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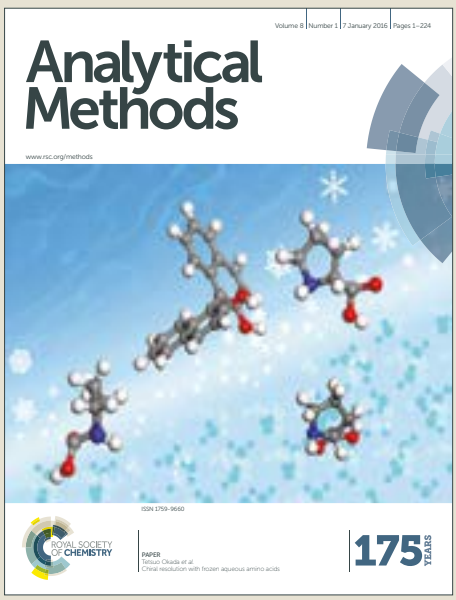
DOI:
10.1039/C7AY00550D

Analytical Methods

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: C. Jenkinson, J. Bradbury, A. Taylor, J. S. Adams, S. He, M. Viant and M. Hewison, *Anal. Methods*, 2017, DOI: 10.1039/C7AY00550D.



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DOI: 10.1039/C7AY00550D

Automated development of an LC-MS/MS method for measuring multiple vitamin D metabolites using MUSCLE software

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Key terms: Vitamin D analysis, MUSCLE software, LC-MS/MS, Automated method development, Chiral separation.

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Disclosure statement: The authors have nothing to disclose.

View Article Online
DOI: 10.1039/C7AY00550D

Abstract

Manual development of liquid chromatography tandem-mass spectrometry (LC-MS/MS) methods is a rate limiting step in analytical laboratories, particularly if several compounds have the same multiple reaction monitoring (MRM) transitions. This study describes the application of Multi-platform Unbiased optimisation of Spectrometry via Closed-Loop Experimentation (MUSCLE) software to automate the development of an LC-MS/MS method to measure multiple metabolites of vitamin D. Comparison with a manually developed method for the same compounds was used to evaluate the effectiveness of MUSCLE in improving method parameters. LC and MS parameter ranges were set up in MUSCLE, which optimised the method during a fully-automated 200 sample sequence. Visual scripts altered method parameters after each sample run while a closed-loop multi-objective optimisation approach identified optimum instrument parameters throughout the sequence to improve sensitivity and run time. The optimised sample run developed using MUSCLE shortened analysis time for 10 metabolites from 8.2 minutes to 6.2 minutes. This was achieved by increased initial methanol concentration in the mobile phase and an altered gradient that increased the in-run organic mobile phase. However, MS parameters could not be optimised further to improve analyte sensitivity over manual optimisation, although in most cases MUSCLE confirmed the manually optimised conditions. Comparison between each of the developed methods showed no significant analyte bias between methods. MUSCLE has been shown here to automate and improve the throughput of a multiple analyte vitamin D LC-MS/MS method. Utilisation of this software could be applied to industries requiring fast automated method development such as clinical and pharmaceutical laboratories.

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1 Introduction

Liquid chromatography tandem-mass spectrometry (LC-MS/MS) has become the primary method of choice for quantitation of compounds such as endocrine and pharmaceutical owing to fast sample analysis and improved accuracy over other techniques.[1] Increased demand has been placed on developing high throughput multi-analyte LC-MS/MS methods, particularly in clinical laboratories requiring rapid results.[1] The development of LC-MS/MS methods can often be challenging as both LC and MS parameters need to be optimised to produce accurate and sensitive methods to achieve the necessary user outputs. To determine optimum multiple reaction monitoring (MRM) transitions it is usually necessary manually tune each sample within the method into the mass spectrometer. Optimisation of LC parameters are also required to achieve high resolution and hence separation of the analytes, particularly those with equal mass-to-charge ratios (*m/z*) and MRM transitions.[2, 3] The development of LC-MS/MS methods can therefore be time consuming and labour intensive to gain optimal method conditions.

MUSCLE (Multi-objective Unbiased optimisation of Spectrometry via Closed Loop Experimentation) is a software platform that has been developed to aid LC-MS/MS method development. The software fully automates the development of LC-MS/MS methods for targeted analytes, gaining optimum runtime, resolution and sensitivity during the course of a sample sequence.[4] MUSCLE applies visual scripting to enable the software to operate the LC-MS/MS system independently, whilst a closed loop optimisation approach using a genetic algorithm is used to optimise the method throughout each sample run.[4] The current study describes the application of MUSCLE software for the automated development of an LC-MS/MS method to measure multiple vitamin D metabolites. A comparison between optimised method conditions

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obtained by MUSCLE with a method that was previously developed manually,[5] was used to assess the effectiveness of MUSCLE in optimising LC and MS conditions. Previous reports have described automated method development using a closed-loop strategy to optimise method parameters for non-targeted analysis of metabolites on gas chromatography mass spectrometry (GC-MS) and LC-MS/MS platforms.[4, 6-8] The development of a six steroid LC-MS/MS method was also developed using MUSCLE, which improved the run time and sensitivity of steroid analytes.[4] However, to our knowledge, this is the first automated approach for LC-MS/MS method development that has been applied to vitamin D metabolites that include analytes with equal *m/z*.

The metabolic pathway of vitamin D is complex, providing several challenges in developing an LC-MS/MS method to target multiple vitamin D metabolites. There are two forms of vitamin D, D3 and D2, which are respectively produced by the action of ultraviolet light on skin, or obtained from plants.[9] The metabolite 1α,25-dihydroxyvitamin D (1α,25$(OH)_2$D3), present at low picomolar endogenous concentrations, is the biologically active form which binds to intracellular vitamin D receptor.[10] However, the precursor to 1α,25$(OH)_2$D3, 25-hydroxvitamin D (25(OH)D) is the principal determinant of vitamin D-sufficiency/deficiency, and is the most commonly quantified metabolite of vitamin D. Several metabolites that form other parts of the vitamin D metabolic pathway are normally found at low endogenous concentrations, which is challenging when developing methods for accurately measuring these compounds. Stereoisomers and chiral metabolites of vitamin D require baseline separation by LC in order to be quantified by mass spectrometry. This includes separating D3 and D2 C3-epimers, 3-epi-25OHD from 25(OH)D,

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formed from epimerisation.[11] Chiral metabolites 23R,25(OH)$_2$D3 and 24R,25(OH)$_2$D3 which are thought to be non-active, have the same *m/z* as 1α,25(OH)$_2$D3, also require separation.[10]

Previously we have reported a manual method for quantification of 10 metabolites of vitamin D, including separation of compounds with equal *m/z*.[5] Routine analysis of serum samples demonstrated quantitation of several metabolites along the vitamin D metabolic pathway including 25OHD3, 3-epi-25OHD3, 24R,25(OH)$_2$D3, 1α,25(OH)$_2$D3 and 25OHD2. The application of MUSCLE for developing this method significantly reduced the optimisation time for method development, as well as improving assay throughput and sensitivity. As this software can work independently,[4] it is anticipated that a further benefit will be reduced labour requirements needed for the development of this method.

2 Experimental

2.1 Reagents and chemicals

Vitamin D reference standards (**Table 3)** and formic acid were purchased from Sigma Aldrich (Pool, UK). 7αC4 reference standard was purchased from LGC standards (Teddington, UK). A Lux cellulose-3 chiral column (100 mm, 2 mm, 3µm) was purchased from Phenomenex (Macclesfield, UK) and a 2µm inline filter was purchased from Waters Corporation (Manchester, UK). LC-MS grade water was purchased from Fisher Scientific (Leicestershire, UK) and LC-MS grade methanol was purchased from Greyhound Chromatography (Merseyside, UK). Vitamin D depleted serum was purchased from Golden West Biologicals Inc. (Temecula, US).

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2.2 Manually developed LC-MS/MS method for measuring multiple vitamin D metabolites

The optimisation steps and method parameters for a manually developed LC-MS/MS method to quantify 10 vitamin D metabolites has been described previously.[5] Each metabolite was manually tuned into the mass spectrometer in methanol at 1 µg/mL to determine optimum MRM transitions. A Lux cellulose-3 chiral column achieved separation of analytes with equal MRM transitions with an overall run time of 8.2 minutes. Validation of this method was performed in accordance to US Food and Drug Administration (FDA) guidelines using a Waters ACQUITY UPLC coupled to a Waters Xevo-MS and TQ-S mass spectrometer.

2.3 MUSCLE software

A detailed description of the MUSCLE software and applications has been described previously.[4] The software works on a stand-alone desktop application. For the MUSCLE sequence a sample has been defined as an individual run in the sequence were a 1 µL injection is taken from the same standard solution. The software package MUSCLE uses closed-loop optimisation to automate LC-MS/MS method development. A multi-objective genetic algorithm (GA) simultaneously optimises a set of user defined LC and MS parameters. The software utilises user-defined visual scripts to change the values of each of the instrument parameters (LC & MS) that are being optimised, with the values being entered for the parameters being decided by the GA. The GA chooses values for each run based upon the previous runs in the sequence that have produced favourable results. Each LC-MS/MS run is evaluated based upon three objectives; minimisation of run time (measured by the retention time of the last eluting peak), maximisation of total number of detected peaks and maximisation of total peak area. MUSCLE maintains a set of preferred samples, each with a specific set of LC and MS parameters, which is

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updated every generation of the GA. The maintained set of samples will be updated every generation if an improvement is made based upon the objectives. As MUSCLE optimises for multiple objectives simultaneously, the set of preferred samples can contain many runs which represent a trade-off between the objectives of the GA, e.g. one run may have a very short run time but not detect all of the peaks, whereas another run may have good sensitivity, detect all the peaks, but have a much longer run time. It is up to the user to select from this set the run which suits best.

2.4 Experiment and sequence set up

The optimisation of this vitamin D method using MUSCLE was performed on a Waters Xevo mass spectrometer coupled to a Waters AQUITY UPLC. An electrospray ionisation source was used in positive ionisation mode. A Phenomenex Lux cellulose-3 chiral column (100 mm, 2 mm, 3 µm) was used, heated at 60 ^{o}C with a flow rate of 0.330 mL/min. The mobile phase solution was water/methanol/0.1% formic acid, which had been previously shown to be the optimal phases when the method was developed manually.[5]

User defined LC and MS conditions were set up within software, setting minimum and maximum value ranges to be used by the genetic algorithm when altering method conditions. The user defined LC and MS conditions for this application are displayed in (**Table 1).** The total number of samples in the sequence was 200 with a 1 µL injection of the same standard solution for each sample run. The first 20 samples were set to have random LC and MS values determined by the software, within the minimum and maximum ranges set. The remaining

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samples in the sequence applied the genetic algorithm for optimising method parameters. The genetic algorithm crossover rate was set at 0.7 and the mutation rate was 0.2.

2.5 Sample preparation and method validation

Sample collection and analysis was approved by the Scientific Committee of the NIHR-Wellcome Trust Clinical Research Facility at Queen Elizabeth Hospital Birmingham. Ethical approval was obtained from the NRES Committee West Midlands (REC reference no. for CHHIP 14/WM/1146). Written informed consent was obtained for sample collection.

Sample preparation and analysis of samples was performed as previously described.[5] Briefly, supported liquid-liquid extraction was performed following protein precipitation prior to LC-MS/MS analysis. Internal standards 25OHD3-d3, 3-epi-25OHD3-d3 and 1α,25$(OH)_2$D3 were added to samples and used for quantitation.

Method validation was carried out with the optimised methods obtained by MUSCLE, compared with the manually developed method for accuracy, precision, assessing matrix effects. Validation was performed based on US FDA guidelines.[12] The analysis of 10 routine serum samples was performed on each of the three methods to compare the quantified concentration of each analyte to inform of the standardisation of measurements. Serum samples were collected as previously described.[5]

2.6 Data analysis

Data analysis was performed using MUSCLE in tandem with Waters MassLynx software. The MUSCLE software was used to identify sample run times, peak areas and numbers of separated

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peaks of each sample. Waters MassLynx software was used to confirm chromatographic conditions. SPSS statistics software V22 was used for comparing methods using regression analysis, Bland-Altman plots and independent T-tests. SigmaPlot V13 was used to assess mass spectrometry conditions on peak area.

3 Results and discussion

3.1 Optimisation of MUSCLE method

MUSCLE was used to optimise the development of a vitamin D LC-MS/MS method, determining whether the optimum LC and MS conditions can be enhanced with the use of this automated software. The assay sequence took approximately 30 hours to complete the 200 sample runs. The selected run revealed an improved set of LC conditions that shortened the retention times of all compounds in the method and the overall run time, when compared with a manual method. The shortened run time of 6.2 minutes for MUSCLE compared with 8.2 minutes for the manual method did not compromise the separation of compounds with equal *m/z*. The sample with the optimal LC conditions was run number 117 in the sequence. During analysis of data an additional high throughput method was highlighted for accurately quantifying 25OHD3. This method separated 25OHD3 from 3-epi-25OHD3 and an isobar 7αC4 which has the same molecular weight. The total run time for this method was 3 minutes.

Figure 1 displays overlaid chromatograms comparing the method developed with MUSCLE and the manually developed method, along with the chromatogram of the high throughput 25OHD3 method. Details of the mobile phase methods optimised using MUSCLE and the manually developed method are displayed in **Table 2** and an overlaid comparison of mobile phase gradients is shown in **supplementary information Figure 1**. The mobile phase associated with

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the preferred MUSCLE method had a higher starting methanol concentration compared to the manual method, 72% compared with 64%. The mobile phase gradients were also different. The MUSCLE method was characterised by a convex gradient, whereas a linear gradient was used for the manual method. The convex gradient shortened the time for reaching maximum methanol required to elute the most polar vitamin D analogue, which also reduced retention times for each of the hydroxylated forms of vitamin D. Both the manual method and all samples run during the MUSCLE sequence could not completely separate the 24OHD2 and 25OHD2 compounds, although partial separation was achieved. **Table 3** summarises the vitamin D retention times between methods. The mean shortening of analyte retention time using the MUSCLE method was 0.99 ±0.26 minutes. The mobile phase method which produced a high throughput method for 25OHD3 had a higher starting methanol mobile phase concentration (80%) and a concave gradient which rapidly increased the methanol at the beginning of the method.

Cone voltage and collision energies of three principal metabolites in the method, 1α,25$(OH)_2$D3, 3-epi-25OHD3 and combined 24OHD2 and 25OHD2 were assessed for improvement in sensitivity based on the results obtained using MUSCLE, compared with manually optimised values. Cone voltage and desolvation temperatures for the entire method were also monitored and compared to manually optimised conditions. The collision energy and cone voltage effects on peak areas of 1α,25$(OH)_2$D3, 3-epi-25OHD3 and combined 24OHD2 and 25OHD2 are displayed in **Figure 2**, along with the effects of the method cone voltage and desolvation temperatures on the peak area of all analytes in the method. These results confirmed that optimised values had already been obtained manually, as no significant improvement in peak area was observed. Although the peak area was increased at desolvation temperatures above 600

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^{o}C, further analysis revealed no improvement in detection limits of analytes when the desolvation temperature was increased. The method therefore incorporated all of the mass spectrometry parameters that were optimised manually.

A number of mass spectrometry conditions are being altered in tandem for each sample, as optimising one parameter each time would take an excessive length of time. This means individual compound results will be affected by the changes in desolvation temperatures along with their individual cone voltage and collision energy altered values. The accuracy of these mass spectrometry results could therefore be affected by the multiple parameters being optimised at the same time. A limitation observed with utilising this software was that the results listed by generation plateaued towards the higher generation values and the samples towards the end of the sequence, suggesting the full 200 samples was not required.

The development of this method was performed on a Waters Xevo TQ-MS mass spectrometer. The limits of detections achieved with this system have restricted the quantitation of certain analytes within this method. Hence, under these parameters the observed limits of quantitation using MUSCLE do not achieve the required levels for assessing clinical reference concentrations ranges for all analytes assessed using this method. However incorporating these analyties into the method was still essential to ensure accurate quantitation by separating analytes of equal mass. The overall improved assay performance assessed for separation and quantitation does not apply to reducing quantitation limits to a point at which they can be routinely quantified for some of the analytes in this method. Specifically routine clinical measurements of 1α,25$(OH)_2$D3 (15-60 pg/mL)[13] and to a lesser extend 24,25$(OH)_2$D3, which clinically can be measured at low pg/mL

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concentration ranges in patients with abnormalities.[14, 15] It would also not be possible to measure circulating levels of 24OHD2 and 3-epi-25OHD2 which were incorporated into this method development to ensure 25OHD2 measurements are accurate. It was also important to demonstrate the separation of 23,25$(OH)_2$D3 for accurate quantification of 1α,25(OH)2D3 and 24,25(OH)2D3. Future studies are required to establish a MUSCLE method capable of routinely quantifying these analytes at levels below the quantitation limits achieved on this platform. These studies would focus on applying MUSCLE to develop this method on a later generation mass spectrometer platform that will improve limits of detection. In addition, the application of MUSCLE to develop a derivitisation method of these analytes on a later generation mass spectrometer would ensure the lower limits of quantitation are achieved.

3.2 Method validation

The accuracy and precision of both the MUSCLE and manual methods are displayed in **Table 4**. Both accuracy and precision were within acceptable guidelines outlined by the FDA. Post-column infusion analysis of 25OHD3 was performed with method optimised manually, MUSCLE optimised method and 25OHD3 MUSCLE optimised method to assess any enhancement or suppression of the 25OHD3 signal owing to different retention times. The signal of 25OHD3 was compared in a vitamin D depleted serum, water sample and routine serum sample. The results from these infusion studies, displayed in **supporting information Figure 2**, showed no changes in signal enhancement or suppression, suggesting 25OHD3 analysis will not be affected by the altered mobile phase methods and retention times.

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Analysis of 10 routine human serum samples was performed using the manual method, MUSCLE optimised method and 25OHD3 MUSCLE optimised method to show standardisation and ensure no analyte bias between methods. Regression analysis between the methods is shown in **Figure 3** and the deviation between methods is shown in **supporting information Figure 3**. Comparison of quantified 25OHD3 using the manual and MUSCLE methods gave a mean difference of 0.76% (95% CI 1.888 - -3.403) bias towards the MUSCLE method however this was not significant (t =0.937, P =0.768). There was a mean difference of 1.38% (95% CI 2.864 - -5.614) bias towards the 25OHD3 high throughput method in comparison to the manually developed, although this was not significant (t =0.935, P =0.995). All other metabolites quantified had varying degrees of mean bias (0.15-9.92%), however none of these differences were found to be significant and this bias was not always varied towards one particular method.

4. Conclusion

Data in this study confirm the efficacy of MUSCLE software in automating the development of a multiple vitamin D LC-MS/MS method. Incorporating this software into the development of this method shortened the chromatographic run time, whilst confirming the majority of the manually optimised mass spectrometry parameters. The MUSCLE software optimised the LC-MS/MS method for measuring 10 vitamin D metabolites, without affecting baseline separation of the metabolites. Baseline separation of compounds with equal *m/z* was also unaffected by the MUSCLE method. Applying this method improved the overall throughput by approximately 3.3 hours per 100 samples, whilst reducing the method development time and labour. The optimum mass spectrometry results from the MUSCLE sequence matched with those that were previously

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optimised manually, suggesting that the software is capable of achieving optimum mass spectrometry without the need for manual optimisation.

In the current study a MUSCLE software strategy was used to further optimise a previously optimised method performed manually. A key advantage of MUSCLE has been demonstrated by the improvement of existing methods to enhance method throughput, and potentially improve sensitivity. In particular MUSCLE may help in the development of other vitamin D methods, particularly for alternative vitamin D pathways that are likely to have compounds with identical *m/z* .[5, 16] MUSCLE has also been used previously to develop methods without prior knowledge of optimised method conditions.[4] This would be beneficial for the development of LC-MS/MS methods in a number of fields such as clinical, pharmacology and metabolomics laboratories, in which high throughput methods are required while reducing the time and labour to develop these methods.

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Tables

Table 1 User defined minimum and maximum LC and MS optimisation parameters.

LC mobile phase conditions	Minimum value	Maximum value	Intermediate value
Initial methanol mobile phase %	60.0	80.0	2.0
Final methanol mobile phase %	90.0	100.0	1.0
Ramp curve	3.0	9.0	1.0
Ramp end (min) time	3.0	8.0	0.5
MS conditions	**Minimum value**	**Maximum value**	**Intermediate value**
1α,25$(OH)_2$D3 cone voltage	12	32	2
1α,25$(OH)_2$D3 collision energy (V)	24	44	2
25OHD2+24OHD2 cone voltage (V)	16	36	2
25OHD2+24OHD2 collision energy (V)	22	42	2
3-epi-25OHD3 cone voltage (V)	16	36	2
3-epi-25OHD3 collision energy (V)	22	42	2
Cone voltage (V)	14	34	2
Desolvation temperature (^{o}C)	400	700	50

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Table 2 Method inlet gradients.

Manual method			
Time	**%Water**	**%MeOH**	**Gradient**
Initial	36.0	64.0	
5.25	5.5	94.5	6 - Linear
5.60	36.0	64.0	6
5.60-8.20	36.0	64.0	

MUSCLE method			
Time	**%Water**	**%MeOH**	**Gradient**
Initial	28.0	72.0	
3.00	3.0	97.0	7 – Convex
3.90	2.2	97.8	6- Linear
4.40	28.0	72.0	6
4.40-6.20	28.0	72.0	

25OHD3 MUSCLE method			
Time	**%Water**	**%MeOH**	**Gradient**
Initial	20.0	80.0	
2.20	7.0	93.0	3 - Concave
2.50	20.0	80.0	6
2.50-3.00	20.0	80.0	

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Table 3 List of vitamin D metabolites analysed and retention time changes between methods.

Compound	Abbrev.	Manual Method RT (min)	MUSCLE Method RT (min)	25OHD3 MUSCLE Method RT (min)
25-HydroxyvitaminD3	25OHD3	3.65	2.76	1.51
3-Epi-25-hydroxyvitaminD3	3-Epi25OHD3	4.28	3.28	1.82
1α,25-DihydroxyvitaminD3	1α,25$(OH)_2$D3	2.62	1.81	-
23R,25-DihydroxyvitaminD3	23R,25$(OH)_2$D3	3.21	2.36	-
24R,25-DihydroxyvitaminD3	24R,25$(OH)_2$D3	2.73	1.95	-
25-HydroxyvitaminD2	25OHD2	3.83	2.93	-
24-HydroxyvitaminD2	24OHD2	3.74	2.84	-
3-Epi-25hydroxyvitaminD2	3-Epi-25OHD2	4.52	3.45	-
1α,25- DihydroxyvitaminD2	1α,25$(OH)_2$D2	3.16	2.32	-
1α,24- DihydroxyvitaminD2	1α,24$(OH)_2$D2	2.67	1.86	-
Ergocalciferol	Vitamin D2	4.90	3.40	-
Cholecalciferol	Vitamin D3	5.25	3.60	-
7α-Hydroxy-4-cholesten-3-one	7αC4	4.01	3.08	1.70

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Table 4 – Accuracy and precision for the manual and MUSCLE methods.

			Manual method			MUSCLE method		
			Precision (%) RSD			Precision (%) RSD		
Compound	Conc. (ng/mL)	Level	Intra-day N=6	Inter-day 6+6+6	Accuracy (%)	Inter-day 6+6+6	Inter-day 6+6+6	Accuracy (%)
25OHD3	1.00	Low				7.7	9.6	98.9
	2.40	Low	5.4	9.8	99.5	3.7	4.7	104.4
	12.0	Medium	6.8	7.4	95.3	3.5	4.4	106.3
	30.0	High	5.6	10.9	100.2	6.9	4.1	103.6
3-Epi-25OHD3	0.40	Low				9.4	8.0	95.1
	1.20	Low	5.1	5.4	106.2	4.6	5.9	109.0
	6.00	Medium	8.8	8.0	98.1	4.4	4.4	106.0
	15.0	High	8.0	7.4	93.1	4.3	5.6	103.8
1α,25(OH)$_2$D3	0.30	Low				8.8	10.4	97.9
	0.480	Low	2.5	3.2	97.2	2.9	4.4	97.1
	2.40	Medium	7.1	3.9	93.5	2.2	3.0	98.8
	12.0	High	7.5	5.9	95.9	2.6	2.7	100.6
24R,25(OH)$_2$D3	0.50	Low				5.4	9.5	108.2
	1.20	Low	6.1	8.1	101.9	4.4	4.5	98.2
	6.00	Medium	7.2	8.2	103.6	1.9	3.6	100.1
	15.0	High	6.0	8.4	103.8	6.4	7.1	94.9
25OHD2	0.40	Low				4.3	5.0	89.0
	1.20	Low	8.3	10.3	97.1	5.1	3.7	101.4
	6.00	Medium	7.8	7.9	93.1	6.0	3.5	98.4
	15.0	High	5.1	4.8	92.6	2.9	4.3	95.8
24OHD2	0.40	Low				10.0	9.7	101.3
	1.60	Medium				20.6	22.5	96.2
	6.40	High				9.4	10.1	108.8
3-Epi-25OHD2	0.40	Low				7.9	7.1	104.6
	1.60	Medium				9.6	8.2	94.3
	6.40	High				13.3	11.4	98.3
1α,25(OH)$_2$D2	0.30	Low				9.2	10.9	94.7
	1.20	Medium				9.9	10.4	96.6
	4.80	High				6.2	6.9	107.6
1α,24(OH)$_2$D3	0.30	Low				13.7	13.8	90.9
	1.20	Medium				8.7	7.7	100.6
	4.80	High				8.7	8.3	97.9

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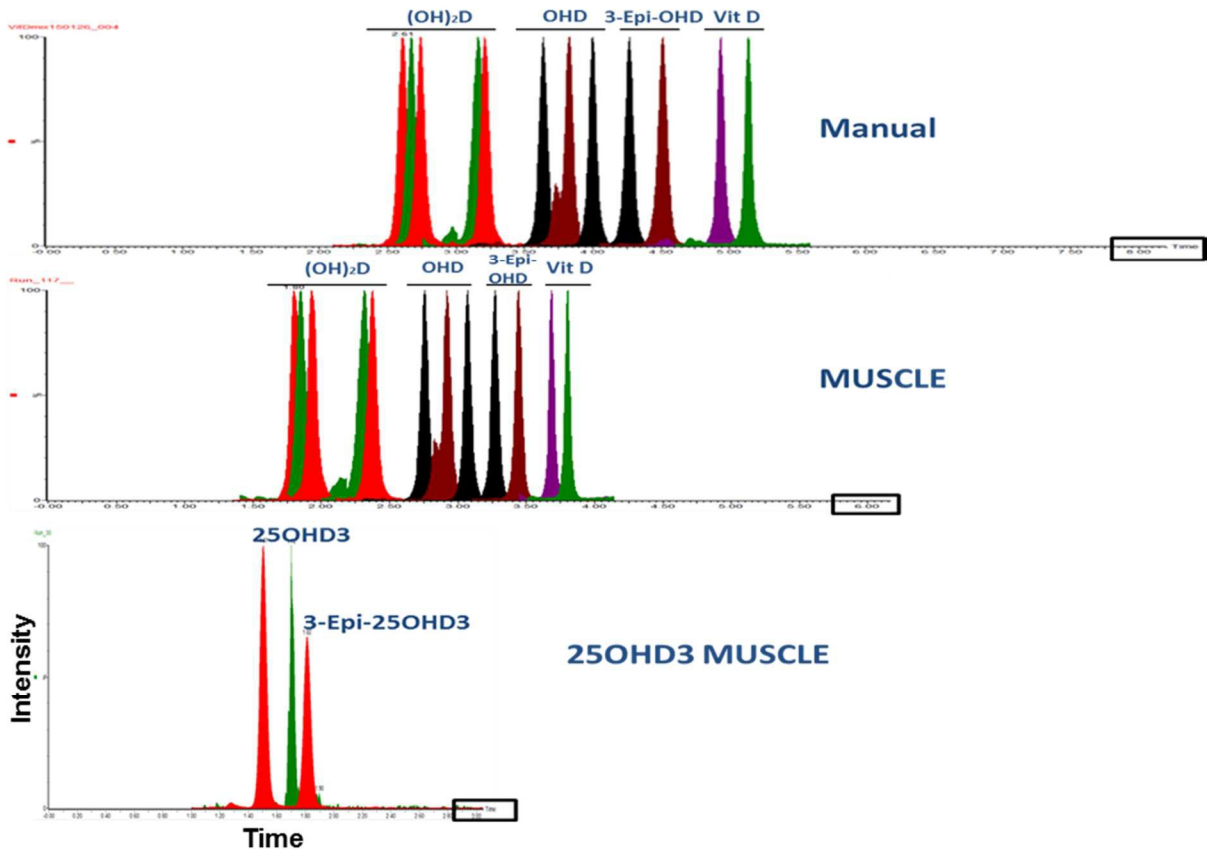
Figures

Figure 1 Chromatograms showing a) overlaid analyses comparing the manually developed method, optimised MUSCLE method and 25OHD3 MUSCLE method of spiked analytes in methanol and water (50/50), b) individual compounds listed comparing manual and optimised MUSCLE methods of spiked analytes in charcoal stripped serum following an SLE extraction

a)



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b)

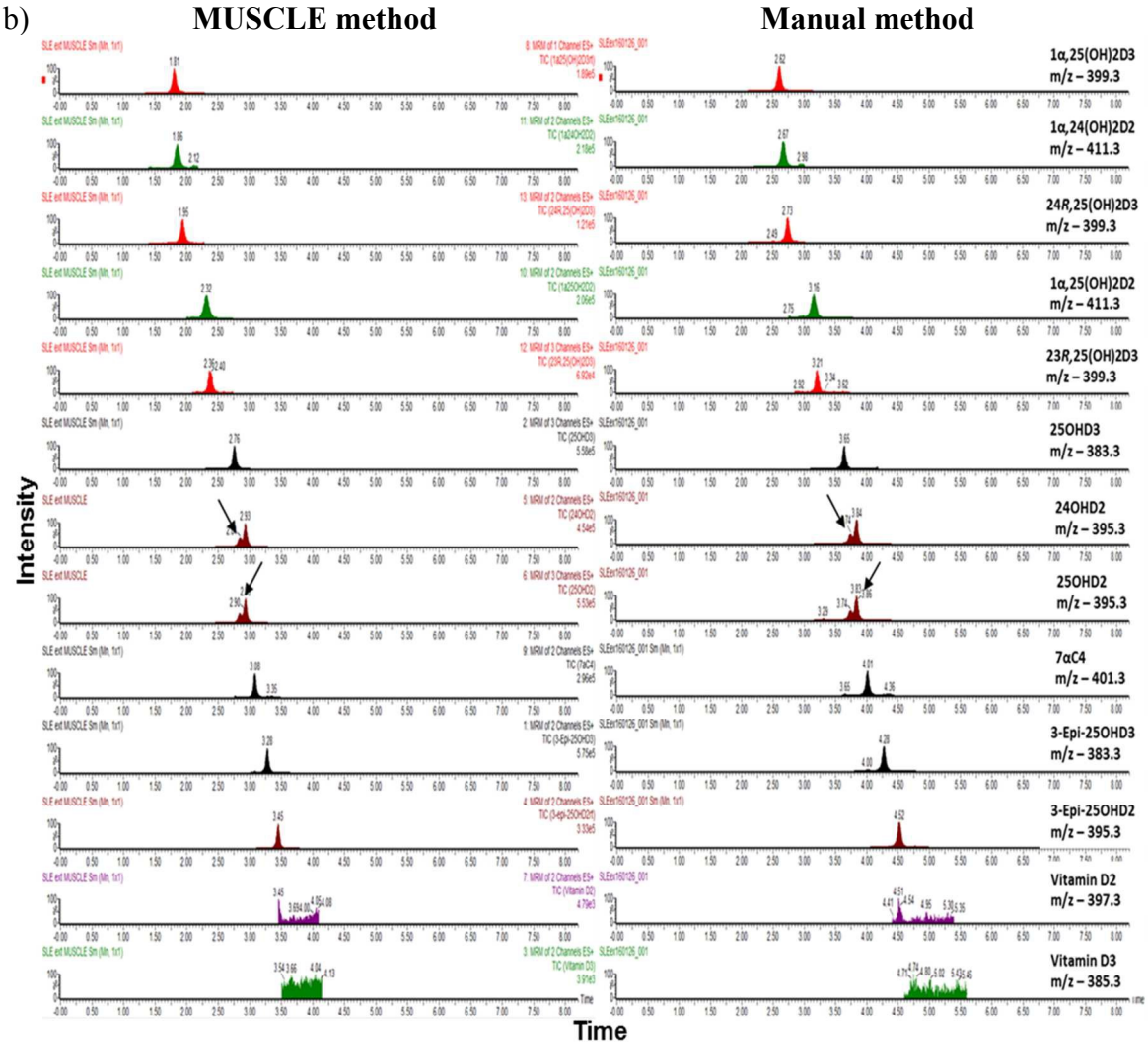

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Figure 2 MUSCLE mass spectrometry optimisation of cone voltage and collision energies on peak areas of individual compounds, along with the cone voltage and desolvation temperatures used for the entire method on peak areas of all analytes in the method. The manually optimised values were 1α,25(OH)$_2$D3 cone voltage 24, collision energy 24; 3-epi-25OHD3 cone voltage 26, collision energy 28; 25OHD2 cone voltage 22, collision energy 26; method cone voltage 24, desolvation temperature 550 ^{o}C

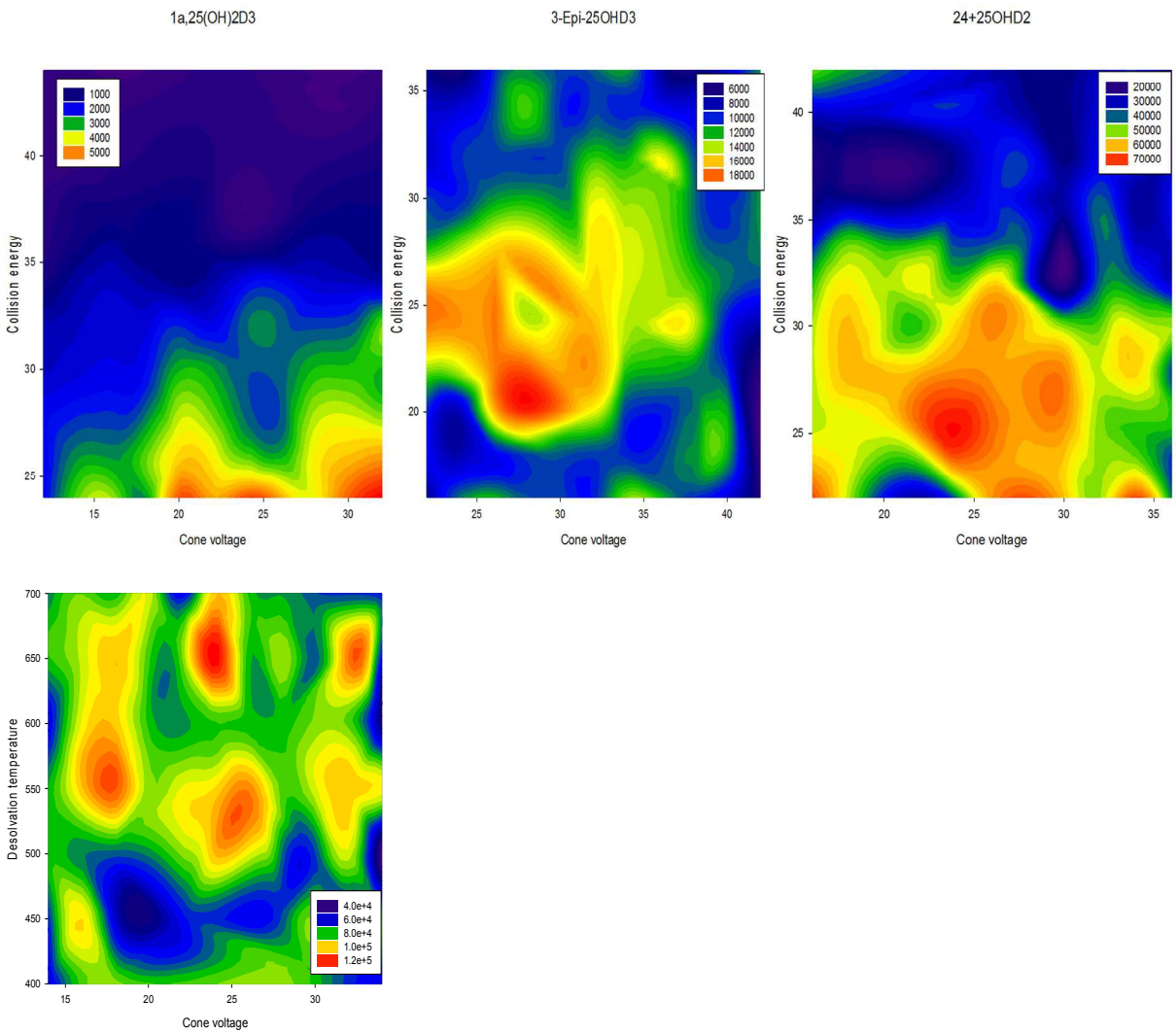

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Figure 3 Regression analysis of the MUSCLE optimised methods compared with the manually developed method for a) 25OHD3, b) 3-epi-25OHD3, c) 25OHD2 and d) 24,25(OH)$_2$D3 concentrations analysed with routine serum samples (n=10). Two results for 25OHD2 were below the LLOQ have not been included. Regression lines and equations between the manual method and MUSCLE (dashed line) and 25OHD3 MUSCLE method (dotted line) are shown in a) and b). The regression line (solid line), 95% confidence intervals (dotted line) and regression equations are shown in c) and d) for comparison between the manual and MUSCLE method for 25OHD2 and 24,25(OH)$_2$D3 respectively