Application Note

Clinical & Diagnostic

The most selective LC-EC applications for Clinical & Diagnostics analysis

Catecholamines
Serotonin
Metanephrines
VMA
HVA
5-HIAA

PET imaging tracer
Fluorodeoxyglucose (FDG)
FDG impurities

Sulfides
Homocysteine
Glutathione
Disulfides

Vitamins, minerals
A, C, D, E, and K
Iodide
Q10
Ubiquinols

DNA adducts
8-hydroxy-2'-deoxyguanosine
O^6-methylguanine
8-Hydroxydeoxyguanosine
7-Methylguanine

Vitamins, ubiquinols and precursors

- **Vitamins A, C, D, E, K, ubiquinols and related components**
- **ALEXYS Analyzers for analysis of vitamins**
- **Reproducible and robust**

This note shows a selection of example chromatograms related to vitamins from the work of a few of our many detector-users to support the applicability of the electrochemical approach and the use of our detectors in particular.

Introduction

Water soluble vitamin C

Vitamin C (ascorbic acid, AA) is an essential nutrient involved in various biochemical processes, like the repair of tissue, the enzymatic production of certain neurotransmitters, the functioning of several enzymes, immune system function, and it also functions as an antioxidant.

Fat soluble vitamins A, D, E and K, and Q_{10}

Vitamin A (retinol) plays a role in vision and bone growth. It comes from animal sources such as eggs, meat and dairy products. Beta-carotene, a precursor, comes from fruit and vegetables (paprikas, carrots).

Vitamin D (ergocalciferol, D_2, cholecalciferol, D_3) promotes the adsorption of calcium in the body, and is essential for development of bones and teeth. Vitamin D is made by the body when exposed to sun light, or taken up via our diet of cheese, butter, fish, and milk.

Vitamin E or tocopherol is an important antioxidant. Antioxidants protect cells against the effects of free radicals, which are potentially damaging by-products of the body's metabolism.

Vitamin K denotes a group of several closely related molecules, consisting of a naphthoquinone skeleton with an isoprene side-chain of differing lengths. They are needed for the posttranslational modification of certain proteins, mostly required for blood coagulation. Normally it is produced by bacteria in the intestines. The K vitamins occur at very low concentrations in plasma (0.1 - 4 ng/ml).

Coenzyme Q_{10} is one of a number of naturally occurring ubiquinones that act as electron acceptors in mitochondrial oxidative phosphorylation. The reduced forms, the ubiquinols, are strong anti-oxidants. Oxidation of LDL (low-density lipoproteins) is believed to play an important role in early atherosclerosis. According to this oxidation hypothesis, LDL is protected against oxidative stress by i.a. the Q_{10} antioxidants, thereby slowing down the formation of modified LDL. More specifically, the potent lipophilic antioxidants α-TOH (tocopherol, vitamin E), QH_2-10 (ubiquinol-10, the reduced form of ubiquinone-10 or Q_{10}), β-carotene and lycopene are supposed to be the important factors in this protection process. There is no unanimity about the relative physiological importance of these compounds.

LC-ECD analysis of vitamins and Q_{10}

Water soluble vitamin C

The analysis with LC-ECD is very straightforward and highly reproducible with the use of hydrochinon as internal standard. The separation is reversed phase isocratic separation with a highly aqueous mobile phase, followed by EC detection in amperometric mode. Specific considerations, a human plasma sample preparation method and an example chromatogram areis given in the next page.

Fat soluble vitamins A, D, E and K, and Q_{10}

Due to the high lipophilicity of the compounds of interest the reversed phase separation requires a highly apolar mobile phase. To maintain sufficient electrical conductivity of the mobile phase (for the subsequent EC detection), $LiClO_4$ is added, which is dissolvable in highly apolar solutions (in contrast to many other salts). Under these extremely apolar conditions the HyREF is the recommended choice for a reference electrode.

Most of the fat soluble vitamins are directly detectable in amperometric mode, but vitamin K and Q_{10} require additional reduction before they can be detected with EC in oxidative mode. With the use of a second flow cell, the quinone moiety will be reduced to a quinol (which is then detectable in oxidative mode in the second cell). The use of the additional flow cell (set to a reductive potential) upfront the oxidative detector flow cell is necessary only specific for vitamin K and Q_{10} analysis.

Various examples of chromatograms of the fat-soluble vitamins and Q_{10} are given in the next sections.

Vitamin C (ascorbic acid) in human plasma

Ascorbic acid (AA) is normally present in human as well as in animal plasma and tissue. The daily need is considered to be at least 70 mg, and normal blood levels are between 28 and 85 µM. For example, the analysis of AA in plasma of geriatric patients reflects the feeding status quality of such patients.

Precautions for sample storage

Since it is quite labile, accurate measurement of AA in biological matrices highly depends on a reliable method to stabilize AA [1, 2]. So, if samples are stored without specific precautions, auto-oxidation of AA may occur already after 15 minutes, and erroneous measurements will result. It appears that di-thiothreitol (DTT) and metaphosphoric acid (MPA) are quite effective as an aid for long-term stabilization (up to one year) of AA in biological samples and, in addition, DTT appears to reduce dehydro-ascorbic acid [1, 2].

Chromatographic profile

The peak of AA already elutes after less than 3 min, but the run -time of the isocratic method is determined by DTT, which elutes after about 15 min (conditions in Table 1). Uric acid is usually present in the chromatograms but does not interfere.

Sample preparation method

Blood samples of at least 1 ml are centrifuged and 0.2 ml of the supernatant is thoroughly mixed with 0.9 ml MPA (3%) and immediately frozen (-20 °C). After thawing, 0.2 ml phosphate buffer (pH 7.4) with a known amount of DTT is added, mixed and left for 30 min. If samples do not need storage, 0.2 ml sample is mixed with 0.2 ml of the phosphate/DTT mixture and left for 30 min. Then these non-stored samples are mixed with 0.9 ml MPA 3%. Subsequently, both types of samples are centrifuged (5 min, 10,000 g), 0.1 ml is transferred to an autosampler sample vial containing 0.9 ml MPA 3%, capped and mixed. A volume of 10 µl is injected into the LC-ECD system for analysis. An example chromatogram is given in Fig. 1.

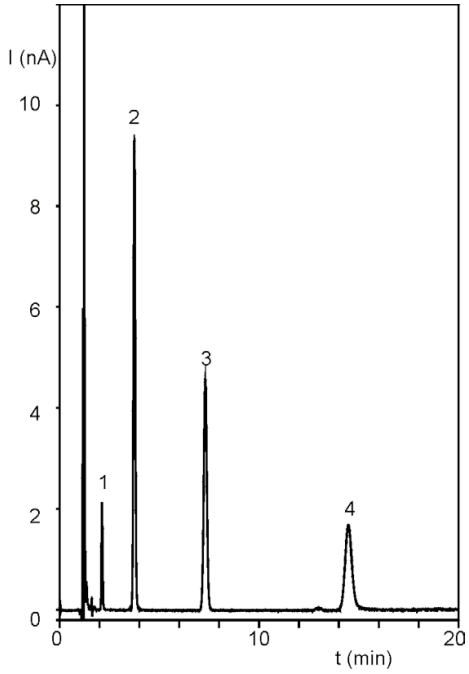

Figure 1: Chromatogram of ascorbic acid (1) in pretreated plasma. Plasma concentration is 41 µM AA. Other peaks are: Urate (2), Hydrochinon (IS, 3) and DTT (4). Conditions Table 1. Courtesy: M. van der Horst, M.G.J. Lunenborg and E. Oeben, Scheper Ziekenhuis, Emmen, The Netherlands.

Table 1

LC-ECD conditions

Columns	Supelco C18-BDS, 5 µm, 150 x 4.6 mm
Mobile phase (MP)	50 mM phosphate buffer pH 2.8, 200 mg/L EDTA, 2% methanol (v/v)
Flow rate	1.3 mL/min
Temperature	30 °C
E_{cell}	0.60 V on glassy carbon (vs. Ag/AgCl sat'd)
I_{cell}	Ca. 0.4 nA

References

1. S.A. Margolis and T.P. Davis, Clin. Chemistry, 34 (1988) 2217 – 2223.
2. S.A. Margolis, R.C. Paule and R.G. Ziegler, Clin. Chemistry, 36 (1990) 1750 – 1755.

Ubiquinols, vitamin E, and ß-carotene in human LDL

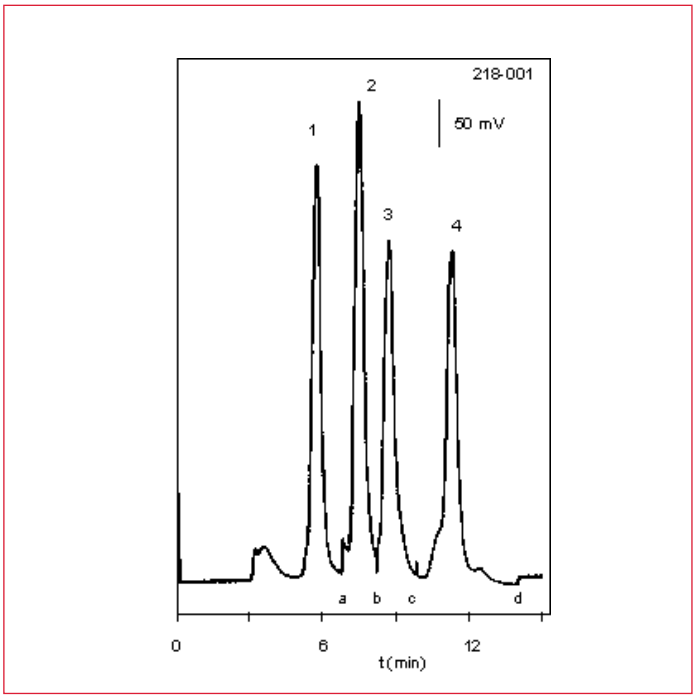

Figure 2: Analysis of human LDL. Concentrations (amounts) are: 1. α-tocopherol (vitamin E) 3.6 µM (72.9 pmol), 2. ubiquinol-9 0.77 µM (15.5 pmol), 3. ubiquinol-10 0.21 µM (4.2 pmol), and 4. β-carotene 0.11 µM (2.2 pmol). The detector range setting was set to 10 nA/V at the start of the run, and changed to 1 nA/V at *a*, 0.5 nA/V at *b*, 1 nA/V at *c*, and 10 nA/V at *d*. Conditions Table 2. Courtesy ref [1].

Table 2

LC-ECD conditions	
Columns	Inertsil® ODS-2, 10 x 2 mm + 200 x 3 mm, 5 µm (Chrompac)
Mobile phase	22.5% methanol, 77.5 % ethanol/isopropanol (95/5), 20 mM $LiClO_4$
Flow rate	0.35 mL/min
Temperature	30 °C (separation and detection)
E_{cell}	0.6 V on glassy carbon (vs. Ag/AgCl sat'd with LiCl)
Injection volume	20 µL

Vitamins A, D, E, K (standard)

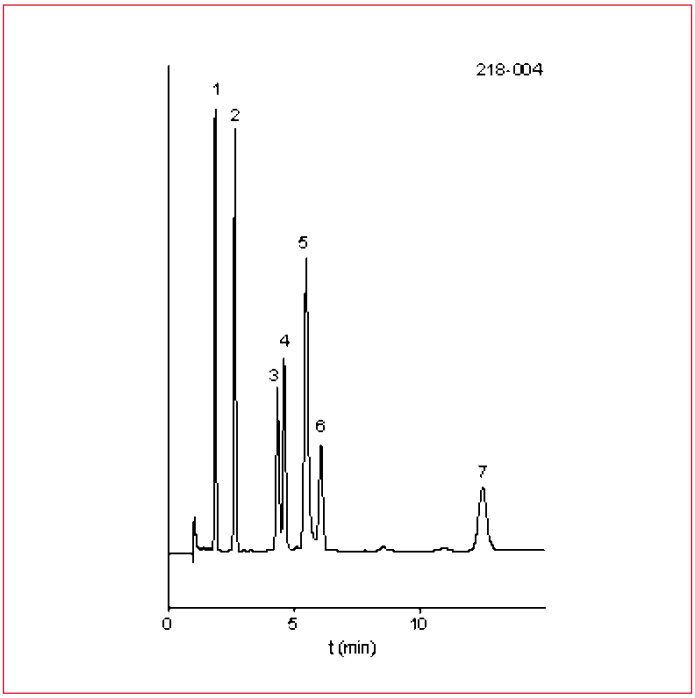

Figure 3: Analysis of a standard mix of 5 µM vitamin A (1), 5 µM vitamin A acetate (2), 100 µM vitamin D_2 (3), 100 µM vitamin D_3 (4), 10 µM vitamin E (5), 100 µM vitamin K_2 (6) and 100 µM vitamin K_1 (7). Scale: 240 nA full scale. Conditions Table 3

Table 3

LC-ECD conditions	
Columns	Spherisorb ODS 100 x 4.6 mm, 3 µm
Mobile phase	100 mM $LiClO_4$, 96% MeOH
Flow rate	1.0 mL/min
Temperature	30 °C (separation and detection)
E_{cell}	1.05 V on glassy carbon (vs. HyREF) Only necessary for detection of vit K: pre-reduction on additional cell at -400 mV upfront detection

References

1. Yolanda B. de Rijke et al., Arterioscler. Thromb. Vasc. Biol. 17 (1997) 127-133

Vitamin K in plasma

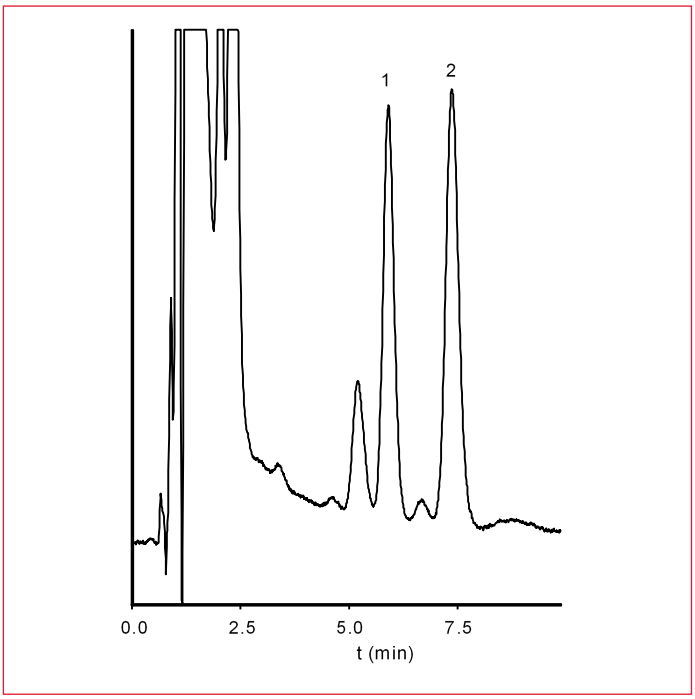

Figure 4: Analysis of vitamin K_1 (1) in plasma extract after LLE, SPE, evaporation and reconstitution in mobile phase. Concentrations are 2.5 ng/ml for vitamin K_1 and 5 ng/ml for the internal standard 2,3 hydrophylloquinon. Conditions in Table 4

Q_{10} (standard)

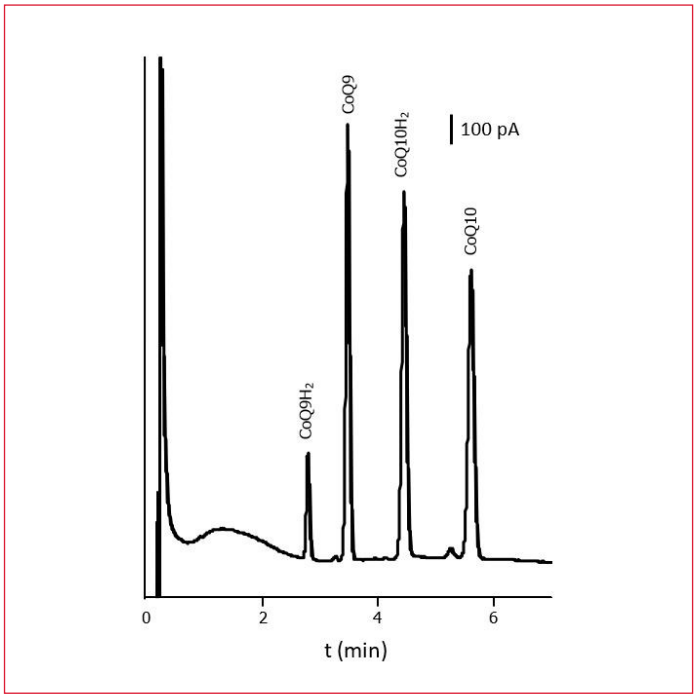

Figure 5: Example chromatogram obtained with a standard mix of 5µM ubiquinone-9 and ubiquinone-10 that was partially reduced to form ubiquinol-9 and ubiquinol-10 prior to analysis. Many methods reported in literature use CoQ9 as an internal standard. Conditions used to record the chromatogram are listen in Table 5

Table 4

LC-ECD conditions	
Columns	Spherisob ODS-2, 50 x 2.1 mm, 5 µm (Higgins)
Mobile phase	0.1 M lithiumperchlorate in methanol with 4% water
Flow rate	0.2 mL/min
Temperature	30 °C (separation and detection)
$E_{cell\ 1}$ (reduction)	-0.5 V on glassy carbon (vs. HyREF)
$E_{cell\ 2}$ (detection)	0.3 V on glassy carbon (vs. HyREF)

Table 5

LC-ECD conditions	
Columns	Acquity BEH C18 column, 100 x 1 mm ID, 1.7 µm
Mobile phase	0.1 M lithiumperchlorate in 80% acetonitrile and 20% methanol
Flow rate	0.25 mL/min
Temperature	45 °C (separation and detection)
Injection volume	1 µL
$E_{cell\ 1}$ (reduction)	-1.3 V on glassy carbon (vs. HyREF)
$E_{cell\ 2}$ (detection)	+1.1 V on glassy carbon (vs. HyREF)

References

1. M.J. Shearer, Adv. in Chromatogr. 21 (1983) 243 - 301
2. J.P. Langenberg and U.R. Tjaden, J. Chromatogr. 305 (1984) 61 – 72

Recommended LC-ECD hardware

The advised LC configuration for the analysis of fat-soluble vitamins is the ALEXYS UHPLC SCC base system in combination with a SenCell 2 mm GC HyREF. For analysis which require pre-reduction (Vitamin K and Q10) a DCC system with additional FlexCell GC HyREF is advised.

Figure 6: ALEXYS analyzer for the analysis of vitamins consisting of a AS110 autosampler, P6.1L pump with integrated degasser & solvent selection valve, and DECADE Elite detector.

Ordering information

Detector only (connection to 3rd party HPLC)	
176.0035B	DECADE Elite SCC electrochemical detector
176.0035DB	DECADE Elite DCC electrochemical detector
116.4320	SenCell 2 mm GC HyREF
102.4305*	Flexcell GC HyREF
Recommended ALEXYS analyzer (add columns and flow cells)	
180.0097W	ALEXYS UHPLC base SCC
180.0098W*	ALEXYS UHPLC base DCC
116.4320	SenCell 2 mm GC HyREF
102.4305*	Flexcell GC HyREF
Columns+	
250.1165	Acquity UPLC in-line filter kit + 6 frits (205000343)
250.1163	Acquity UPLC BEH C18, 1.7µm,1 x 100 mm (186002346)

*) DCC system and FlexCell only required for pre-reduction (Vitamin K and Q10).

+) Columns are products of Waters Corporation (Milford, USA). The Waters part numbers are given between parenthesis.

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