



**MULTI-PREP® GV-65C MIXED BED (CATION EXCHANGE/NON IONIC) METHOD
FOR THE ANALYSIS OF 25-HYDROXY-VITAMIN D₂ AND
25-HYDROXY-VITAMIN D₃ IN SERUM BY GC/MS or LC/Tandem MS**

September 2008

SAMPLE PREPARATION - (Please see Notes and Supplemental Information before proceeding)

1. Pipette 0.2-0.5 mL of serum plus internal standard into a disposable borosilicate glass tube. Mix.

HARDWARE SETUP - (Please refer to the Detectabuse Hardware Setup Instructions)

COLUMN CONDITIONING

1. Wash column with 1.0 mL of Methanol. Allow to flow by gravity.
2. (Optional) Wash column with 1.0 mL Methanol:Water (75:25). Allow to flow by gravity.
3. Wash column with 1.0 mL Methanol:Water (50:50). Allow to flow by gravity.

SAMPLE DEPROTEINIZATION

1. Prepare a "crash" solution of Acetonitrile:Formic Acid (98:2).
2. Add 1 mL of this solution to prepared sample. Mix.
3. Wait 5 minutes.
4. Centrifuge for 3 min. @ 4500 RPM.

SAMPLE EXTRACTION - (Please see Notes at end of this section before proceeding)

1. Carefully pour supernatant from spun down tube onto column. Allow to flow by gravity.
2. Add 0.5 mL Methanol:Water (50:50). Allow to flow by gravity.
3. Add 0.5 mL Methanol:Water (75:25). Allow to flow by gravity.
4. Vacuum dry for a few seconds.
5. Add 0.2 mL Heptane.
6. Wait 1 min.
7. Vacuum dry for 5 min.

Note: If liquids do not elute freely by gravity flow, there is probably air trapped within the column bed or frits. Tapping the column mounting plate onto the vacuum box should initiate flow. Any columns that have not emptied within 3 or 4 min. may be induced with a low vacuum from a small vacuum pump.

SAMPLE ELUTION

Elute with 0.4 mL Methanol

Note: If a sample does not elute freely by gravity flow, there is either air trapped within the column bed or frits or aqueous phase remaining on the column because of weak vacuum during the column drying step. In most cases, tapping the column will initiate flow. If this does not do the job, use a rubber bulb to gently push a few drops of elution solvent and trapped air into the collection tube. Allow the remainder of solvent to flow by gravity.

A. FOR LC/MS/MS

1. Add 0.1 mL of Water to eluate,
2. Mix and inject .

B. FOR GC/MS DERIVATIZATION

1. Dry down eluate at less than 50°C
- 2 Add 75 µL Ethyl Acetate, 25 µL TMSI to each dried extract
3. Incubate at 70°C for 30 min.

SUPPLEMENT - When using an automated robotic system all liquids may be allowed to flow unassisted through the column or may be pulled through the column with vacuum or pushed through with positive pressure.

Assisted flow parameters may be set as follows:

Column Conditioning - Pass through column in approximately 20 seconds (± 20%).

Sample, Sample Washes and Elution Solvent - Pass through column in approximately 60 seconds (± 20%).

Column Drying Steps – Use 12 – 15 PSI positive pressure for 40 seconds or vacuum set at 15" Hg for 30 seconds (These drying parameters are for individual columns).

GC/MS ANALYSIS

GC/MS: Hewlett-Packard equipped with Mass Selective Detector

GC Column: H.P. Ultra 2 Capillary Column (or equivalent), 15 m x 0.25 mm, 0.25 um film thickness.

Acquisition Mode: SIM

Injector Temp.: 290°C

Detector Interface Temp.: 305°C

Temperature Program:

Initial: 150°C, program at 25°C/min. to 300°C

Final Time: Hold for 2.5 min.

Equil. Time: 1.0 min.

Splitless

He Flow: 1.0 mL/min. @ 200°C

Septum Purge: 2.0 mL/min.

Purge Off Time: 1.0 min.

Solvent Delay: 4.0 min.

Dwell: 20

Start Acq.: 4.0 min.

Stop Run: 8.5 min.

MSD PROGRAM

<u>Compound</u>	<u>Ions Monitored</u>	<u>Ret. Time</u>
25-Hydroxy-Vitamin-D ₃	<u>439</u> , 440, 544	7.24
25-Hydroxy-Vitamin-D ₂	<u>451</u> , 452, 541	7.49

LC/MS/MS ANALYSIS

HPC: Agilent 1200 LC with Rapid Resolution Package
Mobile phase 87% methanol and 13% water each containing 0.1% Formic Acid.

Flow rate: 0.5 mL/min.

HPLC Column: Agilent C18 Eipses 2.1 x 50mm, 1.8 micron particle size.

MS/MS: Agilent 610 MS/MS with Hot Box upgrade

Source: Multi-Mode @ ESI-APCI mode

Collision energy ; 3volts

<u>Compound</u>	<u>Ions Monitored</u>	
	<u>Precursor</u>	<u>Transition</u>
25-Hydroxy-Vitamin-D ₃	401	383
25-Hydroxy-Vitamin-D ₃ -d ₆	407	386
25-Hydroxy-Vitamin-D ₂	413	395

NOTE: Specifics presented by M.P. George, Agilent Technology at a poster session on 11/5/08 at the 2008 Mass Spectrometry: Applications to the Clinical Laboratory Conference
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We thank M.P. George of Agilent Technology for his extensive involvement in the development and evaluation of this procedure in collaboration with a well known , highly respected reference laboratory .

This method is a preliminary procedure for investigational use only. Although it has performed well in our laboratories It must be validated by your laboratory before it is used to report patient values.

We would appreciate your comments on it's performance and welcome your suggestions for improvements or enhancements.