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Fully automated method for the determination of 24,25(OH)$_2$ and 25(OH) D$_3$ hydroxyvitamins, and Vitamins A and E in human serum by HPLC

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Abstract

A new fully automated method for the determination of metabolites of Vitamin D$_3$ and Vitamins A and E has been developed. A robotic station for liquid–liquid extraction, connected on line with an automatic system for solid-phase extraction (Prospekt) and a liquid chromatograph were used and the complexity of the overall method was overcome by full automation. The eluate from the chromatograph was monitored by a photodiode-array detector at three wavelengths, namely, 265 nm for Vitamin D$_3$ metabolites, 291 nm for Vitamin E and 325 nm for Vitamin A—which are the maximum absorption wavelengths for the analytes. The time required per sample analysis was 35 min because of the overlapping development of the steps. The linearity obtained for serum samples (standard addition method) gives correlation coefficients (r^2) ranging between 0.996 and 0.989, with standard deviation of the slope between 4.0 and 4.9%. The repeatability was between 4.0 and 6.0% and the within-laboratory reproducibility was lower than 10.1% in all cases—both expressed as relative standard deviation—for low concentrations of the analytes, namely, 3 ng/ml for 24,25(OH)$_2$ dihydroxyvitamin D$_3$, 10 ng/ml for 25(OH) hydroxyvitamin D$_3$, 100 ng/ml for Vitamin A and 2 μg/ml for Vitamin E. The method has been validated using a CRM (NIST, SRM968c).

Keywords: Fat-soluble vitamins; Retinol; 24,25(OH)$_2$ hydroxyvitamin D$_3$; 25(OH) hydroxyvitamin D$_3$; α-Tocopherol; Serum; Prospekt; HPLC

1. Introduction

Fat-soluble vitamins, i.e. Vitamins A, E, K, and D play an important role in preventing cancer, cardiovascular diseases, coagulation (γ-carboxylation of inactive clotting factors), and bone calcification [1]. These vitamins are regarded as useful clinical indicators of the incidence of cancer, myocardial infarction, haemorrhage and osteomalacia diseases. The toxicity of fat-soluble vitamins has been studied in precision-cut liver-slice cultures [2,3]. Over the last decade, nutritionists and clinicians have been increasingly interested in measuring these fat-soluble vitamins in human plasma in order to evaluate their

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status, fat malabsorption disorders or their toxic levels.

The Vitamin D endocrine system is unique among endocrines, as its precursor molecule (7-dehydrocholesterol) is dependent upon sunlight for its endogenous synthesis [4]. Furthermore, this endocrine system also requires multi-organ participation in the activation and biological expression of the metabolically active hormone 1,25-dihydroxyvitamin D [5–7]. Vitamin D is considered essential for life, as this pleiotropic hormone regulates cell proliferation and differentiation, mineral metabolism, immune responses, and brain function in addition to its influence on many intracellular processes [7–10]. Vitamin D status is of crucial importance in calcium metabolism because the active hormone regulates active calcium uptake in the gastrointestinal tract, renal calcium excretion, and skeletal calcium turnover [5,7,11]. Because of its ubiquitous role in mineral metabolism, it is generally accepted that in states of chronic Vitamin D deficiency, skeletal integrity, and cellular functions are compromised.

In most vertebrates, the metabolite produced in the liver, i.e. 25-hydroxyvitamin D is the main circulating form and its concentration is used clinically to assess Vitamin D status [11,12].

Vitamin A is crucial for morphogenesis, vision, immune function, reproduction, neuronal and neural development and maintenance of differentiation functions [13–15]. Recent interest has focussed on the potential role of Vitamin A status in modulating the effects of HIV infection, particularly in the vertical transmission from mother to infant [16]. Clinical interest in the relationship between antioxidant micronutrients, such as Vitamin E, in several degenerative human health conditions [17], and retinol deficiency with abnormal retinoids metabolism, which is implicated in neural degeneration associated with ageing and certain brain pathologies such as Alzheimer's diseases [18,19] has been growing in the last few years. In addition, recent epidemiological and clinical studies have suggested that the fat-soluble Vitamins A and E protect against coronary heart disease and they may play a key role in the prevention of some cancers [20–22]. Reduced plasma α-tocopherol levels have also been reported in the Smith–Lemli–Opitz syndrome [23].

Moreover, monitoring the status of Vitamins A and E is of great help in detecting an inadequate dietary intake of these vitamins or a possible deficiency of either retinol-binding protein, which protects retinol from degradation and cells from its amphypathic properties, or of α-tocopherol transfer protein (α-TTP), which incorporates α-tocopherol into very low-density lipoproteins in the liver [24].

Recently published methods allow the simultaneous determination of retinol and tocopherol in human plasma or serum [25–29]. For Vitamin D_3, it is significant that the method allows the determination of the 25-hydroxylated metabolite since it is the main circulating form of this vitamin [30].

In this paper, we report an automated method for the simultaneous determination of 25-hydroxyvitamin D_3, 24,25$(OH)_2$ hydroxyvitamin D_3 and Vitamins A and E, consisting of liquid–liquid extraction with isopropanol and *n*-hexane, solid-phase extraction connected on line with a liquid chromatograph and photodiode-array detector.

2. Material and methods

2.1. Apparatus and instruments

The experimental set-up consists of a Zymate II Plus robot (Zymark Co, Hopkinton, Massachussets) with the necessary peripherals; a ProStar 410 autosampler equipped with a 7.5 ml sample loop (Varian, Palo Alto, CA, USA) and a Prospekt automated solid-phase extractor (Spark Holland, Emmen, The Netherlands) both connected on-line with a liquid chromatograph (Varian 240 pump; Varian PDA detector 330). Data processing was carried out using a Star Chromatography Workstation version 6.0 software running on a personal computer.

An Ultrabase C_{18} column (250 mm × 4.6 mm; 5.0 μm particle size, Scharlau Science, Barcelona, Spain) and a cartridge packed with Oasis HLB as sorbent material were used as analytical column and for solid phase extraction (SPE), respectively. Ultrabase C_{18} guard columns (15 mm × 4.0 mm; 5 μm particle size; Scharlau Science) were also used.

The robot station consists of a Zymate II Plus robot with the necessary peripherals (namely, a master laboratory station, a centrifuge, a vortex, a liquid–liquid

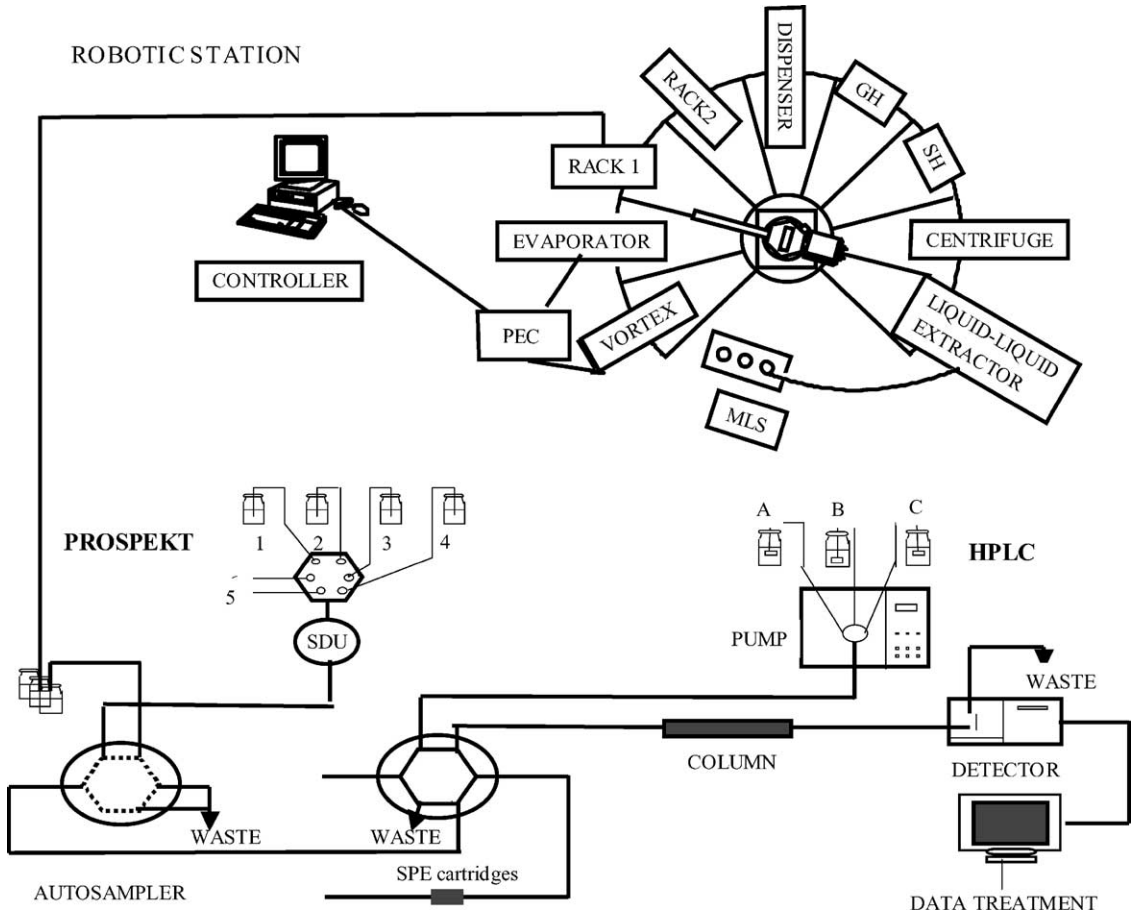

Fig. 1. Experimental set-up. Robotic station, GH: general hand; SH: syringe hand; MLS: master laboratory station; PEC: power event controller. Prospekt, SDU: solvent delivery unit.

extractor, an evaporator and two racks) in a circular arrangement (see Fig. 1).

2.2. Solutions and reagents

Acetonitrile, *n*-hexane, isopropanol and methanol (Panreac, Barcelona, Spain) were of HPLC grade. Demineralised water was purified in a Milli-Q filtration system to obtain water of HPLC grade. Dipotassium hydrogen phosphate (Panreac) was used to prepare a 10 mM buffer solution by dissolving 1.742 g in 1 l of Milli-Q water and adding HCl solution up to pH 6.5. Reagent A was a 20:80 acetonitrile–water mixture.

Individual standard solutions of 40 μg/ml 24,25 $(OH)_2$ Vitamin D_3 and 25(OH) Vitamin D_3 and 1000 μg/ml of Vitamins A and E (Sigma–Aldrich, St. Louis, MO, USA) were prepared separately by dissolving the content of each vial in methanol.

Retinyl acetate (Sigma, St. Louis, MO, USA) was used as internal standard and the solution prepared by dissolving the amount necessary for a concentration of 500 μg/ml in 1:1 methanol–water. The certified reference material was SRM 968c (NIST).

The serum samples were supplied by a local hospital and stored at −20 °C until use. No decrease in the concentration of the target analytes was observed when the samples were stored at −20 °C for 3 months. The methanol standard solutions were stored for 6 months at −20 °C without degradation. The standard solutions in phosphate buffer were prepared daily.

2.3. Sample pre-treatment

This step is done by the robotic station, which treated six samples each time. Two millilitres of serum sample is placed into each 10-ml glass tube in rack 1 and spiked with 20 μl of internal standard solution. The robot, fitted with a syringe hand, adds 2 ml of 2-propanol to each tube. Then, it changes to the all-purpose hand and takes the tube from the rack 1 to place it in the vortex rack, where, after the vortex rack has been filled with all tubes, agitation is applied at 200 rpm for 3 min in order to precipitate the proteins. Next, the robot takes the tube from the vortex rack to place it in the centrifuge, where, after filled with the tubes, centrifugation is applied at 4500 × *g* for 10 min. After the robot takes the tube from the centrifuge to place it in the liquid–liquid extractor, the supernatant is transferred into other glass tube, which is placed in rack 2. The tube with the solid is placed in rack 1 to repeat the process twice, but with 1 ml of 2-propanol each. After last extraction, the robot places the glass tube with the 4 ml of 2-propanol extract in the evaporator rack, which is filled with six tubes, and the extractant evaporated to 1 ml. Next, the robot takes the tube from the evaporator rack to place in the rack 1, where, with the syringe hand, adds 2 ml of *n*-hexane to all tubes for extracting the analytes followed by agitation at 130 rpm for 3 min. Then, the robot changes the syringe hand to the all-purpose hand and takes the tube from the vortex rack to place in the centrifuge, where, after it has been filled with six tubes, centrifugation is applied at 4500 × *g* for 3 min. After the robot takes the tube from the centrifuge to place in the liquid–liquid extractor, the *n*-hexane layer is transferred into other glass tube, which was placed in rack 2. This process is repeated three times, after which the extract is placed in the evaporator rack, where, after the evaporator rack has been filled with six tubes, the extracts are evaporated to dryness under a stream of N_2. The robot takes the tube from the evaporator rack to the "dilute & dissolve" peripheral, which dispenses 3 ml phosphate buffer and put the tube in the vortex rack. When this is repeated with all samples, agitation is applied at 130 rpm for 3 min. This process is repeated twice. The reconstituted sample is transferred to an 8-ml sample vial for the autosampler injecting 7.5 ml.

2.4. Clean-up/pre-concentration

Solid-phase extraction of the samples is done with Oasis HLB cartridges, which are activated with 1 ml methanol and equilibrated with 1 ml water and 1 ml 10 mM phosphate buffer of pH 6.5. The flow rate for activation and equilibration is 4 ml/min. The injection volume is 7.5 ml and the flow rate for injection is 1 ml/min. A 5:95 methanol–water solution is used for the washing step. The volume of the washing mixture is 1.5 ml and the flow rate is 1 ml/min. Then, the analytes are eluted by the mobile phase. The cartridges can be reused up to 15 times, after cleaning with 4 ml water and 4 ml methanol. The 8 ml reconstituted extract volume is necessary because the target analytes are not dissolved completely with volumes smaller than 8 ml. This fact involves a 4-times dilution with respect to the serum sample, but the subsequent liquid–solid extraction yields a 21-times pre-concentration; so, the final pre-concentration factor is 6.66.

2.5. Chromatographic separation and detection

The initial mobile phase (70:30 reagent A-methanol) passes through the Oasis HLB cartridge, elutes the analytes at a flow rate of 0.3 ml/min for 1 min and transports them to the analytical column. Then, a linear gradient in 5 min for obtaining 30:70 reagent A-methanol with a flow rate of 0.6 ml/min is applied. Afterwards, a second gradient is programmed for 3 min to obtain 90:10 methanol–isopropanol at a flow rate of 1 ml/min. At minute 15, other gradient is programmed in order to obtain a 1:1 methanol–isopropanol mixture for 7 min at a flow rate of 1.2 ml/min, maintained until the end of the run. Finally, 5 min is necessary for re-establishing and equilibrating the initial conditions. The analysis time is 35 min.

The eluate is monitored photometrically with a photodiode-array detector at the maximum absorption wavelength for each analyte (namely, 265 nm for Vitamin D_3 metabolites, 325 for Vitamin A and the internal standard, 291 nm for Vitamin E).

3. Results and discussion

The liquid–liquid extraction step was adapted from the manual procedure established by Ortiz-Boyer et al. [31,32].

3.1. Optimisation of the solid-phase extraction system

The type of sorbent was studied using two materials (namely, C_{18} and HLB Oasis). The assays were carried out using both standard solutions and serum samples. The highest pre-concentration factor was provided by the Oasis HLB, which is made for the copolymer divinilbencene-*N*-vinilpirrolidone. This sorbent has a retention efficiency three times higher than C_{18}.

The effect of pH was also studied. Oasis HLB can be used within a wide range of pH (1–14) and this was used for optimisation. For pH 1–2 the signal from Vitamin E decreased. When the range of pH was between 12 and 14 the signal from all analytes also decreased. In the range of 3–12 the retention of the analytes did not change. A sample pH of 7.0 adjusted with phosphate buffer was selected for further experiments.

For cartridge conditioning, both the volume and flow rate of methanol were studied. The volumes assayed were from 1 to 4 ml at 3 ml/min. The optimum volume was 1 ml, as lower volumes decreased the signal and larger ones did not increase the signal. The flow rate was studied between 1 and 5 ml/min. The activation was efficient within the range 1–4 ml/min. The last value was selected for a shorter activation time. After activation, 1 ml of water was passed through the cartridge for equilibration as the sample was in phosphate buffer.

The washing step was studied in depth because of the compounds with similar features present in serum coelute with the analytes. First, different mixtures were assayed with the aim of removing the interferences with minimum or nil loss of the analytes by including a washing step before analytes elution. For this study, an experimental design with three variables, namely, flow rate, volume and acetonitrile:water percentage and three centre points was used. The volume was between 0.5 and 2.5 ml, the flow rate between 1.0 and 3.0 ml/min and acetonitrile percent in the water mixture between 5 and 15. Very significant losses of the analytes occurred under all the conditions assayed; so other experimental design for the methanol–water mixture, in which the methanol percent in water was between 5 and 15, was done. The best results were obtained for 1.5 ml of 5% methanol at a flow rate of 1.0 ml/min (Table 1).

Table 1
Ranges assessed and optimal values for the variables[a]

Step	Variable	Tested range	Optimum value
Pre-concentration			
Activation			
	Methanol		
	Flow rate (ml/min)	1.0–5.0	4.0
	Volume (ml)	1.0–4.0	1.0
Sample injection			
	Flow rate (ml/min)	1.0–3.0	1.0
	Volume (ml)	2.5–7.5	7.5
Clean-up			
	Methanol (%)	0–20	5
	Volume (ml)	1.0–5.0	1.5
	Flow rate (ml/min)	1.0–3.0	1.0

[a] For the robotic treatment and separation-detection steps, see text.

3.2. Optimisation of the individual chromatographic separation and detection

The analytical column used was Ultrabase C_{18} (250 mm × 4.6 mm; 5 μm) [31,32].

The mobile phase was optimised in order to obtain the best separation of the analytes in the shortest time. The experimental variables were composition of the mobile phase and flow rate. Several mobile phases (namely, methanol, acetonitrile, water, isopropanol) were assayed. The best results were obtained for the sequence explained under "Experimental" (Section 2.5).

Retinyl acetate was used as internal standard. [33].

Under the optimum working conditions, the chromatogram obtained with serum as sample is shown in Fig. 2.

3.3. Features of the method

3.3.1. Linearity

For the study of linearity, calibration curves were run, covering the concentration range of the analytes usual in serum samples, namely, 1–4 ng/ml for 24,25$(OH)_2$ D_3 hydroxyvitamin, 10–40 ng/ml for 25(OH) D_3 hydroxyvitamin, 300–700 ng/ml for Vitamin A and 5–10 μg/ml for Vitamin E.

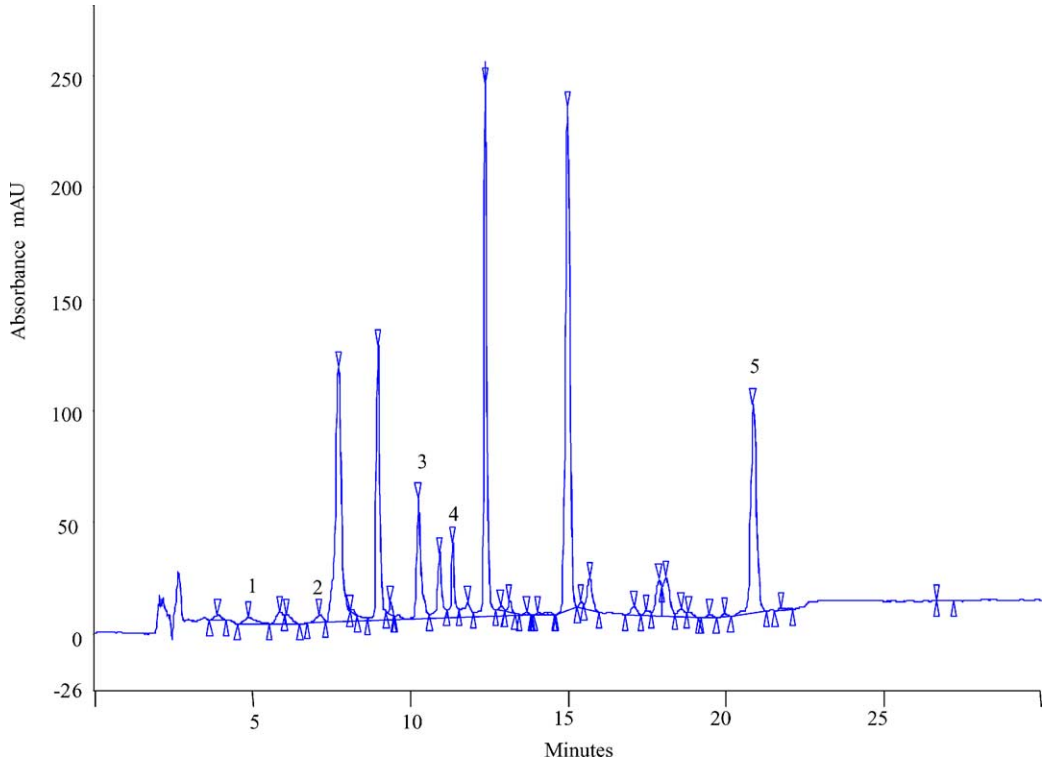

Fig. 2. Chromatogram from a serum sample: (1) 24,25(OH)$_2$ Vitamin D$_3$, (2) 25(OH) Vitamin D$_3$, (3) Vitamin A, (4) internal standard and (5) Vitamin E.

Regression analysis was performed using the analyte area/internal standard area ratio versus concentration of analyte. After routine use of the method, the relative standard deviation (R.S.D.) vlaues of the slope were 4.6% for 24,25(OH)$_2$ Vitamin D$_3$, 4.9% for 25(OH) Vitamin D$_3$ and 4.0% for Vitamins A and E. Ten calibration curves obtained from different samples, days and the correlation coefficient, r^2, was between 0.996 and 0.989 (Table 2).

Table 2
Features of the method

Analyte	Limit of detection	Limit of quantitation	Linear range
24,25(OH)$_2$ Vitamin D$_3$[a]	0.10	0.5	1.0–100
25(OH) Vitamin D$_3$[a]	0.05	0.2	1.5–100
Vitamin A[a]	0.2	0.5	1.0–1500
Vitamin E[b]	0.02	0.1	0.1–20

[a] Expressed as ng/ml.
[b] Expressed as μg/ml.

3.3.2. Precision

To evaluate the precision of the proposed method, within-laboratory reproducibility and repeatability were estimated in a single experimental set-up with duplicates [34]. The experiments were carried out using a serum pool. The optimal values obtained for the variables were used in all experiments. Two measurements of each analyte signal per day were carried out on 7 days. The repeatability, expressed as R.S.D., was between 3.2 and 6.0%; meanwhile, the R.S.D. for within-laboratory reproducibility was between 4.1 and 10.2% (Table 3) for analyte concentrations of 10, 300 ng/ml and 5 μg/ml of Vitamin D$_3$ metabolites, Vitamins A and E, respectively.

3.3.3. Accuracy

The accuracy was estimated carrying out six measurements of a serum pool spiked with 3 ng/ml of 24,25(OH)$_2$ dihydroxyvitamin D$_3$, 10 ng/ml of 25(OH) hydroxyvitamin D$_3$, 500 ng/ml of Vitamin

Table 3
Precision and accuracy of the method

Analyte	Repeatability[a]	Within-laboratory reproducibility[a]	Accuracy[b]
24,24(OH)$_2$ Vitamin D$_3$	6.0	10.1	3.0
25(OH) Vitamin D$_3$	4.5	8.8	2.2
Vitamin A	3.3	7.5	1.8
Vitamin E	3.2	7.0	1.8

[a] Expressed as relative standard deviation (%).
[b] Expressed as relative error (%).

Table 4
Validation of the method with the CRM 968c, at two concentration levels

	Level I		Level II	
	Certified value (μg/ml)	Found value (μg/ml)	Certified value (μg/ml)	Found value (μg/ml)
25(OH) D$_3$	0.015 ± 0.002	0.0156 ± 0.0016	0.016 ± 0.002	0.0155 ± 0.0016
Vitamin A	0.841 ± 0.027	0.842 ± 0.020	0.484 ± 0.0012	0.486 ± 0.0010
Vitamin E	7.47 ± 0.47	7.51 ± 0.42	16.79 ± 0.76	16.79 ± 0.54

A and 5 μg/ml of Vitamin E. Then, the difference between the average concentration in both the spiked and non-spiked serum is the added concentration [34]. The accuracy, expressed as relative error (RE) in the estimation of the added concentration, was between 1.8 and 3.0% (Table 3).

The method was validated by the certified reference material (NIST, SRM 968c). This is a reference material certified for Vitamins A and E and a reference value for 25-hydroxyvitamin D$_3$, which consists of two different levels of the target compounds, as can be seen in Table 4, where the results of this study are summarised.

3.3.4. Detection and quantification limits

For the purpose of this method, the limits of detection and quantification are defined as $3s_b$/slope and $10s_b$/slope (where s_b is the standard deviation of 11 blank measurements). In this case, the blank is the noise measured before each analysis (Table 2).

4. Conclusions

Human intervention is minimised in this method thus making easy to apply it. Using the described automated on-line clean-up step, most compounds with structure similar to that of the analytes are removed; those remaining with the analytes after this step appear in the chromatogram either in between the analytes peaks or after them.

The reusability of the clean-up/pre-concentration cartridges drastically reduces the cost of the analyses. This fact is of enormous interest as it makes the potential adoption of the method for routine analysis more affordable.

This fully automated method works 24 h per day.

The real time required for the routine process is 35 min per sample, because the steps can overlap.

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