Quantitation of Vitamin K in Milk Products by Pre-column Reduction HPLC–Fluorescence

Harvey E. Indyk[1*], Brendon D. Gill[1], Shane Wei[1], Lisa Harvey[1], David C. Woollard[2]

[1] Fonterra Co-operative Group Ltd, P.O. Box 7, Waitoa 3380, New Zealand

[2] Hill Laboratories, Private Bag 3205, Hamilton 3240, New Zealand

* Corresponding author

Abstract

Vitamin K is a dietary component that, as a cofactor, has critically important physiological roles in the body. As it is present in milk at very low levels, it is fortified at higher levels in paediatric milk-based formulae. Currently, the predominant analytical strategy for both food database surveys and routine compliance testing is high-performance liquid chromatography with post-column zinc reduction and fluorescence detection. This study presents a single-laboratory validation of a pre-column reduction modification that provides enhanced sensitivity (> 2×) and increased analytical throughput. Accuracy was confirmed by analysis of a certified reference infant formula and milk proficiency samples, and by comparison against the reference method for a wide range of dairy products containing vitamin K_1 at both natural and supplemented levels. The method offers several performance advantages and is suitable for routine product compliance release testing.

Keywords:

Vitamin K, High-performance liquid chromatography, Fluorescence, Milk

Introduction

Vitamin K is a fat-soluble vitamin consisting of both natural phylloquinone (K_1) and menaquinones (MK_n), and synthetic menadione (K_3) forms, all of which contain a common 2-methyl-1,4-naphthoquinone nucleus. Naturally occurring vitamin K congeners exist exclusively in the biologically active 2'-trans configuration, whereas synthetically produced vitamin K_1 typically contains

Indyk, H.E.; Gill, B.D.; Wei, S. Harvey, L.; Woollard, D.C. (2021) *Food Analytical Methods* 14, 984–988. doi.org/10.1007/s12161-020-01922-0

a minor proportion of the biologically inactive 2′-cis isomer. The predominant form of vitamin K in the human diet is K_1, which is derived principally from green leafy vegetables and several plant oils, with a minor contribution of 2′,3′-dihydro-K_1 from partially hydrogenated oils, whereas, with the exception of MK_4, MK_n are derived from both fermented foods and colon microflora. The comparative structures, sources, bioactivities, physiological functions and recommended dietary intakes have been reviewed comprehensively (Booth 2009; EFSA2017; Eitenmiller et al. 2008; Gröber et al. 2015; Hamidi and Cheung 2014; Indyk et al. 2003; Shearer et al. 2012; Shearer and Okano 2018).

Nutritionally significant amounts of K_1 are present at natural levels in a wide range of foods, with dairy products considered to be an important source within a balanced diet (Fu et al. 2017; Schurgers and Vermeer 2000). In fact, bovine milk is known to contain comparable endogenous levels of both K_1 and MK_4, whereas fermented dairy products such as cheese and yoghurt also contain significant levels of higher menaquinones ($MK_{8–10}$) (Booth 2012; Fu et al. 2017; Indyk and Woollard 1997; Koivu-Tikkanen et al. 2000; Schurgers and Vermeer 2000;Woollard et al. 2002). Infant formulae are the only milk-based foods that are routinely supplemented with synthetic K_1 to comply with international regulatory requirements that are intended to protect the newborn prophylactically; in this context, it is notable that the low K_1 content of breast milk is a recognised neonatal risk factor (Indyk et al. 2003; Shearer et al. 2012; Thijssen et al. 2002).

Currently, the analytical technique that is utilised predominantly for the estimation of the vitamin K content of foods, including milk and paediatric formulae, is high-performance liquid chromatography with fluorescence detection (HPLC-Fl), although electrochemical redox methods and, more recently, tandem mass spectrometry are viable detection alternatives (Card et al. 2009; Eitenmiller et al. 2008; Fanali et al. 2017; Gentili et al. 2016; Indyk and Woollard 1997, 2000; Karl et al. 2014; Koivu-Tikkanen et al. 2000; Schurgers and Vermeer 2000; Woollard et al. 2002; Zhang et al. 2019). HPLC-Fl is implemented utilising post-column zinc reduction of K_1 to its fluorescent hydroquinone derivative (K_1H_2), in preference to sodium borohydride or platinum reduction alternatives. The predominant chromatographic mode prescribed is reversed-phase exploiting C_{18} column chemistry, which facilitates quantitation of the *cis*- and *trans*-K_1 isomers present in supplemented paediatric formulations as a single peak. Alternatively, the separate quantitation of these isomers can be accomplished utilising either a C_{30} phase under reversed-phase conditions or unmodified silica under normal-phase conditions (Cook et al. 1999; Schimpf et al. 2019; Woollard et al. 2002).

Stannous chloride has been reported to be an alternative reductant in a non-chromatographic spectrofluorimetric assay and in the only example of a pre-column reduction HPLC-Fl method for vitamin K congeners in plasma (Ahmed and Mahmoud 2015; Nevado and Laguna 1998). Pre-column reduction may confer both performance and environmental advantages in comparison with the

conventional post-column HPLC-Fl format. Thus, the aim of this study was to investigate its potential to enhance the routine product compliance testing of the K_1 content in milk products.

Materials and Methods

Vitamin K was extracted from samples with hexane, subsequent to enzymatic lipid digestion with lipase and precipitation of fatty acids; details of apparatus, reagents, vitamin K standards and sample preparation were as previously reported (AOAC 2019; Indyk and Woollard 2000). The LC system (Nexera X2, Shimadzu, Kyoto, Japan) was equipped with an Ascentis Express C_{18} end-capped, fused-core particle analytical column (5 µm, 150 × 4.6 mm, Merck, Darmstadt, Germany). Under the conventional reference method post-column reduction conditions, isocratic elution was implemented with mobile phase A (methanol, 900 mL; dichloromethane, 100 mL; methanol, 5 mL containing zinc chloride, 1.37 g; sodium acetate, 0.41 g; acetic acid, 0.30 g) and reduction was accomplished with a stainless steel assembly (20 × 4 mm) dry packed with zinc powder (< 60 µm) that was configured between the column and the detector. The injection volume was 50 µL, fluorescence detection at λ_{ex} = 243 nm and λ_{em} = 430 nm and quantitation of the K_1 content of a sample was achieved by interpolation of a multi-level (0–40 ng mL^{-1}) external standard calibration. Under the alternative pre-column reduction conditions evaluated, the zinc column assembly was positioned between the injector and the analytical column and the mobile phase was modified for optimised separation; all other operating conditions were unchanged. Pre-column reduction experiments were also performed in an off-line mode with either zinc or stannous chloride.

The estimated vitamin K_1 contents of prepared sample extracts were compared by both the candidate pre-column and the reference post-column zinc reduction strategies, with method accuracy further evaluated by analysis of a certified infant formula reference material (NIST SRM 1849a, Gaithersburg, MD, USA) and two international proficiency milk powder samples (NurtureChek, Global Proficiency, Hamilton, New Zealand). Routine milk, dairy and infant formula powders were obtained from local production (Fonterra Co-operative Group Ltd., New Zealand) and a representative infant formula powder was used to determine assay stability and intermediate precision.

Results and Discussion

Method Development

Preliminary investigations of an off-line reduction strategy were based on a previously reported study of the reductive efficiency of stannous chloride compared with either sodium borohydride or zinc metal reagents; this study reported that stannous chloride yielded higher fluorescence intensity for reduced K_1, MK_4 and MK_7 standards (Ahmed and Mahmoud 2015). In the present study, both stannous

chloride (in either ethanolic hydrochloric acid or ethanolic glycerol solution, 50 mg mL^{-1}) and zinc powder were evaluated for their ability to effect the reduction of multi-level K_1 standards and sample extracts, achieved off-line in glass vials (10 min incubation at ambient temperature). Subsequent HPLC separation and fluorescence detection of the K_1H_2 derivative demonstrated equivalent reduction efficiencies for both stannous chloride and zinc reagents, yielding equivalent calibration linear regression slopes. However, when applied to infant formula sample extracts, these off-line reduction strategies yielded inconsistent quantitative K_1 results; consequently, further studies targeted on-line reduction techniques.

The comparative performances of on-line pre- and post-column reduction formats using the dry-packed zinc column positioned either before or after the analytical column respectively were evaluated. These trials were initially conducted under identical elution (mobile phase A) and detection conditions, with chromatography illustrated in Fig. 1.

Pre-column-reduced K_1H_2 eluted with a significantly shorter retention time than K_1 under post-column format conditions, consistent with the increased polarity of the hydroquinone structure. Nonetheless, peak areas and linear regression slopes for calibration standards and the K_1 contents estimated for a range of paediatric formula extracts were demonstrated to be statistically equivalent under both instrument configurations. Further, the equivalence in fluorescence response for equimolar K_1 calibration standards obtained for both reduction formats under identical mobile phase A eluent conditions illustrates the capacity of the zinc column to effect reduction of the maximum on-column injection of approximately 2 ng of K_1.

Under pre-column conditions, a progressive reduction in the dichloromethane content of the mobile phase was found to moderately increase the retention of K_1H_2, facilitating improved resolution from early-eluting peaks. These trials demonstrated that a modified mobile phase A containing no dichloromethane yielded optimal retention and resolution, as well as a significant increase, by a factor of > 2, in fluorescence response as revealed in the slopes of typical linear regression calibration curves (y = 99,108x versus y = 45,297x), thereby enhancing the sensitivity of the assay compared with the conventional post-column configuration. The significant improvement in assay sensitivity accruing from the removal of dichloromethane was due to the well-known electron-withdrawing properties of chlorinated organic solvents, which effectively quench the fluorescence of dissolved analytes (Bolinova et al. 2014).

The optimised pre-column reduction protocol was also effective in resolving the MK_4 and 2′,3′-dihydro-K_1 congeners that may be present, with their retention times relative to K_1 consistent with conventional post-column reduction methodology. However, and as previously reported, the C_{18}

analytical column does not resolve the *cis*- and *trans*-isomers in either reduction format, thereby facilitating quantitation of total K_1 as a single aggregated peak.

Method Validation

This study describes a pre-column reduction modification to the chromatographic protocol prescribed in the reference method that was previously validated through an international multi-laboratory collaborative study (AOAC 2019; Indyk and Woollard 2000). Sample preparation, incorporating enzymatic digestion with lipase in phosphate buffer, extraction with hexane and reconstitution in methanol, was implemented without modification in the present study. It was therefore appropriate to evaluate those performance parameters that were specifically associated with the modified chromatographic procedure.

Detector linearity was established over independent days (n = 10) by least-squares linear regression of multi-level K_1 calibration curves (0–40 ng mL^{-1}) for both pre- and post-column reduction, with both method variants yielding mean correlation coefficients (r^2) > 0.9995 and F-test *p*-values > 0.05, confirming an absence of residual heteroscedasticity.

The method detection limit (MDL) was estimated from the precision of the complete method applied to a sample containing low levels of analyte (Su 1998) and was estimated for K_1 by replicate analysis (n = 7) of a whole milk powder containing endogenous levels. The estimated MDL values for pre- and post-column reduction method variants were 0.53 and 0.26 μg hg^{-1}, respectively, confirming that both analytical techniques may be applied to infant formulae that contain K_1 at supplemental levels approximately two orders of magnitude higher.

Within-run repeatability (RSD_r) was estimated by the analysis of seven replicates of an infant formula that was used as a quality control sample and measured 3.28 and 3.81% for pre- and post-column formats respectively. Between-run intermediate precision was assessed with replicates of the same quality control sample over 10 days (n = 24) and yielded RSD_{iR} values of 3.61 and 3.49% for pre- and post-column formats respectively.

Method bias was evaluated by duplicate analyses of NIST SRM 1849a and two NurtureChek proficiency milk powder samples. For NIST 1849a, the mean results for the pre- and post-column reduction variants were 105.9 and 101.8 μg hg^{-1} respectively, compared with the certified value and expanded uncertainty of 106 ± 17 μg hg^{-1}. For the two proficiency samples, pre-column reduction yielded 80.6 and 49.2 μg hg^{-1} and post-column reduction yielded 75.2 and 43.9 μg hg^{-1}, results that were consistent with the assigned values of 76.7 ± 7.7 and 47.1 ± 3.9 μg hg^{-1}. These data confirm an absence of bias as estimated against samples containing known concentrations of K_1.

The accuracy and potential analytical bias of the proposed pre-column reduction technique was further evaluated by direct comparison against the commonly used reference post-column reduction method (AOAC 2019; Indyk and Woollard 2000) A paired *t*-test applied to multiple between-run replicate data for the infant formula quality control sample (n = 24) demonstrated an absence of bias between the two reduction strategies ($p = 0.384$, $\alpha = 0.05$). The comparative analytical performance of the two techniques was also assessed by the routine compliance release analysis of 70 diverse dairy product samples (paediatric formulae, whole milk, skim milk and buttermilk powders, certified reference dairy powders and liquid UHT milk), containing a wide range of K_1 concentrations (< 1–100 μg hg^{-1}) representing either endogenous or supplemental levels, as presented in Table 1. A paired *t*-test result applied to the entire data set ($p < 0.01$, $\alpha = 0.05$) indicated a statistical bias, although the mean difference for all samples was < 5.0%. In addition, the almost complete overlap of confidence intervals ($\alpha = 0.05$) around the two population means suggests that the null was not rejected, and that any bias was of negligible practical significance. The wide range of K_1 content across this sample set also allowed data examination by linear regression, yielding good correlation between the two method variants ($y = 1.026x + 0.159$, $r^2 = 0.9969$). Further, a Bland-Altman plot demonstrated an equal distribution around zero difference, suggesting an absence of systematic bias across the concentration range, as illustrated in Fig. 2.

Although reliable LC-MS techniques have recently become available, and with electrochemical redox detection methods being less favoured, HPLC-Fl remains the predominant platform for analysis of the K_1 content of both clinical and food samples. It is also apparent that, amongst the many HPLC-Fl methods reported, the reduction of K_1 is accomplished exclusively by the post-column technique, although an early study reported the enhanced reduction efficiency of zinc, in the presence of zinc ions, in both pre- and post-column modes (Haroon et al. 1987). Indeed, the reference post-column reduction HPLC-Fl method has been successfully implemented internationally for approximately 20 years for the routine compliance testing of milk-based paediatric formulae. The proposed method variant, based on a pre-column reduction modification, has been demonstrated in this single laboratory study to provide an equivalent quantitative estimate of the K_1 content in a wide range of dairy products, and provides several advantages including (i) a faster chromatographic cycle time, (ii) a reduced analyte elution volume, (iii) significantly enhanced assay sensitivity and (iv) a “greener” eluent composition.

Conclusions

An HPLC-Fl method, incorporating a pre-column reduction modification of the conventional reference post-column reduction protocol, has been single-laboratory validated. The technique provides several performance advantages, and is appropriate for use in high-throughput routine compliance testing of

the K_1 content of manufactured dairy foods, including both supplemented and non-supplemented nutritional products.

Compliance with Ethical Standards

Conflict of Interest: Harvey Indyk declares that he has no conflict of interest. Brendon Gill declares that he has no conflict of interest. Shane Wei declares that he has no conflict of interest. isa Harvey declares that she has no conflict of interest. David Woollard declares that he has no conflict of interest.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent: Not applicable.

References

Ahmed S, Mahmoud AM (2015) A novel salting-out assisted extraction coupled with HPLC-fluorescence detection for trace determination of vitamin K homologues in human plasma. Talanta 144:480–487

AOAC (2019) AOAC Official Method 999.15. Vitamin K in milk and infant formulas, liquid chromatographic method. In: Official methods of analysis of AOAC International, 21st edn. AOAC, Gaithersburg

Bolinova P, Šrámková I, Sklenarova H, Acebal CC, Fernandez Band BS, Solich P (2014) A study of the effect of organic solvents on thecfluorescence signal in a sequential injection analysis system. Anal Methods 6:9392–9396

Booth SL (2009) Roles for vitamin K beyond coagulation. Annu Rev Nutr 29:89–110

Booth SL (2012) Vitamin K: food composition and dietary intakes. Food Nutr Res 56:5505. https://doi.org/10.3402/fnr.v56i0.5505

Card DJ, Shearer MJ, Schurgers LJ, Harrington DJ (2009) The external quality assurance of phylloquinone (vitamin K1) analysis in human serum. Biomed Chromatogr 23:1276–1282

Cook KK, Mitchell GV, Grundel E, Rader JI (1999) HPLC analysis for *trans*-vitamin K1 and dihydro-vitamin K_1 in margarines and margarine-like products using the C_{30} stationary phase. Food Chem 67:79–88

EFSA European Food Safety Authority (2017) Dietary reference values for vitamin K. EFSA J 15:4780. https://doi.org/10.2903/j.efsa.2017. 4780

Eitenmiller RR, Ye L, Landen WO Jr (2008) Vitamin K. In: Vitamin analysis for the health and food sciences, 2nd edn. CRC Press, Boca Raton, pp 193–227

Fanali C, D'Orazio G, Fanali S, Gentili A (2017) Advanced analytical techniques for fat-soluble vitamin analysis. Trends Anal Chem 87: 82–87

Fu X, Harshman SG, Shen X, Haytowitz DB, Karl JP, Wolfe BE, Booth SL (2017) Multiple vitamin K forms exist in dairy foods. Curr Dev Nutr 1:e000638. https://doi.org/10.3945/cdn.117.000638

Gentili A, Miccheli A, Tomai P, Baldassarre ME, Curini R, Pérez- Fernández V (2016) Liquid chromatography–tandem mass spectrometry method for the determination of vitamin K homologues in human milk after overnight cold saponification. J Food Comp Anal 47:21–30

Gröber U, Reichrath J, Holick MF, Kisters K (2015) Vitamin K: an old vitamin in a new perspective. Dermatoendocrinol 6:e968490. https://doi.org/10.4161/19381972.2014.968490

Hamidi MS, Cheung AM (2014) Vitamin K and musculoskeletal health in postmenopausal women. Mol Nutr Food Res 58:1647–1657

Haroon Y, Bacon DS, Sadowski JA (1987) Chemical reduction system for the detection of phylloquinone (vitamin K_1) and menaquinones (vitamin K_2). J Chromatogr 384:383–389

Indyk HE, Woollard DC (1997) Vitamin K in milk and infant formulas: determination and distribution of phylloquinone and menaquinone-4. Analyst 122:465–469

Indyk HE, Woollard DC (2000) Determination of vitamin K in milk and infant formulas by liquid chromatography: collaborative study. J AOAC Int 83:121–130

Indyk HE, Shearer MJ, Woollard DC (2003) Vitamin K: properties and determination. In: Caballero B, Finglas P, Toldra F (eds) Encyclopedia of food sciences and nutrition, vol 4, 2nd edn. Academic press, London, pp 6032–6038

Karl JP, Fu X, Dolnikowski GG, Saltzman E, Booth SL (2014) Quantification of phylloquinone and menaquinones in feces, serum, and food by high-performance liquid chromatography-mass spectrometry. J Chromatogr B 963:128–133

Koivu-Tikkanen TJ, Ollilainen V, Piironen VI (2000) Determination of phylloquinone and menaquinones in animal products with fluorescence detection after post-column reduction with metallic zinc. J Agric Food Chem 48:6325–6331

Nevado JJB, Laguna MAG (1998) Spectrofluorimetric determination of vitamin K_3. Analyst 123:287–290

Schimpf KJ, Thompson LDB, Pan S-J (2019) Determination of *trans* and total vitamin K_1 in infant, pediatric, and adult nutritionals by HPLC with post column reduction and fluorescence detection: multi-laboratory testing study, AOAC Final Action 2015.09. J AOAC Int 102:222–232

Schurgers LJ, Vermeer C (2000) Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations. Haemostasis 30:298–307

Shearer MJ, Okano T (2018) Key pathways and regulators of vitamin K function and intermediary metabolism. Annu Rev Nutr 38:127–151

Shearer MJ, Fu X, Booth SL (2012) Vitamin K nutrition, metabolism, and requirements: current concepts and future research. Adv Nutr 3: 182–195

Su GCC (1998) A comparison of statistical and empirical detection limits. J AOAC Int 81:105–110

Thijssen HHW, Drittij M-J, Vermeer C, Schoffelen E (2002) Menaquinone-4 in breast milk is derived from dietary phylloquinone. Br J Nutr 87:219–226

Woollard DC, Indyk HE, Fong BY, Cook KK (2002) Determination of vitamin K_1 isomers in foods by liquid chromatography with C_{30} bonded-phase column. J AOAC Int 85:682–691

Zhang Y, Bala V, Mao Z, Chhonker YS, Murry DJ (2019) A concise review of quantification methods for determination of vitamin K in various biological matrices. J Pharm Biomed Anal 169:133–141

Table 1. Comparison of vitamin K_1 content of dairy products by precolumn and post-column reduction methods (μg hg^{-1})

Sample[a]	n	Pre-column	Post-column
Fluid milk[b]	2	23.1 (1.11)	20.8 (1.23)
Whole milk[c]	16	4.5 (0.49)	4.0 (0.40)
Skim milk[c]	2	0.2 (0.02)	0.2 (0.02)
Buttermilk[c]	2	1.0 (0.09)	1.8 (0.12)
Infant formula[b]	24	52.4 (1.73)	52.1 (1.82)
Follow on-GUMP[b]	20	60.4 (6.76)	58.8 (7.36)
NIST 1849a[b]	2	105.9 (3.32)	101.8 (3.75)

Vitamin K_1 values as mean, with standard deviation in parentheses

[a] All products are powders except fluid milk

[b] Fortified

[c] Unfortified

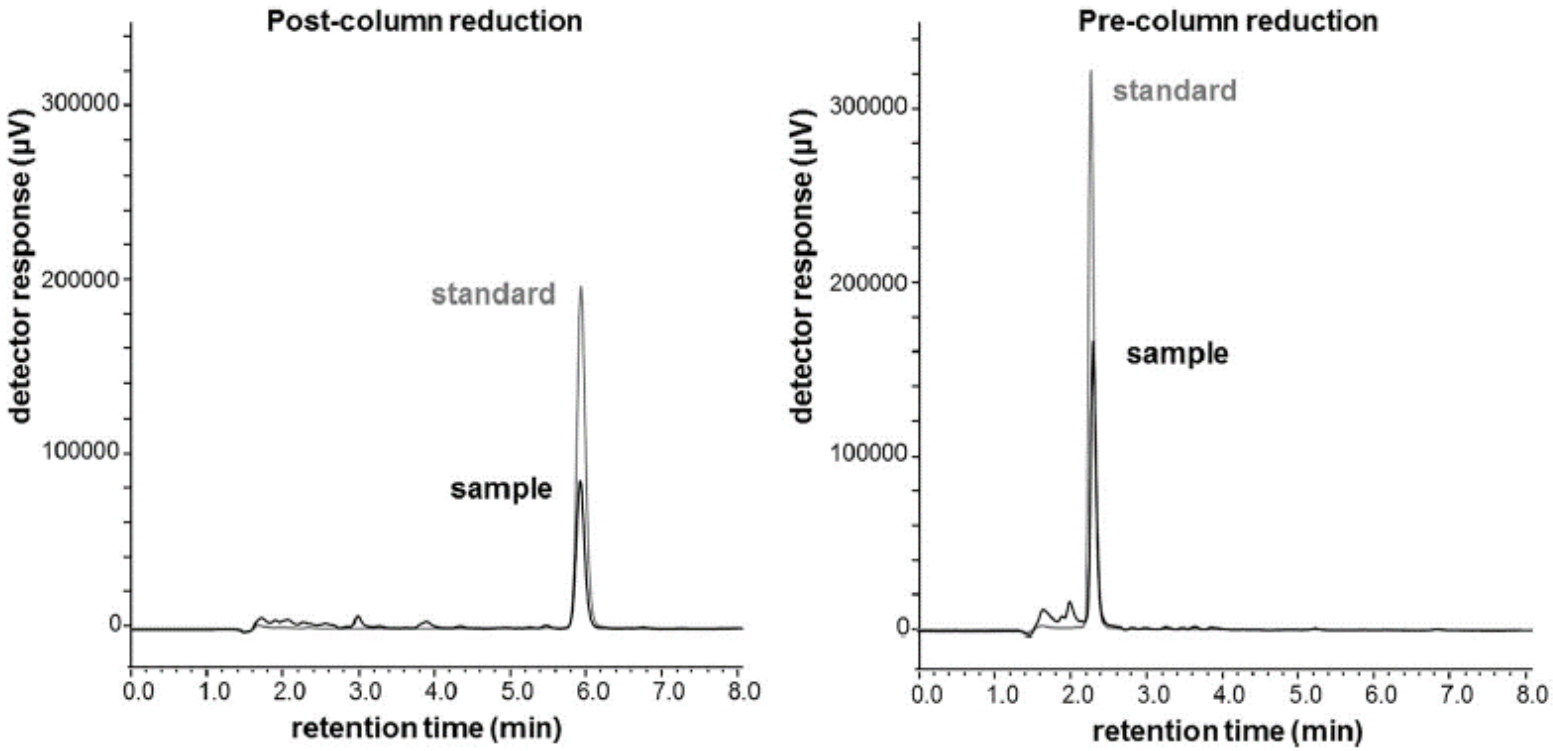

Fig. 1 Superimposed chromatograms of a K_1 calibration standard (45 ng mL^{-1}) and an infant formula sample extract under post-column and pre-column reduction formats. Mobile phase: methanol:dichloromethane 900:100 v/v, incorporating 5 mL of methanol containing 1.37 g of zinc chloride, 0.41 g of sodium acetate and 0.30 g of acetic acid; injection volume: 50 µL; fluorescence detection: λ_{ex} = 243 nm, λ_{em} = 430 nm

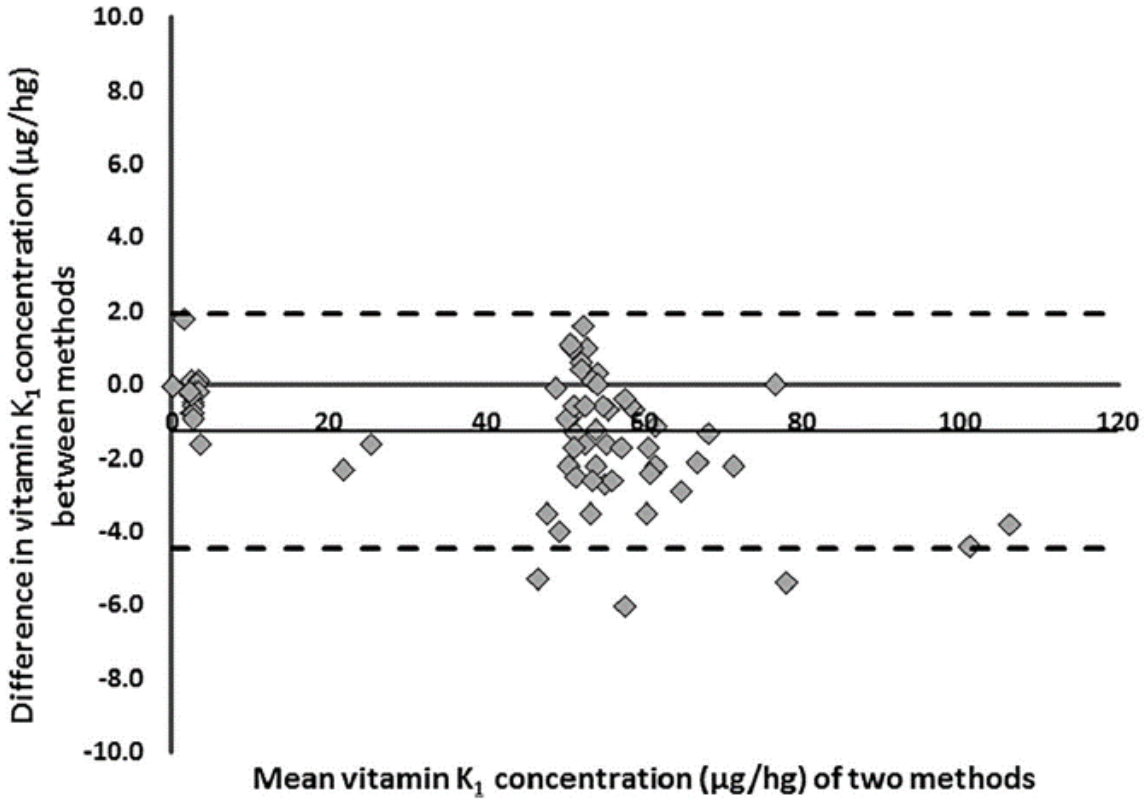

Fig. 2 Bland–Altman plot comparing the pre-column reduction method with the conventional post-column reduction method as a function of K_1 concentration