Dimethyl Sulfoxide Extraction Method for the Liquid Chromatographic Analysis of Microencapsulated Vitamin D_3

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A more time efficient and simpler method than methods based upon AOAC procedure 979.24 was developed for the analysis of concentrated microencapsulated vitamin D_3. The improved efficiency and simplicity begins with the use of a 10% water in DMSO solution to dissolve the sample matrix. A 2.0 mL DMSO volume is combined with 200 mg of microencapsulated vitamin D_3 in a flask or bottle. The mixture stands without agitation for 10 min when testing a starch matrix sample and for 30−60 min when testing a cellulose matrix sample. Five minutes of sonication or heating is required for vitamin A/D combinations. Next, 2,2,4-trimethylpentane (isooctane) is pipetted into the DMSO solution−sample mixture. The isooctane volume used was proportional to the sample concentration; specific isooctane volumes relative to sample concentrations are provided. The mixture is stirred for *ca.* 5 min using a magnetic stirrer. The supernatant is filtered through a 0.2 μm PTFE membrane into a suitable LC vial and chromatographed. Validation data which support equivalence to traditional AOAC 979.24-based methodology are presented.

Keywords: *Vitamin D; DMSO extraction; vitamin analysis*

INTRODUCTION

The first standard analytical method for vitamin D_3 was the chick bioassay commonly used from 1932 to 1968 (Deluca, 1988). The chick bioassay was a poor analysis procedure because several days were required to run a sample and the results were quite inaccurate; results ± 10% of the actual vitamin D_3 potency were not uncommon.

In the early 1970s, the colorimetric method for vitamin D_3 was used by industry (Kirk-Othmer, 1984). Antimony(III) chloride was added to give vitamin D_3 a strong visible light absorbance at 500 nm (*Official Methods of Analysis*, 1995). The colorimetric method was faster and more accurate than the chick bioassay but could not resolve the vitamin D_3 absorbance signal from impurity signals present. A false positive signal for vitamin D_3 would be indicated when detectable levels were not present. This resulted in positively biased analytical results.

In 1975, normal phase liquid chromatography methods for vitamin D_3 were submitted to the AOAC and accepted as standard methods of analysis (Hofsass *et al.*, 1978). AOAC methods 979.24 and 982.29 are for concentrated and low-level vitamin D_3 samples (*Official Methods of Analysis*, 1995) Hofsass *et al.*, 1978). Concentrated vitamin D_3 samples range in potency from 100 000 to 30 000 000 IU/g. Low-level samples are found in the range of 220−13 200 IU/g vitamin D_3. Vitamin D_3, a nutrient commonly used in the poultry industry as an antirachitic agent, is present as a concentrated powder in the range of 200 000−1 000 000 IU/g. Concentrated vitamin D_3 can be assayed in industry by AOAC 979.24, Vitamin D in Vitamin Preparations.

The nutrient is commonly microencapsulated in a carbohydrate matrix before blending into animal feed in order to stabilize the vitamin component. Analysis of microencapsulated vitamin D_3 requires a means of dissolving this carbohydrate matrix and a method of extracting the nutrient. AOAC method 979.24 can be referenced for the analysis of these microencapsulated vitamin D_3 materials. In this method samples are saponified with ethanol, sodium ascorbate, and potassium hydroxide under reflux for 30 min. The samples are then extracted once with ether and twice with pentane.

The use of dimethyl sulfoxide (DMSO) can be referenced from the literature as a solvent for dissolving the matrices of fat soluble and water soluble vitamin concentrates (Al-Ashmawi *et al.*, 1992; Dalbacke and Dahlquist, 1991; Qureshi *et al.*, 1990; Beaulieu *et al.*, 1989; Indyk, 1988). Based upon these references and some in-house development work, an alternate sample preparation method has been optimized using 10% (v/v) aqueous DMSO. This method greatly reduces the time needed to prepare microencapsulated vitamin D_3 concentrate samples for analysis.

EXPERIMENTAL PROCEDURES

Traditional Sample Preparation Methodology for Vitamin D_3 Feed Additives. Analytical procedures referenced from AOAC method 979.24 for concentrated powders require multiple steps and heating of the sample. The vitamin D_3 concentrate sample is heated with a base to decompose the matrix, extracted into an organic solvent (such as hexane), and heated again to evaporate the organic solvent. The remaining residue is reconstituted with an organic solvent compatible with the mobile phase.

Though AOAC 979.24 is the traditional reference procedure for commercial vitamin D_3 products, minor modifications to address current sample matrices and instrumentation have been validated and implemented in our laboratory. The modifications presented were developed over the course of several years of vitamin D_3 analysis and differ solely in the reagents used for hydrolysis and in the solvents used for partitioning. The AOAC procedure stipulates refluxing the sample with a solution of potassium hydroxide; however, equivalent procedures have been developed for carbohydrate matrices refluxing with a sodium bicarbonate−water slurry. Though some laboratories use ethanol with the reflux step, 2-propanol has proven to be an adequate substitute. Rather than using a single ether extraction followed by two pentane

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Table 1. Optimization of Aqueous DMSO Volumes for Vitamin D_3 Potency

trial	vol of DMSO solutn (mL)	vitamin D_3 potency (IU/g)
1	2	411 900
	5	408 400
	10	403 100
	15	397 500
2	2	412 000
	5	410 300
	10	403 900
	15	399 500
3	2	410 500
	5	406 100
	10	400 900
	15	398 300
4	2	417 200
	3	416 300
	4	415 800
	5	413 400
5	2	415 100
	3	414 200
	4	416 500
	5	414 400
6	2	414 600
	3	408 700
	4	409 900
	5	406 000

extractions, two extractions with hexane have been validated to yield equivalent results.

With AOAC 979.24, the extract solution is evaporated under a vacuum; however, evaporation of the extract at atmospheric pressure under N_2 works as well with the added advantage of not subjecting the vitamin D_3 sample matrix to unnecessary heating. Isooctane was substituted for both the use of toluene and mobile phase for the final dilution because it resembles the mobile phase (1% ethanol/1% butanol in isooctane) and thus provides a baseline with less noise. Approximately 90 min is required to prepare a single sample for analysis using the traditional AOAC-based methodology.

Optimized Sample Preparation for Concentrated Microencapsulated Vitamin D_3. An aqueous solution of DMSO was found to quickly dissolve the carbohydrate matrix of commercial vitamin D_3 additives at room temperature. Two parameters concerning the water−DMSO solution needed to be optimized for recovery of the vitamin D_3: (1) the percentage of water in the DMSO solution and (2) the volume of DMSO solution relative to the volume of isooctane extracting solvent.

The percentage of water in the water−DMSO solution was optimized by performing liquid−liquid extractions on several identical weights of a single homogeneous sample using aqueous DMSO solutions of various concentrations to break the sample matrix. A 10% water in DMSO solution was found to provide the highest amount of vitamin D_3 extracted for the commercial samples investigated.

The volume of DMSO solution relative to the volume of extracting solvent was optimized by running a single lot of vitamin D_3 concentrate using various DMSO solution volumes. The first volume range investigated was from 2 to 15 mL of DMSO solution followed by a more narrow solution range of 2−5 mL (Table 1). Volumes of extracting solvent used were 10 mL for 200 000 IU/g, 20 mL for 400 000 IU/g, 25 mL for 500 000 IU/g, and 50 mL for 1 000 000 IU/mL. A ratio of 1 part DMSO solution to 10 parts extractant solvent was found to optimally extract vitamin D_3 from commercial samples of concentrated vitamin D_3 powder.

Apparatus. Liquid chromatograph from Thermo Separation Products consisting of a P100 pump, AS3000 autosampler, and UV150 detector was used. A Shimadzu 601 integrator was used for signal processing. Column: Whatman Partisil 10, 4.6 mm × 25 cm. Reagents: cholecalciferol, 99% crystalline from Aldrich (Milwaukee, WI); water, nanopure grade or equivalent; dimethyl sulfoxide, ACS reagent, Fisher Scientific (Fair Lawn, NJ) (Warnings—Irritating to eyes, respiratory system, and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable gloves and eye/face protection.)

Sample Preparation. To 200 mg of sample containing starch- or cellulose-microencapsulated vitamin D_3 in a 125 mL Erlenmeyer flask or other suitable container was pipetted 2.0 mL of a 10% (v/v) aqueous DMSO solution. The mixture was allowed to stand for at least 10 min without agitation. Agitation was not required to dissolve the sample matrix in the 10% (v/v) aqueous DMSO solution for starch-microencapsulated products. Longer DMSO solution treatment was required for all cellulose-encapsulated samples tested. Heating and agitation was required for one cellulose sample which contained a combination of vitamins A and D. The appropriate volume of isooctane was pipetted into the container, and the container was sealed with a rubber stopper. The mixture was stirred using a magnetic stirrer for approximately 5 min. Then the supernatant was filtered through a 0.2 μm PTFE membrane into a suitable HPLC vial and chromatographed.

Standard Preparation. Crystalline vitamin D_3 (cholecalciferol; 50 mg) was weighed into a low-actinic 100.0 mL volumetric flask and dissolved and diluted to volume with isooctane. This is the 20 000 IU/mL stock solution. A 5.0 mL aliquot of stock solution was transferred using a volumetric pipet to a 25.0 mL actinic volumetric flask and diluted to volume with isooctane. This is the 4000 IU/mL working standard solution.

Instrument Conditions. The liquid chromatograph was operated with a flow rate of 1.0 mL/min and a wavelength of 254 nm. Column temperature was ambient and a 50 μL injection volume was used. A pressure of *ca.* 300 psi was observed.

RESULTS AND DISCUSSION

Samples were analyzed with the DMSO method in a fraction of the time needed for the AOAC methods. The DMSO method presented here yielded results which correlate well to results obtained with the traditional AOAC-based methodology. Chromatographic profiles from the DMSO extraction were equivalent to profiles resulting from the extracts obtained using the traditional AOAC-based methodology.

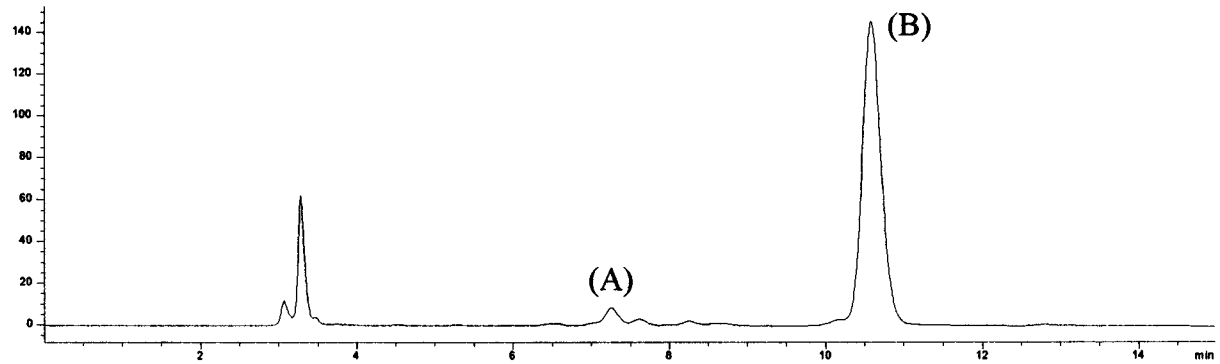

Figure 1. LC analysis of microencapsulated vitamin D_3: (A) previtamin D_3 and (B) *cis*-vitamin D_3.

Table 2. Accuracy of the DMSO Method

sample	matrix	claim (IU/g)	traditional AOAC methodology (IU/g)	DMSO method (IU/g)
1	starch	200 000	214 900	211 600
2	starch	400 000	409 200	417 900
3	starch	400 000	423 200	425 300
4	starch	400 000	429 100	425 100
5	starch	500 000	508 700	514 900
6	starch	500 000	509 300	507 300
7	starch	500 000	506 200	509 400
8	starch	1 000 000	1 021 200	1 048 600
9	starch	1 000 000	1 018 400	1 034 000
10	cellulose	500 000	572 000	568 000
11	cellulose	500 000	516 000	508 000
12	cellulose	200 000	277 000	278 000

Table 3. Summary of DMSO Method Precision Results

sample	*N*	claim (IU/g)	mean (IU/g)	SD (IU/g)	%RSD
1	6	200 000	210 800	1100	0.5
2	6	400 000	413 200	1300	0.3
3	6	500 000	506 100	500	0.1
4	6	1 000 000	1 017 000	4300	0.4

Table 4. Summary of DMSO Method Ruggedness Results

lot	claim (IU/g)	analyst	potency (IU/g)
1	200 000	1	211 600
		2	224 700
		3	208 600
2	400 000	1	425 300
		2	416 400
		3	425 100
3	400 000	1	425 100
		2	425 100
		3	430 500
4	400 000	1	423 300
		2	422 500
		3	427 500
5	500 000	1	514 900
		2	512 400
		3	515 700
6	500 000	1	507 300
		2	508 100
		3	517 400
7	500 000	1	506 800
		2	505 700
		3	507 500
8	1 000 000	1	1 048 600
		2	1 053 200
		3	1 012 300
9	1 000 000	1	1 034 000
		2	1 055 200
		3	1 005 000

The DMSO sample extraction method was validated to illustrate equivalent accuracy, reproducibility, and ruggedness to the modified AOAC method. The accuracy of the DMSO method was established using 12 samples covering the concentration range from 200 000 to 1 000 000 IU/g. Table 2 shows a comparison of results between the modified AOAC method and the DMSO method. Statistical analysis of the DMSO method data showed no significant difference to the results from the modified AOAC method. The Wilcoxon *T*-test (Massart *et al.*, 1988) was used for the statistical analysis, as this test is recommended for sample sizes less than 30 and for sample sets where the presence of a Gaussian distribution is not proven. Application of the Wilcoxon *T*-test to the data from Table 2 gives an experimental *T* value of 29 and a critical *T* value of 14 (95% confidence level). The null hypothesis (equivalence of the two methods compared) is accepted when the experimental *T* value is greater than the critical *T* value; therefore, equivalence of the two methods is shown. Method precision was determined for each sample concentration by analyzing six replicates of the same lot of sample. The relative standard deviation for each sample concentration was under 1.0%; see Table 3 for details. The ruggedness of the DMSO method was established by comparing results obtained by three different analysts. The results between analysts correlated well, and Table 4 is a summary of the ruggedness results.

CONCLUSION

The DMSO extraction method for vitamin D_3 encapsulated in starch is an excellent alternative to traditional AOAC 979.24-based methods. The analysis of microencapsulated vitamin D_3 can now be performed in a fraction of the time previously required.

LITERATURE CITED

Al-Ashmawi, M. I.; Metwally, M. F.; Saleh, H. M.; Mansour, O. Determination of ascorbic acid (vitamin C) in the presence of dehydroascorbic acid using PMR (proton magnetic resonance) spectroscopy. *Anal Lett.* **1992**, *25*, 1065.

Beaulieu, N.; Curran, N. M.; Gagne, C.; Gravelle, M.; Lovering, E. G. Liquid-chromatographic methods for vitamins A and D in multivitamin-mineral formulations. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 247.

Dalbacke, J.; Dahlquist, I. Determination of vitamin B12 in multivitamin-mineral tablets by high-performance liquid chromatography after solid-phase extraction. *J. Chromatogr.* **1991**, *541*, 393.

Deluca, H. F. The vitamin D story: a collaborative effort of basic science and clinical medicine. *FASEB J.* **1988**, *2*, 224.

Hofsass, H.; Alicino, N. J.; Hirsch, A. L.; Ameika, L.; Smith, L. D. Comparison of High Pressure Liquid Chromatographic and Chemical Methods for Vitamin D_3 Concentrates. II. Collaborative Study. *J. Assoc. Off. Anal. Chem.* **1978**, *61* (3), 735.

Indyk, H. Photoinduced reduction and simultaneous fluorescence detection of vitamin K1 (phylloquinone) with HPLC. *J. Micronutr. Anal.* **1988**, *4*, 61.

Kirk-Othmer, *Encyclopedia of Chemical Technology*; Wiley: New York, 1984; Vol. 24, p 201.

Massert, D. L.; Vandeginste, B. G. M.; Deming, S. N.; Michotte, Y.; Kaufman, L. *Data Handling in Science and Technology 2 Chemometrics: a textbook*; Elsevier Science: New York, 1988; pp 51−2.

Official Methods of Analysis, 16th ed.; AOAC International: Arlington, VA, 1995.

Qureshi, S. Z.; Saeed, A.; Haque, S.; Rahman, N. Spectrophotometric method for the estimation of vitamin C in drug formulations. *Anal. Lett.* **1990**, *23*, 995.

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