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Vitamin D analysis in plasma by high performance liquid chromatography (HPLC) with C_{30} reversed phase column and UV detection – Easy and acetonitrile-free

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ABSTRACT

Two physiologically important forms of vitamin D exist: vitamin D_2 and vitamin D_3, which by liver based hydroxylase enzymes are converted to 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, respectively. These hydroxylated metabolites of vitamin D are measured in plasma to assess the vitamin D status of animals and humans. Therefore cheap and reliable analytical methods are very much in demand in nutritional and physiological research. After saponification and extraction of plasma or serum samples the current method uses reverse phase high performance liquid chromatography on a C_{30} column and with UV detection at 265 nm for quantifying vitamin D_2, vitamin D_3, 25-hydroxyvitamin D_2, and 25-hydroxyvitamin D_3. The method proved versatile with respect to plasma lipid content, sample amount, and plasma concentration of the vitamin D metabolites as it was tested using plasma from six different species: cattle, pigs, poultry, mink, horses, and humans. In cattle plasma recoveries were between 86.6 and 101.0%, within day error between 0.9 and 5.9%, and between day error between 0.2 and 1.7%. However, depending on species and sample amount error percentages varied. When running the method on standard reference material® 972 "Vitamin D in human serum" from the National Institute of Standards and Technology (NIST) (Gaithersburg, USA) the results for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 concentrations were within the boundaries provided by NIST, reflected by *Z*-scores between 0.1 and 0.9.

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1. Introduction

Vitamin D is important for maintaining skeletal health, controlling blood calcium levels, and carrying out other functions in the body, e.g., immune-modulation, etc. It exists in two physiologically significant forms, vitamin D_2 (D_2) and vitamin D_3 (D_3), which are both hydroxylated by liver enzymes into 25-hydroxyvitamin D_2 (25(OH)D_2) and 25-hydroxyvitamin D_3 (25(OH)D_3), respectively. The level of 25(OH)D_2 and 25(OH)D_3 in plasma is considered the best indicator of physiological vitamin D status; hence there is great need for easy and accurate methods for establishing plasma content of vitamin D metabolites in nutritional and physiological research [1].

One of two analytical methods are usually applied, either commercial immune binding assay kits mainly of radio immuno assay (RIA) type or various high performance liquid chromatography (HPLC) applications [2,3]. Unfortunately immune binding kits are in most cases unable to distinguish between neighbouring metabolites of vitamin D, e.g., 25(OH)D_2 and 25(OH)D_3 [2,4], a problem also encountered in normal phase HPLC. Reverse phase HPLC with UV detection is the most convenient and easiest HPLC procedure to work with, but analysing lipid soluble compounds as vitamin D metabolites in biological samples provides a great challenge in order to ensure an efficient sample clean up and a proper separation from interfering compounds. Therefore, today liquid chromatography tandem mass spectrometry (LC MS/MS) often is the method of choice for assessing troublesome compounds as vitamin D metabolites [3,5], although the use of LC MS/MS equipment is costly and requires a great deal of technical experience.

Thorough sample cleanup and concentration is usually accomplished through solid phase extraction (SPE), preparative HPLC or liquid–liquid extraction with or without alkaline saponification [3]. Furthermore inclusion of an appropriate internal standard is a very efficient way of producing accurate results and minimizing variation between samples [6].

The outcome of a saponification process depends on the lipid content of the sample matrix. Soaps formed during the saponification step increase the risk of formation of soap micelles, which decrease the polarity of the aqueous phase making it difficult to extract even slightly polar compounds into the organic phase [7], which in the case of vitamin D, can compromise extraction of hydroxylated vitamin D metabolites into the organic phase. Hence it is important to consider how resilient the analytical method is to varying lipid content of the sample matrix.

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Table 1
Plasma cholesterol, triglyceride, and phospholipids in different species.

Species	Cholesterol (mM)	Triglyceride (mM)	Phospholipids (mM cholin)
Cattle	4.35	0.33	2.38
Pigs	2.00	0.37	1.11
Poultry	3.71	0.77	3.85
Mink	5.19	3.36	6.32
Horses	1.19	0.26	1.43
Humans	4.82	1.88	2.92

Reversed phase C_{18} hydrocarbon columns are the most common HPLC columns for vitamin D analyses [3]. Recently reversed phase C_{30} columns have proven their efficiency in separation of closely related long chain hydrophobic compounds and are used more and more in, i.e., carotenoid analysis, where the best chromatographic conditions are obtained with almost water free mobile phases [8]. This higher possible content of organic solvents in the mobile phase due to the highly hydrophobic nature of a C_{30} column allows for a much more flattened elution gradient making it easier for closely related compounds to be separated with a good resolution [9,10]. On C_{18} columns retinol often co-elute with 25(OH)D_2 which increases the need for time consuming sample clean up. Another advantage of the C_{30} column is that ethanol and methanol have proven to be efficient eluents, whereby the use of expensive and harmful acetonitrile can be diminished [11].

The objective of the present study was to develop an HPLC method with UV detection, which can be applied in plasma and serum from various species, for simple, yet accurate and precise, analysis of vitamin D metabolites in plasma and serum.

2. Materials and methods

2.1. Chemicals and reagents

Water quality was at all times secured by treatment on a Millipore Synergy® UV water treatment system from Millipore S.A.S. (Molsheim, France) and methanol from Poch S.A. (Gliwice, Poland), ethanol (96%) (EtOH) from Kemetyl (Køge, Denmark), and heptane from Sigma Aldrich (Steinheim, Germany) were of HPLC grade. L(+)-Ascorbic acid from VWR International (Leuven, Belgium) was prepared in a 20% (w/v) solution with water (ASC) weekly and potassium hydroxide from Merck (Darmstadt, Germany) was prepared in a 50% (w/v) solution with water (KOH) monthly.

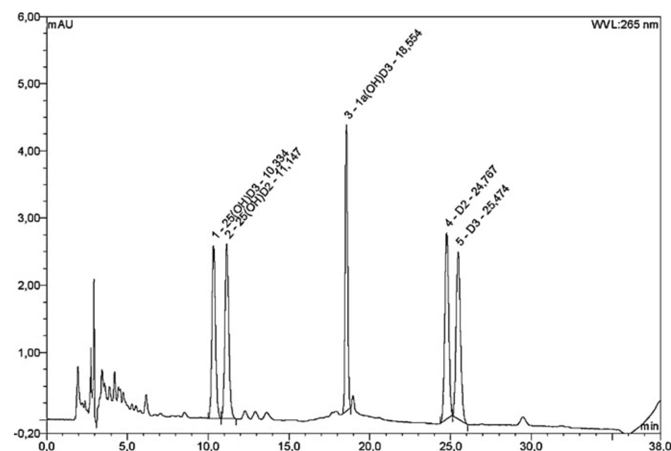

Fig. 1. Chromatogram after saponification of 100 ng of 25(OH)D_2, 25(OH)D_3, D_2, and D_3 standard with 450 μL of KOH.

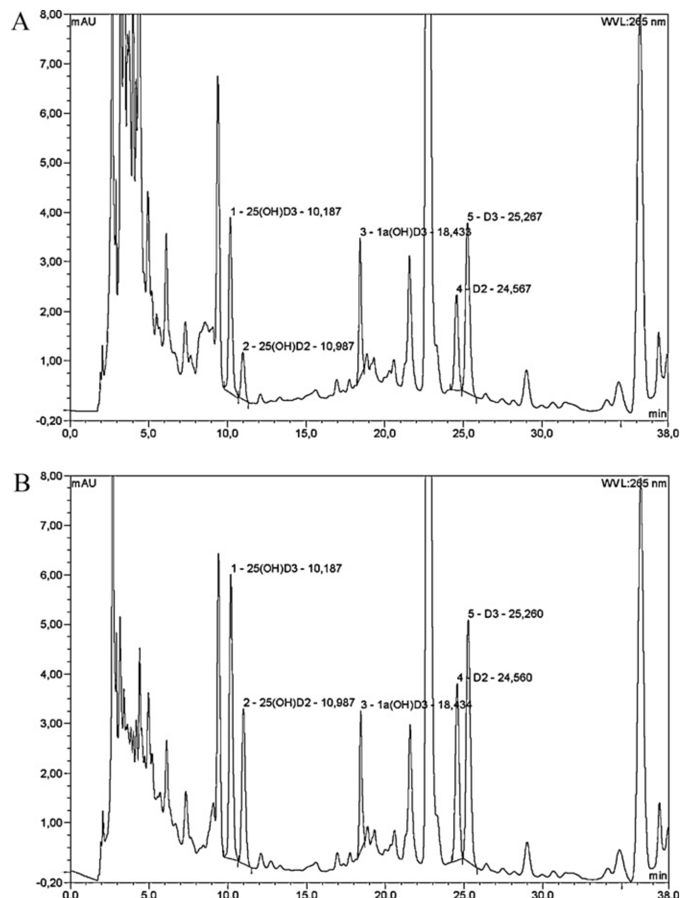

Fig. 2. Chromatograms from recovery (A) pure plasma from cattle obtained approximately 20 h after feeding a single oral dose of 250 mg D_2 and D_3 and the same plasma (B) added 60 ng of 25(OH)D_2, 25(OH)D_3, D_2, and D_3.

2.2. Vitamin D standards

HPLC grade 1α-hydroxyvitamin D_3 (1αOHD_3) (≥97% pure), used as internal standard, and standard metabolites of 25(OH)D_2 (≥98% pure), 25(OH)D_3 (≥99% pure), D_2 (≥98% pure), and D_3 (≥98% pure) were purchased from Sigma Aldrich (Steinheim, Germany) and dissolved in EtOH. 25(OH)D_2, 25(OH)D_3, D_2, and D_3 were stored as 0.1 mg/mL stock solutions and 1αOHD_3 as a 2.5 μg/mL stock solution at −80 °C. For analysis internal standard 1α(OH)D_3 was prepared in a 25 ng/mL solution fresh every week while solutions of other vitamin D metabolites were prepared prior to use.

Exact concentrations of standard solutions were determined according to their reported extinction coefficients: 1α(OH)D_3: $\varepsilon_{264\,nm} = 18000\,M^{-1}\,cm^{-1}$ [12]; 25(OH)D_2: $\varepsilon_{264.5\,nm} = 19400\,M^{-1}\,cm^{-1}$ [13]; 25(OH)D_3: $\varepsilon_{265\,nm} = 18000\,M^{-1}\,cm^{-1}$ [14]; D_2: $\varepsilon_{264.5\,nm} = 18167\,M^{-1}\,cm^{-1}$ [15]; and D_3: $\varepsilon_{264.5\,nm} = 17886\,M^{-1}\,cm^{-1}$ [15] on a Shimadzu UV-1650pc UV-VIS spectrophotometer from Shimadzu Corporation (Kyoto, Japan).

2.3. Plasma samples

Pooled plasma samples were obtained from six different species to secure a wide variety in concentration of, and relation between, different vitamin D metabolites together with various lipid contents in the plasma. The species were: lactating dairy cattle (*Bos taurus*), slaughter pigs (*Sus scrofa*), slaughter poultry (*Gallus domesticus*), mink at skinning (*Mustela vison*), Danish warmblood horses (*Equus caballus*), and humans (voluntary healthy subjects) (*Homo sapiens*) (Table 1).

Table 2
Response factors relative to the internal standard $1\alpha(OH)D_3$, equations (through 0.0), and correlations (R^2) for concentration curves from saponified pure vitamin D metabolites after adding different amounts of KOH.

Vitamin D metabolite	Response factor [a] ($1\alpha(OH)D_3 = 100$)	450 μL KOH		300 μL KOH		150 μL KOH		0 μL KOH	
		Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2
$25(OH)D_2$	97	$Y = 9.3 \times 10^{-3}\ X$	0.993	$Y = 9.6 \times 10^{-3}\ X$	0.989	$Y = 9.3 \times 10^{-3}\ X$	0.994	$Y = 8.0 \times 10^{-3}\ X$	0.993
$25(OH)D_3$	90	$Y = 8.5 \times 10^{-3}\ X$	0.992	$Y = 9.1 \times 10^{-3}\ X$	0.994	$Y = 8.7 \times 10^{-3}\ X$	0.995	$Y = 7.6 \times 10^{-3}\ X$	0.992
D_2	107	$Y = 10.2 \times 10^{-3}\ X$	0.993	$Y = 10.6 \times 10^{-3}\ X$	0.991	$Y = 10.3 \times 10^{-3}\ X$	0.994	$Y = 8.6 \times 10^{-3}\ X$	0.992
D_3	104	$Y = 9.9 \times 10^{-3}\ X$	0.991	$Y = 10.4 \times 10^{-3}\ X$	0.991	$Y = 10.0 \times 10^{-3}\ X$	0.995	$Y = 8.1 \times 10^{-3}\ X$	0.991
$1\alpha(OH)D_3$	100	$Y = 9.5 \times 10^{-3}\ X$	0.994	$Y = 10.0 \times 10^{-3}\ X$	0.992	$Y = 9.7 \times 10^{-3}\ X$	0.994	$Y = 8.0 \times 10^{-3}\ X$	0.993

[a] Calculated as $\frac{(\alpha_{150}+\alpha_{300}+\alpha_{450})/3}{(\alpha_{150}+\alpha_{300}+\alpha_{450})_{1\alpha(OH)D_3}/3} \times 100\%$.

Table 3
Saponification of plasma from cattle with increasing amounts of KOH.

Vitamin D metabolite	KOH (μL)	Peak height[a] (μAU)	Peak area[a] (μAU × min)	Concentration[a] (ng/mL)
$25(OH)D_2$	0	420 ± 65	112 ± 21	14.0 ± 2.8
	150	536 ± 19	143 ± 6	10.7 ± 0.4
	300	488 ± 16	134 ± 6	11.2 ± 0.5
	450	498 ± 29	138 ± 12	11.1 ± 0.5
$25(OH)D_3$	0	2402 ± 154	635 ± 41	79.7 ± 6.3
	150	2918 ± 65	714 ± 20	53.3 ± 1.7
	300	2911 ± 61	772 ± 20	64.1 ± 1.1
	450	3033 ± 118	809 ± 28	65.5 ± 1.2
D_3	0	64 ± 4	19 ± 1	2.3 ± 0.1
	150	60 ± 6	17 ± 4	1.3 ± 0.3
	300	79 ± 2	28 ± 1	2.3 ± 0.1
	450	77 ± 3	25 ± 2	2.0 ± 0.2
$1\alpha(OH)D_3$	0	1629 ± 78	282 ± 11	
	150	2724 ± 59	473 ± 12	
	300	2528 ± 53	426 ± 7	
	450	2593 ± 110	437 ± 20	

[a] Results are presented as mean ± sd, $n = 5$.

2.4. Standard human serum

Standard reference material® 972 "Vitamin D in human serum" was purchased from the National Institute of Standards and Technology (NIST) (Gaithersburg, USA). The reference material consisted of four serum samples: level 1, human serum; level 2, human serum diluted with horse serum to achieve a lower $25(OH)D_x$ concentration; level 3, human serum fortified with $25(OH)D_2$; and level 4: human serum fortified with 3-epi-$25(OH)D_3$ [16].

2.5. Sample preparation

Samples were at all times protected from light during preparation. 1.5 mL of plasma and 2.0 mL of internal standard solution was placed in a culture tube with a Teflon-covered screw cap and added 0.5 mL methanol, 1.0 mL ASC, and 400 μL KOH. Samples were saponified for 20 min in a water bath at 80 °C and subsequently rapidly cooled in cold water. If less than 1.5 mL of plasma was used, or in case of using pure standards, water was added up to a sample volume of 1.5 mL.

Samples were extracted with 5.0 mL heptane two times and extracted fractions were quantitatively transferred to a clean culture tube after centrifugation at 1500 × *g* for 10 min. The heptane fraction was evaporated to exact dryness over nitrogen (N_2) from Air Liquide (Horsens, Denmark) at 40 °C and the samples were redissolved in 200 μL of methanol–water (85% v/v) (MeOH85), vortex mixed, centrifuged at 1500 × *g* for 10 min, and transferred to 300 μL micro injection vials.

2.6. High performance liquid chromatography equipment and conditions

The HPLC equipment from Dionex Corporation (Sunnyvale, USA) consisted of a Dionex UltiMate 3000 vacuum degasser, auto

Table 4
Recovery percentage, within, and between day error from analysis of plasma from cattle, which received large oral doses of D_2 and D_3 (250 mg of each) prior to collecting the plasma samples.

Vitamin D metabolite	Added amount (ng)	Measured amount[a] (ng)	Recovery (%)	CV% between day
$25(OH)D_2$	0	25.6 ± 0.6		
	30	56.1 ± 1.7	101.0	
	60	83.4 ± 0.8	97.4	0.2
$25(OH)D_3$	0	93.8 ± 1.8		
	30	124.1 ± 2.7	100.3	
	60	145.7 ± 1.8	94.8	0.6
D_2	0	52.1 ± 3.1		
	30	71.1 ± 2.7	86.6	
	60	97.7 ± 2.5	87.2	1.7
D_3	0	107.8 ± 4.0		
	30	124.9 ± 4.9	90.6	
	60	152.5 ± 3.1	90.9	1.7

[a] Results are presented as mean ± sd, $n = 5$.

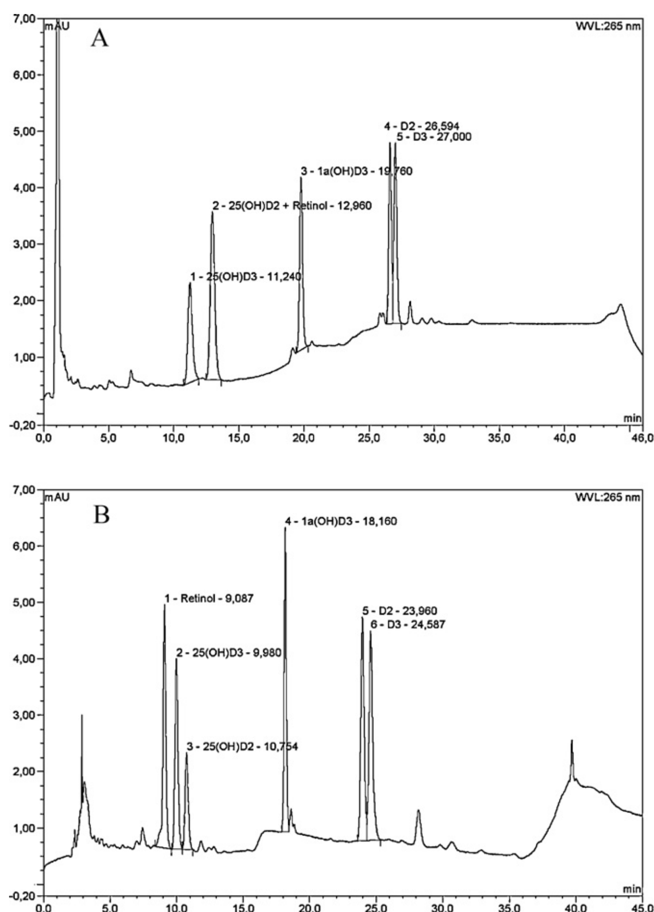

Fig. 3. Chromatograms from separation of vitamin D metabolites on a (A) C_{18} and a (B) C_{30} column, respectively, note the co-elution of $25(OH)D_2$ and retinol on the C_{18} column (peak number two).

sampler, pump, and thermostat controlled column compartment. Detection was carried out using a Dionex UltiMate 3000 variable wavelength UV detector at a fixed wavelength of 265 nm. Data was collected and stored in the Chromelion software from Dionex Corporation (Sunnyvale, USA).

The analytical column was a C_{30} column (250 mm × 4.6 mm ID) with 5.0 μm particle size from YMC Europe GmbH (Dinslagen, Germany) and the guard column was a C_{30} column (10 mm × 4.0 mm ID) with 5.0 μm also from YMC Europe GmbH (Dinslagen, Germany). The temperature was held at 50 °C for both columns.

50 μL of sample was injected and gradient elution performed at a flow rate of 1.0 mL/min with MeOH85 and EtOH during 55 min. During the first 12 min 5% EtOH was pumped through the column and subsequently the EtOH content was increased to 40% during three min. Seventeen minutes later the amount of EtOH was increased to 90% during 3 min and held constant for 10 min to clean the column. The gradient was returned to the initial setting during 5 min and 5 min later the next sample could be injected. Peaks of interest eluded during the first 25 min.

The detection limit established as three times the baseline noise was calculated to 0.14 ng/mL plasma and the quantification limit established as ten times the baseline noise to 0.45 ng/mL plasma.

3. Results and discussion

3.1. Vitamin D standards

Pure solutions containing 1000 ng/mL $25(OH)D_2$, $25(OH)D_3$, $1\alpha(OH)D_3$, D_2, and D_3 in MeOH85 were injected on the HPLC system in equal amounts based on the UV absorption of the vitamin D standards. However, this gave rise to slightly different peak areas between vitamin D metabolites, which indicated that either the vitamin D standards were not as pure as expected or that their extinction coefficients were not precisely determined. Response factors based on peak areas relative to the peak area of the internal standard $1\alpha(OH)D_3$ were $25(OH)D_2$ = 97%, $25(OH)D_3$ = 95%, D_2 = 105%, and D_3 = 110%.

3.2. Robustness of vitamin D metabolites against saponification

The robustness of the pure vitamin D metabolites against the saponification process was tested by adding 0, 150, 300, or 450 μL KOH to standard solutions of the five vitamin D standards. Irrespective of the amount of KOH added a given amount of vitamin D standard gave rise to the same peak area and no significant interfering peaks were present in the chromatograms (Fig. 1). However, omission of KOH reduced the amount of extracted vitamin D metabolites with 20%. This was probably due to the reduced polarity of the aqueous phase, caused by lack of ions, impairing the extraction of the vitamin D metabolites to the heptane phase. Linear fits through (0.0) and correlations of concentration curves are presented in Table 2. Saponification of increasing amounts of $1\alpha(OH)D_3$, $25(OH)D_2$, $25(OH)D_3$, D_2, and D_3 with 150, 300, and 450 μL KOH, respectively, gave rise to linear concentration curves. The slopes of the concentration curves for $25(OH)D_2$, $25(OH)D_3$, D_2, and D_3 varied relative to the slope of the $1\alpha(OH)D_3$ concentration curve with response factors similar to the response factors encountered when using un-saponified standards.

Table 5
Standard reference material® 972 Vitamin D in human serum from the National Institute of Standards and Technology (NIST) (Gaithersburg, USA) analysed by the current method.

Serum sample	Analysed $25(OH)D_2$ (ng/mL)	Certified $25(OH)D_2$[a] (ng/mL)	Z-score[b]	Analysed $25(OH)D_3$ (ng/mL)	Certified $25(OH)D_3$[a] (ng/mL)	Z-score[b]	Certified 3-epi-$25(OH)D_3$[a] (ng/mL)
NIST level 1[c]				23.2	23.9 ± 0.8	0.9	
NIST level 2[d]	1.69	1.71 ± 0.08	0.3	11.9	12.3 ± 0.6	0.7	
NIST level 3[e]	26.2	26.4 ± 2.0	0.1	19.3	18.5 ± 1.1	0.7	
NIST level 4[f]	2.46	2.40 ± 0.21	0.2	68.4	33.0 ± 0.8	–	37.7 ± 1.2

[a] The certified concentration is a weighted mean from analyses performed at the National Institute of Standards and Technology (NIST) (Gaithersburg, USA) and Centers for Disease Control and Prevention (CDC) (Atlanta, USA). The uncertainty (U) is calculated as $U = ku_c$, where u_c is the combined effect of between-laboratory and within-laboratory uncertainty at the level of one degree of freedom and the coverage factor (k) is determined from the Student's t-distribution corresponding to the corresponding degrees of freedom and a 95% confidence interval [16].
[b] Calculated as $\frac{\text{mean}_{\text{certified}} - \text{mean}_{\text{analyzed}}}{\text{sd}_{\text{certified}}}$.
[c] Level 1: human serum (0.9 mL sample).
[d] Level 2: human serum diluted with horse serum to achieve a lower 25(OH)D concentration (0.9 mL sample).
[e] Level 3: human serum fortified with $25(OH)D_2$ (0.9 mL sample).
[f] Level 4: human serum fortified with 3-epi-$25(OH)D_3$ (0.9 mL sample).

Table 6
Plasma concentration of vitamin D metabolites in different species assessed using different amounts of plasma.

Species	Vitamin D metabolite	0.5 mL plasma		1.0 mL plasma		1.5 mL plasma	
		ng/mL[a]	n	ng/mL[a]	n	ng/mL[a]	n
Cattle	25(OH)D_2	10.5 ± 0.3	4	10.9 ± 0.6	5	11.0 ± 0.2	5
	25(OH)D_3	65.2 ± 1.4	4	65.3 ± 1.4	5	62.3 ± 1.0	5
	D_2	nd		nd		nd	
	D_3	2.0 ± 0.7	4	2.6 ± 0.3	3	2.4 ± 0.1	5
Pigs	25(OH)D_2	nd		nd		nd	
	25(OH)D_3	9.6 ± 0.1	3	9.5 ± 0.3	5	9.6 ± 0.6	5
	D_2	nd		nd		nd	
	D_3	1.5 ± 0.4	4	0.9 ± 0.2	5	1.2 ± 0.04	5
Poultry	25(OH)D_2	4.0 ± 0.2	5	4.0 ± 0.1	5	4.4 ± 0.2	5
	25(OH)D_3	21.3 ± 1.0	5	24.0 ± 0.7	5	23.5 ± 1.0	5
	D_2	nd		nd		nd	
	D_3	nd		nd		nd	
Mink	25(OH)D_2	0.4 ± 0.1	4	0.4 ± 0.1	5	0.5 ± 0.02	5
	25(OH)D_3	53.3 ± 1.4	5	47.0 ± 0.1	5	46.3 ± 0.3	5
	D_2	nd		nd		nd	
	D_3	nd		nd		nd	
Horses	25(OH)D_2	nd		0.1 ± 0.03	4	0.1 ± 0.03	5
	25(OH)D_3	nd		0.5 ± 0.2	5	0.6 ± 0.1	5
	D_2	nd		nd		nd	
	D_3	nd		nd		nd	
Humans	25(OH)D_2	0.7 ± 0.1	5	0.6 ± 0.06	5	0.6 ± 0.04	5
	25(OH)D_3	19.7 ± 0.6	5	20.5 ± 0.5	5	20.2 ± 0.8	5
	D_2	nd		nd		nd	
	D_3	nd		nd		nd	

[a] Results are presented as mean ± sd.

Since pure vitamin D metabolites were shown to be resilient to saponification at the suggested concentrations of KOH, the only concern when saponifying plasma samples was to secure an alkaline sample and achieve sufficient saponification of plasma triglycerides and other fatty acid esters to prevent soap micelle formation due to excessive formation of mono, di and triglycerides, cholesterol esters, etc. The most stable peak areas and peak heights of vitamin D metabolites in cattle plasma after saponification with increasing levels of KOH were found at 300 and 450 μL added KOH (Table 3). Since vitamin D metabolites were unaffected by saponification at the 450 μL KOH level (Table 2), a general level of 400 μL KOH was chosen for saponification of plasma from all species, to also secure complete saponification of plasma with higher plasma lipid content than cattle plasma (Table 1).

3.3. Recovery and reproducibility in plasma

Recovery of 25(OH)D_2, 25(OH)D_3, D_2, and D_3 was checked by adding 30 or 60 ng of the pure vitamin D metabolites to pooled plasma samples from cattle, which were fed 250 mg D_2 and D_3, respectively, approximately 20 h prior to obtaining the plasma samples. Chromatograms showed excellent separation of the vitamin D metabolites of interest at both levels of added vitamin D metabolites (Fig. 2). Recoveries of 25-hydroxylated metabolites were close to 100% for the 30 ng level whereas the recovery decreased slightly at the 60 ng level. Recoveries of D_2 and D_3 were stable regardless of added level but were generally lower than the recoveries for the 25-hydroxylated metabolites. Within day error percentages were between 0.9 and 5.9% and between day error percentages between 0.2 and 1.7% depending on the vitamin D metabolite (Table 4). Recoveries and error percentages are within the boundaries of other recently published HPLC UV methods with intra assay CV% between 4.9 and 5.3, inter assay CV% between 8.2 and 8.7, and recoveries between 92.0 and 103.2% [17] and LC MS/MS methods with intra assay CV% between 0.9 and 11, inter assay CV% between 0.1 and 14 [18,19], and recoveries between 92 and 97% [19].

3.4. Performance of the C_{30} column

Analysing plasma or serum for vitamin D metabolites by C_{18} reversed phase columns implies the use of a steep gradient because the solvent initially must be polar to secure a good separation, but apolar by the end of the run to secure proper column cleaning between consecutive runs. Hereby it is difficult to achieve a good resolution between closely related compounds. In contrast a good resolution can be obtained on a C_{30} column because the slope of the gradient can be flattened. The high content of organic solvents in the initial eluent implies that vitamin D metabolites are easily solubilised and that the column is easily cleaned after each sample.

We compared the current method using a C_{30} column for separation to a method previously published by our laboratory using a C_{18} column. In this C_{18} method, samples were prepared as previously described, but were re-dissolved in 200 μL of acetonitrile–water (60% v/v). The guard column was a Supelcogel ODP-50 Supelcoguard cartridge (20 mm × 4.0 mm ID) with 5.0 μm particle size. The analytical column was a C_{18}, Supelcogel ODP-50 (150 mm × 4.0 mm ID) with 5.0 μm particle size both from Supelco (St. Louis, USA). The columns were during elution kept at 30 °C and gradient elution was performed at a flow rate of 1.0 mL/min with acetonitrile, methanol, and water [20].

Chromatograms from separation on the C_{18} and the C_{30} column, respectively, are shown in Fig. 3. On the C_{18} column 25(OH)D_3 were completely separated from 25(OH)D_2 and retinol (resolution = 2.8) and easily identified and quantified, while 25(OH)D_2 could not be quantified because it completely co-eluted with retinol (resolution = 0.0). D_2 and D_3 were separated on the C_{18} column (resolution = 1.0), but the resolution was too low for proper quantification as this requires resolution >1.0 [21]. Complete baseline separation requires a resolution ≥1.4 [21], which was obtained using the current C_{30} column method between retinol and 25(OH)D_3 (resolution = 2.4) and between 25(OH)D_2 and 25(OH)D_3 (resolution = 1.8). The separation of D_2 and D_3 (resolution = 1.3) also allowed for quantification of these metabolites on the C_{30} column (see Fig. 3).

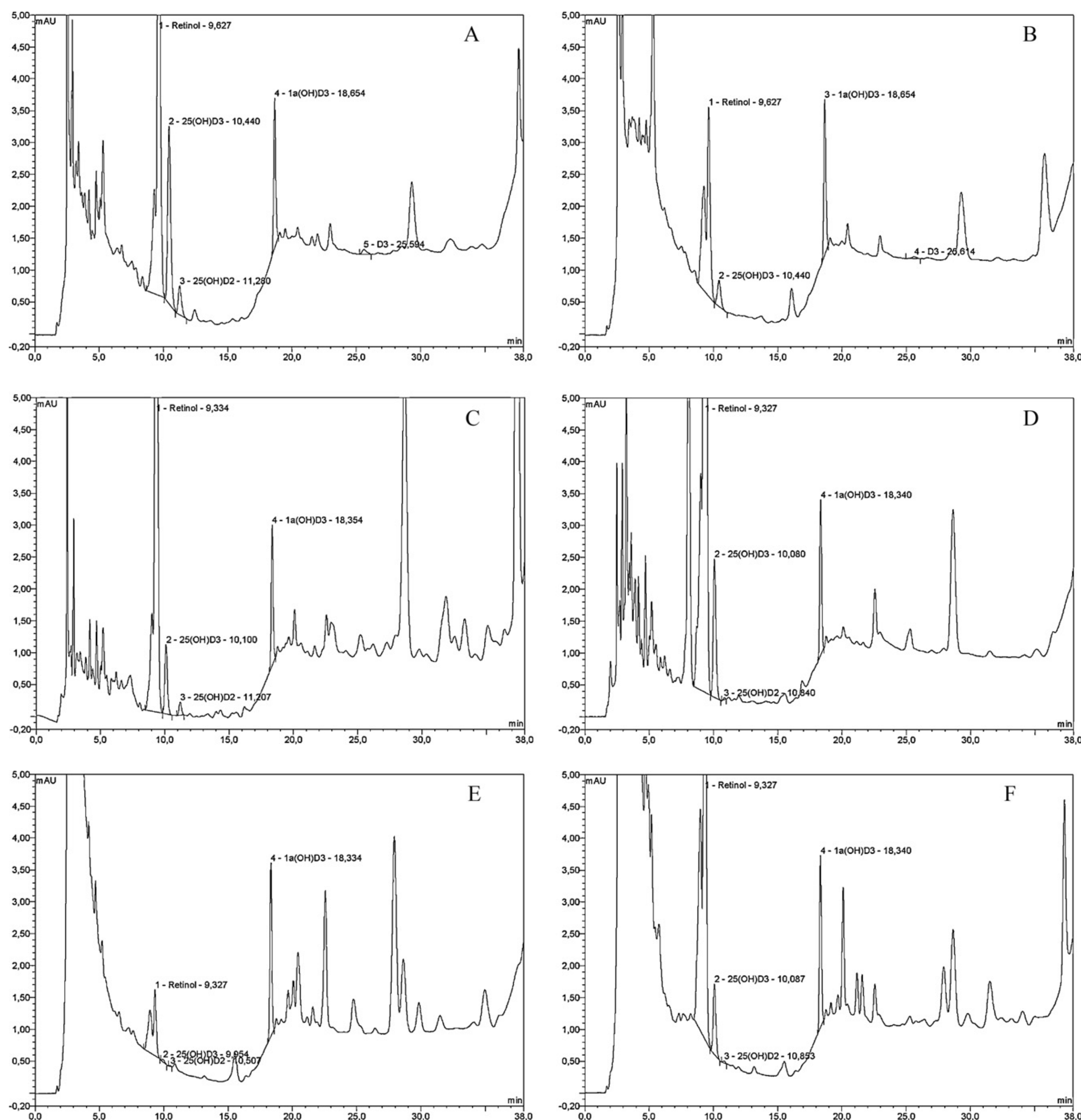

Fig. 4. Chromatograms from analysis of plasma from six different species: (A) cattle, (B) pigs, (C) poultry, (D) mink, (E) horses, and (F) humans.

3.5. Standard human serum

Results from analysing the NIST standard reference material for vitamin D analysis in human serum using the current method were within the acceptable boundaries for 25(OH)D_2 and 25(OH)D_3 provided by NIST for the level 1 to 3 samples [16] (Table 5). However it must be stated that the current C_{30} column did not allow separation of 3-epi-25(OH)D_3 from 25(OH)D_3 in the level 4 sample. Since 3-epi-25(OH)D_3 may occur in plasma or serum from infant humans [22] the current method should not be applied to plasma and serum from infant humans without taking this into consideration.

3.6. Vitamin D metabolites in plasma from different species

The method described was applied to pooled plasma from six different species which showed various contents and composition of both plasma lipids (Table 1) and vitamin D metabolites (Table 6). In all pool samples 25(OH)D_2 and 25(OH)D_3, indicative of physiological vitamin D status, were separated and quantified if present in the sample. D_2 was not detected in any samples while D_3 were detected and quantified in the samples from cattle and pigs. D_2 and D_3 could also easily be detected and quantified in mink and humans if present in the sample. Chromatograms from poultry and horses showed irregular base lines in the area of the chromatogram where D_2 and D_3 were eluted, rendering separation and quantification of D_2 and D_3 difficult in these samples (Fig. 4).

4. Conclusions

The current HPLC method is simple and accurate and assesses the content of 25(OH)D_2, 25(OH)D_3, D_2, and D_3 accurately and pre-

cisely in plasma and serum from a large variety of species. Hence the method is versatile and able to handle plasma and serum containing a wide concentration range of the four vitamin D metabolites and with very different properties with respect to plasma lipid composition. Furthermore, the UV based method utilises equipment and chemicals that are easily obtained and handled and available in most laboratories.

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