



# Application of an LC-UV Method to Estimate Lutein Recovery During Infant Formula Manufacture

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## Abstract

Lutein is a carotenoid that is considered to be important to the integrity of the retina and is therefore increasingly being supplemented into bovine milk-based paediatric formulae to levels equivalent to those found in human milk. A simple analytical method has been developed and intra-laboratory validated to facilitate routine in-process control of lutein addition. The method involves dilution of a carotenoid premix, followed by C<sub>30</sub> reversed-phase liquid chromatographic separation of lutein, zeaxanthin and  $\beta$ -carotene, with detection and quantitation at 450 nm. The method performance parameters include range (0–700 ng mL<sup>-1</sup>), method limit of detection (0.08  $\mu$ g g<sup>-1</sup>), recovery (95.5–109.5%) and precision (1.2% RSD<sub>r</sub>). The method has been applied to an evaluation of lutein recovery (95.6–104.2%) through the manufacture of paediatric formulae, which confirms that lutein loss through the entire process is insignificant.

## Introduction

Lutein ( $\beta$ - $\epsilon$ -carotene-3,3'-diol) belongs to a class of oxygenated carotenoids (xanthophylls) and is present in many foods, particularly vegetables and fruits. Specifically, it is a dihydroxy derivative of  $\alpha$ -carotene and the structure of lutein and its importance in protecting against oxidative and blue-light damage in the retina have been described previously (Bone, Landrum, Cao, Howard, & Alvarez-Calderon, 2007; Canfield et al., 2003). Indeed, only lutein and its structural isomer zeaxanthin are specifically accumulated in eye tissue.

Although no direct evidence has shown benefits of lutein supplementation to infant vision, lutein has been added to selected infant formulae in recent years to levels equivalent to those found in human milk; however, formula supplementation with carotenoids is currently not routine (Capeding et al., 2010; European Food Safety Authority, 2008; Jewell, Mayes, Tubman, Northrop-Clewes, & Thurnham, 2004; Mackey et al., 2013). Human milk is known to contain several dietary carotenoids, including  $\alpha$ - and  $\beta$ -carotenes, lutein, zeaxanthin,  $\beta$ -cryptoxanthin and lycopene, whereas bovine milk carotenoids are dominated by  $\beta$ -carotene and lutein. A recent study reported that the feeding of carotenoid-supplemented infant formula achieved elevated neonatal concentrations of plasma lutein that were comparable with levels found in breast-fed infants, albeit with evidence that the bioavailability of carotenoids from human milk exceeds that from infant formula (Mackey et al., 2013). Lutein is approved as safe for nutrient fortification use; however, few bovine milk-based infant formulae are currently supplemented with lutein despite the putative protective role of this micronutrient in the neonatal retinal epithelium (Capeding et al., 2010; Jewell et al., 2004; Mackey et al., 2013).

Dietary forage carotenoids are expressed in bovine milk following ruminal digestion, intestinal absorption, metabolism and mammary gland secretion, and ultimately contribute to the colour of the milk fat of pasture-grazed animals, with  $\beta$ -carotene and lutein being quantitatively dominant (Nozière et al., 2006). Nonetheless, the mechanisms controlling the significantly higher uptake of plasma  $\beta$ -carotene relative to the xanthophylls lutein in the mammary gland remain unknown (Calderón et al., 2007).

Analytical methods for carotenoids in foods have previously been reviewed (Barua, Olson, Furr, & van Breemen, 2000; Eitenmiller & Landen, 1999; Oliver & Palou, 2000; Rodriguez-Amaya, 2010; Su, Rowley, & Balazs, 2002). Direct solvent extraction is the preferred technique for samples with low lipid content or samples that are free of xanthophyll esters. However, in samples with high fat content, such as milk and infant formulae, alkaline hydrolysis has generally been applied prior to solvent extraction (Indyk, 1987; Jewell et al., 2004; Schweigert, Hurtienne, & Bathe, 2000), although enzymatic digestion has also been described (Khachik et al., 1997; Liu, Xu, & Canfield, 1998).

Reversed-phase chromatography utilising both high performance liquid chromatography (HPLC) and ultra HPLC platforms, with either  $C_{18}$  or  $C_{30}$  column chemistries, are most commonly used for instrumental analysis (Rivera & Canela-Garayoa, 2012). However, normal phase separations have also been reported because of the superior retention of polar xanthophylls and the resolution of carotenoid isomers (Panfili, Fratianni, & Irano, 2004). Due to subtle differences in the characteristic ultraviolet (UV)-visible absorbance of various carotenoids, photodiode array detection is considered to be essential for unambiguous identification. However, mass spectrometric detection methods have increasingly been used in both atmospheric pressure chemical ionisation and electrospray ionisation modes to identify constituent carotenoids (Breithaupt, 2004; Dachtler, Glaser, Kohler, & Albert, 2001; Rivera & Canela-Garayoa, 2012).

Methods for the analysis of lutein in infant formulae to support label claims have recently been reported (Gill & Indyk, 2008; Yuhas et al., 2011), and the analysis of lutein and zeaxanthin in vegetable oils, using solvent extraction with quantitation by either reversed phase or normal phase LC, has also been described (Franke, Fröhlich, Werner, Böhm, & Schöne, 2010; Ranalli, Contento, & Di Simone, 2011).

As lutein is a highly unsaturated compound, it is inherently susceptible to the oxidative stresses associated with thermal and UV exposure. Several studies have investigated the potential for thermal and oxidative degradation of lutein in organic solvent, oil-in-water emulsion and dosed vegetable oil systems (Henry, Catignani, & Schwartz, 1998; Lavecchia & Zuorro, 2008; Losso, Khachatryan, Ogawa, Godber, & Shih, 2005; Subagio, Wakaki, & Morita, 1999). These studies have demonstrated that the degradation of lutein complies with a first-order kinetic model with the formation of epoxide and cis-isomers, and have confirmed that, although lutein has greater stability than  $\beta$ -carotene and lycopene, free lutein is significantly less stable than its esters.

In view of these considerations, the stability of supplemental lutein during the manufacture of milk-based paediatric formulae is an important concern for compliance and nutritional purposes. Therefore, the purpose of the current study was to develop a method for the determination of lutein in dosed vegetable oil and to apply the method to the investigation of its stability under the thermal processing conditions that are required to manufacture paediatric nutritional products.

## 2. Materials and methods

### 2.1. Apparatus

The Prominence LC-20 series HPLC system included an LC-20AT low pressure gradient solvent delivery module, an SIL-20A HT sample injector and 50 mL injection loop, a DGU-20A degasser unit, a CTO-20AC column oven, and an SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan). Instrument control and data processing were accomplished using LCSolution version 1.25.

Spectral absorbance measurements were obtained with a model UV-1601 spectrophotometer (Shimadzu). Other apparatus included a Super Vario-N centrifuge (Funke Gerber, Berlin, Germany), 3 mL disposable syringes (Terumo Corporation, Laguna, Philippines) and cellulose acetate and nylon membrane 0.2  $\mu$ m syringe filters (Minisart, Sartorius, Gottingen, Germany).

Eluents used for HPLC analysis were prepared using a filtration apparatus with 0.45  $\mu$ m nylon membranes (All-Tech, Deerfield, IL, USA).

## 2.2. Reagents

Lutein and zeaxanthin were obtained from ChromaDex (Irvine, CA, USA). HPLC-grade hexane, methanol, dichloromethane, ethanol and tetrahydrofuran were from Merck (Darmstadt, Germany) and water of > 18 M $\Omega$  resistivity was produced by an E-pure system (Barnstead, Dubuque, IA, USA).

## 2.3. Standards

A subdued incandescent light environment was maintained to protect the photosensitive carotenoids. Stock standards (approximately 40  $\mu\text{g mL}^{-1}$ ) and intermediate standards (approximately 1.2  $\mu\text{g mL}^{-1}$ ) were separately prepared, and concentrations of the individual lutein and zeaxanthin intermediate standards established spectrophotometrically, as described previously (Gill & Indyk, 2008). Spectrophotometric concentrations were further corrected by chromatographic determination of the minor contribution of lutein in zeaxanthin (and vice versa).

Working lutein external calibration standards (approximately 0.12, 0.60, 1.20  $\mu\text{g mL}^{-1}$ ) were prepared for the quantitation of the lutein content of premix-dosed oil blends by serial dilution of the lutein intermediate standard. Mixed working calibration standards containing zeaxanthin as internal standard were prepared for the quantitation of lutein in finished nutritional products, as described previously (Gill & Indyk, 2008).

## 2.4. Sample collection

Spray-dried paediatric formulae were manufactured by a semicontinuous commercial process. Briefly, incoming raw milk was separated into chilled skim milk and cream fractions and the skim milk was thermalised and recombined to achieve a standardized macro component (protein, fat and carbohydrate) composition. A high-concentration fat-soluble vitamin premix containing lutein in a safflower oil carrier was batch dispersed with agitation in a mixed vegetable oil blend that was designed to substitute for milk fat in the final formula product.

The dosed oil blend was added to the standardised milk and subsequently pasteurised. The manufacturing process incorporated low pressure preheating, direct steam injection, multi-stage evaporation and spray drying followed by a fluid-bed finish.

Lutein-dosed oil blend and spray-dried nutritional powder product were sampled under steady-state conditions based on the traceable flow characteristics of the closed system plant, with a residence time of approximately 30 min.

## 2.5. Sample preparation

A 2.0 g aliquot of lutein-dosed oil blend was accurately weighed into a 50 mL disposable centrifuge tube, 5 mL of tetrahydrofuran was added, the tube was capped and the mixture was vortex mixed and ultrasonicated for 30 min. The contents were made to volume with ethanol and mixed thoroughly. Approximately 1.5 mL was transferred to a syringe, filtered through a 0.2  $\mu\text{m}$  nylon membrane into an amber HPLC vial, capped and positioned in the HPLC autosampler.

Paediatric formula powders were prepared for analysis as described previously (Gill & Indyk, 2008). Briefly, approximately 1 g was subjected to mild saponification following the addition of zeaxanthin internal standard. Lutein was extracted and an aliquot was evaporated and reconstituted with mobile phase prior to reversed-phase chromatographic analysis.

## 2.6. Chromatography

For the analysis of extracts separately prepared from both lutein-dosed oil blends and nutritional products, a YMC Carotenoid C<sub>30</sub>, 250  $\times$  4.6 mm, 3  $\mu\text{m}$  column (Waters, Milford, MA, USA) with a mobile phase of methanol:dichloromethane (70:30, v/v) was used under isocratic conditions at a flow rate of 0.5 mL min<sup>-1</sup>. Following the elution of lutein, the flow rate was increased to elute  $\beta$ -carotene, which may be present in certain products. The injection volume was 50  $\mu\text{L}$  and detection was by photodiode array (370–600 nm) with quantitation at 450 nm. These conditions were as previously reported (Gill & Indyk, 2008).

The lutein content of dosed oil blends and nutritional powdered product samples were interpolated from the appropriate calibration curve.

## 3. Results and discussion

### 3.1. Method development

The method utilised for the quantitation of lutein in spray-dried infant formulae has previously been validated and was implemented during this study as reported (Gill & Indyk, 2008). However, the analytical procedure for the measurement of the lutein content of dosed oil was developed and validated during this study, as described below.

Sample preparation involved the initial dilution of a representative oil test sample with tetrahydrofuran, a step that was found to be necessary to ensure the complete solubilisation of lutein. In a comprehensive study, lutein was reported to be of low solubility in many organic solvents that are commonly used for carotenoid analysis, and tetrahydrofuran was demonstrated to provide significantly enhanced solubility for both  $\beta$ -carotene and lutein (Craft & Soares, 1992). Therefore, in the method developed, an initial

solubilisation of the oil in tetrahydrofuran, aided by ultrasonication, was found to be expedient. Following further dilution with ethanol and membrane filtration, the extract was ready for chromatographic analysis.

Quantitation of the lutein content of vegetable oil blends was accomplished by a three-level external standard technique and under the same chromatographic conditions as described for paediatric formulae. The chromatography obtained for authentic lutein, an oil blend and a dosed oil blend is illustrated in Figure 1.

Baseline separation of lutein, zeaxanthin and  $\beta$ -carotene was optimal under the described conditions, with peak identities confirmed by equivalence of retention times and UV-visible spectra against authentic standards. A number of undosed oil blends were found to contain low, but significant endogenous levels of lutein ( $0.3\text{--}0.4\ \mu\text{g g}^{-1}$ ), which should be accounted for during product formulation. The absence of chromatographic interferences in unsupplemented oils confirmed the selectivity of the analytical separation system for lutein. Previous studies have also reported variable levels of endogenous lutein in cold-pressed vegetable oils, with evidence that the xanthophylls are susceptible to processes associated with oil refining (Franke et al., 2010). Premixes were also found to contain zeaxanthin at significant levels, i.e., between 5 and 10% of the declared lutein content, an observation that is consistent with the presence of zeaxanthin in lutein preparations most commonly derived from the petals of the marigold (*Tagetes erecta*) flower (Capeding et al., 2010).

### 3.2. Method validation

A linearity study, where the measurement response is linearly proportional to the analyte concentration, verified that sample extracts contain lutein within a specified concentration range. Linearity of the detector response for lutein was therefore confirmed by the analysis of multi-level lutein calibration standards and yielded a linear least-squares regression with an  $r^2$  of 1.0000 over the concentration range  $0\text{--}700\ \text{ng mL}^{-1}$ . The method detection limit ( $\text{MDL} = \text{sd} \times t_{[n-1, 0.01]}$ ) was estimated to be  $0.08\ \mu\text{g g}^{-1}$  from replicate analysis of an undosed vegetable oil blend containing a low level of endogenous lutein (mean =  $0.35\ \mu\text{g g}^{-1}$ ,  $n = 14$ ).

Method accuracy is the closeness of agreement between results and an accepted true value. In the absence of a suitable standard reference material or reference method, accuracy was confirmed by estimating spiked recovery under laboratory-scale conditions. Duplicate determinations at 50, 100 and 150% of the target lutein content of a dosed oil blend yielded recoveries of 95.5–109.5%, values that comply with the accepted criteria of 80–115% at this concentration interval (AOAC International, 2002).

Analytical recovery was further interrogated through the measurement of lutein in four different vegetable oil blends dosed under large scale manufacturing conditions. Oils were dosed to various lutein contents ( $5\text{--}9\ \mu\text{g g}^{-1}$ ) and recoveries were estimated as 84–97% against theoretical target levels, with a relative standard deviation ( $\text{RSD}_r$ ) of 0.9%.

Method precision, as repeatability, was determined using independent replicate analyses ( $n = 7$ ) of an oil blend supplemented with lutein at  $4.0 \mu\text{g g}^{-1}$  under laboratory-scale conditions, and the  $\text{RSD}_r$  was estimated to be 1.2%. The  $\text{HorRat}_r$  function is a recognized performance criterion for the evaluation of the precision of an analytical method and the measured  $\text{HorRat}_r$  value of 0.14 exceeded the accepted guidance limits of 0.3–1.3 for repeatability (Horwitz & Albert, 2006). Further precision estimates were obtained from oil blends ( $n = 3$ ) that were independently dosed with premix to contain lutein at the higher level of  $8.0 \mu\text{g g}^{-1}$ . Within-sample and between-sample repeatability estimates were equivalent, each with an  $\text{RSD}_r$  of 2.3% and a  $\text{HorRat}_r$  value of 0.29. These data, in addition, confirm that lutein is distributed uniformly during premix dosing, and that a 2 g test sample is representative of the bulk oil.

### 3.3. Lutein recovery in manufactured paediatric formulae

In view of the limited evidence for its protective role in the development of the infant retina, lutein is currently added to specific paediatric formulae to attain plasma levels comparable with those of breast-fed infants. However, as a labile carotenoid, lutein is considered to be vulnerable to the processing conditions that are typically utilised during paediatric formula manufacture (Franke et al., 2010). Few studies have investigated the stability of lutein under such specific processing conditions, although a single report has indicated minor losses during thermal fluid consumer milk manufacture (Nozière et al., 2006). The present study therefore investigated the recovery and stability of lutein during passage through the industrial production of typical lutein-supplemented milk-based paediatric formulae.

An initial production-scale study examined both the homogeneity and the stability of lutein contained in a batch of doped vegetable oil blend, and replicate analysis immediately following supplementation yielded a lutein content of  $5.62 \mu\text{g g}^{-1}$  and an  $\text{RSD}_r$  of 0.58% ( $n = 5$ ). The lutein levels in this oil were continuously monitored at intervals over the 12 h of its incorporation into paediatric product, yielding an overall lutein content of  $5.64 \mu\text{g g}^{-1}$  and an  $\text{RSD}_r$  of 0.72% ( $n = 5$ ). These observations confirm both the effective distribution and the stability of lutein over the duration of a production-scale oil batch. In a further study of the stability of lutein in oil over time, a second discrete production-scale oil blend was dosed with lutein at the higher level of  $7.0 \mu\text{g g}^{-1}$  and the datasets for both oils are illustrated graphically in Figure 2.

Spray-dried paediatric formula was manufactured over time from a single discrete lutein-dosed oil blend and, as a closed system, there is a discrete and measurable residence time through the manufacturing process. The recovery of lutein was therefore quantitatively estimated from the measured content in the final product expressed as a proportion of that predicted from the measured content in the dosed oil blend and oil blend addition rate. These data across the manufacture of two independent paediatric formulae (A, B) containing different lutein levels are presented in Table 1.

Both formula products were manufactured with targeted lutein levels close to the higher range of human breast milk (Capeding et al., 2010). The data illustrated in Table 1 confirm that lutein is stable during the manufacture of spray-dried paediatric formula and is recovered quantitatively in the final powdered product under the described manufacturing conditions. These observations are consistent with independently reported studies of the kinetics of carotenoid degradation in oil systems, confirming that lutein has enhanced stability relative to  $\beta$ -carotene and lycopene, and that stability is dependent on the degree of unsaturation and the tocopherol content of the vegetable oil (Henry et al., 1998; Lavecchia & Zuorro, 2008).

## 4. Conclusions

In view of the available evidence for the role of lutein in the visual development of infants, this nutrient is increasingly being regarded as an important supplemental component of bovine milk-based paediatric formulae. This study presents an analytical method for the in-process quantification of lutein in its oil-based supplement form and the method is sufficiently simple to facilitate real-time in-process control. The method has been applied to the evaluation of lutein recovery in final product through the manufacturing process, with the evidence confirming insignificant loss.

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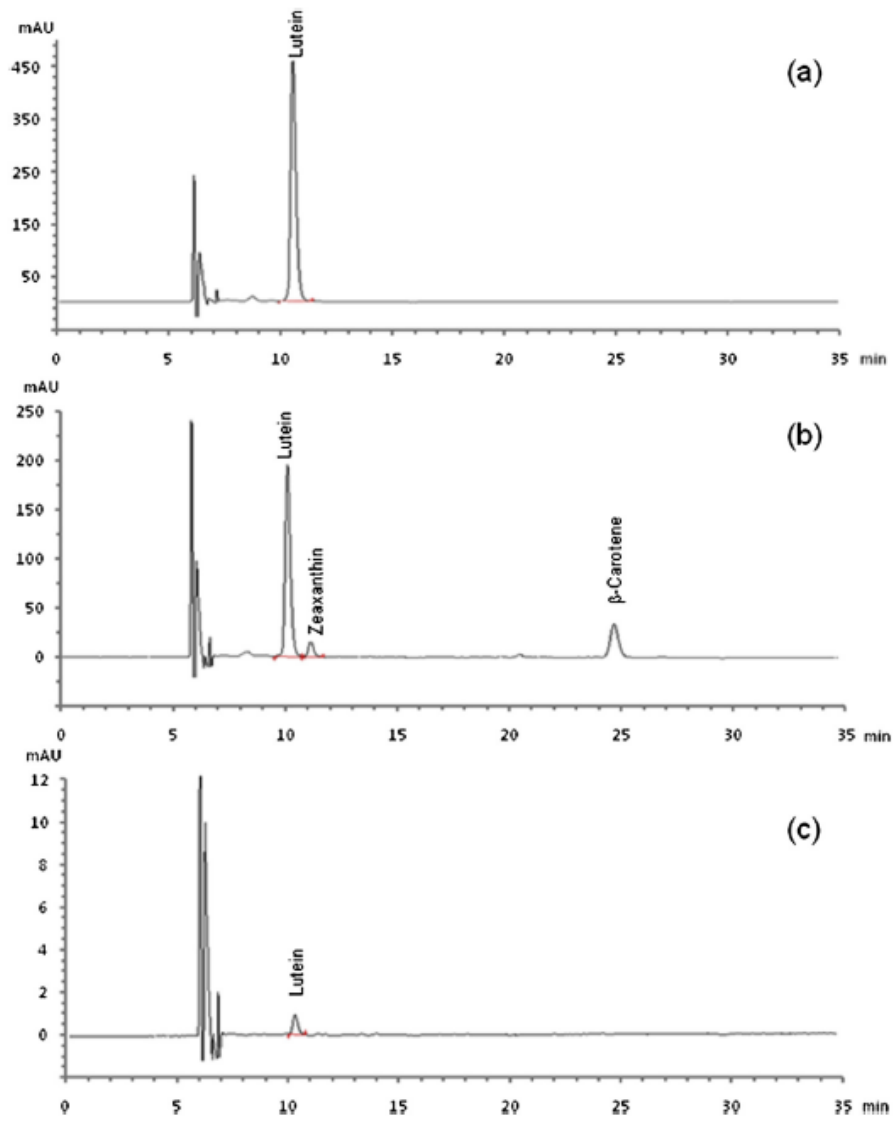
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**Table 1. Recovery of lutein during manufacture of spray-dried paediatric formula**

Sample <sup>a</sup>	Lutein concentration ( $\mu\text{g hg}^{-1}$ )			Recovery (%)
	Oil (measured)	Product (theory)	Product (measured)	
A1	701	156.1	151.0	96.7
A2	700	155.9	149.0	95.6
A3	700	155.9	151.0	96.9
A4	691	153.9	158.0	102.7
A5	703	156.6	153.0	97.7
A6	705	157.0	152.0	96.8
A7	681	151.7	150.0	98.9
B1	562	132.0	134.0	101.5
B2	573	134.6	134.0	99.6
B3	562	132.0	136.0	103.0
B4	566	133.0	135.0	101.5
B5	564	132.5	138.0	104.2
B6	564	132.5	137.0	103.4
B6	563	132.2	134.0	101.3

<sup>a</sup> A and B refer to two independently manufactured paediatric formulae containing different levels of lutein. Each carotenoid-doped oil and finished product was analysed for lutein content over 12 h continuous manufacture ( $n = 7$ ).



**Figure 1.** Chromatography obtained under described conditions. Column was YMC C<sub>30</sub> (250 × 4.6 mm, 3 μm); mobile phase, methanol:dichloromethane (70:30, v/v) at 0.5 mL min<sup>-1</sup>; detection, 450 nm. Samples were (a) authentic lutein standard; (b) carotenoid-dosed oil blend; (c) undosed oil blend

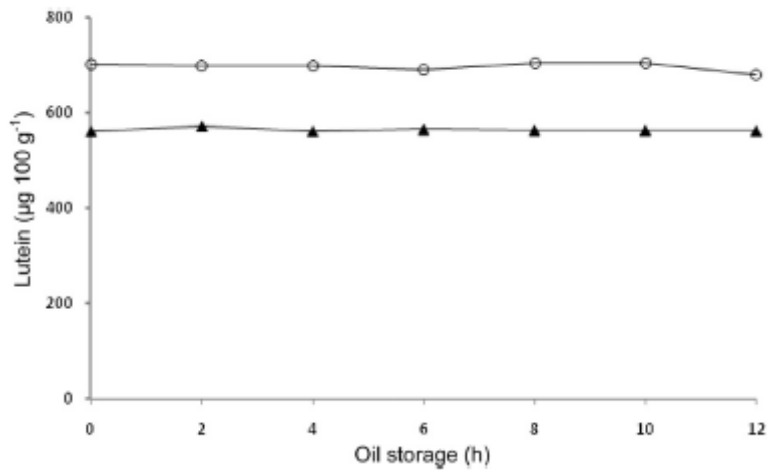


Figure 2. Stability of lutein in carotenoid-dosed oil blend over time (12 h). Lutein content was quantified by HPLC-UV as described. Oil blend was dosed to (○) 7.0  $\mu\text{g g}^{-1}$  or (▲) 5.6  $\mu\text{g g}^{-1}$