Determination of vitamin D in foods by HPLC-UV: The pre-vitamin D problem

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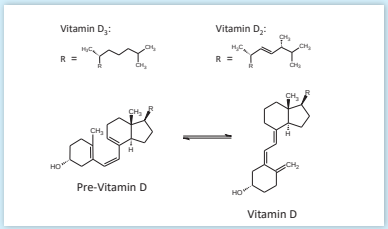
ABSTRACT

Despite the recent proliferation of HPLC-MS methods for the determination of vitamin D in foods, many laboratories continue to use the well-established reference HPLC-UV methods and it is likely that this will continue for some time. One of the potentially confounding issues relates to the reversible conversion of vitamin D to its precursor pre-vitamin D form, to an extent that is dependent on temperature and time. A reliable estimate of the antirachitic vitamin D content of a food should therefore incorporate both pre-vitamin D and vitamin D.

There may be pre-vitamin D_3 present in the sample prior to analysis, and further conversion can occur during analysis. Given that vitamin D_2 is commonly used as the internal standard, the potential presence of pre-vitamin D_2 and pre-vitamin D_3 may compromise unequivocal analytical quantitation, unless all four species can be resolved chromatographically. This study describes the performance of a specific column chemistry that facilitates this and thereby accounts for the contribution of pre-vitamin D vitamers.

INTRODUCTION

The use of HPLC-UV for vitamin D analysis has been used in routine laboratories for over three decades and although superseded by HPLC-MS/MS, remains in common usage in many laboratories. To achieve satisfactory analytical outcomes, it is necessary to incorporate an internal standard. HPLC-UV methods generally rely on vitamin D_2 as internal standard for the determination of vitamin D_3 (and vice versa). Manipulative losses are compensated at each stage in the analysis while thermal isomerisation to the pre-vitamins has been assumed to be the same for both forms of vitamin D.



The majority of commercially available reversed-phase columns are able to achieve the necessary separation of vitamin D_2 and D_3 . However, most are incapable of resolution of all four forms. The implications associated with the temperature and time dependent reversible formation of pre-vitamin D, which may be present in the food or generated during extraction and analysis, is rarely accounted for in quantitative analytical methods.

This study has surveyed several columns under HPLC-UV conditions and identified those few that resolve all four vitamers, thereby facilitating the unequivocal quantitation of total vitamin D as the aggregate of individually measured pre-vitamin D_3 and vitamin D_3 in food.

EXPERIMENTAL

HPLC systems:

i) Agilent HPLC with G1322A degasser, G1311A quaternary pump, G1329B autosampler, G1316A column compartment and G1315A diode array detector, ii) Shimadzu Prominence HPLC with LC-20AD pump and DGU-20A degasser unit, SPD-M20A diode array detector, CTO-20AC column oven with sample injection by a Shimadzu Nexera SIL-30AC cooled autosampler.
Mobile phase was acetonitrile (100%) and column temperature was set at 30°C.

Columns:

i) ACE Excel 5 C18, ii) ACE Excel 5 C18-AR, iii) Agilent Polaris C18-Ether,
iv) Vydac 201TP and v) ACE Excel 5 C18 PFP.

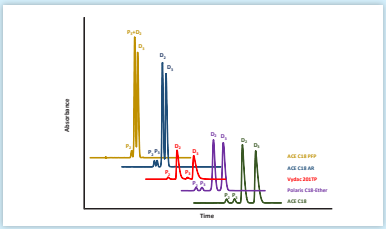
Isomerisation:

Freshly-prepared vitamin D_2 and vitamin D_3 standards were incubated at room temperature and analysed over time for the appearance of their pre-vitamins. At each time-point, extracts were placed in a -20°C freezer until ready for analysis.

RESULTS AND DISCUSSION

Vitamer resolution

The separation of vitamin D_2 and vitamin D_3 ($R_s > 1.8$) could be achieved with many commercial C_{18} columns, with inadequate separation of pre-vitamin D forms. The capabilities of sixteen different columns to separate each pre-vitamin from their respective vitamin D forms, each with various silica packing and C_{18} bonding, resulted in only four columns yielding four baseline resolved peaks:
ACE Excel 5 C_{18}, ACE Excel 5-AR, Agilent Polaris-Ether and Vydac 201TP.

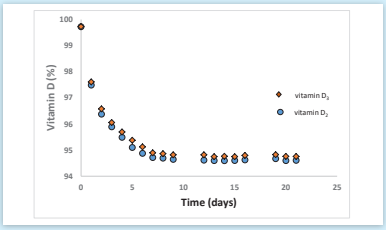


Legend: P_2 =pre-vitamin D_2 ; P_3 =pre-vitamin D_3 ; D_2 =vitamin D_2 ; D_3 =vitamin D_3

While the selectivity of these columns is clearly shown with baseline resolution of the four target vitamin D species, the differing order of elution illustrates very different retention characteristics, with the older Vydac column showing selectivity different from the other three columns.
Accurate quantitation of vitamin D_3 using vitamin D_2 as the internal standard is clearly enhanced when both pre-vitamin D peaks are resolved, the same being true if vitamin D_3 was used as the internal standard for vitamin D_2.

Isomerisation

Measurable pre-vitamin D was absent in freshly-made standard solutions, but appeared quite quickly depending on the temperature of storage. Over 21 days at room temperature, both vitamins D_2 and vitamin D_3 formed their pre-vitamin forms at the same rate and to the same extent.



This confirms the suitability of vitamin D_2 as a surrogate for D_3 , compensating for both manipulative losses during multi-stage sample preparations and importantly, for heat-induced isomerisation during typical extraction and analysis strategies.

CONCLUSIONS

Chromatographic conditions using ACE and Polaris C_{18} columns have demonstrated enhanced vitamin D:pre-vitamin D separations, with a selectivity different from the older Vydac 201TP C_{18} column.
These columns allow the unequivocal speciation of pre-vitamin D_2 , pre-vitamin D_3 , vitamin D_2 and vitamin D_3 , thereby allowing quantitation of the specific contribution of pre-vitamin D to the total vitamin D content of foods.
Vitamin D_2 isomerises to the same extent as vitamin D_3 , compensating for the decrease in vitamin D_3 and attendant increase in pre-vitamin D_3 .
This enhanced chromatographic performance will facilitate a reduction in potential analytical error associated with
i) the contribution of pre-vitamin that may be significantly different between sample and internal standard prior to analysis, and
ii) the known difference in molar absorptivities between pre-vitamin D and vitamin D.