Simultaneous Measurement of Vitamins A, D, E and K Along with CoQ10 and Carotenoids, in Multivitamin Tablets, Infant Formula and Milk

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Key Words
Carotenoids, Food Supplements, HPLC-ECD, Multivitamins, Retinoids, Tocopheryl Acetate

Goal

To develop a sensitive and selective HPLC-electrochemical detection (ECD) method for the simultaneous measurement of numerous fat-soluble nutrients

Introduction

Consumer interest and regulatory concerns for food supplements, fortified food products and dietary nutrients have stimulated the development of improved analytical methods. Fat-soluble nutrients are of particular interest due to their potential health benefits and, in some cases, toxicity. Accurate, simple techniques for comprehensive analysis is important for product labeling, nutritional research, development and quality control.[1]

Most laboratories use individual methods for a single analyte class. Many methods, for example, include a saponification step prior to extraction to convert multiple forms of a compound to a single analyte and to exclude potentially interfering substances.[2-4] This may lead to rapid degradation and/or production of less stable forms of several analytes of interest.[1,5]

Coulometric array electrochemical detection (ECD) utilizes multiple sensors that can be optimized for more than one chemical class.[6] Resolution is increased by electrochemical screening of possible interfering solutes based on differences in oxidation-reduction behavior.

This Application Note shows the use of the Thermo Scientific™ Dionex™ CoulArray™ Coulometric Array Detector for the measurement of a variety of lipid-soluble nutrients including vitamins A (retinol), D2 (ergocalciferol), D3 (cholecalciferol), E (α- and γ-tocopherol), and K1 (phylloquinone), along with carotenoids (α- and β-carotene), retinoids (retinyl palmitate), tocopheryl acetate, and coenzyme Q10 (ubiquinone) (Figure 1). The analysis of multivitamin tablets, infant formula and milk are given as examples. Since extraction efficiency is highly dependent upon sample matrix the procedures described here are only preliminary and need further investigation.

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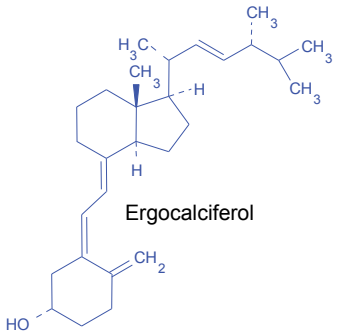
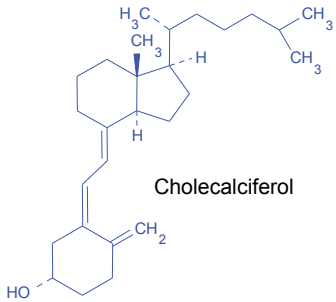


Ergocalciferol



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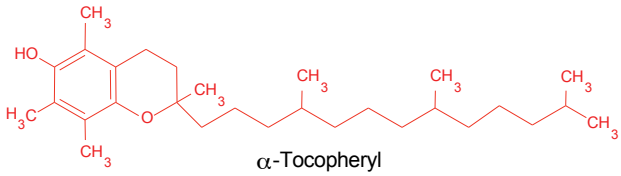
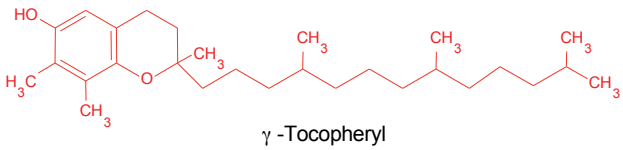


α-Tocopheryl



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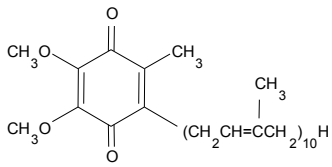
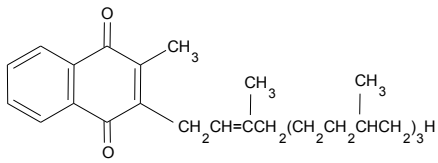
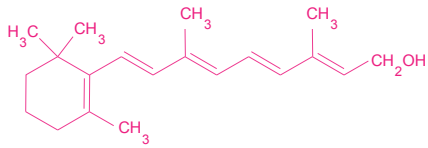


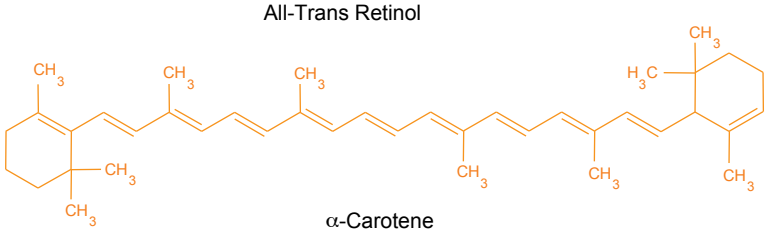
Ubiquinone-10



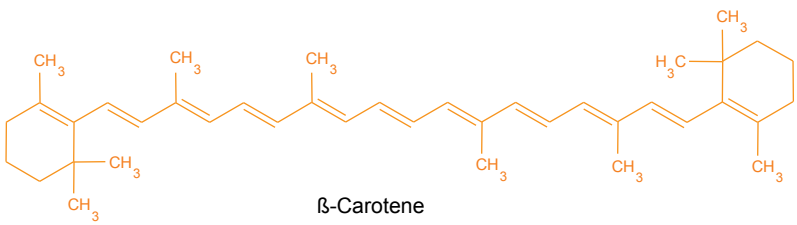
Phylloquinone



All-Trans Retinol



α-Carotene



ß-Carotene

Figure 1. The structure of fat soluble vitamins and nutrients measured using this method.

Materials and Methods

The gradient system consisted of two pumps, an autosampler, a thermal chamber and an eight channel CoulArray detector.

LC Conditions	
Column:	C18 Keystone, 4.6 × .2150 mm, 5 µm
Mobile Phase A:	Acetonitrile-Water, 90:10 (v/v) containing 20 mM Sodium Perchlorate and 5.0 mM Perchloric Acid
Mobile Phase B:	Acetonitrile-1-Propanol, 65:35 (v/v) containing 20 mM Sodium Perchlorate and 10 mM Perchloric Acid.
Gradient Conditions:	20 minutes linear gradient from 10 to 100% B followed by a 5 minutes hold at 100% B before returning to initial conditions for 5 minutes. Total run time was 30 minutes.
Flow Rate:	1.5 mL/min
Temperature:	32 °C
Injection Volume:	Sample dependent
Detector and Conditions	
Detector:	Model 5600A, CoulArray
Applied Potentials:	-700, 100, 250, 400, 550, 750, 800, 850 (vs. Pd)

Standards

Standards were obtained from Sigma-Aldrich® (St. Louis, MO). Stock solutions were made by dissolving approximately 10 mg in 10 mL of ethanol (EtOH) with the exception of the carotenoids and coenzyme Q10. These more lipophilic compounds (ca 1 mg) were dissolved in 5.0 mL hexane followed by dilution with 15 mL ethanol. Solutions were assigned a concentration value, based on molar absorbtivity, before addition of 10 mg/L butylated hydroxyanisole (BHA) as preservative. Stock solutions were stored at -20 °C for up to 6 months. Further dilutions were made weekly in EtOH containing 10 mg/L BHA (diluent) and stored, protected from light at -20 °C.

Sample Preparation

Multivitamin Tablets

A single tablet was powdered and then sonicated in 10 mL hexane for 10 mins. Following the addition of 90 mL EtOH the sample was sonicated for 30 minutes. The solution was centrifuged at 3000 g for 10 minutes. A 25 µL volume was analyzed.

Infant Formula

The concentrate was diluted with water (according to label instructions) and extracted as for unsaponified milk samples below.

Milk Samples (Unsaponified)

A 1.0 mL volume, augmented with 10 µL of 1.0 µg/mL D2 (internal standard), was thoroughly mixed with 3.0 mL diluent and 0.1 g magnesium sulfate. The resulting mixture was extracted 2 times with 4.0 mL hexane. Combined hexane extracts were evaporated under a stream of nitrogen and residue was dissolved in 1.0 mL of diluent. The solution was centrifuged as above.

Milk Samples (Saponified)

A 1.0 mL volume of milk was mixed with 1.75 mL 85% aqueous EtOH containing 75 mg/mL potassium hydroxide and 0.25 mg/mL ascorbic acid. The sample was then placed in a heated water bath for 45 minutes. at 95 °C. Saponified samples were then extracted as for unsaponified milk samples.

Results and Discussion

Multi-component analysis using ECD has previously been limited by its poor compatibility with gradient elution chromatography. Furthermore, since the electrochemical properties of lipid soluble nutrients are very diverse, single channel detectors must be used at potential settings that are suitable for only a few analytes at the expense of others.[7] Coulometric array detection utilizes several high efficiency sensors in series and maintained at different fixed potentials. This enables the concurrent detection of different chemical classes, each at their optimal potential settings. Selectivity is increased through electrochemical screening of possible interfering compounds whose oxidation-reduction behaviors differ from the analytes of interest. Sensor, electronic and software design enables the routine use of gradient elution.

Chromatographic and detector conditions were optimized for the measurement of a wide range of lipid soluble nutrients. Figure 2 shows a 12 component standard mixture. Sensor 1 (not shown) was maintained at -700 mV (vs. Pd) to reduce vitamin K_1 and coenzyme Q10 which were then measured oxidatively on sensors 2 and 3, respectively. Low oxidizing compounds (e.g. tocopherols, retinoids) were oxidatively screened at upstream electrodes and higher oxidizing compounds (calciferols, tocopheryl esters) responded only at higher potentials. Using an array of potentials along the oxidative curve of each analyte allowed generation of response ratios for each peak. Comparison of ratios between authentic standard and unknown sample provided an estimate of the purity of each analyte peak in the samples.

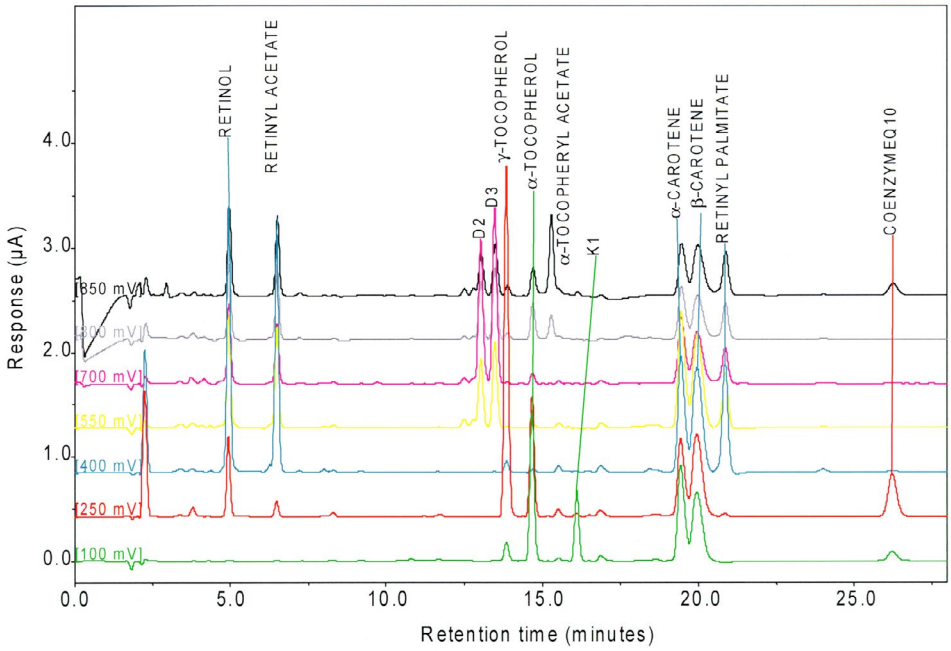


Figure 2. Chromatogram showing the simultaneous measurement of fat-soluble vitamins and nutrients.

Food supplements, fortified food and natural products contain a wide range of fat-soluble nutrients and within a single, often complex, matrix there exists a wide range of analyte levels. Multi-component analysis therefore requires high selectivity, sensitivity and a wide response range. Based on a signal/noise ratio of 5:1 the lod for standard compounds was 4, 9, 8, 7, 5 and 12 pg (on column) for vitamins A, D2, D3, E, and retinyl palmitate, respectively. The assay showed excellent linearity over 4 orders of magnitude for all analytes (e.g., correlation coefficients [0 to 250 ng on column] for vitamins A, D2 and D3, were 0.9988, 0.9992 and 0.9983, respectively).

The analysis of a multivitamin tablet is shown in Figure 3. The simple dissolution technique enabled the measurement of vitamins D and K well above their lower limit of detection. In the same analysis, higher level analytes (retinyl acetate, β-carotene and tocopherols) were detected well within their linear range. Response ratios obtained by comparison of authentic standard and sample peaks demonstrated high peak purity for all analytes.

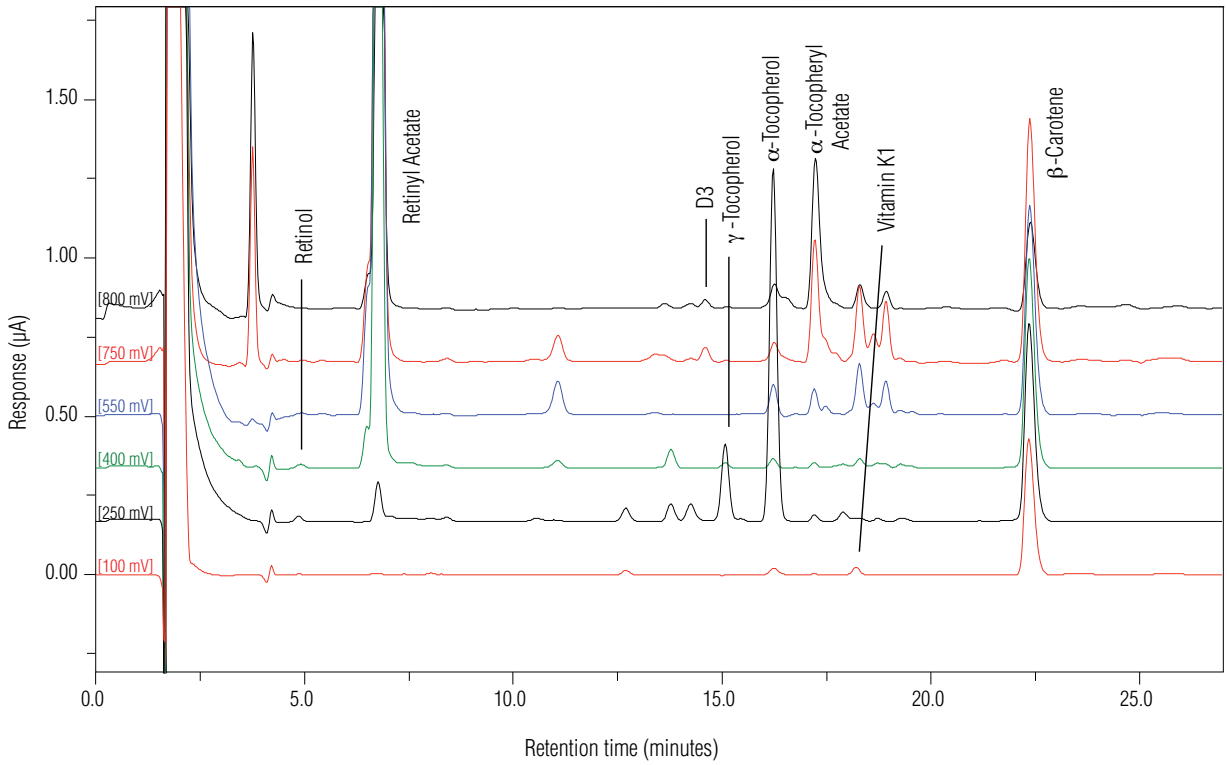


Figure 3. Analysis of a multivitamin tablet.

Infant formula is a complex sample matrix containing low levels of vitamins D and K. Current methods for vitamin D analysis are complex and include a saponification step which can cause the degradation of lipid soluble nutrients (e.g., vitamins E and K, and even vitamin D itself). The feasibility of the analytical method for the measurement of multiple nutrients in infant formula using a simplified extraction method that avoids saponification is illustrated in Figures 4 and 5.

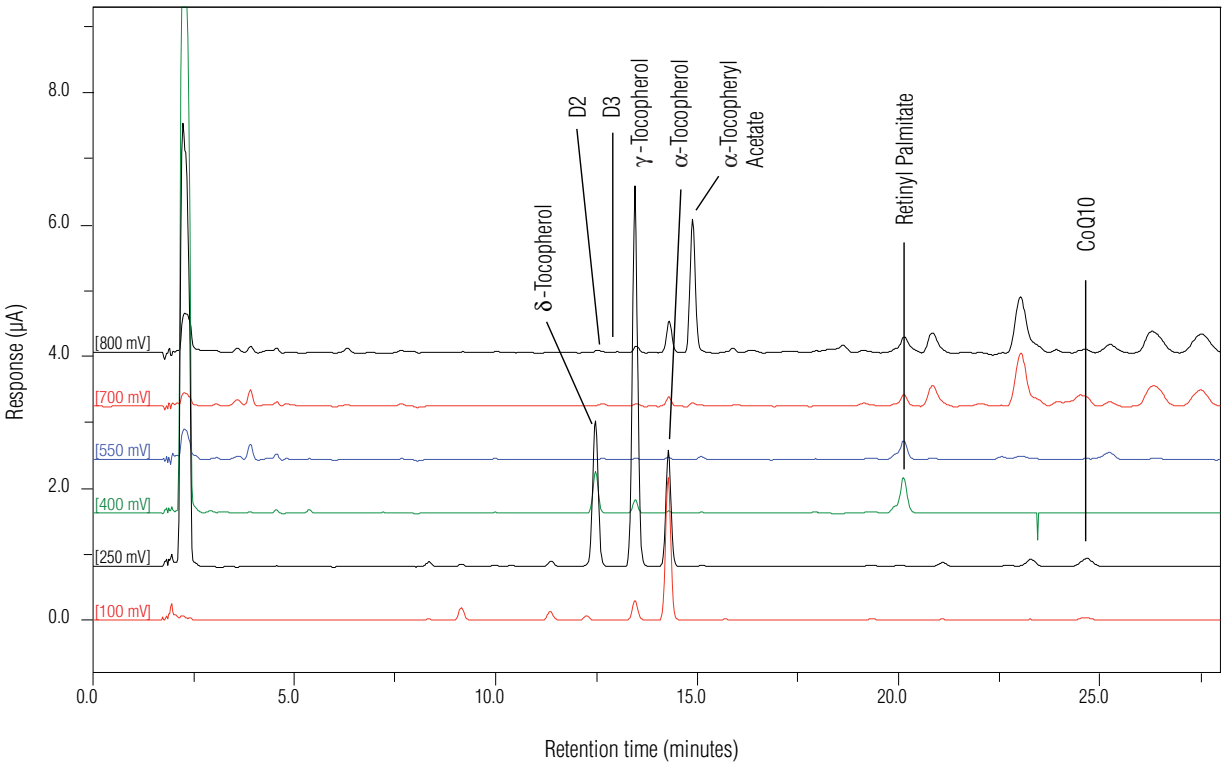


Figure 4. Analysis of infant formula presented at low amplification.

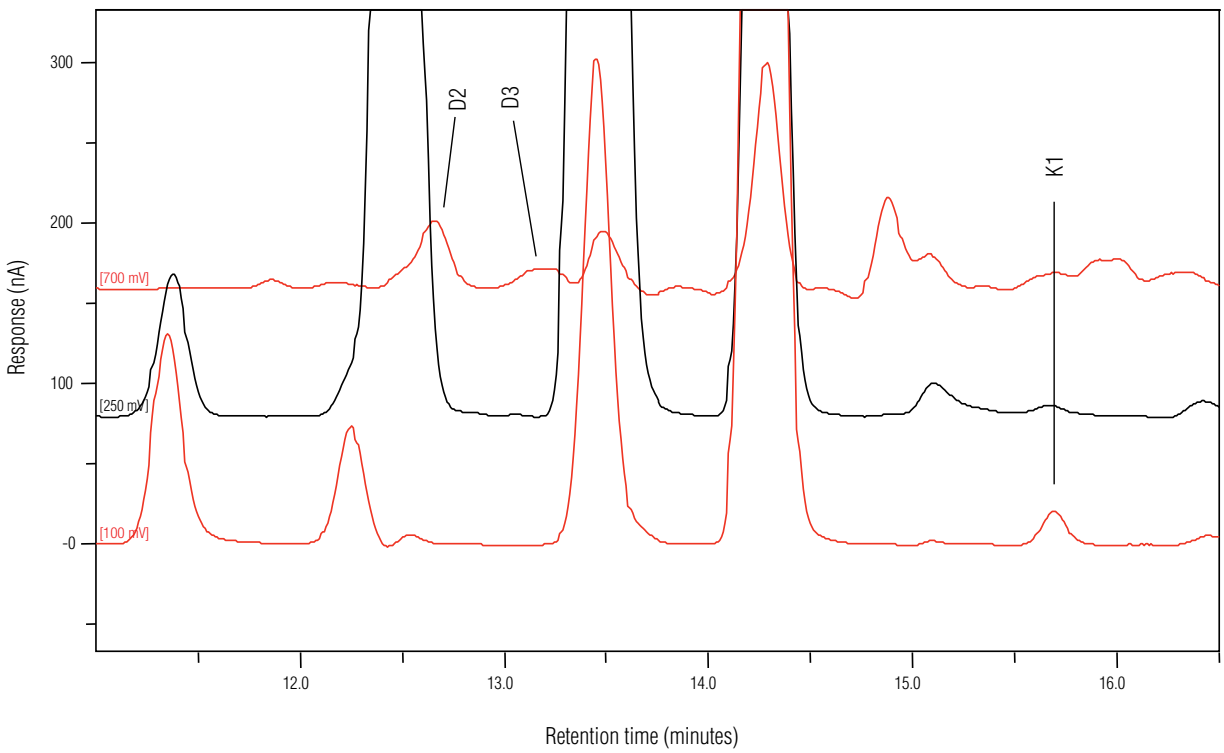


Figure 5. Analysis of infant formula presented at high amplification (approximately 20-fold dilution higher than data presented in Figure 4).

The feasibility of using the analytical method for multi-nutrient analysis, including vitamins A and D, in whole and fortified low fat milk is illustrated in Figures 6 and 7. Figure 6 shows the detection of vitamin A (as retinol), and vitamin D3 (D2 as internal standard) in fortified milk after saponification and extraction.

Figure 7 shows detection of vitamin D, α-tocopherol, γ-tocopherol, α-carotene, β-carotene, retinyl palmitate and coenzyme Q10 in 2% milk in an unsaponified extract. While the saponified extracts are less complex carotenoids, tocopherols and coenzyme Q10 are lost during saponification.

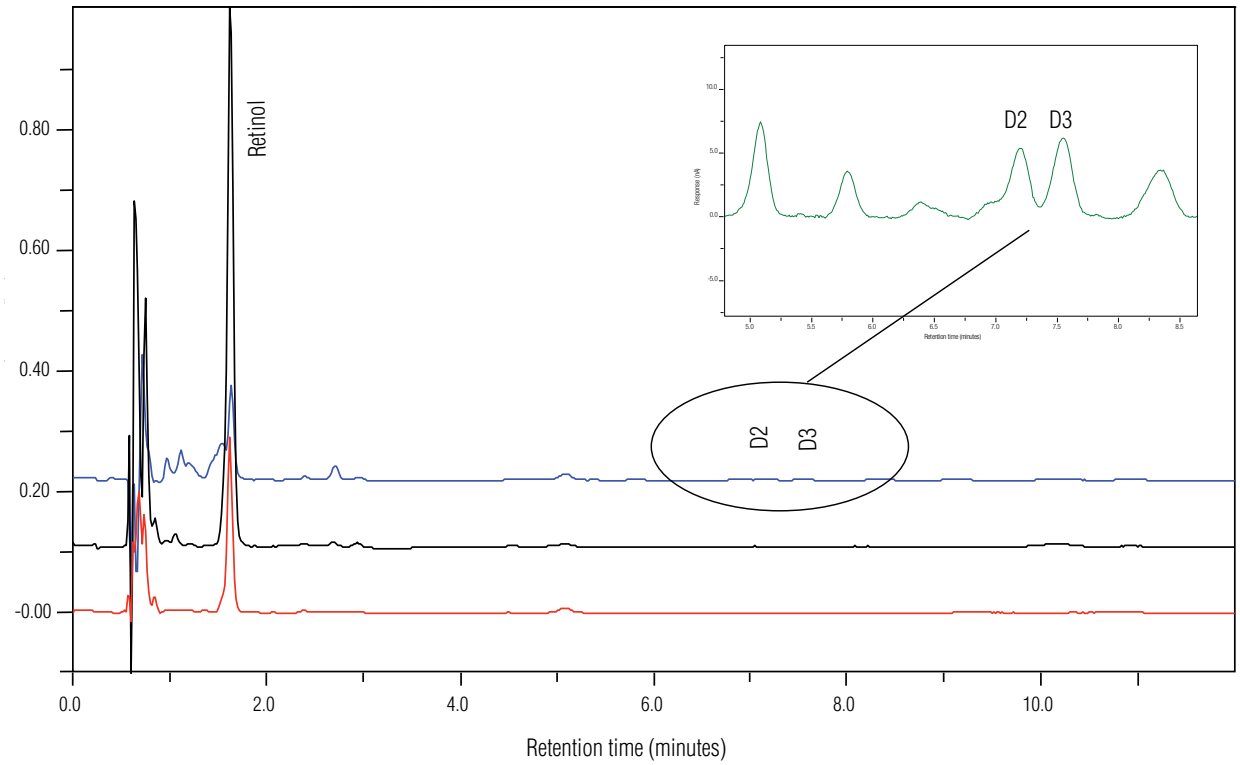


Figure 6. Saponified milk extract. The inset presented at higher amplification shows measurement of Vitamins D2 and D3.

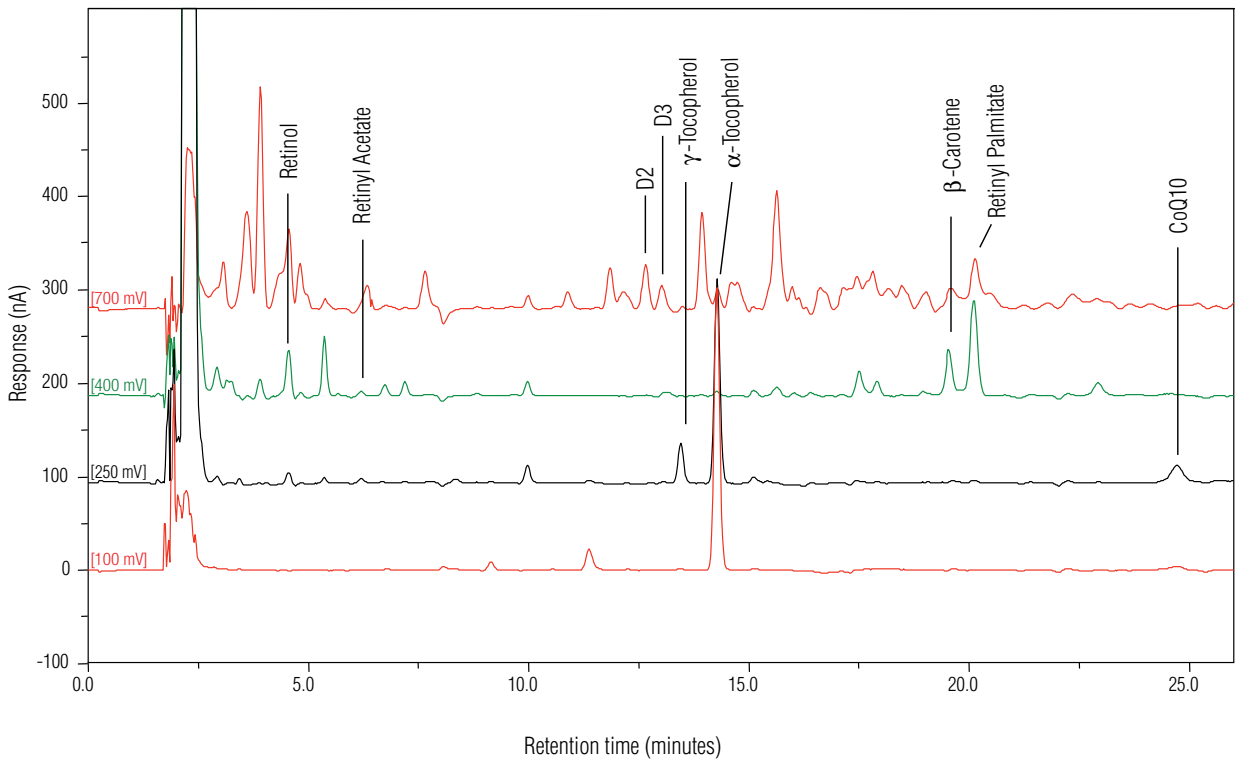


Figure 7. Unsaponified milk extract. Saponificaiton causes the loss of many fat-soluble nutrients as shown in Figure 6.

The absolute recovery of analytes from augmented milk samples ranged from 95 to 105% for all analytes with the exception of carotenoids and retinyl palmitate (data not shown). Preliminary data demonstrate high peak purity, based on response ratios, for most analytes including D3. An overlay of the dominant response channel from 4 'private label' milk samples is shown in Figure 8. These data indicate the feasibility of quantification of vitamin D and several other fat-soluble nutrients in milk with simplified sample preparation.

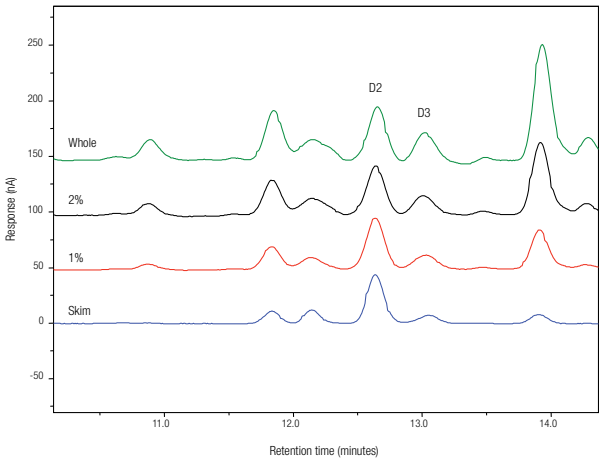


Figure 8. Overlay of unsaponified milk extracts. Comparison of dominant channel 6.

Conclusion

Gradient HPLC with coulometric electrochemical array detection is a powerful approach for the simultaneous measurement of numerous fat-soluble nutrients. Although the sensitivity and selectivity of this technique overcome many of the issues inherent in current analytical methodologies, the ability to use this approach in different foods and supplements requires further examination of sample preparation procedures.

References

1. Curlee, J. (1997) Food Quality, Nov./Dec., 30–35.

2. DeVries, E. J., and Borsie, B. (1982). J. Assoc. Off. Anal. Chem., **65**, 1225–1227.

3. DeVries, E. J., and Borsie, B. (1982) J. Assoc. Off. Anal. Chem., **65**, 1228–1234.

4. Sertl, D. C., and Molitor, B. E. (1985). J. Assoc. Off. Anal. Chem., **68**, 177–182.

5. Liu, Y., Xu, M. J., and Canfield, L. M. (1998). J. Nutr. Biochem., **9**, 178–183.

6. Acworth, I. N., and Gamache, P. H. (1996). Am Lab., **28**, 33–38.

7. MacCrehan, W. A., and Schonberger, E. (1987). Clin. Chem., **33**, 1585–1592.

Ordering Information

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CoulArray Organizer with Temp. Control	70-4340T
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