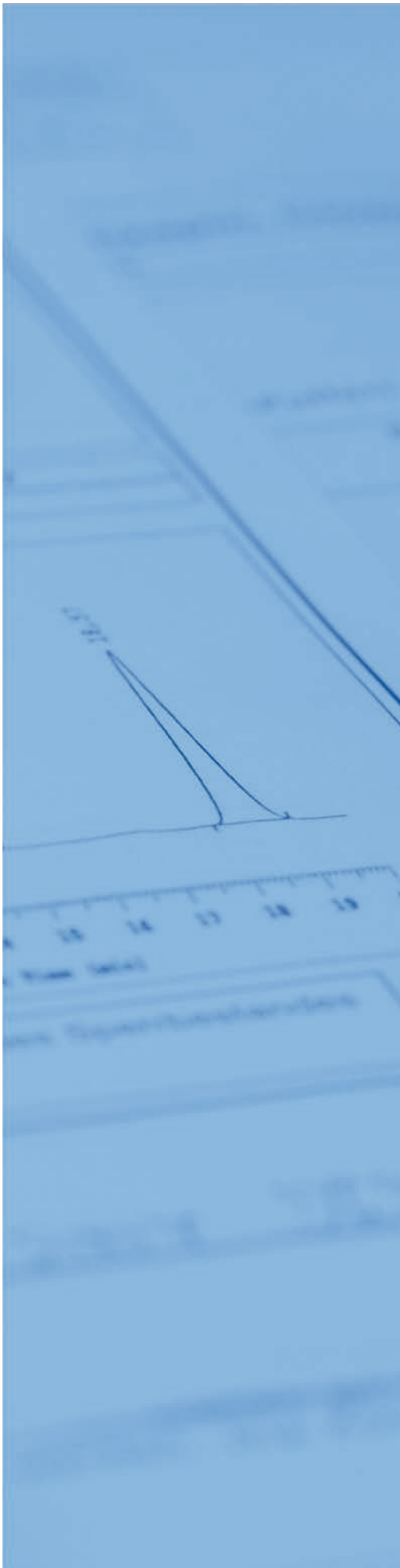
RECIPE

Instruction Manual

ClinRep® UHPLC Complete Kit

Vitamin A and E in Serum / Plasma



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IVD For in vitro diagnostic use

CE IVDD, 98/79/EC

RECIPE Chemicals + Instruments GmbH
Dessauerstraße 3, 80992 Munich / Germany
Phone: +49 / 89 / 54 70 81 - 0
Fax: +49 / 89 / 54 70 81 - 11
info@recipe.de
www.recipe.de

REF 22100

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Contents

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1 Introduction

1.1 Information on changes in this instruction manual

This instruction manual (version 1.2) was revised and replaces the previous version 1.1.

The changes are marked on the page margin.

1.2 Intended use

This ClinRep® Complete Kit is intended for the determination of Vitamin A and E in Serum or Plasma of human origin by UHPLC. The assessment of the vitamin status is used for aid in diagnosis of clinical conditions that are related to an inappropriate vitamin A / E blood level or to a generalised vitamin deficiency. Clinical background is provided in section 1.3.

The ClinRep® Complete Kit is intended for use in professional diagnostic laboratories. The kit components have to be used in accordance with this user manual. The kit is not designed for combination with components from other manufacturers.

1.2.1 IVD symbols

Symbols according to EU directive 98/79/EC for in vitro diagnostic medical devices (IVDD), which are used on the product labels and in this user manual:

For in vitro diagnostic use


Manufacturer

Order number

Lot number


Upper temperature limit: … °C

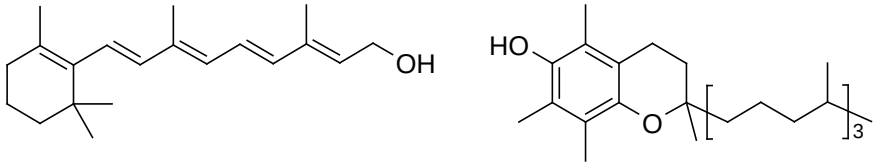

Temperature limits: … °C to … °C


Expiry date


See instructions for use

1.3 Clinical background

Vitamin A (retinol) and vitamin E (α-tocopherol) (see Figure 1) belong to the fat-soluble class of vitamins and are essential for humans.



Vitamin A Vitamin E

Figure 1. Chemical structure of vitamin A (Retinol) and vitamin E (α-Toxopherol)

Vitamin A:

Humans are not capable of synthesising vitamin A and therefore have to cover their demand by nutrition. Pre-formed vitamin A is obtained from animal-derived foods, whereas provitamin A carotenoids, mainly ß-carotene, are obtained from vegetables [1].

The biochemical functions of vitamin A and its metabolites, the retinoids, are manifold. The best known function of vitamin A is its particular significance in vision [1]. In addition vitamin A is required for normal development and growth, e.g. regulation of cell division, bone remodeling and enhancement of immune response [2]. Clinical symptoms of a vitamin A deficiency include degenerative changes in eyes and skin. These are mostly reversible (e.g. night blindness) but in serious cases the changes can lead to blindness (keratomalacia). According to the WHO about 21 % of preschool-age children and approximately 6 % of pregnant women worldwide are affected by a malnutrition related vitamin A deficiency [3]. Besides to malnutrition fat malabsorption, particularly caused by celiac disease or chronic pancreatitis predispose to vitamin A deficiency. Furthermore hepatic damage, often induced by alcohol abuse, is a cause for an insufficient vitamin A status [1]. Conversely an excessive dietary vitamin A supplementation can result in the toxic effects of a hypervitaminosis. During pregnancy high vitamin A concentrations are known to be teratogenic with the risk of fetal malformation [1, 2].

For healthy adolescents and adults a daily dietary intake of 0.8–1.1 mg of retinol equivalents (RE) is recommended by the DACH Societies for Nutrition (common reference values of DGE Germany, ÖGE Austria, SGE/SVE Switzerland), with increased needs during pregnancy (1.1 mg RE) and lactation (1.5 mg RE). Hereby, a ratio equivalence of 1 : 6 : 12 is defined, corresponding to 1 mg retinol (vitamin A), 6 mg all-trans-β-carotene or 12 mg of other provitamin A carotenoids [4].

Age-related reference ranges for children and adults should be used for the monitoring of the vitamin A serum and plasma level. These can be found in section 7.2.

For the determination of β-Carotene in serum and plasma an additional ClinRep® HPLC Complete Kit is available (order no. 30000). The according reference ranges are provided in the user instruction manual of the kit.

Vitamin E:

Vitamin E is the nutritional term for a group of naturally occurring tocopherols and tocotrienols that have biological activity similar to α-tocopherol. In the human organism α-tocopherol represents the main isomer with a percentage of 90 % [5] and thus is used for the assessment of the vitamin E status [1, 5].

α-Tocopherol is an important anti-oxidant which protects from lipid peroxidation. It is involved in immune function in cell signaling, regulation of gene expression and other metabolic processes, e.g. muscle platelets, monocytes and muscle cells [6]. Vitamin E deficiency caused by impaired fat absorption can produce oedema and hemolytic anemia especially to premature babies and newborns [7]. In adults a severe vitamin E deficiency is rarely seen but may occur as a result of serious malabsorption. Compared to vitamins A and D vitamin E is less toxic. However, hypervitaminosis E may lead to a reduced uptake of the fat soluble vitamins D and K [7].

Age-related reference ranges for children and adults should be used for the monitoring of the vitamin E (α-tocopherol) serum and plasma level. These can be found in section 7.2.

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* For the determination with conventional HPLC systems a ClinRep® HPLC Complete Kit with order no. 22000 is available

1.4 General description of the analytical method

In this analytical method, 25-OH-vitamin D is determined from serum or plasma by UHPLC with UV detection.

Prior to UHPLC analysis a short sample clean-up is performed in order to remove the sample matrix and to spike with the internal standard (sample preparation, see section 5.2).

Afterwards the prepared samples are injected into the analytical system. The sample components are chromatographically separated on the analytical column and the analytes are detected by the UV detector.

For the check-up of the analytical system a matrix-free ClinTest® Standard Solution is available (see section 5.3.1).

When the analytical system has been tested successfully samples (calibrator, control, patient) are injected for calibration and measurement (see section 5.3.2). The obtained chromatograms are evaluated with the internal standard method via peak areas (see section 6).

Quality control is performed with ClinChek® Controls. These controls are available in three different concentrations (see section 5.3.3).

2 Components of the complete kit and accessories

2.1 Ordering information

Order No.	Description	Quantity
22100	**ClinRep® Complete Kit for Vitamin A and E in Serum / Plasma by UHPLC** for 100 assays	1 pce.
	Contents:	
	Mobile Phase	1 x 22010
	Standard Solution	1 x 22011
	Precipitant P with Internal Standard	1 x 22112
	Serum Calibrator, lyophil.	1 x 22013
	Sample Preparation Vials	1 x 22120
	Stabilising Reagent S	1 x 22121
	Manual	
	Quick Reference	
	Separately available components:	
22010	Mobile Phase	1000 ml
22011	Standard Solution	3 ml
22112	Precipitant P with Internal Standard	10 ml
22013	Serum Calibrator, lyophil.	3 ml
22120	Sample Preparation Vials	100 pcs.
22121	Stabilising Reagent S	5 ml
	Start Accessories:	
22130	Analytical Column with test chromatogram	1 pce.
	ClinChek® Controls:	
8870	Serum Control, lyophil. Level I	10 x 5 ml
8871	Serum Control, lyophil. Level II	10 x 5 ml
8872	Serum Control, lyophil. Level III	10 x 5 ml
8873	Serum Control, lyophil. Level I, II, III	3 x 3 x 5 ml

2.1.1 Safety Information

Components such as mobile phases and reagents are chemical preparations and may contain hazardous substances. For safety information please consult the respective safety data sheet (SDS) for each component.

The calibrator and control materials are manufactured from human serum. Although the products are tested for the absence of common infection markers, they still should be considered as potentially infectious. For this reason we recommend the product to be handled with the same precautions as patient samples. Detailed safety information is indicated in the respective SDS.

2.1.2 Storage conditions and life time of kit components

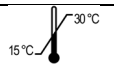
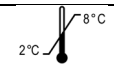
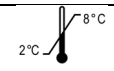
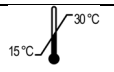
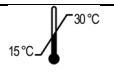
Please unpack all components from the transport packaging **immediately upon receipt** and follow the storage instructions indicated on the product labels and in Table 1.

Unused components, stored under appropriate conditions, can be used until the expiry date indicated on the product label.

After use of ClinRep® Reagents and ClinRep® Mobile Phases the bottles must be closed tightly and stored immediately under the required conditions. Provided that instructions for proper use and storage procedures are followed, the lifetime of the reagents is the same as for the unused products.

Storage conditions and lifetimes of the ClinTest® Standard Solution, the ClinCal® Calibrator as well as ClinChek® Controls (lyophilised and after reconstitution) are indicated in the respective product data sheets.

Table 1. Storage conditions of kit components

Order no.		**Product description**	**Storage conditions**	
REF	22010	Mobile Phase		Store at 15–30 °C
REF	22011	Standard Solution		Store at 2–8 °C
REF	22112	Precipitant P with Internal Standard		Store at 2–8 °C
REF	22013	Serum Calibrator, lyophil.		Store below -18 °C*
REF	22120	Sample Preparation Vials	Store at ambient temperature	
REF	22121	Stabilising Reagent S		Store at 15–30 °C
REF	22130	Analytical Column		Store at 15–30 °C
REF	8870	Serum Control, lyophil., Level I		Store below -18 °C*

Order no.		Product description	Storage conditions	
REF	8871	Serum Control, lyophil., Level II	-18 °C	Store below -18 °C*
REF	8872	Serum Control, lyophil., Level III	-18 °C	Store below -18 °C*
REF	8873	Serum Control, lyophil., Level I, II, III	-18 °C	Store below -18 °C*

*Refers to the lyophilised product. For storage conditions after reconstitution please consult the product data sheet.

2.1.3 Disposal of laboratory waste

For disposal laboratory waste should be collected separately according to the different chemical properties. Recommendations for the disposal of the product and the respective packaging are indicated in section 13 of the respective Safety Data Sheet (SDS).

3 Required instruments

Using this test kit requires a UHPLC system with UV detector and evaluation software.

Required UHPLC modules:

- Isocratic UHPLC pump
- UHPLC autosampler
- UHPLC column heater (30 °C)
- UHPLC UV detektor (325 nm; after 0.4 min: 295 nm)

For sample preparation the following laboratory instruments are required:

- Pipettes, pipette tips
- Tabletop centrifuge
- Vortex mixer

4 Operation of the analytical system

4.1 Installation of the analytical column and equilibration of the UHPLC system

For information regarding the installation of the analytical column and the equilibration of the analytical system, please refer to the user manual of the instrument manufacturer.

4.2 Starting the analytical system

Table 2 provides the parameters for the UHPLC system. To ensure appropriate usage of the analytical system, please consult the user manual of the instrument manufacturer. User trainings, provided by the manufacturer, may also be advisable.

For test run, calibration and measurement please refer to section 5.3.

Table 2: UHPLC parameters

UHPLC pump:	Flow rate: 0.8 ml/min
Mobile phase:	Make sure that the bottle is closed well to avoid alteration of the retention times through evaporation of components of the mobile phase. Recycling: The mobile phase may be circulated through the system for 100 analyses. A new bottle of mobile phase has to be used after 100 analyses.
Column:	The analytical column* is installed within the column heater (30 °C). The backpressure of the analytical column should not exceed 500 bar. *Please note section 4.3 for the de-installation and storage of the analytical column.
Column heater:	30 °C
UV detector:	Set the UV detector at first to a wavelength of 325 nm, then change the wavelength after 0.4 min to 295 nm. For further information on detector settings please refer to the user manual of the instrument manufacturer.
Autosampler:	Injection volume: 5 µl Injection interval: 1.2 min Needle flushing: The injection needle needs to be flushed after the sample extraction (minimisation of sample carryover). Please refer to the recommended needle wash settings in the use manual of the autosampler manufacturer. For flushing please use the mobile phase with order no. 22010.

Evaluation unit:	Integration stop has to be set at 1.2 min.
	Retention times: Vitamin A: approx. 0.25 min Internal Standard IS: approx. 0.59 min Vitamin E: approx. 0.82 min Please note: Depending on the UHPLC system used, differences to the indicated retention times may be observed. These differences do not influence the efficiency of the analytical procedure, however they must be considered with regard to the settings for autosampler and evaluation unit. Also, a shifting of retention times may occur due to an aging of the column or its improper use.

4.3 Standby mode

In case no analyses are being carried out **for several hours**, the flow rate of the UHPLC pump can be reduced to 0.1 ml/min in order to save mobile phase. For an operational pause **until the next day**, pump, column heater and detector can be switched off. The analytical column needs not to be de-installed (column heater switched off!).

In case of a de-installation, the analytical column must be stored tightly closed in mobile phase (order no. 22010).

5 Implementation of the analytical procedure

5.1 Collection and storage of samples

The determination of vitamins A and E can be performed from EDTA-serum or –plasma.

At room temperature (15–30 °C) and stored in the dark the stability of the samples is at least one day. At temperatures between 2–8 °C the samples can be stored at least 7 days and at temperatures below -18 °C at least three months (multiple freeze-thaw cycles should be avoided).

Please note:
The samples are light sensitive and therefore need to be stored in the dark.

5.2 Sample preparation

5.2.1 Reconstitution of the serum calibrator and the serum controls

The ClinCal® Serum Calibrator and the ClinChek® Serum Controls (see section 2.1) are lyophilised and thus must be reconstituted before use. Information on reconstitution, analyte concentrations as well as on storage and stability, is given in the appropriate product data sheets.

5.2.2 Work flow

Sample preparation:

Precipitation:

50 µl Serum / Plasma
(calibrator, control, patient)

⇩

100 µl Precipitant P with Internal Standard (**cooled***)

⇩

50 µl Stabilising Reagent S

mix ⇩ (30 sec, Vortex mixer)

centrifuge ⇩ (5 min, > 1000 x g)

UHPLC Analysis: Inject 5 µl of the supernatant

*Note 1:
Precipitant P with Internal Standard should be used cooled, i.e. at temperatures between 2–8 °C (=storage temperature). For this purpose, the reagent should not be withdrawn from the refrigerator until immediate use (see section 5.2.2.1).

Note 2:
The analytes are light sensitive. Therefore, please protect the samples from direct sun light during the entire sample preparation.

5.2.2.1 Precipitation

Pipette 50 µl of the sample (calibrator, control, patient) into a sample preparation vial (order no. 22120) and add 100 µl of the Precipitant P with Internal Standard (order no. 22112). This reagent should be used **cooled**, i.e. at temperatures between 2–8 °C (=storage temperature). For this purpose, the reagent should not be withdrawn from the refrigerator **until immediate use**.

Subsequently pipette 50 µl Stabilising Reagent S to the sample.

Mix the sample for 30 sec on a vortex mixer. Afterwards centrifuge for 5 min at a minimum rotation speed of 1000 x g.

5.2.2.2 UHPLC Analysis

Inject 5 µl of the supernatant into the UHPLC system.

Please note:
The analytes are light sensitive. Therefore please protect the samples from direct sun light during the entire sample preparation (section 5.2.2).

5.2.3 Stability of the prepared samples

Stored at room temperature (15–30 °C) and in the dark the stability of the prepared serum and plasma samples is at least 3 days. Stored at 2–8 °C the stability is at least 7 days and at temperatures below -18 °C at least 28 days. Avoid repeated freezing and thawing of the samples.

Please note:
The samples are light sensitive and therefore need to be stored in the dark.

5.3 UHPLC Analysis

5.3.1 Test run

Prior to the injection of prepared samples (calibrator, control, patient) the UHPLC system should be checked by the use of the matrix-free ClinTest® Standard Solution (order no. 22011).

Repeatedly inject 2 µl of the standard solution until two consecutive chromatograms are identical in respect of retention times and peak resolution (see example chromatograms in section 5.3.4).

Now check the integration parameters (e.g. run time, peak identification, marks for peak start and end). Correct the parameters if necessary and inject the standard solution once again for verification.

5.3.2 Calibration

For calibration of the UHPLC system the ClinCal® Serum Calibrator (order no. 22013) has to be used. After reconstitution (see section 5.2.1), the calibrator must be prepared as described for the patient samples (see section 5.2).

Inject 5 µl of the serum calibrator several times; this enables a single-point calibration with averaging. When carrying out large series of analyses, we recommend injecting a calibrator every tenth patient sample as well as at the end of the series. This way the chromatographic conditions can be checked and in case of changes (e.g. shifts in retention times) corrections are possible without having to repeat the sample analysis (see also section 6.1).

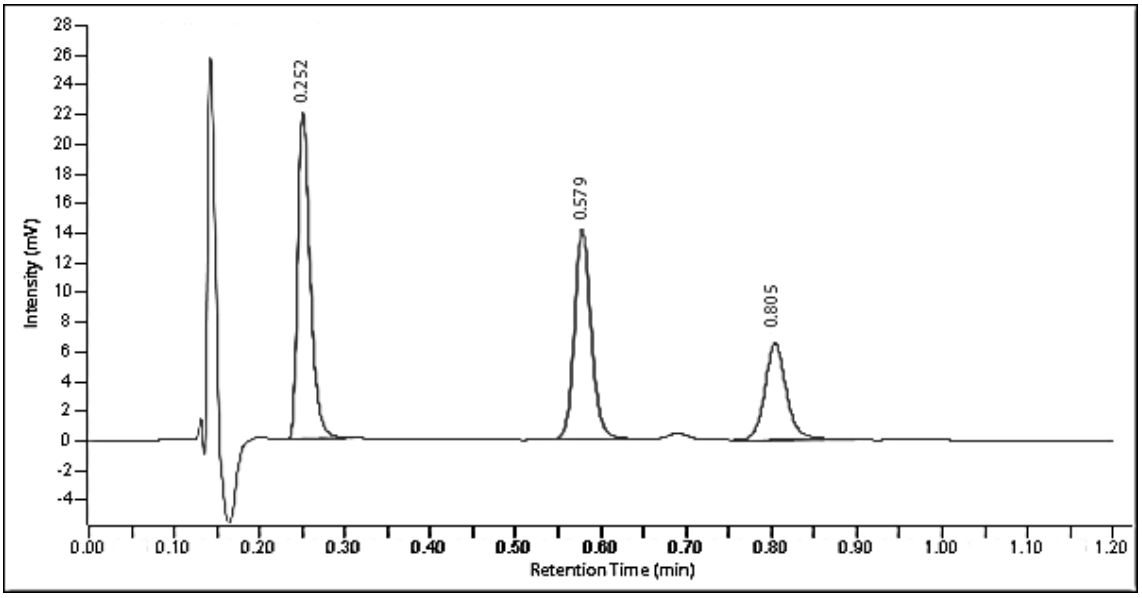
5.3.3 Quality control

For the internal quality assurance of the analytical measurements, ClinChek® Serum Controls in three different concentrations are available (level I, order no. 8870; level II, order no. 8871; level III, order no. 8872, level I, II, III, order no. 8873).

These controls are lyophilised and, subsequent to reconstitution, must be prepared as described for the patient samples (see section 5.2). The controls are analysed within the analytical series. In case of large analytical series, we recommend to insert control samples repeatedly within the series.

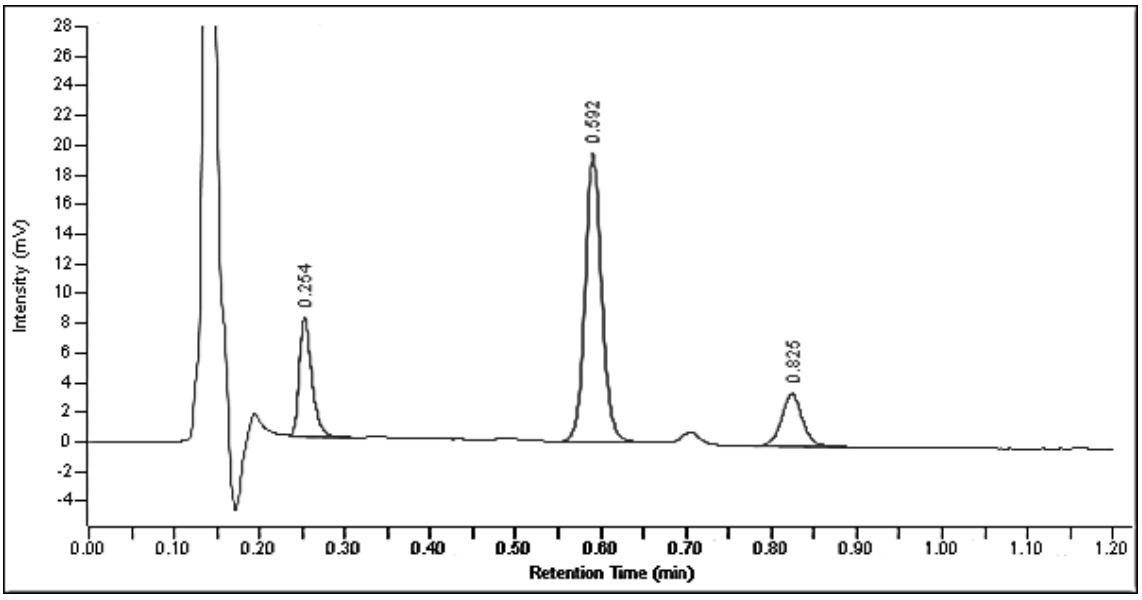
5.3.4 Example chromatograms

The figures below show chromatograms of the ClinTest® Standard Solution (order no. 22011) and of the ClinChek® Serum Control Level I (order no. 8870).



Vitamin A: 0.252 min, Internal Standard IS: 0.579 min, Vitamin E: 0.805 min

Figure 2. Chromatogram of the ClinTest® Standard Solution (order no. 22011)



Vitamin A: 0.254 min, Internal Standard IS: 0.592 min, Vitamin E: 0.825 min

Figure 3. Chromatogram of the ClinChek® Serum Control Level I (order no. 8870)

6 Evaluation

6.1 General

In order to check for stable chromatographic conditions during an analytical series, the chromatograms of the measured samples (control, patient) are compared with those of the serum calibrators, particularly with regard to correlation of peaks and retention times. Temperature fluctuations may lead to shifted retention times and false peak identifications. If calibrators have been run between samples, you can recalibrate without having to repeat the analysis (see also section5.3.2)

6.2 Evaluation method

Calculation of unknown samples has to be done using the internal standard method via peak areas.

According to the internal standard method, each sample is spiked with a so-called "internal standard" prior to the sample preparation. The internal standard is similar to the analytes in terms of behaviour during sample preparation and chromatography. Any losses during the sample preparation hence can be determined by calculating the recovery. Extrapolation to 100 % recovery allows establishing the concentration of the unknown substances in the sample.

Please consult the UHPLC software manual of the instrument manufacturer in order to ensure correct evaluation of the results.

For the calculation of mass concentrations [mg/l] into molar concentrations [µmol/l], and vice versa, the analytical results should be multiplied with the following factors:

Table 3. Conversion factors

Analyte	**Molecular weight [g/mol]**	**Conversion factor: µmol/l → mg/l**	**Conversion factor: mg/l → µmol/l**
Vitamin A (all-trans-Retinol)	286.5	0.287	3.49
Vitamin E (α-Tocopherol)	430.7	0.431	2.32

6.2.1 Manual calculation

Calculation of the recovery rate (REC):

$$\text{REC} = \frac{\text{Area (IS, sample)}}{\text{Area (IS, calibrator)}}$$

Calculation of the analyte concentration c [mg/l]:

$$\text{c (Analyte, sample) [mg/l]} = \frac{\text{Area (analyte, sample)} \times \text{c (analyte, calibrator) [mg/l]}}{\text{Area (analyte, calibrator)} \times \text{REC}}$$

7 Test data

7.1 Test performance

The results were obtained with a modular UHPLC system.

7.1.1 Linearity, detection limit and quantitation limit

Linearity, detection limit and quantitation limit are listed in Table 4.

Table 4. Linearity, detection and lower quantitation limits (LOD, LLOQ)

Analyte	**Linearity [mg/l]**	**LOD [mg/l]**	**LLOQ [mg/l]**
Vitamin A (all-trans-Retinol)	0.04–3.96	0.013	0.040
Vitamin E (α-Tocopherol)	1.02–104	0.535	1.07

7.1.2 Trueness

The trueness rate for vitamin A and E lies between 96–109 %.

7.1.3 Precision

The intra- and interassay precisions of the method were determined with samples in three different concentrations. Analyte concentrations and precision results are indicated in Table 5.

Table 5. Precision results

	Concentration [mg/l]			**Intraassay Precision [%]**			**Interassay Precision [%]**		
Analyte	**Level**			**Level**			**Level**		
	I	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
Vitamin A (all-trans-Retinol)	0.537	1.02	1.49	2.4	1.9	4.0	3.1	3.2	1.9
Vitamin E (α-Tocopherol)	10.8	17.7	21.1	3.1	1.4	4.0	1.4	2.3	1.6

7.2 Reference ranges

The following reference ranges are taken from [5].

Table 6. Reference ranges for vitamin A and E in serum / plasma

Vitamin A		
	[mg/l]	[µmol/l]
Children		
Newborns	0.1–0.3	0.35–1.0
Infants until 1 year	0.15–0.4	0.53–1.4
Children until 10 years	0.2–0.5	0.66–1.7
Adolescents	0.3–0.6	1.0–2.1
Adults	0.3–0.6	1.0–2.1
Vitamin E		
Children		
Premature infants	2.5–3.7	5.8–8.6
Children (1–12 years)	3–9	7–21
Adolescents (13–19 years)	6–10	14–23
Adults	5.5–18	12.8–42

The indicated reference ranges are taken from thoroughly selected and current scientific literature. Their actuality corresponds to the printing date of this document. Please note that these ranges do not reflect any recommendations by the manufacturer of this product, but may be used as a guideline for the assessment of the reference range by the clinical laboratory.

8 References

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[3] Ezzati M, Lopez AD, Rodgers A, Murray CJL (Eds.): Comparative quantification of health risks. Chapter 4: Rice AL, West Jr KP, Black RE: Vitamin A deficiency. World Health Organization 2004; 211–256.

[4] DACH Nutrition Reference Values for Vitamin A and β-Carotene (Download: www.dge.de/wissenschaft/referenzwerte/vitamin-a-b-carotin/; 27.03.2018).

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[6] Maya W, Mayur K, Ashar S. Pharmaceutical Profile of Alpha-Tocopherol – A Brief Review. Int J Pharm Chem Sci 2012;1(3):1023–1039.

[7] Greiling H, Gressner AM (Eds.): Lehrbuch der klinischen Chemie und Pathobiochemie, 3. Edition, Schattauer-Verlag Stuttgart New York 1995; S. 444–446.

9 Troubleshooting

Problem	Possible Cause	Corrective Measure
Pressure fluctuation	Air in the pump	Open the purge valve of the pump and aspirate the mobile phase by use of a syringe. Switch the pump for delivery at enhanced flow rate.
	Defective pump check valves	Clean the pump check valves (ultra sonic bath) or renew them (to be carried out by a service technician).
	Air in the pump, leakage	Check the pump
Spikes on the baseline	Air bubbles in the detector cell	Disconnect the column and flush the detector cell with mobile phase.
	Air bubbles in the mobile phase	Degas the mobile phase
Baseline drift	The analytical system is not equilibrated	Equilibrate the analytical system (see section 4.1)
	Mobile phase contaminated	Renew the mobile phase (see section 4.2)
Noisy baseline	Detector cell contaminated	Flush the detector cell with a suitable solvent (to be performed by a service technician)
	Pressure fluctuation	See „problem caused by pressure fluctuation“
	Contamination of the analytical column	Flush the analytical column with mobile phase (no recycling!). Renew prefilter
Peak splitting	Defective analytical column (column packing)	Replace the analytical column
	Defective injection valve	Maintenance and cleaning of the injection valve (to be carried out by a service technician)
Broad peaks, tailing	The capability of the analytical column has been exhausted	Replace the analytical column
	Overload of analytical column	Reduce the injection volume (see section 4.2)
	Dead volume within the analytical system	Check the analytical system

Problem	**Possible Cause**	**Corrective Measure**
Fluctuation of recovery	Defective pipettes	Check the pipettes
	Injection volume not constant	Check the autosampler
	Incorrect sample preparation	Take care of correct operation
Interfering peaks within the chromatogram	Expiry of samples, reagents, etc., passed	See notes on storage and stability
	Incorrect sample preparation	Take care of correct operation
	Contamination of mobile phase or reagents for sample preparation	Renew the mobile phase and reagents
	Analytical column contaminated	Replace analytical column
	Injection system contaminated	Clean the whole injection system (needle, washing station, etc.) with water, followed by isopropanol.
	Interfering peaks, despite of measures named above: Flush the UHPLC system with acid. Please refer to the user manual of the instrument manufacturer	
High backpressure	Obstruction of components like capillaries, filters, columns, etc.	Localise obstructed components by successive disconnection
Alteration of retention times	Temperature fluctuation	Check the column heater
	Leakage within the UHPLC system	Eliminate the leakage
	Pressure fluctuation	See „problems by pressure fluctuation“
Inappropriate detector sensitivity	Contamination of detector cell	Flush the detector cell with a suitable solvent (to be performed by a service technician)
	Detector lamp exhausted	Renew the detector lamp

10 EC Declaration of Conformity

The EC Declaration of Conformity is available upon request (info@recipe.de).

RECIPE Chemicals + Instruments GmbH
Dessauerstraße 3
80992 München

Tel. +49 89 54 70 81 - 0
Fax. +49 89 54 70 81 - 11

info@recipe.de
www.recipe.de

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ISO 13485