AUTOMATED DETERMINATION OF VITAMIN A AND E IN SERUM BY ON-LINE SPE-HPLC

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**Don't go the roundabout way,
go on-line!**

INTRODUCTION

Vitamin A and E are fat-soluble vitamins which are mainly stored in the liver and fatty tissue of the human body. The most thoroughly investigated physiological functions of vitamin A and E are their contribution to visualization, regulation of reproduction, of growth and the synthesis of steroid hormones. In serum, vitamin A and E are partly bound to proteins which makes a direct solid phase extraction impossible. They are extremely light- and air-sensitive and therefore samples have to be maintained in the dark as much as possible.

Vitamin A and E are often analyzed using commercially available kits based on LLE, centrifugation and HPLC analysis (see table 3).
Alternatively, serum samples are mixed with acetonitrile/water solutions until a final concentration of 17% acetonitrile [1]. The acetonitrile makes vitamin A completely free from the proteins, but vitamin E remains partly bound. A higher acetonitrile concentration can not be used because it would cause protein precipation. To release more vitamin E from proteins, Sodium Dodecyl Sulfate (SDS) can be added [2].

In this poster a method is described for fully automated determination of vitamin A and E in raw serum, using on-line SPE-HPLC.

EXPERIMENTAL

Instrumentation

HPLC experiments were carried out with an isocratic pump (118, Beckman) and a variable wavelength UV detector (655A, Hitachi). For automatic sample preparation an integrated system was used, comprising a Basic-Marathon autosampler, a Prospekt solid phase extraction unit and a SDU solvent delivery unit (Spark Holland). The system configuration is shown in figure 1. It is essential that the content of the transfer tubing between the autosampler and the Prospekt is approximately 1 ml with a 1 mm I.D.

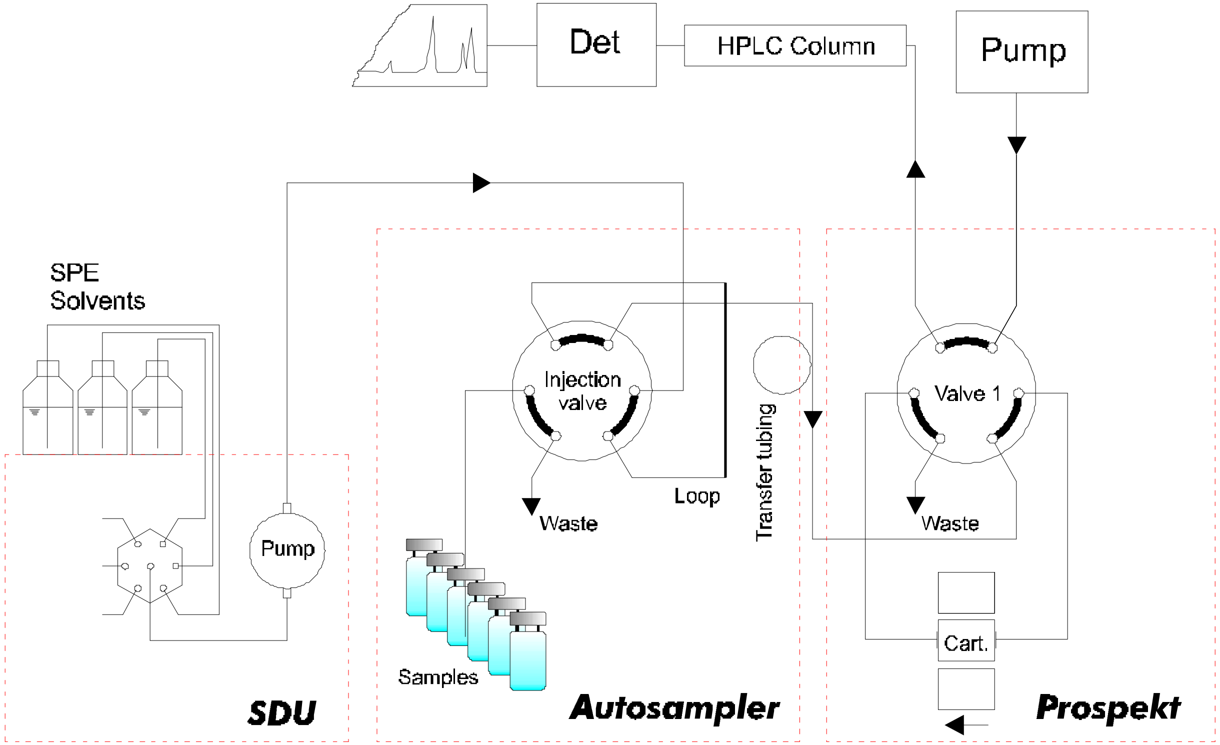

Figure1: System configuration On-line SPE-HPLC

Chromatographic conditions.

Anal. Column : 5 µm Inertsil ODS-2; 150 × 4.6 mm ID
SPE cartridge : C18 IST 10 × 2 mm ID
Mobile phase : Methanol/Tetrahydrofuran/Water 93:5:2 (v/v %)
Flow rate : 1.0 ml/min
Detection : 294 nm (UV max vitamin E)
Injection vol. : 100 µl

Sample Preparation.

- *Activation with 2 ml methanol at a flowrate of 4.0 ml/min*
- *Conditioning with 4 ml 34% Acetonitrile at a flowrate of 4.0 ml/min*
- *Equilibration with 0.4 ml 34% Acetonitrile 15 mM SDS at a flowrate of 0.4 ml/min.*
- *Load sample (serum) with 2.0 ml 34% Acetonitrile 15 mM SDS at a flowrate of 0.4 ml/min.*
- *Clean-up with 1.0 ml 34% Acetonitrile 15 mM SDS at a flowrate of 1.0 ml/min.*
- *Elute cartridge with 1.0 ml mobile phase.*
- *Purge tubing with 2.0 ml 34% Acetonitrile at a flowrate of 4.0 ml/min.*
- *Purge tubing with 2.0 ml water at a flowrate of 4.0 ml/min.*
- *Start next sample preparation.*

The total SPE time is 9:30 min and runs concurrently with the chromatography.

RELEASING THE VITAMINS FROM THE PROTEINS

The sample transfer from the autosampler to the Prospekt module is used to release vitamin A and E from the proteins. During the transfer the serum sample is mixed with the transfer solvent from the SDU. Therefore, the composition of the transfer solvent is very important. In previous experiments, we optimized the acetonitrile concentration (34%) and the flowrate for the release of vitamin A [1]. However, the release of vitamin E is negligible under these conditions. To improve the release of vitamin E from the proteins, we added SDS to the transfer solvent. A significant increase in recovery of vitamin E was observed. We optimized the concentration of SDS and noticed that the peak area of vitamin E did not further increase beyond concentrations of 15 mM SDS.

VALIDATION

Figure 2 shows a chromatogram of a patient sample. Obviously the sample clean up is very efficient.

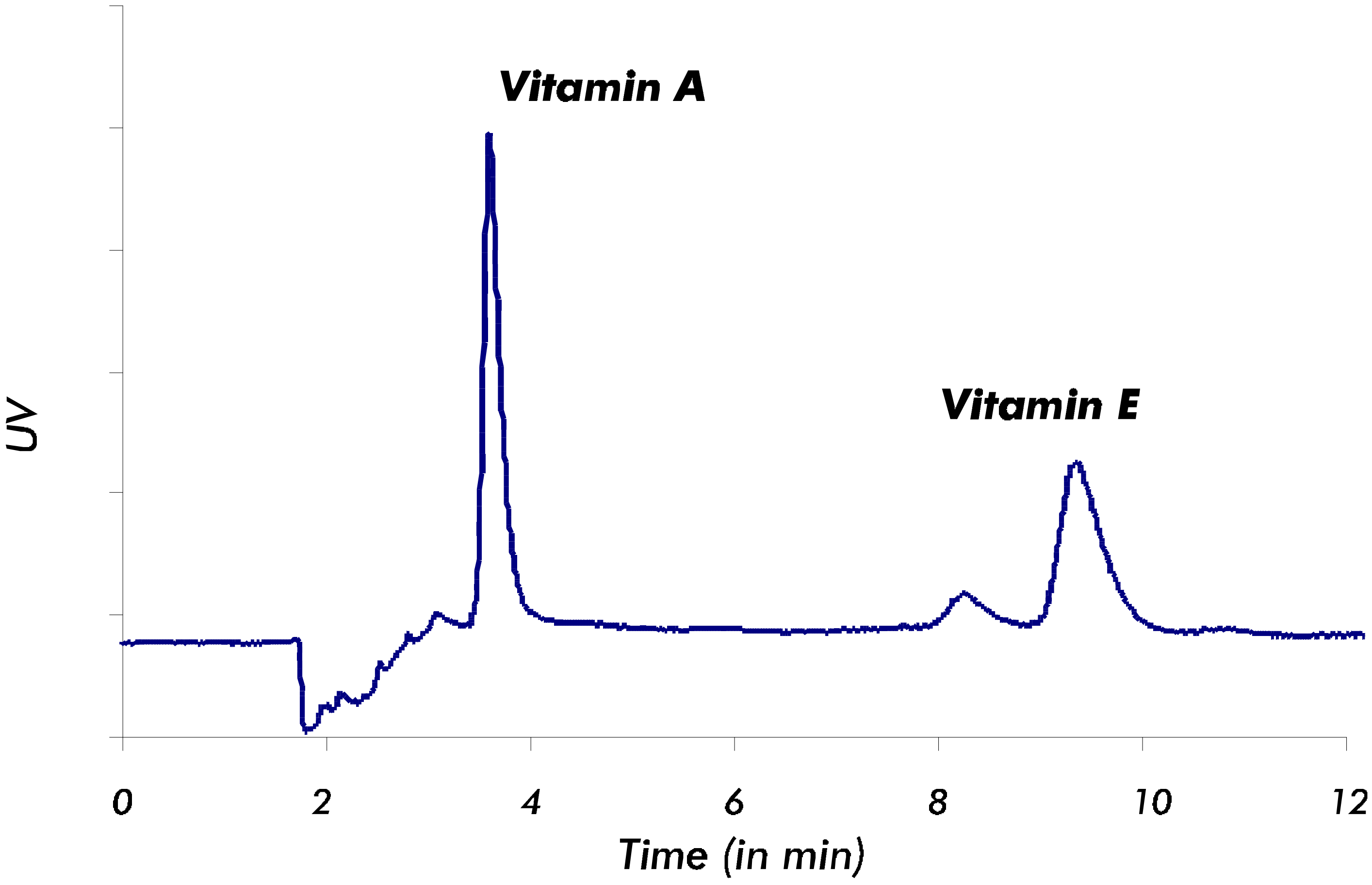

Figure 2: Chromatogram of Vitamin A and E in a patient sample.

The precision was good (see table 1). We found that spiking of serum samples for calibration plots is not feasible, because to much vitamin E is lost by adsorption to glassware/plastic ware when preparing the calibration samples. Apparently, the adsorption proceeds much faster than protein binding. Therefore, calibration standards were prepared in mobile phase rather than in serum. To correct the calibration line for incomplete recovery of vitamin E (vitamin A is completely recovered from serum), several calibration standards with "known" concentrations [2] were analyzed. From these analyses we concluded that the recovery of vitamin E is 70.3 % with an RSD of 5 % . Vitamin E values in serum, obtained using the calibration plot for vitamin E in mobile phase, must therefore be multiplied by a factor of 1.42 to obtain the true vitamin E concentration.

Table 1: Performance of the On-line SPE-HPLC method

	Repeatability	Linearity (R^2)	Detection limit	Recovery
Vitamin A	RSD = 1.7 % (n=6)	0.999	0.051µM	100%
Vitamin E	RSD = 4.1 % (n=6)	0.999	2.74 µM	70%

Using this correction factor, we analyzed a number of serum standards from the SKLZ and compared the values to results of other participants in the vitamin quality assessment program of the SKZL. The results are given in table 2.

Table 2: Results Spark vs. mean results conventional methods

	Result Spark	Mean Value other participants
Vitamin A low	1.03 µM	0.97 µM
Vitamin E low	12.2 µM	12.5 µM
Vitamin A medium	1.95 µM	1.87 µM
Vitamin E medium	21.5 µM	23.5 µM
Vitamin A high	4.99 µM	5.33 µM
Vitamin E high	49.3 µM	57.2 µM

The results are in good agreement with the mean values of the results of all other participants, except for the high vitamin E concentration. Possibly the calculated correction factor needs adjustment in the high concentration range. Validation is continued to finetune and adjust the assay.

THE BENEFITS OF ON-LINE SPE

Conventional methods include a lot of manual steps which all can lead to loss of vitamin A and E. Using on-line SPE, the only manual action is placing the vial with serum in the autosampler tray. During analysis of a sample, the Prospekt already prepares the next sample, and so the total assay cycle time is not longer than the chromatography time, i.e. 9 minutes.

Table 3: Comparison on-line SPE vs. conventional methods

On-line SPE	***Conventional method***
Place serum sample in autosampler	*Precipate proteins with ethanol*
	Extract lipids with n-Hexaan
	Centrifuge sample
	Evaporate supernatant
	Reconstitute sample in eluent
	Place sample in autosampler
Start analyze sample	*Start analyze sample*

A clear benefit of the on-line SPE method is that the raw sample, containing the light- and air-sensitive vitamin A and E is analyzed in a closed system reducing the risk of vitamin degradation significantly. Obviously, the On-line SPE method is superior considering labor time.

CONCLUSION

- *A simple and fast on-line SPE-HPLC assay for Vitamin A and E in serum has been developed.*
- *The assay is robust and precise because serum samples are analyzed in a closed system, fully automatically, with a minimum of manual sample treatment.*
- *The recovery of the assay for vitamin A is 100 % with a RSD value of 1.7 % (n=6).*
- *The recovery of the assay for vitamin E is 70.3 % with a RSD value of 4.1 % (n=6).*
- *The technique used to release analytes from serum proteins is promising as a general approach for other protein bound analytes.*

REFERENCE

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