Fat soluble vitamin detection in Food by LC/MS/MS

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INTRODUCTION

Vitamins are needed for healthy growth and these include a group of fat soluble vitamins including A, D, E and K. Vitamin A is important, it is a source of metabolites which are important growth factors and also in the form of retina it is needed by the eye for both low-light and colour vision. Vitamin E is again a group of vitamins of similar structure. There are eight different forms of vitamin E, of which γ-tocopherol is common in the diet and found in for example margarine but is not absorbed as efficiently by people as α-Tocopherol, the most biologically active form of vitamin E which is the second most common form of vitamin E (found in for example wheat germ) and is the form normally used as an additive either in it's native form or as the acetate ester. Vitamin E is an antioxidant that stops the production of reactive oxygen species formed when fat undergoes oxidation and helps reduce cancer risks from such species which can cause DNA damage. Vitamin K is also a group of structurally similar, fat-soluble vitamins that are needed for the post-translational modification of certain proteins required for blood clotting and is involved in metabolic pathways of bone and other tissue. The two naturally occurring forms are vitamin K_1 and vitamin K_2.

The final fat soluble vitamin is Vitamin D and this can be ingested as cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2) and the body can also synthesize it from cholesterol when sun exposure is sufficient. Vitamin D_2 can actually be synthesized in adequate amounts by all humans from sunlight, however when sun exposure is low and vitamin D in the diet is absent diseases can occur namely, e.g. rickets the childhood form of osteomalacia. So like other vitamins, vitamin D is added to staple foods such as milk to avoid disease due to deficiency.

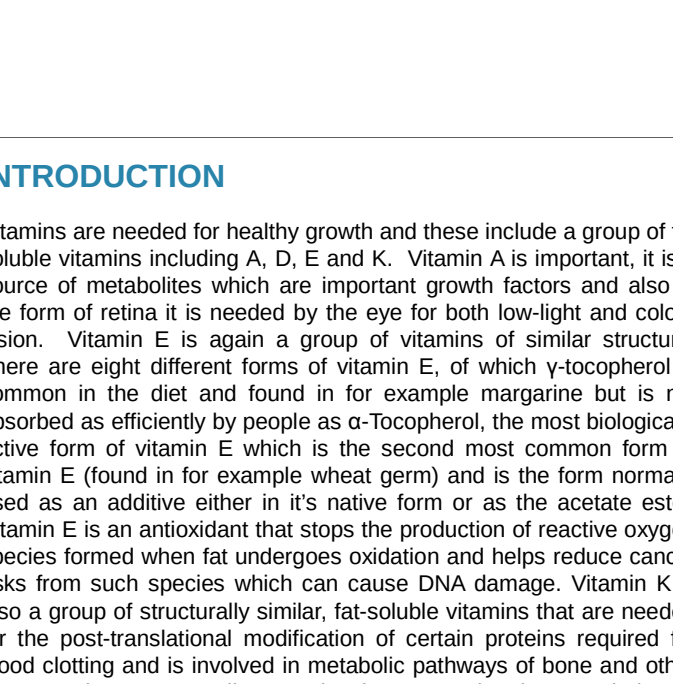

Figure 1. The chemical structures of the Fat soluble vitamins.

Traditionally individual methods have been used to screen for each vitamin so one method that is capable to screen for all fat soluble vitamins would be beneficial. Here we present some new data acquired by LC/MS/MS with a screening method which contains Vitamin K1, K2, D2, D3, A and E. Detection of these vitamins has been shown to be at the low ppb level by LC/MS/MS and reverse phase chromatography using positive mode atmospheric pressure chemical ionisation. The mass spectrometry methods utilises *Scheduled* MRM™ and a small particle size HPLC column and some food samples were obtained and then extracted by and analysed by LC/MS/MS to show the applicability of this method to routine sample analysis.

MATERIALS AND METHODS

Sample preparation: Sample (1 g) was mixed with methanol (1 mL) containing pyrogallol (3%, w/v) and 50% aqueous methanol was added (3 mL) together with internal standard solution (concentration 1ppm, 100 µL). The sample was sonicated (10 minutes) and shaken or roller mix for a further 20 minutes. KOH was then added and the sample vortex mixed. Saponification, to break up the fats and release the vitamins, was then performed at 40 ºC for 30 min during which the samples were briefly vortex mixed. The samples were then cooled and acidified with a solution of HCL

Hexane (15 mL) was added and the samples vigorously shaken and vortex-mixed for 20 s three times with the tubes kept cool between mixing. At the end of the hexane extraction the tubes were centrifuged (3000 rpm, 5 minutes). The top layer (3 ml) was taken and evaporate to dryness. The sample was reconstituted with acetonitrile (0.5 ml) and vortexed (30 s), sonicated (5 mins) and vortexed (30 s) and then this extract was diluted with 50% aqueous acetonitrile (2 ml). This sample preparation was designed to detect vitamins at levels found in foods (0.1 – 50 ppm) and for lower levels a SPE extraction has also been developed to detect these vitamins but this is not discussed in this presentation.

Chromatography: Samples were separated by reversed-phase HPLC on a small particle size C18 column (Kinetex C18 50x 2.1mm, 2.6 µm, Phenomenex) at 600 µl/min using a Shimadzu UFLC System over a 7.5 minute gradient from 20% methanol in water to 100% methanol. The column temperature was maintained at 60 ºC and an injection volume of 50 µL was used.

Mass Spectrometry: Analysis was performed on an AB SCIEX QTRAP® 5500 LC/MS/MS system. The source conditions were a standard set up of curtain gas = 40 psi, Nebuliser current = 5 kV (positive mode), gas 1 = 30 psi with the source temperature set at 400 ºC. The MRM conditions used are shown in Table 1 with the resolution kept at unit for both Q1 and Q3 and *Schedule*d MRM™ used to acquire all the data.

Q1	Q3	RT	ID	DP	EP	CE	CXP
269.2	93	4.1	Vitamin A 1	100	11	35	5
269.2	157.2	4.1	Vitamin A 2	100	11	41	15
269.2	119.1	4.1	Vitamin A 3	100	11	31	5
451.4	187.1	4.7	Vitamin K1 1	65	12	37	15
451.4	128	4.7	Vitamin K1 2	65	12	116	9
451.4	199.2	4.7	Vitamin K1 3	65	12	45	5
458.4	136	4.7	Vitamin K1 IS 1	65	12	116	9
458.4	199.2	4.7	Vitamin K1 IS 2	65	12	45	5
445.4	187.1	4.5	Vitamin K2 1	110	2	33	19
445.4	105	4.5	Vitamin K2 2	110	2	90	9
445.4	80.9	4.5	Vitamin K2 3	110	2	71	11
431.4	165.1	4.5	Vitamin E 1	120	9	40	15
431.4	137.1	4.5	Vitamin E 2	120	9	68	19
437.4	171.1	4.5	Vitamin E IS 1	120	9	40	15
437.4	143.1	4.5	Vitamin E IS 2	120	9	68	19
397.2	69.1	4.4	Vitamin D2 1	96	10	51	8
397.2	91.1	4.4	Vitamin D2 2	96	10	83	8
400.2	69.1	4.4	Vitamin D2 IS 1	96	10	51	8
400.2	91.1	4.4	Vitamin D2 IS 2	96	10	83	8
385.4	90.9	4.4	Vitamin D3 1	115	5	98	9
385.4	367.4	4.4	Vitamin D3 2	115	5	21	17
385.4	259.2	4.4	Vitamin D3 3	115	5	21	17
388.4	90.9	4.4	Vitamin D3 IS 1	115	5	98	9
388.4	259.2	4.4	Vitamin D3 IS 2	115	5	21	17

Table 2. The MRM conditions used to detect the Fat soluble vitamins.

RESULTS

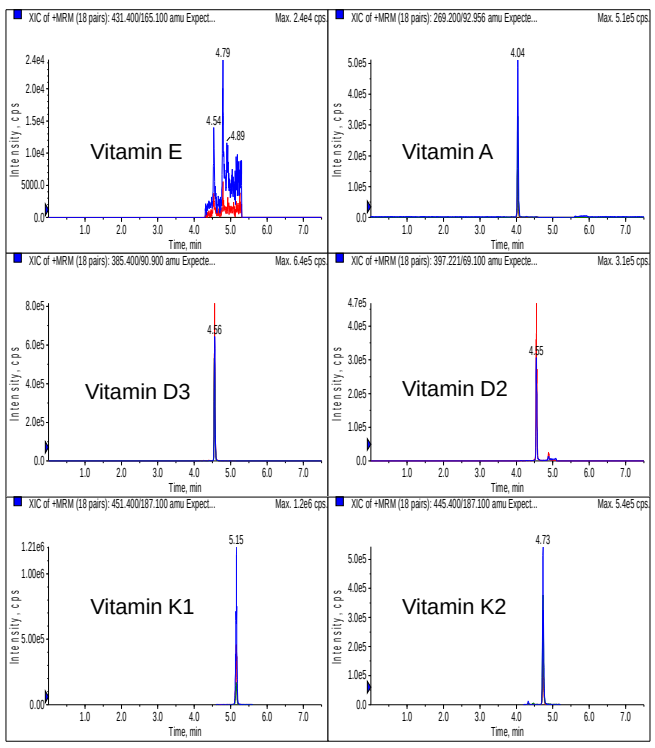

Figure 2. The 20 ppb solvent standard above is a mixture of the fat soluble vitamins. In this figure you can see that the response for each vitamin varies with Vitamin E the weakest with 20ppb it' s limit of detection (LOD). However vitamin E's recommended daily does (RDA) is a 1000 x higher at 12 mg compared to the most active vitamin, vitamin D, whose RDA is 5 µg while vitamin A RDA is 800 µg and vitamin K is 75 µg[1]. These RDA are set in Europe by the European Commission (Commission Directive 2008/100/EC). Therefore the vitamin E level will be over a 1000 times higher than vitamin D in food so it's LOD can be set a lot higher. In this method vitamin D's LOD, in both the D2 and D3 form, is below a ppb.

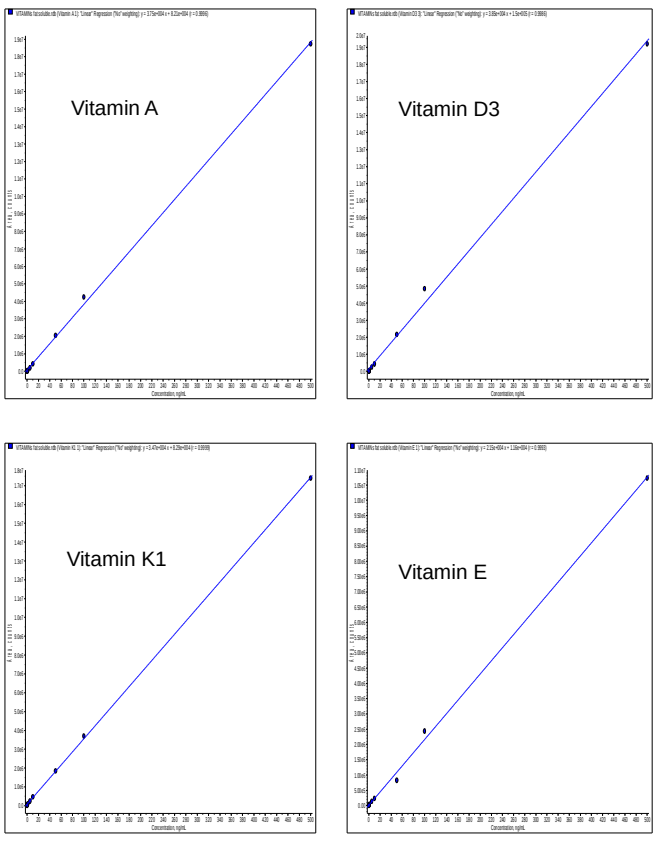

Figure 3. The above figures are typical calibration lines that can be produced in APCI mode when analysing fat soluble vitamin standards. All calibration lines were linear over the range tested (1 – 250 ppb) and had 'r' values over 0.99.

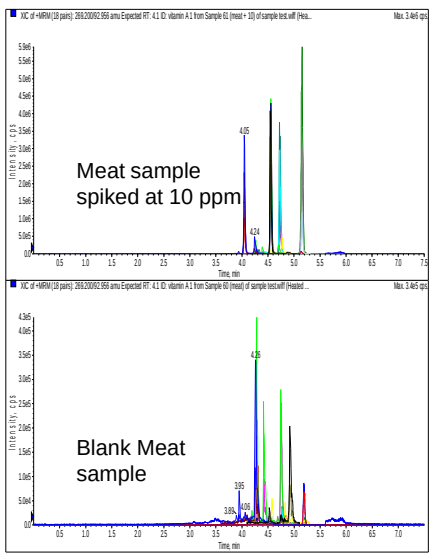

Figure 4. The opposite figure shows an experiment where a sample of minced meat bought from a local shop had been extracted and analysed (bottom pane). A subset of the same sample was then spiked at 10 ppm with a mixture of the fat soluble vitamins and analysed. The results clearly shows the detection of A, D, E and K in the spike although the meat was not completely blank. 10ppm was used as this is the level at which some pet foods are spiked with vitamin A and below the level of vitamin E additives.

CONCLUSIONS

This work has clearly demonstrated that Fat soluble vitamins can be detected using APCI and LC/MS/MS analysis. Although the responses seen for each vitamin vary with the weakest being vitamin E, these responses actually align with the RDA of these vitamins in food so the approach of a multi vitamin assay by LC/MS/MS is valid with detection levels of some vitamins below 1 ppb. In this original work a method to develop the native vitamins has been developed and the detection limit in food will depend mainly on the extraction techniques used. For low level vitamins e.g. vitamin D, a solid phase extraction may be required (which has been developed but not discussed in this presentation) while for high level vitamins such as A and E the simple saponification and liquid / liquid extraction is sufficient. In future work the ester forms of vitamin A and E are planned to be added to this method as these are often the forms at which the vitamins are used as additives[2].

REFERENCES

1. http://www.food.gov.uk/multimedia/pdfs/labellingfoodsupplements.pdf.
2. Directive 2002/46/EC of the European Parliament.

TRADEMARKS/LICENSING