Chromatography Technical Review Version 1.0

The Anatune Clinical Workstation – Technical Review Part 1 – Vitamin D

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Outline

- Simple to operate push button end to end solution.
- Maximises productivity with minimal operator intervention improving data quality
- System features a sample capacity of 96 sample vials.
- 96 Batch cycle time of twelve hours and fifty five minutes
- Automated addition of deuterated standards and reagents
- Automated protein crash
- Automated sample clean-up using C8 Solid Phase Extraction
- Direct injection and analysis by Liquid Chromatography Tandem Mass Spectrometry
- Vitamin D consumables and method start-up kit included with purchase
- Full training and instrument support available to get you started

Introduction

The monitoring of vitamin D levels in patients is important for the prevention and control of disease. Vitamin D, specifically 25-hydroxy vitamin D (25-OH D) plays a critical role in regulating calcium and phosphorus levels in the body. If these levels are not adequately controlled, bone mineralization conditions, such as rickets in children or osteoporosis in adults may occur. Recent studies identifying a role for vitamin D in prevention of cancer and cardiovascular disease have generated renewed interest in monitoring vitamin D levels in serum also. 25-OH D is a hydrophobic, fat soluble vitamin that is absorbed like a fat in the intestines. 25-OH D also promotes reabsorption of calcium in the kidneys. It is commonly used to diagnose conditions that interfere with fat absorption, such as Crohn's disease.

Vitamin D is produced in the skin of humans as D3, and by plants, invertebrates and fungus as D2. These forms are very similar and differ only in one methyl group and a double bond (Figure 1). Vitamin D3, cholecalciferol is derived from the conversion of 7-dehydrocholesterol by sunlight (ultraviolet B and radiation) in the skin. Vitamin D2, ergocalciferol which is the form found in many nutritional supplements is derived mainly from plants, ergocalciferol can be synthetically produced by UV irradiation of the fungal sterol, ergosterol. In general blood circulating levels of vitamin D3 are higher than those of vitamin D2. Each form is metabolized by the liver to its 25-hydroxy derivative which is the active form. Measurement of 25-OH D the most abundant vitamin D metabolite in the circulation is the single best indicator of nutritional vitamin D status. This is due to its long serum half-life (approximately 3 weeks) and because the hepatic 25-hydroxylation step is unregulated. Both 25-OH D2 and 25-OH D3 are measured in serum and are collectively referred to as vitamin D in studies. It is critical that assays be capable of measuring total circulating vitamin D (D2 and D3). Traditionally, competitive binding experiments and immunoassays have been used to measure vitamin D. However, the cross-reactivity of the antibodies used in these assays can be less than 100 %, and the results may not reflect total D2 and D3. More recently, liquid chromatography tandem mass spectrometry has gained acceptance as the method of choice due to its ability to reliably quantify both vitamin D forms.

Existing sample preparation techniques include liquid/liquid extraction with hexane or heptane; this generally is then followed by a drying step and reconstitution in LC mobile phase. Other techniques include both offline and online solid phase extraction (SPE). Offline SPE generally involves large volumes of solvents and much user processing. Whereas online SPE coupled directly to LC compromises throughput as samples are processed and analysed one at a time and the SPE cartridge must be eluted, reconditioned and re-equilibrated between samples.

Testing for vitamin D deficiency is becoming increasingly important in hospital clinical laboratories, so a reliable, robust, high throughput method is needed to offer fast turnaround for medical diagnosis. It is with this in mind that a fully automated push button end to end solution, the Anatune Vitamin D Workstation (Figure 2), featuring an automated internal standard addition, protein precipitation stage, automated centrifugation and supernatant handling, miniaturized solid phase extraction stage to remove matrix impurities, all coupled directly to liquid chromatography tandem mass spectrometry has been developed.

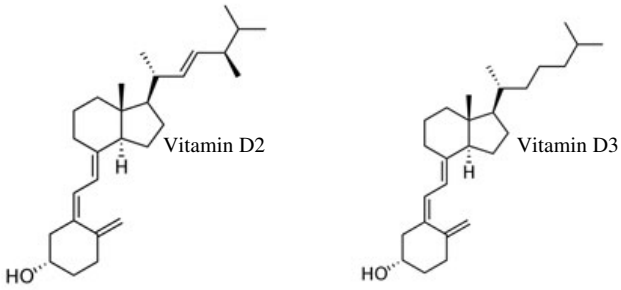

Figure 1 Structures of vitamins D2 and D3.

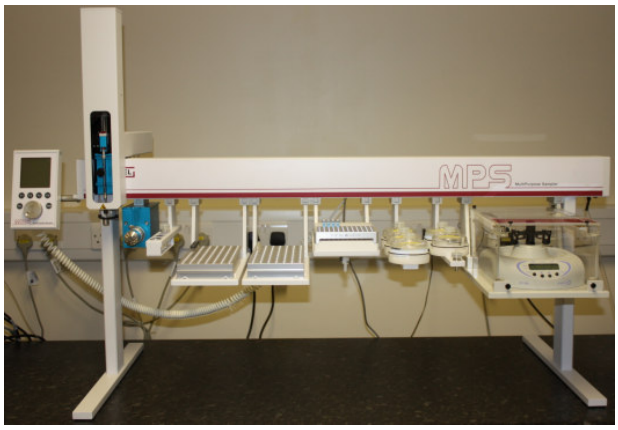

Figure 2 The Anatune Vitamin D Workstation.

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Multi Purpose Sampler (MPS)

The GERSTEL Multi Purpose Sampler (MPS) is more than just a widely used and highly productive autosampler. The MPS offers more capabilities for introducing samples into and LC or GC instrument than any other autosampler, providing unparalleled versatility, ease of use and productivity. The MPS boats a variety of unique features;

Complete software integration – one method and one sequence list control the complete system: MPS, LC or GC and mass spectrometer. The GERSTEL Maestro software is completely integrated with the Agilent Chemstation or Masshunter software for easy and error free operation.

Modular design, easily upgradeable – Exchangeable MPS syringe modules enable fast change over between techniques and enable precise liquid handling operation.

Sample Prep by mouse click- Sample prep steps are easily selected by mouse click from a drop down menu: Standard addition, dilution, heating, mixing and centrifugation. Automated sample prep ensures repeatable, just in time sample preparation for best results and optimal reproducibility

The Anatune CF-100 Centrifuge

The Anatune CF-100 (Figure 3) centrifuge plays an important role in the Vitamin D Workstation, it is used to vortex samples during solvent protein precipitation and then to spin down samples prior to SPE. Solvent protein precipitation is where a water miscible solvent such as methanol, ethanol, acetone or acetonitrile is added to a protein solution to cause the proteins to precipitate out. These proteins are then separated from the residual liquid using centrifugation, leaving the supernatant suitable for further sample processing or analysis.
The CF-100 was designed by Anatune as an add on accessory for the Gerstel Multi Purpose Sampler (MPS) autosampler, The CF-100 centrifuge features full hardware and software integration with the Gerstel MPS using Maestro software. Control of the CF-100 is via Gerstel Maestro software which means it can be integrated into any Chemstation or Masshunter sequence. The Gerstel Maestro prep-ahead software eliminates instrument idle time since the next sample is prepared during the previous sample analysis. External control lines permit the centrifuge to be started and stopped automatically. The centrifuge rotor comes to rest in exactly the same position every time, enabling vial addition and removal using the MPS robotic Z head, in a fully automated fashion. The CF-100 uses the magnetic transport features of the MPS to move vials to and from the rotor. The centrifuge features a hardware safety interlock which prevents the MPS gaining access to the rotor whilst it is still turning.

The centrifuge can independently switch between vortex and centrifuge and is capable of adjustable speed up to 3000 rpm.

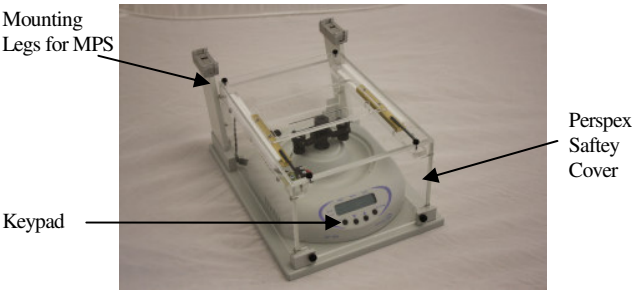

Figure 3 The Anatune CF-100 centrifuge and Perspex safety cover.

As part of the vitamin D protocol proteins are precipitated from serum samples by the addition of zinc sulphate and methanol, the vial is then centrifuges at 3000 rpm for 1 minute to separate the proteins from the supernatant. (Figure 4).

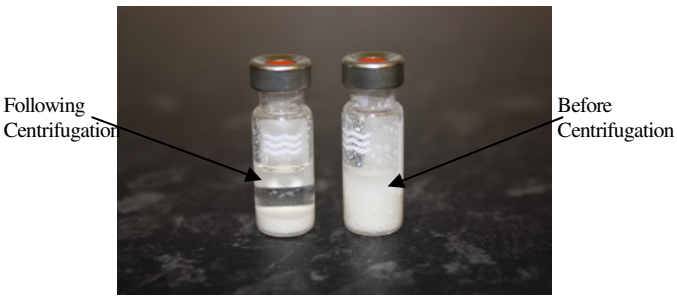

Figure 4 Showing the protein precipitate prior and after centrifugation.

The left vial in figure 4 clearly shows the pelletized proteins in the bottom of the vial and the clear supernatant above, ready for further sample processing, in this case SPE using Instrument Top Sample Preparation.

Instrument Top Sample Preparation (ITSP)

ITSP from Microliter Analytical Supplies (Figure 5) is a consumable device designed to automate SPE and filtration on autosamplers such as the Gerstel MPS. It offers significant benefits over other non-automated SPE procedures; it reduces variability and improves consistency, reduces the cost of consumables and solvents, improves efficiency and productivity and finally reduces risks and errors. The MPS is able to move the ITSP device anywhere on the autosampler deck enabling flexibility in methodology. ITSP uses the analytical syringe of the autosampler as a reservoir above a filter or sorbent material in the case of SPE, the syringe is also used to move the ITSP device around. The autosampler can use hydraulic pressure of the syringe to pass sample across the sorbent media in either direction. Solvent use is minimised due to the small bed volumes of sorbent used, which in turn leads to the use of small elution volumes to give good concentration factors in the final extracts

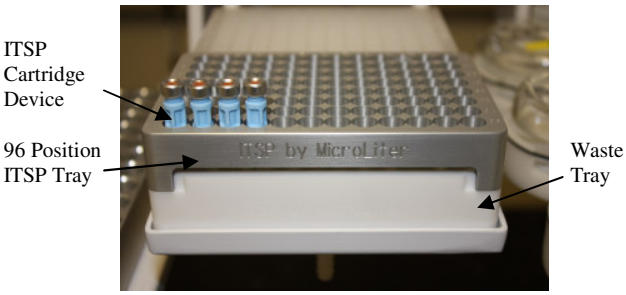

Figure 5 Instrument Top Sample Preparation (ITSP) by Microliter.

In order to implement ITSP on an MPS, a modified needle guide attachment needs to be fitted to enable correct centering of the needle on the ITSP cartridges. (Figure 6). The standard ITSP system features a 96 cartridge capacity, big enough for daily high throughput applications such as Vitamin D.

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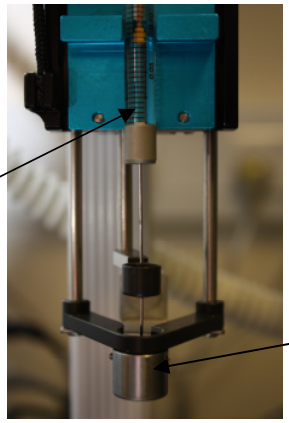

Figure 6 Syringe and modified needle guide.

The ITSP device features an 8 mm crimp top seal manufactured from Teflon and red rubber, this grips the syringe needle during transport and is crowned concavely to mate with the self centering modified needle guide attachment. The ITSP cartridge features a narrow needle guide channel which stabilizes the device in a vertical orientation during transport, and the sorbent material or filter membrane is housed in a filter cup at the bottom of the device.
The analytical syringe acts as the standard column reservoir found in other SPE and filter media formats. The needle penetrates the Teflon rubber septum and creates a seal so that the sample is forced through the media by positive pressure when the plunger is depressed.

Instrumentation

Gerstel MPS 2 fitted with 250 µl syringe
Anatune CF-100 Centrifuge
ITSP Hardware Kit
Maestro Version 1.3.7.69 or later.
Agilent 6410 Triple Quadrupole Mass Spectrometer with HotBox and Multimode Source
Agilent 1200 Series HPLC
G1312B Binary Pump
G1316B Thermostatted Column Compartment SL
G1379B Degasser

Methodology

A Working calibration was prepared by diluting a commercially available serum calibration 25-OH D2/D3 (Chromsystems, Munchen, Germany) 1 in 2, in 0.9 % saline to produce four calibration levels (Table 1). For sample preparation 200 µl of serum sample is placed in a standard 2 ml glass autosampler vial and the vial crimped using a magnetically transportable crimp cap. The sample is then placed on the vial tray of the MPS.

Cal Level	Analyte µg/L	
	25-OH D2	25-OH D3
Std_01	3.8	4.7
Std_02	7.6	9.4
Std_03	15.2	18.8
Std_04	30.4	37.5

Table 1 Serum calibration levels.

The following aspects of sample preparation are fully automated, conducted via the MPS, ITSP or the CF-100. (Figure 8 & 9)

40 µl of internal standard solution (25-OH Vitamin D3-d6 50 ng/ml) is added to the sample, followed by 200 µl of a 0.2 M zinc sulphate solution to enhance the sensitivity of the assay. Following this, 500 µl of methanol is added to the vial to precipitate the proteins. The vial is then moved using magnetic transport to the CF-100 centrifuge whereby the contents are thoroughly vortexed for 1 minute to assist in the protein precipitation. The vial is then centrifuged at 3000 rpm for 1 minute to separate the proteins from the supernatant.

A 10 mg C8 ITSP SPE cartridge is solvated with 100 µl of methanol and then equilibrated with 100 µl of HPLC grade water. 500 µl of the supernatant is then loaded onto the SPE cartridge, before the cartridge is washed with 100 µl of 60 % methanol in water. The cartridge is then dried with 250 µl of air. Analytes are eluted with one 100 µl aliquot of methanol into a 300 µl high recovery vial. The polarity of the final solution is then adjusted by the addition of 40 µl of HPLC grade water.

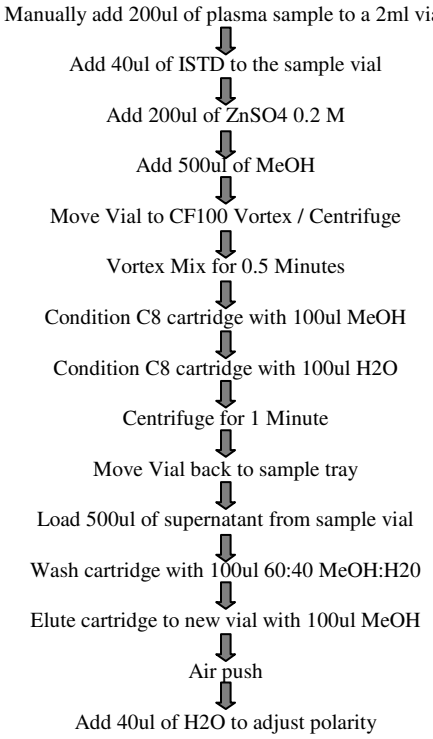

Figure 8 Flow diagram of Vitamin D extraction.

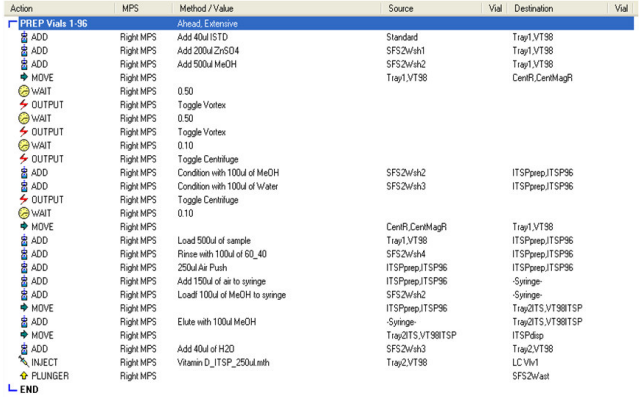

Figure 9 Vitamin D Prepsequence.

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Quantitative Analysis

Sample analysis is fully automated by means of an external injection valve and loop fitted onto the MPS, 20 µl of extracted sample is injected using the valve and loop, filled using the analytical syringe. Separation is achieved by means of an Agilent Eclipse Plus C18 2.1 x 50 mm; 3.5 µm particle size column. The chromatographic mobile phases consisted of 0.1 % acetic acid (v/v) in water (eluent A) and 0.1 % acetic acid (v/v) in methanol (eluent B). A gradient elution was performed from 80 % B to 90 % B in 2 minutes, with an isocratic hold at 90 % for 0.5 minutes; the column was then equilibrated to baseline conditions. Column flow rate was 0.5 ml/min throughout the chromatographic run whilst the column temperature was maintained at 50 °C.

An Agilent 6410B tandem mass spectrometer with multimode source was used in positive simultaneous ESI/APCI mode. Instrument analysis time was 5.5 minutes per sample using the conditions listed (Table 2)

Parameter	25-OH D2	25-OH D3	25-OH D3-d6
Precursor ion	413.3	401.3	407.3
Product ion (Q)	395.3	383.3	389.3
Product ion (q)	159.1	159.1	159.1
Dwell	50	50	50
Fragmentor (V)	120	120	120
Collision Energy (Q)	5	5	5
Collision Energy (q)	25	25	25

Gas Temp (°C):200
Vaporizer Temp (°C):170 Capillary (v):2500
Corona Current (µA):6 Gas Flow (l/min):5
Nebulizer (psi):40 Charging Voltage (V):2000

Table 2 Selected MS conditions for analysis

Experimental Results

Calibration curves were constructed for 25-OH D2 and 25-OH D3. Linear calibrations were achieved from the Chromsystems four point serum calibration standards. Correlation coefficients of 0.999 and 0.998 were obtained for 25-OH D2 and 25-OH D3 respectively Figure 10.

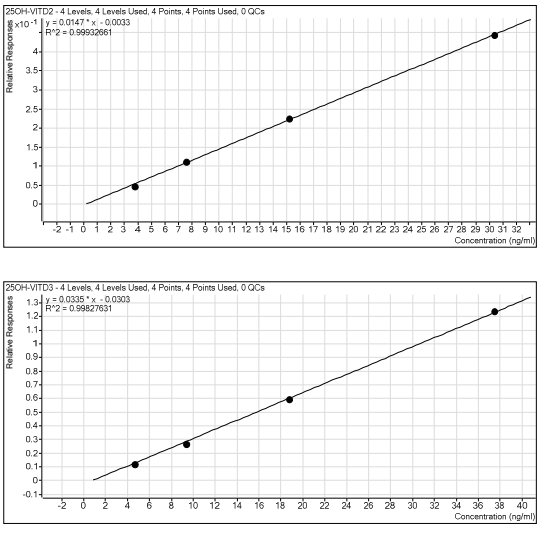

Figure 10 Calibration Curves for 25-OH D2 and 25-OH D3

The method was validated by running tri level vitamin D serum controls obtained from UTAK Laboratories. Dried control materials were reconstituted with water to provide solutions with target concentrations of 10 and 30 ng/ml for 25-OH D2 (8.5-11.5, 26-35) and 25-OH D3 (9.3-12.7, 26-36) (Table 3 & 4)

Sample	Analyte	
	25-OH D2	25-OH D3
QC_Low_01	9.944	10.480
QC_Low_02	9.727	9.512
QC_Low_03	10.672	10.339
QC_Low_04	9.987	10.309
QC_Low_05	9.789	9.951
QC_Low_06	9.277	8.915
QC_Low_07	10.560	9.453
QC_Low_08	10.120	9.840
Average	9.899	9.918
SD	0.455	0.603
% RSD	4.597	6.080

Table 3 Showing results of the low serum control samples.

Sample	Analyte	
	25-OH D2	25-OH D3
QC_Level1_01	30.620	30.219
QC_Level1_02	31.731	34.045
QC_Level1_03	33.516	33.895
QC_Level1_04	30.834	28.473
QC_Level1_05	29.612	29.055
QC_Level1_06	31.228	33.220
QC_Level1_07	29.126	29.395
QC_Level1_08	29.915	28.495
Average	31.257	31.485
SD	1.313	2.528
% RSD	4.201	8.030

Table 4 Showing the results of the Level 1 serum control samples.

The solid phase extraction provided sample extracts free from matrix interferences resulting in clean chromatograms in which 25-OH D2 and D3 are the only major components (Figure 11).

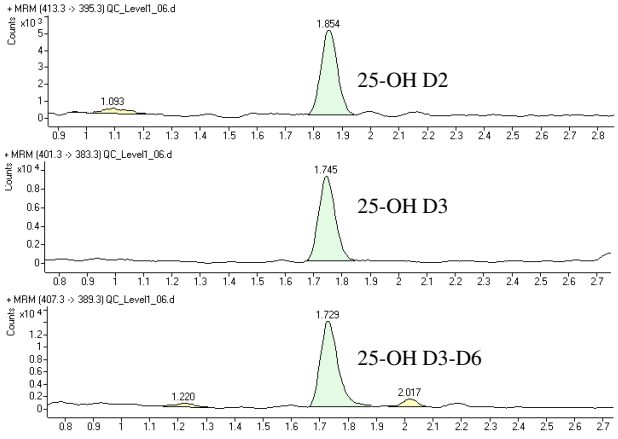

Figure 11 Representative chromatogram from Level 1 QC sample.

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Using the comprehensive prep ahead functionality in Maestro the system is capable of processing 96 samples in twelve hours and fifty five minutes (Figure 12).

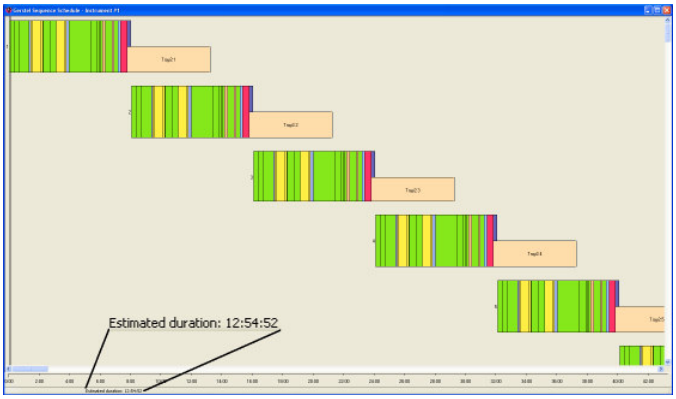


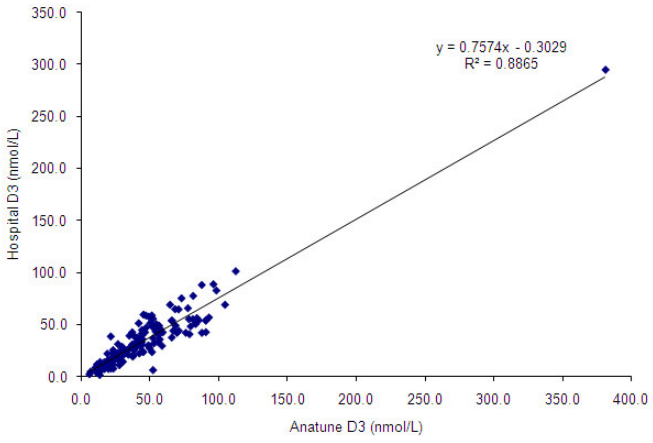
Figure 12 Prepsequence for 96 samples.

Further Work

In addition to the work undertaken with commercially available UTAK QC's and Chromsystems standards, an intercomparison study was undertaken. One hundred and thirty eight samples were provided by a large hospital which had previously been extracted and screened using their SPE and LC-MS/MS procedure.

For the alternative SPE procedure, samples were processed as follows; working standard in acetonitrile was added to each sample and then mixed to ensure full protein precipitation. Following centrifugation the supernatant was introduced onto a Bond Elut C18 cartridge which had previously been solvated and equilibrated. Following sample loading the cartridge was then washed with 50 % methanol/water and the vitamin D eluted with 10 % tetrahydrofuran in acetonitrile. The resulting eluate was then dried under a stream of nitrogen gas, and the residue was reconstituted in 75 % methanol/water. Extracts were then ready for injection and transferred to the autosampler of the LC-MS/MS system for analysis.

Results for vitamin D3 are displayed in the graph below (Figure 13) the data generated by the hospital are plotted against the results achieved using the Anatune Vitamin D Workstation



The data shows a good correlation between the two techniques with a correlation coefficient of 0.8865, indicating the data of the Anatune vitamin D workstation is comparable with that of the hospital.

Finally, work was undertaken to maximise the sensitivity of the assay by investigating alternative ionisation techniques, all Anatune data presented here was acquired using multimode ionisation (MMI), but during this experiment further data was acquired using dedicated electrospray and atmospheric pressure chemical ionisation techniques. Briefly, a calibration standard was extracted using the automated Anatune SPE procedure and then analysed using the three ionisation techniques, the extracted ion chromatograms for 25-OH D3 of each technique are plotted in Figure 14 to compare abundance between the three techniques.

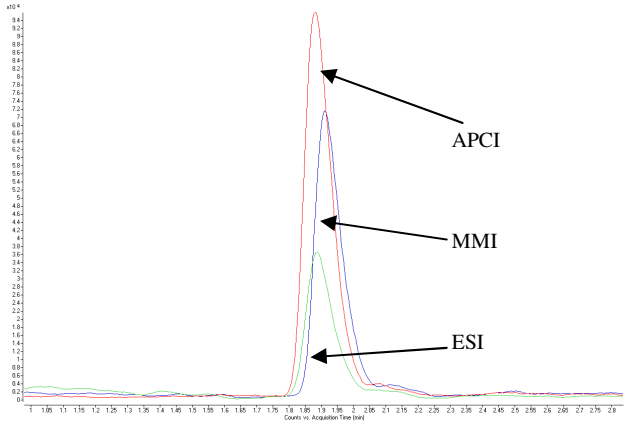


Figure 14 Comparing the three different ionisation techniques.

From the data presented it can be seen that APCI is the most sensitive ionisation technique whilst ESI is the least sensitive. For 25-OH D3 the area counts for the following techniques APCI-591846, MMI-423886 and ESI-234186 were obtained, which show that APCI is over 2.5 times more sensitive than ESI.

Conclusions

Automation allows a lab to scale up their operation for higher throughput since sample preparation, SPE extraction and clean up now occurs automatically during analysis time and can occur 24 hours a day.

Each sample extract is treated in exactly the same way and prepared "just in time", potentially important for light and temperature sensitive Vitamin D samples, improving sample to sample reproducibility when compared to a manual batch process.

Improved data quality due to the consistency gained by automating liquid handling for addition of standards, reagents and by the elimination of manual SPE procedures. Finally sample sequence integration between sample prep system and analytical system, reducing possible transcription errors.

References

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Susan Knox, John Harris, Lisa Calton and A Michael Wallace. A simple automated solid phase extraction procedure for measurement of 25-hydroxyvitamin D3 and D2 by liquid chromatography-tandem mass spectrometry. Annals of Clinical Biochemistry 2009;46:226-230.

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