



Tecan AC Extraction Plate™

Simple sample preparation for the determination of vitamin D in serum by LC-MSMS

Introduction

A growing number of laboratories have moved from the use of immunoassay methods to liquid chromatography coupled with tandem mass spectrometry (LC-MSMS) for the determination of vitamin D. The advantage of the latter method is unparalleled specificity combined with high sensitivity, offering the potential for the determination of multiple analytes, such as 25OH-vitamin D3 and 25OH-vitamin D2.

Prior to any analysis by LC-MSMS, a sample clean-up step is recommended in order to remove matrix components such as proteins, lipids, carbohydrates and salts. The current methods for sample preparation include protein precipitation ('protein crash'), solvent extraction (such as liquid-liquid extraction, LLE) and solid-phase extraction (SPE). However, these methods require at least one labor-intensive and time-consuming step, such as filtration, centrifugation or solvent evaporation. In addition, these steps prevent straightforward automation of the sample preparation process, which is highly desirable in order to cope with the increasing number of vitamin D determinations and sample load experienced by many laboratories.

The AC Extraction Plate simplifies sample preparation by eliminating the need for centrifugation, filtration or solvent evaporation steps. Instead, it reduces the sample preparation procedure to simple liquid manipulation in the form of pipette and shake steps, which can be easily automated with a liquid handling platform such as Tecan's Freedom EVO®. This application note demonstrates the suitability of the AC Extraction Plate for sample preparation prior to the determination of vitamin D in serum by LC-MSMS, along with details of the workflow and how to use the AC Extraction Plate.

The Tecan AC Extraction Plate

The centerpiece of the new sample preparation method is the AC Extraction Plate with TICE™ (Tecan Immobilized Coating Extraction) technology. It is a deep-well microplate, consisting of 96 wells containing a special coating that acts as an extraction phase for small apolar molecules. In an aqueous medium, the coating absorbs these analytes (such as vitamin D2 and D3) with high affinity, while proteins, phospholipids, carbohydrates and salts remain in solution. After rinsing the well with a wash solution to remove the matrix, the analytes are eluted from the coating using a solvent mixture containing a high percentage of organic solvent, and this eluate can be used directly for LC-MSMS analysis. Procedural variations are mitigated by adding an internal standard (in this case an isotopically-labeled analyte, D₆-25OH-vitamin D3) at the beginning of the sample preparation process).

The workflow

The extraction mix containing the internal standard is prepared and pipetted into a well of the AC Extraction Plate, followed by an aliquot of the sample (eg. serum or plasma). After horizontal mixing on a shaker, the supernatant is removed, leaving the analyte(s) of interest retained in the TICE coating on the walls of each well. A wash solution is added, and the AC Extraction Plate is shaken again, then the wash solution is discarded.

To elute the analyte from the coating, an elution solvent is added and the AC Extraction Plate is shaken. The eluate from each well is then transferred to either an HPLC vial or an uncoated microplate, and loaded into an autosampler for sample injection. The process is depicted schematically in Figure 1.

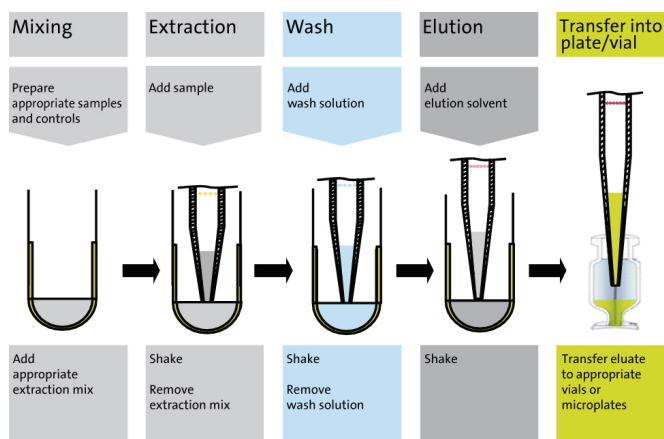


Figure 1 Typical sample preparation workflow using the AC Extraction Plate.

Materials and methods

Instruments

- LC-MS/MS system: Shimadzu Prominence binary gradient HPLC system, autosampler and column oven (Shimadzu, Switzerland) coupled to a 4000 QTRAP® MS/MS system (QTRAP®, USA) in APCI mode
- Shaker: MixMate® microplate shaker (Vaudaux-Eppendorf, Switzerland)

Pipettes

- For serum samples: MICROMAN® M50, 20-50 µl (Gilson, Switzerland)
- For solvents: 8-channel Eppendorf Research® plus pipette for disposable tips, 30-300 µl (Vaudaux-Eppendorf, Switzerland)

Solvents used for sample preparation

- Internal standard working solution: d₆-25OH-Vitamin D₃ (50ng/ml) in acetonitrile
- Modifier buffer: 0.2 M sodium carbonate/sodium hydrogen carbonate 1:1 (v/v) in water/acetonitrile 95/5 (v/v)
- Wash solution: water/methanol 90:10 (v/v)
- Elution solvent: water/methanol 10:90 (v/v)

All solvents used were LC-MS Chromasolv[®] grade (Sigma Aldrich, USA). Internal standard, D₆-25OH-vitamin D₃, was obtained from Cerilliant/USA.

Serum samples

Four serum calibrators and two serum quality controls with two defined concentrations of 25OH-vitamin D₃ and 25OH-vitamin D₂ metabolites were used as samples to cover the typical physiological range (Table 1). These were provided as lyophilized sera, reconstituted with water according to the manufacturer's instructions (Chromsystems, Munich) and stored in a freezer.

Serum sample	25OH-Vitamin D ₃ [ng/ml]	25OH-Vitamin D ₂ [ng/ml]
Cal1	4.3	0
Cal2	20.1	14.1
Cal3	34.5	27.2
Cal4	65.8	54.7
QC1	16.7	17.2
QC2	37.7	37.8

Table 1 Analyte concentrations of the calibrators (Cal) and quality controls (QC) that were used in the experiments.

Sample preparation and analysis

The modifier buffer was mixed with internal standard solution (50 ng/ml) at a ratio of 2:1 (v/v). This mixture represents the actual extraction mix used during the sample preparation procedure.

Calibrators, controls and samples were treated as outlined in Table 2, using manual pipetting.

Step	Procedure
1	150 µl of extraction mix was added to a well of the AC Extraction Plate
2	50 µl of serum sample, serum calibrator or quality control sample was added
3	Extraction plate was shaken horizontally for 10 min at 1200 rpm*
4	Extraction mix was removed using a pipette and 200 µl of wash solution added
5	Extraction plate was shaken horizontally for 2 min at 1200 rpm*
6	Wash solvent was removed and 200 µl of elution solution was added
7	Extraction plate was shaken horizontally for 5 min at 1200 rpm*
8	The resulting eluates were transferred from the AC Extraction Plate to a non-coated well plate. This non-coated plate was covered with a pierceable cover to minimize evaporation of solvent and placed in a cooled autosampler (10 °C) for immediate analysis by LC-MSMS

Table 2 Detailed workflow for the determination of vitamin D by LC-MSMS with the Tecan AC Extraction Plate.

* tested with the specified MixMate[®] shaker

HPLC parameters

Eluent A: water/methanol (90:10, v/v) + 0.1 % formic acid

Eluent B: methanol/acetonitrile (80:20, v/v) + 0.1 % formic acid

Column: Phenomenex[®] Synergi[™] Fusion-RP 80A, 50 x 2 mm, 4 micron, with guard column (Phenomenex, USA)

Column temperature: 40 °C

Injection volume: 20 µl

Flow rate: 0.7 ml/min

Gradient:

Start: 70 % B

From 0.1 min to 1.1 min: 70 % to 98 % B (linear)

From 1.1 min to 2.1 min: 98 % B

From 2.1 min to 2.3 min: 98 % B to 70 % B (linear)

From 2.3 min to 4.0 min: 70 % B (re-equilibration of column)

Total HPLC cycle time (including re-equilibration): 4 min

LC-MSMS parameters

Ionization and probe positioning:

- APCI (heated nebulizer), positive ion mode
- Vertical position of APCI probe: +8 mm
- APCI needle: slightly off-axis

Ionization source parameters:

- CUR 25
- GS2 0
- CAD Medium
- Ihe on
- TEM 275 °C
- NC 5
- GS1 40
- EP 10

MRM transitions and mass dependent parameters:

Analyte	Q1 mass	Q3 mass	DP	CE	CXP
25OH-vitamin D3 (Quantifier)	383	211	60	39	16
25OH-vitamin D3 (Qualifier)	383	229	60	34	10
25OH-vitamin D2 (Quantifier)	395	269	65	29	10
25OH-vitamin D2 (Qualifier)	395	209	65	34	16
D ₆ -25OH-vitamin D3	389	211	60	39	16

Table 3 MRM transitions monitored for the two metabolites and the internal standard.

Each MRM transition was monitored using a dwell time of 50 ms. All data processing was conducted with the quantifier mass transitions.

Performance parameters

All experimental data was obtained by manual pipetting with the AC Extraction Plate using the chemicals and workflow as described above.

1. Linearity

Linearity was determined with four serum calibrators. Each serum calibrator was extracted three times using three different wells of the AC Extraction Plate. Analyte/IS peak

area ratios were used to calculate calibration curves based on a linear regression model (Figure 2) which yielded good linearities ($r > 0.99$) for both analytes.

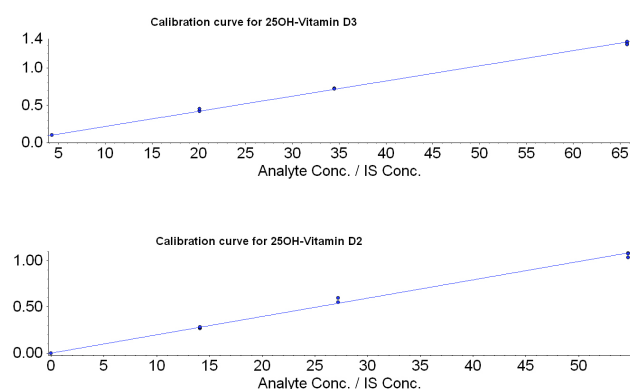


Figure 2 Calibration curves for 25OH-vitamin D3 (top) and for 25OH-vitamin D2 (bottom) determined from four concentrations of serum calibrators – each calibrator was extracted three times using the AC Extraction Plate.

By spiking serum with elevated concentrations of both analytes it was confirmed that the linear range extends at least up to a concentration of 250 ng/ml.

2. Sensitivity

Sensitivity was determined by calculating the signal-to-noise ratio (S/N) of the extracted ion chromatograms from the lowest serum calibrator (Calibrator 1) after sample preparation using the AC Extraction Plate. Calibrator 1 contains 4.3 ng/ml of 25OH-vitamin D3 in serum, and its mass chromatogram yielded a S/N >27 (Figure 3).

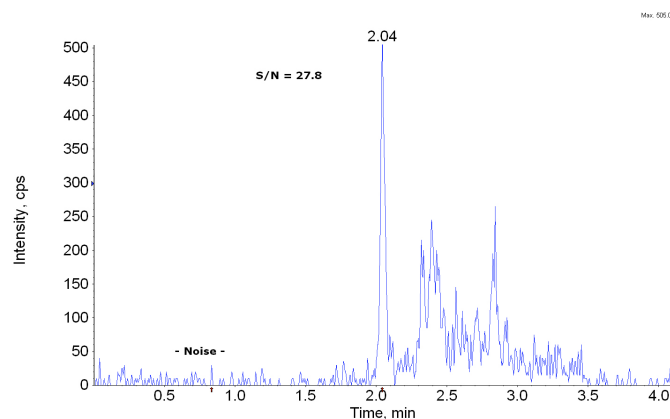


Figure 3 Mass chromatogram of 25OH-vitamin D3 in Calibrator 1 with a concentration of 4.3 ng/ml. Retention time is 2.04 min and calculated S/N >27.

As Calibrator 1 only contains 25OH-vitamin D3, the S/N of the mass chromatograms from Calibrator 2 were also calculated to check for the sensitivity of 25OH-vitamin D2. The mass chromatograms for vitamin D2 and vitamin D3 from the extracted Calibrator 2 yielded S/N >100, confirming a lower limit of quantification (LLOQ) significantly below 5ng/ml for both metabolites (Figure 4).

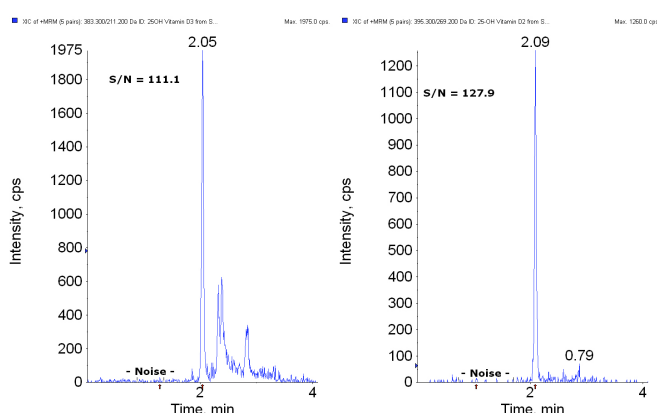


Figure 4 Mass chromatograms after extraction of Calibrator 2 showing 20.1 ng/ml of 25OH-vitamin D3 (left) and 14.1 ng/ml of 25OH-vitamin D2 (right) with their respective S/N values.

3. Accuracy and Precision

The accuracy and precision of vitamin D determinations using the AC Extraction Plate were assessed for well-to-well, plate-to-plate and day-to-day variability.

Figure 5 plots the concentration determined for the low QC for 25OH-vitamin D2 in 40 wells of a 96-well plate. Three replicates were measured per well, and the mean value and precision were calculated. The overall mean concentration value obtained was 16.7 ng/ml, which compares well with the target value of 17.2 ng/ml for the low QC. All the values are comfortably within the $\pm 20\%$ range of 13.8 to 20.6 ng/ml specified for the low QC. The precision also shows a tight cluster around 2-8 %, which is acceptable for the low QC. These values demonstrate that the potential well-to-well variability is within the accepted analytical variances for the determination of vitamin D. These values also include the overall variability of the LC-MSMS system.

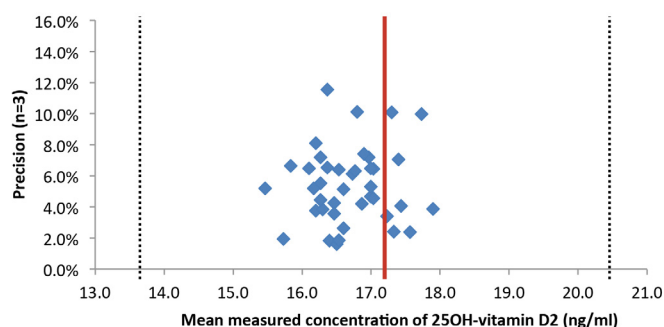


Figure 5 Concentration and precision obtained (n=3) for the determination of 25OH-vitamin D2 in 40 different wells of a single AC Extraction Plate (Low QC target value = 17.2 ± 3.4 ng/ml for 25OH-vitamin D2).

For the determination of plate-to-plate variability, the measured concentration and precision values of QC2 from three different AC Extraction Plates were assessed. All three plates were measured on a single day under the same experimental conditions. The high QC solution was added to ten different wells in each plate, extracted as previously described, and the eluate used for LC-MS analysis (n=3). The measured concentrations of 25OH-vitamin D3 in QC2 obtained for each replicate, as well as the mean values, are shown in Figure 6. This figure shows that there is little variability from plate to plate, and that the values measured are well within the target value ranges for this QC.

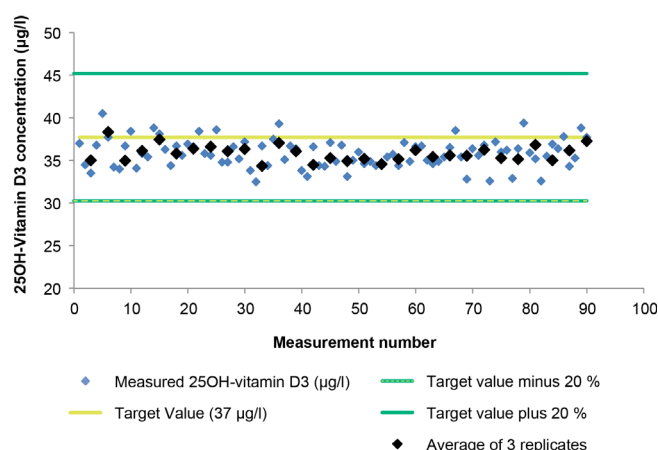


Figure 6 Measured concentrations of 25OH-vitamin D3 in QC2 after sample preparation using three different AC Extraction Plates.

A similar experiment was performed to assess the day-to-day variability. Both the concentration and precision were determined for QC1 and QC2 samples of 25OH-vitamin D3 and 25OH-vitamin D2 over five days using 15 separate AC Extraction Plates. The results for 25OH-vitamin D3 in QC2 are shown in Figure 7.

The slightly lower values obtained on Day 2 are due to variations in daily laboratory performance, and are not related to varying performance of the AC Extraction Plate, as all three plates from Day 2 are affected in the same manner.

The data obtained for the well-to-well, plate-to-plate and day-to-day variability show that the extraction performance of the AC Extraction Plate is acceptable in terms of both accuracy and precision.

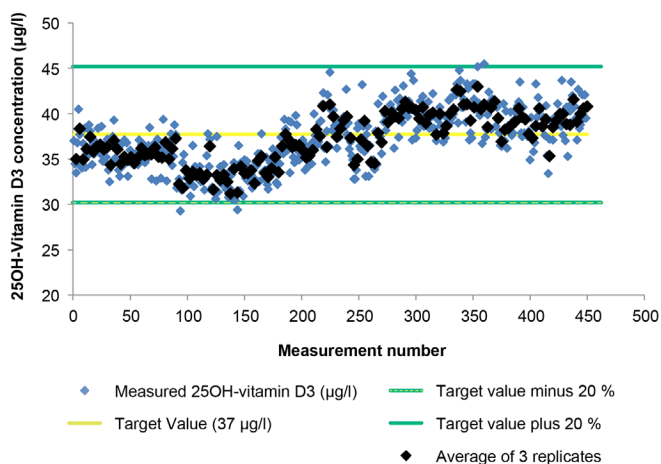


Figure 7 Determined concentration of 25OH-vitamin D3 in QC2 on five different days using three different AC Extraction Plates each day.

5. Recovery

Recovery was determined in six different serum samples by the determination of their endogenous 25OH-vitamin D3 levels and subsequent spiking with three different concentrations of this analyte (Table 4). The final concentrations determined in all sera after sample preparation with the AC Extraction Plate showed values ranging from 92 to 123 %, with one outlying value at 131 %. These recovery rates indicate that there are no serious matrix effects, and that acceptable recovery rates are obtained from actual serum samples over a range of concentrations.

Serum	Endogenous value	15 ng/ml addition	Recovery (%)	30 ng/ml addition	Recovery (%)	50 ng/ml addition	Recovery (%)
Pool 1	16.9	32.4	104	53.7	123	65.3	97
Pool 2	18.9	35.5	111	51.2	108	74.3	111
Pool 3	30.0	47.1	114	61.7	106	87.9	116
Pool 4	40.9	54.8	92	65.8	83	95.9	110
Pool 5	26.0	43.6	117	61.6	118	85.1	118
Pool 6	16.9	36.5	131	52.2	118	76.2	119

Table 4 Concentrations and recovery rates for 25OH-vitamin D3 in endogenous and spiked samples of six different sera.

6. Extraction efficiency and Matrix effects

As all sera contain some amount of the metabolite 25OH-vitamin D3, the extraction efficiency and any potential matrix effects were determined by using D₆-25OH-vitamin D3 as marker. In these experiments, pure acetonitrile (not containing any D₆-25OH-vitamin D3) was used in the extraction step of the sample preparation. Six serum samples from different origins were investigated.

The extraction efficiency was determined by spiking two serum samples with D₆-25OH-vitamin D3 (one before and one after sample preparation) and the extraction efficiency of the AC Extraction Plate was calculated from the ratio of the spike before/spike after peak areas as a percentage. The values are shown in Table 5. The extraction efficiency gave a mean value of 68 %, with an RSD of 3 %.

Sample number	Extraction efficiency %	Matrix effect* %	Matrix effect %
1	66.3	91.4	9
2	71.6	108.3	- 8
3	68.9	109.0	- 9
4	65.6	90.5	9
5	67.2	106.9	- 7
6	68.3	107.1	- 7
Mean (RSD)	68.0 (3%)	102.2 (9%)	± 8

*(signal suppression / enhancement: ≤ 9%)

Table 5 Values for extraction efficiency and matrix effect determined from serum samples of six different origins.

To investigate the potential for any residual matrix effects after extraction, D₆-25OH-vitamin D₃ was spiked into an eluate obtained after sample preparation using the AC Extraction Plate. The resulting peak area was compared to the peak area obtained for pure elution solvent after spiking with the same concentration of D₆-25OH-vitamin D₃. A potential matrix effect was determined by calculating:

$$\frac{[\text{Peak response in the presence of matrix}]}{[\text{Peak response in the absence of matrix}]} \times 100$$

The calculated values from six different serum samples showed that there do not appear to be any residual matrix effects that could impact on the determination (Table 5).

Conclusion

A rapid, sensitive and reliable method for the determination of vitamin D by LC-MS/MS has been developed using Tecan's innovative AC Extraction Plate for sample preparation. This method offers a broad dynamic range covering the physiologically and clinically relevant concentrations, and shows excellent precision and accuracy for both 25OH-vitamin D₃ and 25OH-vitamin D₂. Sample preparation using the AC Extraction Plate has an extraction efficiency around 68 %, with a RSD of 3 %. Well-to-well, plate-to-plate and day-to-day accuracy and precision were found to be acceptable.

The AC Extraction Plate provides a fast, robust and easy-to-use analytical sample preparation method requiring minimal sample pretreatment. More time-consuming and complicated procedures – such as protein precipitation, filtration and centrifugation – are no longer required, accelerating and simplifying the entire sample preparation workflow. This extraction method can be performed manually, or can be easily automated on a liquid handling platform.

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