatic lipolysis have also been described as alternatives to these conventional techniques (Lietz & Henry, 1997; Oliver & Palou, 2000).

The carotenoid profile of milk has been reviewed, and while human milk reportedly contains significant levels of lutein, cryptoxanthin, lycopene and β-carotene, the major forms in bovine milk are lutein and β-carotene (Calderón et al., 2007; Canfield, Giuliano, & Graver, 1995; Canfield et al., 2003; Jensen, 1995). Two recent reviews have described carotenoid metabolism and factors affecting expression in bovine milk (Nozière et al., 2006; Sauvant, Grolier, & Azaïs-Braesco, 2002). In view of their high fat content, saponification has generally been applied to the extraction of carotenoids in milk and infant formulas (Giuliano, Neilson, Kelly, & Canfield, 1992; Granelli & Helmersson, 1996; Indyk, 1987; Jackson, Lien, White, Bruns & Kuhlman, 1998; Jewell et al., 2004; Schweigert, Hurtienne & Bathe, 2000), although enzymatic digestion has also been described (Khachik et al., 1997; Liu, Xu, & Canfield, 1998).

This study describes a simple, robust and validated method for the determination of lutein in bovine and human milk, milk powders, and infant formulas involving a single-tube alkaline hydrolysis and solvent extraction scheme with internal standard quantitation.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a SCL-10Avp system controller, LC-10ADvp pump, FCV-10ALvp low pressure gradient unit, SIL-10AF sample injector unit equipped with a 50 μL injection loop, DGU-14A degasser unit, CTO-10ASvp column oven and a SPD-M10Avp photodiode array detector (Shimadzu, Kyoto, Japan). Instrument control and data processing were implemented using Shimadzu Class-VP version 6.12.

Absorbances for calibration standards were acquired with a model UV-1601 spectrophotometer (Shimadzu) with digital readout to 4 decimal places. Samples were centrifuged in a Funke Gerber Super Vario-N (Berlin, Germany). Terumo 3 mL disposable syringes (Terumo Corporation, Laguna, Philippines) and Minisart 0.2 μm syringe filters with cellulose acetate membranes (Sartorius, Göttingen, Germany) were used for standard preparation.

Prior to use, mobile phases were filtered and degassed using a filtration apparatus with 0.45 μm nylon filter membranes (All-Tech, Deerfield, IL, USA).

2.2. Reagents

Lutein and zeaxanthin were purchased from ChromaDex (Irvine, CA, USA). Potassium hydroxide, pyrogallol and HPLC grade solvents, hexane, diisopropyl ether, ethyl acetate, methanol, dichloromethane, and ethanol, were supplied by Merck (Darmstadt, Germany). Water was purified with resistivity ≥18 MΩ using an E-pure water system (Barnstead, Dubuque, IA, USA).

2.3. Standards

Due to the light-sensitive nature of carotenoids, all steps in this procedure were performed under subdued lighting. Stock standards were prepared by weighing approximately 1 mg of lutein and zeaxanthin into separate 25 mL volumetric flasks and dissolved in approximately 20 mL of ethanol accomplished by shaking and ultrasonication for 5 min. The stock standards were then made to volume with ethanol. Intermediate standards were prepared by filtering approximately 3 mL of the stock solution through a 0.22 μm syringe filter into a 100 mL volumetric flask. Approximately 80 mL of ethanol was added and the flask sonicated for 5 min, then made to volume with ethanol.

The concentration of individual intermediate standards was determined from their measured absorbance against ethanol and published absorptivities ($E^{1\%}_{1\,cm}$: lutein, 2550 at 445 nm and zeaxanthin, 2480 at 452 nm) (Scott, Finglas, Seale, Hart, & de Froimont-Gortz, 1996) and corrected for chromatographic purity.

Five calibration standards were prepared containing a constant concentration of the internal standard zeaxanthin and a variable concentration of lutein. A further correction to each individual standard was computed by subtraction of the minor contribution (<5%) of lutein in zeaxanthin (and vice versa) prior to linear regression of the ratios of corrected peak area against concentration. Lutein content in an unknown sample was interpolated from this calibration curve.

2.4. Sample preparation

Sample preparation was adapted from that used for the rapid analysis of carotenoids in bovine milk (Indyk, 1987). Approximately 1 g of powder or 1 g liquid test sample was weighed accurately into a 60 mL boiling tube to which 10 mL of ethanolic pyrogallol (1% w/v) was added. For heterogenous products, a 4 g aliquot of a slurry (25 g powder in 100 g water) was used. A 1 mL aliquot of zeaxanthin intermediate standard (0.9 μg mL^{-1}) was added to each sample, followed by 2 mL of a 50% w/v KOH solution, then the tubes were incubated at 70 °C for 10 min in a water bath with periodic agitation to avoid powder agglomeration and ensure efficient lipid saponification. Following cooling in a cold water bath, 20 mL of extraction solution (hexane:diisopropyl ether, 75:25 v/v) was added and the tubes placed on a horizontal shaker for 5 min. Water (20 mL) was added, the tubes capped, inverted 10 times and centrifuged for 10 min at 200 × *g*. A 1 mL aliquot of the upper solvent layer was transferred to an HPLC vial, blown to dryness, and reconstituted with 1 mL mobile phase ready for analysis.

2.5. Chromatography

The reversed phase column selected for use was a YMC Carotenoid C_{30}, 250 mm × 4.6 mm, 3 μm (Waters, Milford, MA, USA). The mobile phase consisted of a methanol:dichloromethane (70:30 v/v) run isocratically at a flow rate of 0.5 mL min^{-1}. The injection volume was 50 μL and detection was by photodiode array (370–600 nm) with quantitation at 450 nm.

2.6. Statistical analysis

Statistical evaluation of experimental data for method performance parameters was accomplished with Microsoft Excel 2003 SP1 (Microsoft Corporation, Redmond, WA, USA).

3. Results

3.1. Method performance

Chromatography achieved under the described conditions is illustrated in Fig. 1 for carotenoid standards and representative samples. Baseline separation of lutein and zeaxanthin was achieved under the described conditions, and run time was optimised for the polar xanthophylls. Peak identity for the xanthophylls was confirmed by equivalence of retention time and UV–vis spectra against authentic standards.

The performance of the method was determined as summarised in Table 1. Linearity of detector response for both lutein and zeaxanthin was confirmed by least-squares regression analysis of multilevel calibration standards. In the absence of a currently available

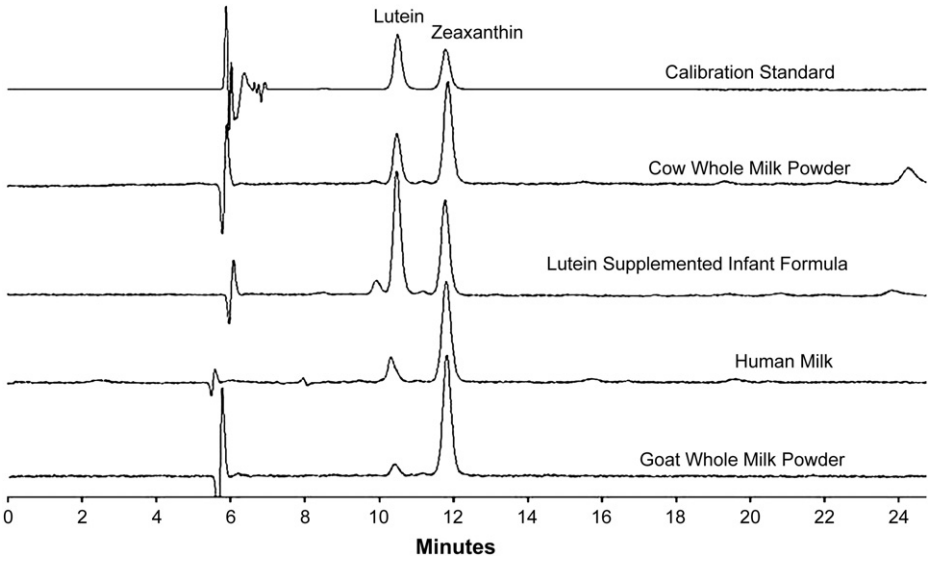

Fig. 1. Overlaid chromatograms of milk and powder samples spiked with zeaxanthin internal standard (column: YMC Carotenoid C_{30}, 250 mm × 4.6 mm, 3 μm; mobile phase: methanol:dichloromethane (70:30 v/v); flow rate: 0.5 mL min^{-1}; detection: 450 nm).

infant formula certified reference material with certified levels of lutein, method accuracy was confirmed by evaluating spiked recovery at 3 levels equivalent to 10, 20 and 30 μg 100 mL^{-1} of ready-to-feed infant formula. In addition, accuracy was evaluated for 2 different lutein-supplemented samples by comparing lutein levels as measured by the described procedure against the values obtained from the aggregate of multiple extractions ($n = 4$). Precision, as repeatability, was determined using 6 replicate analyses of a sample of a lutein fortified infant formula and this was repeated on 3 separate days to provide an estimate of intermediate precision. The method detection limit (MDL) was determined in accordance with US Environmental Protection Agency procedures (USEPA, 1993).

Recoveries of lutein and zeaxanthin were also quantitated independently through each of the separate saponification and extraction steps (Table 2). Recovery through saponification (70 °C for 10 and 30 min) was determined following acid neutralisation after hydrolysis and direct dilution in ethanol without solvent extraction, a modification of a method reported for vitamin A (De Vries & Silvera, 2002). Recovery attributed to the described single solvent extraction procedure was assessed by comparison of peak area of each carotenoid with that of unextracted analyte.

3.2. Milk and milk powder samples

The described method was applied to the analysis of various pediatric formulas, cow and goat whole milk powders, and liquid cow and human milk; results summarised in Table 3. Endogenous lutein levels measured in reconstituted infant formulas and bovine whole milk powders ranged from 1.0 to 5.8 μg 100 mL^{-1}. The levels in fluid bovine and human milk are comparable at 6.0 and 4.3 μg 100 mL^{-1}, respectively.

4. Discussion

It has been recently identified that there is a need for validated methods for the analysis of carotenoids, including lutein, in fortified foods (Blake, 2007). The method presently described was developed predominantly for the compliance testing of lutein-supplemented pediatric formulas, while also being suitable for estimation of endogenous lutein in milk. In a high sample throughput laboratory, simple and rapid procedures for extraction of lipophilic components are preferred over those requiring multiple solvent extractions.

Due to the high fat content of milk products, either alkaline hydrolysis or lipase digestion is generally advocated to both remove lipid and release carotenoids (Oliver & Palou, 2000; Schweigert et al., 2000). Although saponification has been employed in the analysis of lutein in milk (Calderón et al., 2007; Jewell et al., 2004; Lietz et al., 2006), the polar xanthophylls are reportedly vulnerable to alkaline conditions and lutein losses have been noted (Liu et al., 1998; Rodríguez-Bernaldo de Quirós & Costa, 2006; Scott, 1992), since apart from degradation via cleavage, *cis–trans* isomerisation is also possible. It is therefore expedient to apply the mildest conditions necessary to effectively hydrolyse triglycerides. The impact of high temperature alkaline hydrolysis on lutein and zeaxanthin recovery was assessed directly, without solvent extraction, and losses due to either mechanism during the described saponification

Table 1
Method performance parameters

Analyte	Range (ng mL^{-1})	Linear regression	r^2	MDL[a] (μg 100 g^{-1})	RSD_r[b] (%)	RSD_{IR}[c] (%)	Recovery[d] (%)	Recovery[e] (%)
Lutein	15.7–470.9	$y = 1414.4x - 2377.3$	0.9999	1.4	2.2	3.2	101–108	95
Zeaxanthin	20.6–618.5	$y = 1367.7x - 5936.1$	0.9997					

[a] Method detection limit: determined from n replicates at or near the expected detection limit, $MDL = t_{(n-1,\ 1-\alpha)} \times sd$, where $n = 7$ and $\alpha = 0.05$.
[b] Relative standard deviation repeatability: $RSD_r = sd/mean \times 100$, $n = 6$.
[c] Relative standard deviation intermediate reproducibility: $RSD_{IR} = sd/mean \times 100$, $n = 18$ (6 samples tested on 3 different days).
[d] Mean recovery of lutein spiked blank skim-milk samples (3 levels each in triplicate).
[e] Mean recovery of 2 independent samples by the described method, expressed as a percentage of total extractable lutein (measured as the aggregate of 4 pooled extractions quantitated against lutein external standard).

Table 2
Recovery of lutein and zeaxanthin through separate saponification and solvent extraction steps

Sample	Saponification recovery[a] (%)		Extraction recovery[b] (%)
	70 °C, 10 min	70 °C, 30 min	
Lutein	99 ± 3.7	92 ± 2.2	68
Zeaxanthin	102 ± 4.6	99 ± 4.4	69

[a] Mean of 5 replicates ± standard deviation.
[b] Singlicate determinations.

procedure were confirmed as analytically insignificant for both carotenoids.

Hexane-based extraction solvent mixtures containing varying proportions of diisopropyl ether or ethyl acetate were evaluated during extraction of the non-saponifiable carotenoid components. Although the more polar hexane:ethyl acetate system gave predictably higher absolute lutein recoveries, quantitative recovery in a single extraction was not achieved and higher levels of ethyl acetate resulted in degradation of chromatographic performance. In contrast, while the single-extraction procedure incorporating diisopropyl ether was also less than quantitative, the extract yielded unequivocal chromatograph. Both binary solvent systems were found to require at least 3 extractions for quantitative recovery. The inability to quantitatively partition lutein and zeaxanthin in a single extraction was not unexpected in view of their relative high polarity compared to the parent carotenes.

In the absence of quantitative recovery during a single-extraction scheme, or in procedures involving multiple steps, the use of an internal standard is mandatory. Since zeaxanthin is structurally comparable to lutein and further, was found in insignificant levels in test samples, it was therefore considered to be an ideal internal standard for the specific determination of lutein by LC–UV techniques. The utility of zeaxanthin was also supported by baseline resolution from lutein under the described chromatographic conditions, in contrast to several previous reports where aggregate values of lutein and zeaxanthin were a consequence of inadequate resolution (Canfield et al., 2003; Jackson et al., 1998; Liu et al., 1998). Since the solubility of zeaxanthin in ethanol is lower than lutein, it proved necessary to sonicate and filter the stock standard solutions until a stable absorbance (at 450 nm) was achieved prior to calibration.

Recovery and precision were assessed using the criteria developed by AOAC International (2004). The estimated recovery range of 101–108% is within the guideline limits of 75–120% at typical lutein concentration levels found in milk and supplemented formula, while the measured $HORRAT_r$ (0.3) is consistent with the guideline range (0.3–1.3).

Table 3
Lutein levels in various milk samples and infant formulas

Sample	Lutein[a] (μg 100 mL^{-1})
Whey-based, partially oil-filled pediatric formula	1.0
Whey-based, milk-fat pediatric formula	1.8
Whey-based, oil-filled pediatric formula[b]	15.3
Whey-based, oil-filled pediatric formula[b]	6.2
Milk-based, milk-fat pediatric formula	2.6
Milk-based, oil-filled pediatric formula	2.6
Cow whole milk powder	5.8
Goat whole milk powder	1.1
Raw whole milk	6.0
Processed whole milk	3.3
Human milk	4.3

[a] Values are given on a reconstituted basis normalised to 12% solids.
[b] Lutein-supplemented infant formula.

While LC–MS methods may assist in the identification of carotenoids, most reports have utilised the photodiode-array detector for confirmation of peak identity by spectral comparison against authentic compounds. In the method described presently, this technique was found to be both facile and adequate for the unequivocal identification of lutein and zeaxanthin as both share similar but distinct spectral characteristics.

The method was applied to the analysis of various pediatric formulas and milk powders as well as human and bovine milk. The applicability of this method to each matrix was initially assessed by analysing each sample type in the absence of internal standard to reveal the potential contribution of endogenous zeaxanthin. In goat whole milk powder and pediatric formulas, there were negligible amounts of endogenous zeaxanthin. While human and bovine milk showed trace levels of endogenous zeaxanthin, the quantitation of lutein was not significantly affected given the relatively high level of internal standard added.

In contrast to the carotenes, it has been suggested that lutein may be actively secreted into mammalian milk in view of its specific role during early neonatal macular development (Gossage, Deyhim, Yamini, Douglass, & Moser-Veillon, 2002; Jewell et al., 2004). Human milk reportedly contains several predominant carotenoids including α- and β-carotene, lutein, zeaxanthin, β-cryptoxanthin and lycopene, while bovine milk is dominated by β-carotene and lutein (Calderón et al., 2007; Canfield et al., 2003; Giuliano et al., 1992; Gossage et al., 2002; Jackson et al., 1998; Jewell et al., 2004; Khachik et al., 1997; Liu et al., 1998; Nozière et al., 2006; Schweigert et al., 2000). In the present study, a representative human milk sample yielded lutein levels consistent with the range (0.8–5.7 μg 100 mL^{-1}) of previously reported values (Canfield et al., 2003; Gossage et al., 2002; Jackson et al., 1998; Lietz et al., 2006). Similarly, lutein levels measured in raw and processed consumer bovine milk were also comparable and recent reports (trace – 6.6 μg 100 mL^{-1}) (Calderón et al., 2006; Calderón et al., 2007; Indyk, 1987; Nozière et al., 2006). Minimal losses have been reported for lutein through thermal milk processing (Nozière et al., 2006), and indeed, only a small decrease was observed in this study. There are no reported literature values for the lutein content in goat milk, although this species milk is characterised by the absence of β-carotene (Nozière et al., 2006). Prior to the present study, one report only has surveyed xanthophyll content of ready-to-feed formula milk. In that study, variable levels of lutein (trace-18.0 μg 100 mL^{-1}) and zeaxanthin (trace-2.3 μg 100 mL^{-1}) were found, although it was unknown which formulas were intentionally lutein-supplemented, and it was speculated that egg lipid, a commonly used ingredient, may also contribute to the xanthophyll contribution (Jewell et al., 2004). In the present study, 2 formulas were intentionally supplemented with lutein during manufacture, while the remainder contained endogenous levels generally consistent with the contribution of milk fat.

5. Conclusions

Infant formulas are increasingly supplemented with lutein due to its putative role as a protective factor in the neonatal retinal epithelium. A rapid, routine and robust method for the compliance analysis of lutein is therefore essential. The method described incorporates a mild alkaline hydrolysis and single-extraction technique combined with an isocratic chromatographic separation and has been demonstrated to be applicable to both supplemented milk-based pediatric formulas and endogenous lutein in milk.

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