THE DETERMINATION OF VITAMIN D₃ BY HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

YÜKSEK BASINÇLI SIVI KROMATOGRAFİSİ (HPLC) İLE D₃ VİTAMİNİ TAYİNİ

Semih ÖLTEŞ, Yaşar HİŞİL
Ege University, Food Engineering Department İZMİR

SUMMARY: Vitamin D₃ was resolved and quantified by applying reversed-phase high pressure liquid chromatography (RP-HPLC) to the samples of hen eggs. The samples were separated on a reversed-phase C₁₈ column with acetonitrile + methylene chloride (0.001 % triethylamine) + methanol (700 + 300 + 0.5) as the mobile phase. Quantitative determination was accomplished by monitoring the ultraviolet absorbance of vitamin D₃ at 280 nm. The recovery was 94.43 % for vitamin D₃. Details of the accuracy and precision of the method were presented.

ÖZET: Tavuk yumurta örneklerindeki D₃ vitamini, zıt faz yüksek basınçlı sivı kromatografi (RP-HPLC) yöntemiyle tanımlanmış ve miktarları tespit edildi. Örnekle; zıt-faz C₁₈ kolonunda, asetonitril + metilenklorür (0,001 % trietilamin) + metanol (700 + 300 + 0,5)'den oluşan mobil fazda ayrıldılar. Kuantitatif tayin, D₃ vitaminnin ultraviole absorsbbyssının 280 nm. ye ayardanması yoluya gerçekleştirildi. D₃ vitamininin geri kazanımı % 94,43 olarak bulundu. Metodun doğruluk ve kesinliği ile ilgili detaylar burada verilmektedir.

INTRODUCTION

The resolution, identification and determination of the vitamin D and its metabolites in foods was a formidable technical problem and difficult until recently for the following reasons: the unstability of vitamin D to light and heat, and the complex nature of the sample matrix, and the physicochemical properties of the vitamin D (the highest vitamin D activity in foods is small, extinction values used for physicochemical measurement are low and its extraction takes long time, it can be reacted with the some colorimetric reagents together some other compounds, or absorbed radiation in the same regions of the spectrum ) ; and neither a specific chemical reaction nor a specific detector for vitamin D was found, the removal of interfering substances (e.g. vitamin A, E, sterols etc.) is always required (HENDERSON and WICKROWSKI, 1978; KOBAYASHI et al., 1986; PARRISH, 1979; TAKEUCHI et al., 1984).

The hen egg is one of the most important source of vitamin D in the diet. Since the vitamin D is extremely susceptible to oxidation and isomerization during transportation, preparation and storage of eggs; it is very important that its level must be known and controled as accurately as possible (AMMON and DIRCHERL, 1974; ÇOLAKOĞLU and ÖLTEŞ, 1985; ÖLTEŞ and ÇOLAKOĞLU, 1987). The egg, which is the main source of the vitamin D, contains naturally occurring vitamin D₃ and other metabolites. From the point of view of nutritional science, it is important to establish a simple and accurate modified method for determination of the vitamin D present in egg.

EXPERIMENTAL

The experiment was used as described in a previous paper (ÖLTEŞ and HİŞİL, 1990, 1991).

Instrumentation

Apparatus: Waters Associates ALC 200 high performance liquid chromatograph equipped with a M 440 UV detector (280 nm).

Column: µ Bondopak C₁₈ (3.9 i.d. x 300 mm, Waters Associates)

Mobile phase: Methylenecichloride (0.001 % triethylamine) and acetonitrile and methanol (300 + 700 + 0.5).

Flow rate: 0.3 ml/min, AUPS : 0.01, CS : 0.25 cm/min.
Reagents

Analytical standard (Sigma C.C., St. Louis, USA) of vitamin D₃ were used as reference standard. Other chemicals were of extra grades.

Sample Preparation

As described in our papers (ÖLTEŞ and HIŞIL, 1990, 1991), the sample eggs were broken out, and the yolks from albumen were separated: 0.5 g of ascorbic acid (as antioxidant), 20 ml of water, 15 ml of potassium hydroxide solution and 50 ml of ethanol were added to the samples. After saponification on a boiling bath and extraction, the sample dissolved in ethanol was separated on the column. The amount of vitamin D₃ was calculated by comparison of the standard with known amounts according to peak height (REYNOLDS and JUDD, 1984; RÜCKEMANN and RANFFT, 1977; RYCHENER and WALTER, 1985; SÖDERHJELM and ANDERSSON, 1978; WICKROWSKI and MCLEAN, 1984).

Details of the chromatographic parameters were described in previous papers (ÖLTEŞ and HIŞIL, 1990, 1991).

<table>
<thead>
<tr>
<th>Sample no</th>
<th>Vitamin D₃ content (IU per 100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>188</td>
</tr>
<tr>
<td>2</td>
<td>178</td>
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<tr>
<td>3</td>
<td>180</td>
</tr>
<tr>
<td>Mean</td>
<td>182</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>3.3</td>
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</table>

Table 1. The Replicate Assay of Vitamin D₃ in Egg Yolk

RESULTS AND DISCUSSION

The results for the three replicate analysis of vitamin D₃ in egg yolk samples were given in Table 1. The average level of the results was 182 IU per 100 g edible portion (range : 178-188 IU / 100 g). The average value of 182 IU / 100 g was lower than that of recommended dietary allowance of vitamin D for infants (400 IU / day).

In view of the nature of the developments in the methods of vitamin D assay, the discussion in this study was on HPLC methods of vitamin D₃ since total activity of vitamin D in eggs is the sum of vitamin D₃ and other metabolites which may be present. In our research, the identity of vitamin D₃ was confirmed by comparison of retention times of egg samples and the standard solutions of vitamin D₃ (ANTALICK, 1979; BORSJE et al., 1978; COHEN and LAPOINTE, 1978; COHEN and WAKEFORD, 1980; HOFSASS et al., 1978; JACKSON, 1982; JACKSON et al., 1982; LANDEN, 1981; LEIN et al., 1980; MULHOLLAND and DOLPHIN, 1985; NIEKERK and SMIT, 1980; OKANO et al., 1984; OSADCA and ARAUJO, 1977; RAY et al., 1977; SIVELL et al., 1982; STANCHER and ZONTA, 1983; TAKEUSHI et al., 1986; WILLIAMS et al., 1972). The retention time of vitamin D₃ was 7.8 min.

The recovery study of vitamin D₃ was performed by adding vitamin D₃ working standard solution to egg samples. Calibration curve was obtained by injecting the standard solutions in methanol containing vitamin D₃ in different amounts. The recovery of vitamin D₃ calculated from peak heights was 94.43 % (standard deviation 3.3 % for 3 replicates). Figure 1 shows the chromatograms of vitamin D₃ standard, vitamin D₃ in yolk and a mixture of vitamin D₃ standard and vitamin D₃ in yolk.

![Figure 1. Chromatograms of vitamin D₃ reference standard and mixed egg yolk](image-url)

a) vitamin D₃ standard; b) vitamin D₃ in yolk; c) vitamin D₃ standard and vitamin D₃ in yolk
In conclusion, we recommended the high pressure liquid chromatographic method which is rapid, simple, sensitive, good reproducible and precise, to be an very efficient technique for the determination of vitamin D₃ in hen eggs.

REFERENCES