Analysis of Selected Vitamins with HPLC and Electrochemical Detection

Application Note

Food and Clinical

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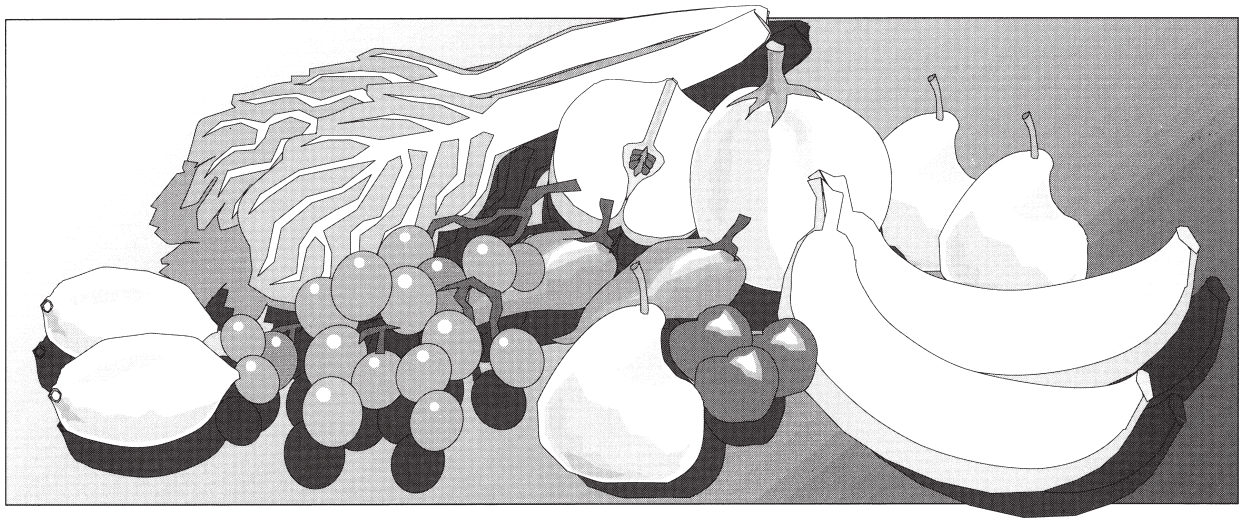

Several oxidizable vitamins (vitamin A, B_6, C, D_3 and E) were analyzed using HPLC and electrochemical detection. Excellent sensitivity was achieved with all vitamins. The minimum detectable level for vitamin A-palmitate was about 80 pg, for β-carotene about 50 pg, for vitamin B_6 about 30 pg, for vitamin C below 1 pg, for vitamin D_3 about 70 pg and for vitamin E about 30 pg. The optimum potentials were evaluated using an automated increment for the potentials. The reproducibility was tested using vitamin B_6 and was found to be 3% RSD of peak height for 100 runs. Due to the high selectivity of electrochemical detection, it was possible to keep sample preparation very simple.

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Introduction

In the last decade, liquid chromatography (LC) with electrochemical detection (ECD) has been used more and more for the determination of electroactive compounds at trace levels and in complex matrices.

For the analysis of electroactive vitamins in food, pharmaceutical preparations, tissue and body fluids, the combination of HPLC and ECD provides high sensitivity and excellent selectivity [1,2,7,8,9,11]. Electroactive vitamins can be determined in the low pg range and moreover, fewer matrix effects are encountered. This enables sample preparation and enrichment to be simplified and reduced [11].

Despite the high sensitivity and selectivity of electrochemical detection for vitamin analysis, it is not widely used. It has a reputation for low response stability due to the fact that the electroactive surface of the detector cell is in direct contact with the mobile phase and all the compounds and products of the oxidative and reductive reaction. This can result in contamination of the working electrode and an unstable response. The response instability can be minimized by using modern electrochemical detectors with electrochemical self-cleaning routines [19].

This note describes a method for analyzing oxidizable vitamins with high sensitivity and reproducibility using a modern electrochemical detector.

Experimental

We used Agilent 1049A electrochemical detector with HP 1050 Series isocratic pump and autosampler. The chromatographic conditions for each experiment are listed with the appropriate figures.

Results and discussion

Analysis of vitamin A (retinol), provitamin A (β-carotene) in fruit juice

Vitamin A is one of the fat-soluble vitamins and is of importance for normal growth and development of the human body.
Malabsorbtion results in disease of the liver [1] and an insufficiency results in extreme dryness of the skin and nightblindness [12]. Sources of vitamin A are liver, fish oil, milk, butter and the yolk of eggs. A further source is provitamin A (β-carotene), which is present in many plants and is converted in the human body to retinol (vitamin A_1).

The analysis of vitamin A is of interest for quality control of food and pharmaceutical products and for diagnosis and therapeutic reasons in clinical routine testing and medical research.

Measurement of this vitamin is nowadays based on HPLC methods [10] using UV-Visible, fluorescence or electrochemical detection. The analysis of vitamin A and/or β-carotene with electrochemical detection has several advantages over UV-Visible or fluorescence detection. UV detection shows a lack of sensitivity and selectivity [1, 2, 4], whereas fluorescence detection normally offers sufficient sensitivity but sometimes (depending on the matrix) insufficient selectivity [1,3].

Using an electrochemical detector, the minimum detectable level for vitamin A-palmitate was about 80 pg and for β-carotene about 50 pg with a signal-to-noise ratio of 2, (figure 1).

In figure 1 the analysis of vitamin A-palmitate and β-carotene is shown. 0.5 µl of a standard mixture was injected and the minimum detectable level was calculated. In order to measure at optimum sensitivity or at the highest possible selectivity, it is necessary to evaluate the optimum detection potential for each compound of interest. This can be done using built-in software routines (auto-increment mode).

The sample is injected as often as specified between various selected potentials. The voltage difference between these potentials can also be determined by the user.

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1.5 ml/min
Oven temperature	30 °C
Injection volume	0.5 µl

Detector parameters

Operation mode	Amperometry
Potential	1 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	8 s.

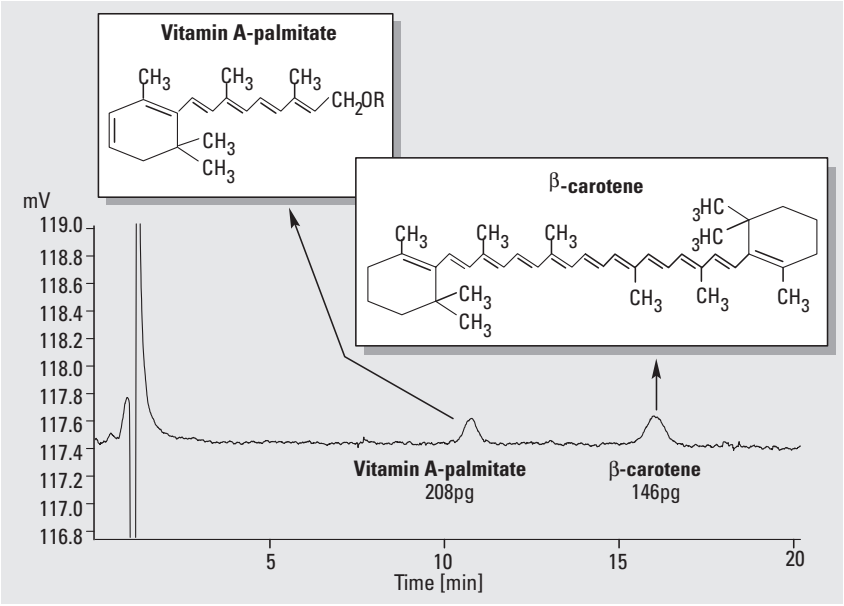


Figure 1
Sensitivity of electrochemical detection with vitamin A and β-carotene

Figure 2 shows the result of the automated optimization procedure. At 1 V the optimum signal-to-noise ratio was obtained. Further increase of the oxidative potential did not increase the signal height but produced more noise and drift, and so no better signal-to-noise ratio could be achieved.

The optimization procedure also showed how easily β-carotene can be oxidized. Oxidation started at 0.5 V. This can be of advantage when high selectivity is more important than high sensitivity.

For the analysis of vitamins in food, the main problem is often not only sensitivity but also selectivity [13-17]. The high selectivity of electrochemical detection means that problems due to the matrix or coeluting peaks can be avoided without time-consuming sample preparation. For example, β-carotene in fruit juice can be analyzed by direct injection into an HPLC instrument, figure 3.

Figure 3 shows that, with 1 µl injection volume and no sample preparation, β-carotene in fruit juice was easily determined. The concentration of β-carotene was found to be 2.7 mg/100 ml fruit juice.

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1.5 ml/min
Oven temperature	30 °C
Injection volume	3 µl
Detector parameters	
Auto-increment parameters	
Start potential	0.4 V
End potential	1.1 V
Increment	0.1 V

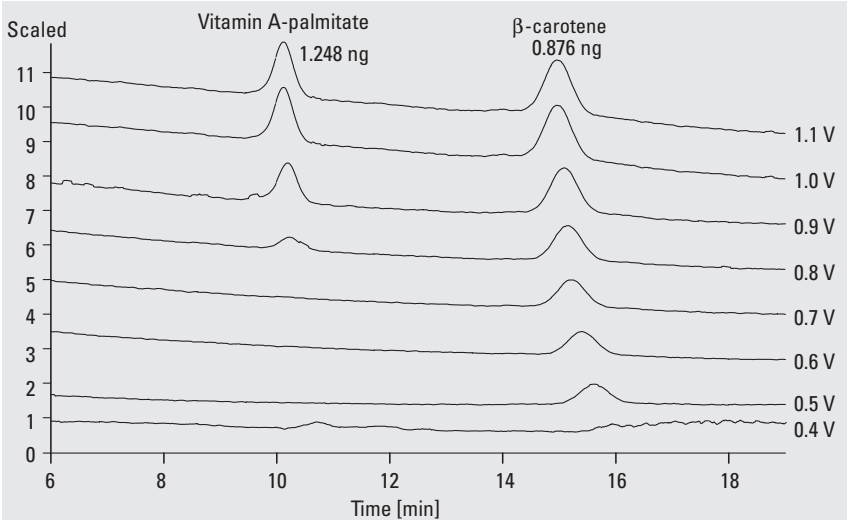


Figure 2
Optimization of detection potentials

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1.5 ml/min
Oven temperature	30 °C
Injection volume	1 µl
Detector parameters	
Operation mode	Amperometry
Potential	1 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	8 s.

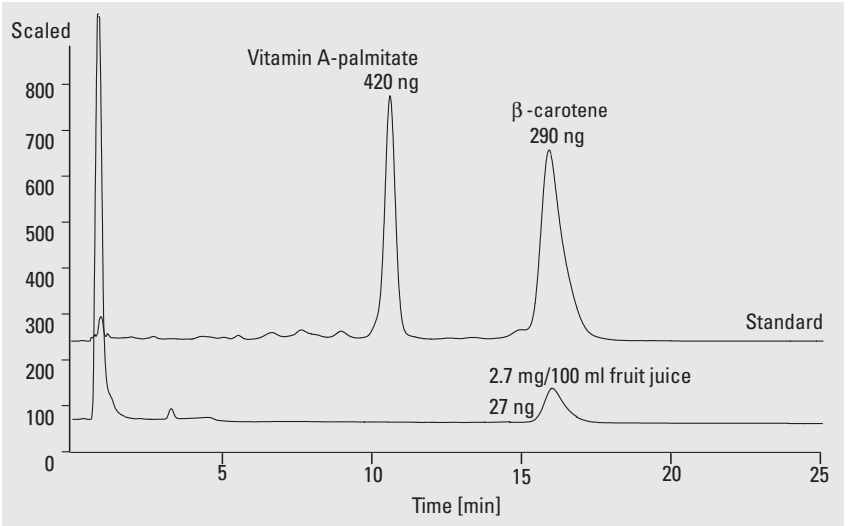


Figure 3
Analysis of β-carotene in fruit juice

Analysis of vitamin B_6 (pyridoxine hydrochloride) in a pharmaceutical vitamin preparation

Vitamin B_6 is a water-soluble vitamin and consists of three interconvertible forms (pyridoxine hydrochloride, pyridoxamine dihydrochloride and pyridoxal hydrochloride). Vitamin B_6 is of great importance for the nervous system [5, 6]. In food, it is present in liver, bananas, wheat and vegetables.

Whereas other methods show lack of sensitivity and selectivity, e.g. the minimum detectable level with HPLC and fluorescence detection is in the low ng range [5], a combination of HPLC and electrochemical detection enables the analysis of vitamin B_6 in the pg range (30 pg with a signal-to-noise ratio of 2), figure 4.

Figure 4 shows, that the determination of vitamin B_6 is possible even when vitamin C is present in large quantities. The sample preparation was kept very simple: 10 µl of the vitamin preparation was diluted with 1 ml distilled water and 0.5 µl of the liquid was injected. The vitamin concentration was 804.16 mg/l vitamin preparation.

In order to evaluate the reproducibility of this type of analysis, 5 µl (450 pg) of the standard sample were injected. The reproducibility was about r.s.d. = 3% for peak heights and about 5% for area counts over 100 runs.

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Water + 0.02M KH_2PO_4 + 0.03M tetrabutylammoniumhydrogensulfate + 0.03M heptanesulfonic acid + 2% acetonitrile
Flow rate	0.8 ml/min
Oven temperature	30 °C
Injection volume	Standard 1µl Sample 0.5 µl
Detector parameters	
Working electrode	Glassy carbon
Operation mode	Amperometry
Potential	1.2 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	1 s

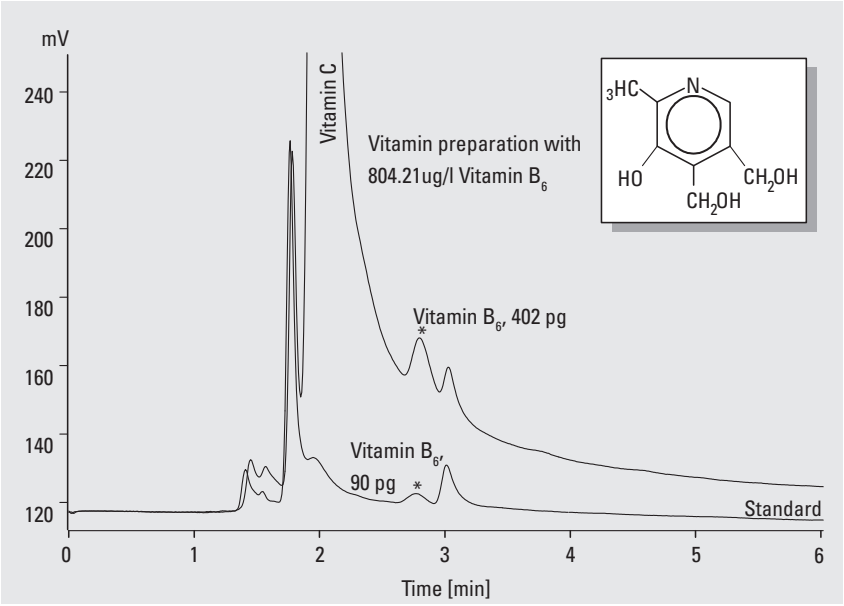


Figure 4
Analysis of vitamin B_6 in a vitamin preparation for chicken feed

Analysis of vitamin C (ascorbic acid) in fruit juice

Vitamin C belongs to the water-soluble vitamins and is of vital importance for mammalian health. Lack of this vitamin is responsible for scorbutus (scurvy) and in former times many people (specially sailors) died because of this deficiency disease. Vitamin C is present in many fruits and vegetables [12].

The analysis of vitamin C in food is mainly performed for quality control purposes. Determination of this vitamin in tissue and body fluids is mainly carried out to increase knowledge of its physiological role in mammalians [7].

There are several methods available for the analysis of vitamin C, but most of them show lack of sensitivity and/or selectivity. Tedious sample preparation steps are needed to remove interfering compounds [7,11,18]. HPLC, in combination with UV-Visible detection for example, shows insufficient sensitivity for the analysis of vitamin C in tissue and body fluids [7,11]. The minimum detectable level for this method is about 4 ng.

Using HPLC and electrochemical detection, the minimum detectable level for vitamin C is below 1 pg. (Due to the low stability of vitamin C, the determination of the minimum detectable level must be done directly after dilution of freshly prepared stock solutions.) The analysis of vitamin C in food such as fruit and fruit juices, is mainly done to control the freshness and proper storage of these products.

Figure 5 shows the chromatogram of a fruit juice which contained 43.16 mg vitamin C in 100 ml fruit juice. The fruit juice was prefiltered, 10 times diluted and 1 µl of the diluted sample was injected. The standard solution contained 1 mg vitamin C in 100 ml water.

Column	100 x 4.6 mm ODS Hypersil, 5 µ (Part No. 79916OD-554)
Mobile phase	Water + 5.4 g/l Na acetate-trihydrate + 3g/l tetrabutylammonium-hydrogensulfate + 3 ml KCl solution (3.3 m), pH 5 with acetic acid.
Flow rate	0.8 ml/min
Oven temperature	30 °C
Injection volume	1 µl

Detector parameters

Working electrode	Glassy carbon
Operation mode	Amperometry
Potential	0.6 V
Range	0.5 µA
Reference electrode	in-situ reference electrode AgCl/KCl in mobile phase
Response time	8 s

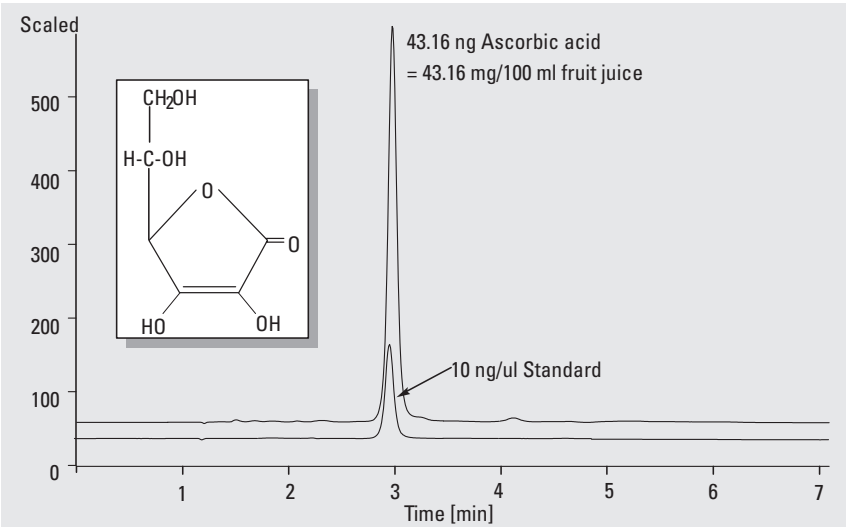


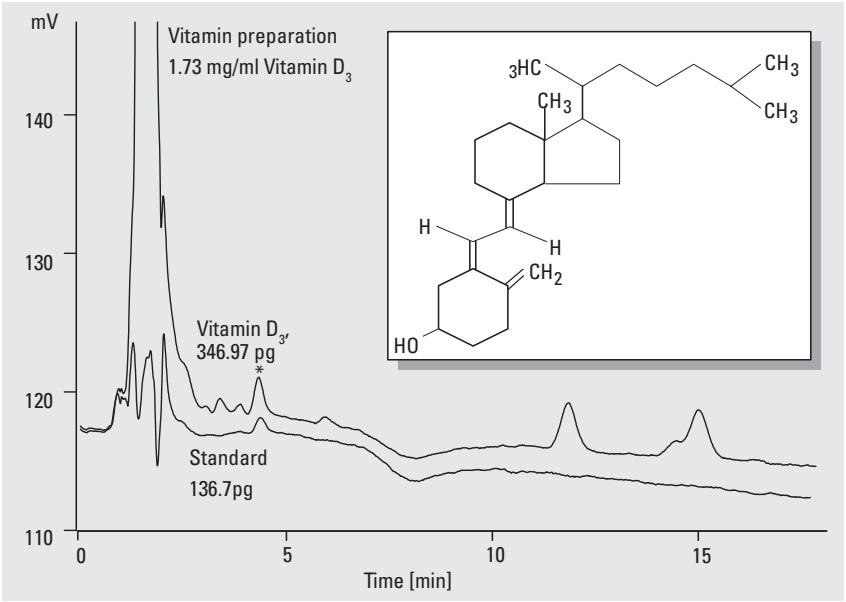
Figure 5
Analysis of vitamin C (ascorbic acid) in fruit juice

Analysis of vitamin D_3 (colcalciferol) in a multi-vitamin preparation

Vitamin D_3 belongs to the fat-soluble vitamins and is of great importance for the metabolism of bone. Lack of this vitamin results in bone deformation from rachitis (rickets).

The determination of vitamin D_3 in pharmaceutical preparations, tissue and body fluids plays an important role in quality assurance procedures and in the understanding of bone metabolism [8]. The methods in use are generally time-consuming, involving several sample preparation steps and some of them are not sufficiently sensitive or selective [8]. The combination of HPLC and electrochemical detection offers excellent sensitivity. The minimum detectable level is about 70 pg with a signal-to-noise ratio of 2.

Figure 6 shows the analysis of vitamin D_3 in a highly viscous liquid multivitamin solution, which is used in poultry farms. Due to the excellent selectivity of electrochemical detections, sample preparation could be kept very simple. 40 µl of the vitamin liquid was shaken with 1 ml of cyclohexane for 1 min and 5 µl of the supernatant liquid were injected. The vitamin D_3 concentration in this preparation was found to be 1,73 mg/l.



Column	125 x 4 mm Lichrospher
RP18, 5 µ	
	(Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1 ml/min
Oven temperature	30 °C
Injection volume	5 µl

Detector parameters

Working electrode	Glassy carbon
Operation mode	Amperometry
Potential	1.3 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	8 s

Figure 6
Analysis of vitamin D3 in a pharmaceutical multivitamin preparation

Analysis of vitamin E (α-tocopherol) in vitamin E capsules

Vitamin E belongs to the fat-soluble vitamins. In mammalians it fulfills the function of a lipid-soluble antioxidant and is necessary for the maintenance and protection of neuronal, muscular and reproductive tissue [9].

The analysis of vitamin E is of interest in the food and cosmetic industry, see for example, the analysis of this vitamin in butter or milkpowder [10] or in beauty creams, where it is used as an antioxidant [12]. The analysis of vitamin E in tissue has become more and more important, because there is a possibility that vitamins A and E may act as cancer-chemopreventive agents [4].

Measurements of plasma vitamin A and E provides an indication of the proper absorbtion of these two compounds. Malabsorbtion of these vitamins occurs in cystic fibrosis and in cholestative liver diseases [1].

The analysis of tocopherols with HPLC and electrochemical detection also offers more sensitivity and selectivity compared with HPLC and UV-Visible or fluorescence detection, figure 7 [9].

A standard solution of 100 pg in 1 µl (figure 7) was injected and the minimum detectable limit was calculated. It was found to be about 30 pg for a signal-to-noise ratio of 2.

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1 ml/min
Oven temperature	30 °C
Injection volume	1 µl

Detector parameters

Working electrode	Glassy carbon
Operation mode	Amperometry
Potential	0.9 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	8 s

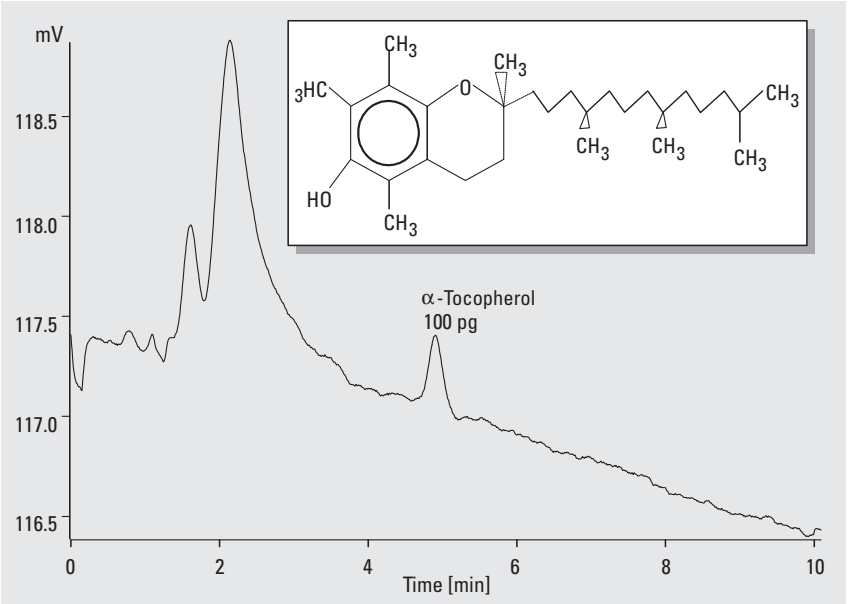


Figure 7
Sensitivity of tocopherol analysis with electrochemical detection

In figure 8, the analysis of α-tocopherol and traces of other tocopherol isomers in a pharmaceutical preparation is shown.

For sample preparation the capsule was cut and extracted with 5 ml of methanol in an ultrasonic bath for 10 minutes. 3 µl of the supernatant liquid was injected. The concentration of a-tocopherol was found to be 270 mg/capsule.

The chromatogram shows that not only α-tocopherol, but also traces of other tocopherol isomers, such as beta, delta and/or gamma tocopherol, were present. Under the chromatographic conditions used, gamma and delta tocopherol could not be separated.

Column	125 x 4 mm Lichrospher RP18, 5 µ (Part No. 799250-564)
Mobile phase	Methanol + 5 g/l lithiumperchlorate + 1 g/l acetic acid
Flow rate	1 ml/min
Oven temperature	30 °C
Injection volume	3 µl
Detector parameters	
Working electrode	Glassy carbon
Operation mode	Amperometry
Potential	0.9 V
Range	0.5 µA
Reference electrode	AgCl/KCl
Response time	8 s

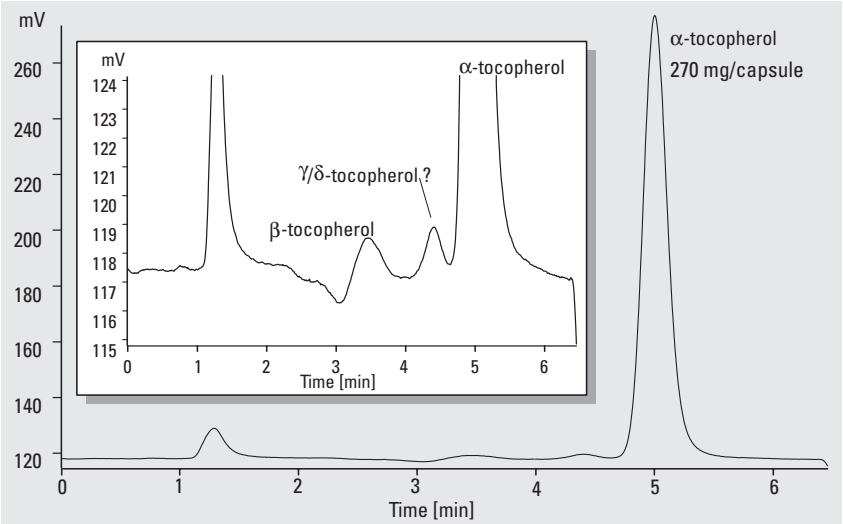


Figure 8
Analysis of tocopherols in vitamin E capsules

References

1
May-Lynn Huang, Gilbert J. Burckart, Raman Venkataramanan, "Sensitive high performance liquid chromatographic analysis of plasma vitamin A using amperometric and ultraviolet detection", *J.Chromatogr.*, **1986**, *380*, 331-338

2
Johanna K.Lang, Lester Packer, "Quantitative determination of vitamin E and oxidized and reduced coenzyme Q by high-performance liquid chromatography with in-line ultraviolet and electrochemical detection", *J.Chromatogr.*, **1987**, *385*, 109-117

3
A.T.Rhys Williams, "Simultaneous determination of serum vitamin A and E by liquid chromatography with fluorescence detection", *J.Chromatogr.*, **1985**, 198-201

4
David W.Nierenberg, Deborah C.Lester, "Determination of Vitamins A and E in serum and plasma using simplified clarification method and high performance liquid chromatography", *J.Chromatogr.*, **1985**, *345*, 275-284

5
Leslie A. Morrison, Judy A. Driskell, "Quantities of B6 vitamers in human milk by high performance liquid chromatography", *J.Chromatogr.*, **1985**, *337*, 249-258

6
Weiying Hou, Huamin Ji, Erkang Wang, "Liquid chromatography of vitamin B6 with electrochemical detection using a carbon fibre electrode", *Anal.Chim.Acta*, **1990**, *230*, 207-211

7
Philip W. Washko, William O. Hartzell, Mark Levine, "Ascorbic acid analysis using high performance liquid chromatography with coulometric electrochemical detection", *Anal.Biochem.*, **1989**, *181*, 276-282

8
A. Sanchez Perez, M. Delgado Zamarreno, J. Hernandez Mendez, R.M. Sanchez Rodriguez, "Flow-injection analysis of vitamin D3 and 25-hydroxy-vitamin D3 by amperometric detection", *Anal.Chim.Acta*, *1989*, *225*, 247-251

9
Michael E. Murphy, James P.Kehrer, "Simultaneous measurement of tocopherols and tocopheryl quinones in tissue fractions using high performance liquid chromatography with redox-cycling electrochemical detection", *J.Chromatogr.*, **1987**, *421*, 71-82

10
W. Kneifel, F. Ulberth, U. Winkler-Macheiner, "Simultaneous determination of retinol and tocopherol in butter and milk powder with high performance liquid chromatography", (original in German) *Z. Deutsche Lebensmittel-Rundschau*, **1987**, 83, 137-139

11
Karel Stulik, Vera Pacakova, "Electroanalytical measurements in flowing liquids", **Prague 1987**, Ellis Horwood Ltd series in analytical chemistry, New York

12
Roempps Chemie Lexikon, 1979, 8. Aufl., *Franckh'sche Verlagshandlung*, *Stuttgart*

13
David W. Nierenberg, "Serum and plasma B-carotene levels measured with an improved method of high performance liquid chromatography", *J.Chromatogr.*, **1985**, *339*, 273-284

14
R. Ohmacht, Gy. Toth, G. Voigt, "Separation of serum carotenoids and vitamin A on Chromsil-Amino and Cyanophases by bi-directional gradient elution technique", *J.Chromatogr.*, **1987**, *395*, 609-612

15
R.J. Bushway, "Separation of carotenoids in fruits and vegetables by high performance liquid chromatography ", *J.Liq.Chromatogr.*, **1985**, *8(8)*, 1527-1547

16
Yen-Ping C. Hsieh, Marcus Karel, "Rapid extraction and determination of a- and B-carotenes in foods", *J.Chromatogr.*, **1983**, *259*, 515-518

17
Adriana Z. Mercadante, Delia B. Rodriguez-Amaya, "Comparison of normal-phase and reversed-phase gravity-flow column methods for provitamin A", *Chromatographia*, **1989**, *28*, 249-252

18
Antal Bognar, "Determination of vitamin C with high performance liquid chromatography", (original in German) *Z. Deutsche Lebensmittel-Rundschau*, **1988**, *84*, 73-76

19
A.G. Huesgen, R. Schuster "Selective HPLC analysis of phenols in river water", *Agilent Technologies Application Note* ,**1990**, 04/90, Pub. no. 5952-1548

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