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New analytical aspects of vitamin D in foods

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A comprehensive study of vitamin D in foods was undertaken during 1991–1994 in Finland to develop high-performance liquid chromatographic (HPLC) methods for analysing cholecalciferol, ergocalciferol and their 25-hydroxylated metabolites in fish, egg yolk, meat, milk, edible mushrooms and fortified foods, and to evaluate which vitamin D compounds should be analysed to estimate the total vitamin D contents in these foods. The procedures included saponification, extraction and purification using one or two chromatographic steps prior to quantification by HPLC using internal standard methods. Ergocalciferol was used as an internal standard for cholecalciferol and vice versa, and, as first presented in the present study, 25-hydroxyergocalciferol was used as an internal standard for 25-hydroxycholecalciferol and vice versa.The methods developed were well suited to determining the contents of ergocalciferol, cholecalciferol and their 25-hydroxylated metabolites in food. Good estimates of the vitamin D content of fish or edible mushrooms are obtained if only cholecalciferol or ergocalciferol, respectively, are determined. On the other hand, in addition to cholecalciferol 25-hydroxycholecalciferol should also be determined from egg yolk, meat and milk. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Vitamin D compounds are naturally present in only a few foods: fish, egg yolk, meat, milk and edible mushrooms. Due to low concentrations the determination of vitamin D-active compounds has been a difficult and laborious task. Previous biological methods were time-consuming, expensive and did not distinguish between different vitamin D compounds. Advances in high-performance liquid chromatographic (HPLC) methods have created new opportunities for assaying individual vitamin D compounds.

Several HPLC methods have been published for supplemental chole- or ergocalciferol. Most of these methods use saponification and chromatographic purification, followed by quantification with HPLC, using an internal standard method (van Niekerk & Smit, 1980; Rychener & Walter, 1985; Johnsson *et al.*, 1989; Sliva *et al.*, 1992; Homberg, 1993). HPLC methods for determining the presence of naturally occurring chole- or ergocalciferol, and especially their hydroxylated metabolites, are quite limited. Applications have mainly been developed for milk samples (Hollis *et al.*, 1981; Reeve *et al.*, 1982; Kunz *et al.*, 1984; Parviainen *et al.*, 1984; McDermott *et al.*, 1983; Takeuchi *et al.*, 1988; Kurman & Indyk, 1994). In these applications the quantification of the vitamin D compounds has usually been done by a biospecific method and HPLC has been used only for purification. Because of limited studies, knowledge of the contribution of different vitamin D compounds to the vitamin D activity in foods other than milk can be regarded as poor.

The present study, carried out in 1991–1994, describes a set of HPLC methods for determining the concentration of chole- and ergocalciferol and their 25-hydroxylated metabolites in different foods. Although the general approach previously developed for analysing chole- or ergocalciferol in fortified foods (see above) was adopted in this study, considerable development and optimization was required in order to be able to quantify the low concentrations of naturally occurring chole- and/or ergocalciferol and their 25-hydroxylated metabolites in different food matrices. The purpose of this paper is to introduce HPLC methods developed, and to clarify which vitamin D compounds should be determined if the entire vitamin D activity of different foods is to be estimated.

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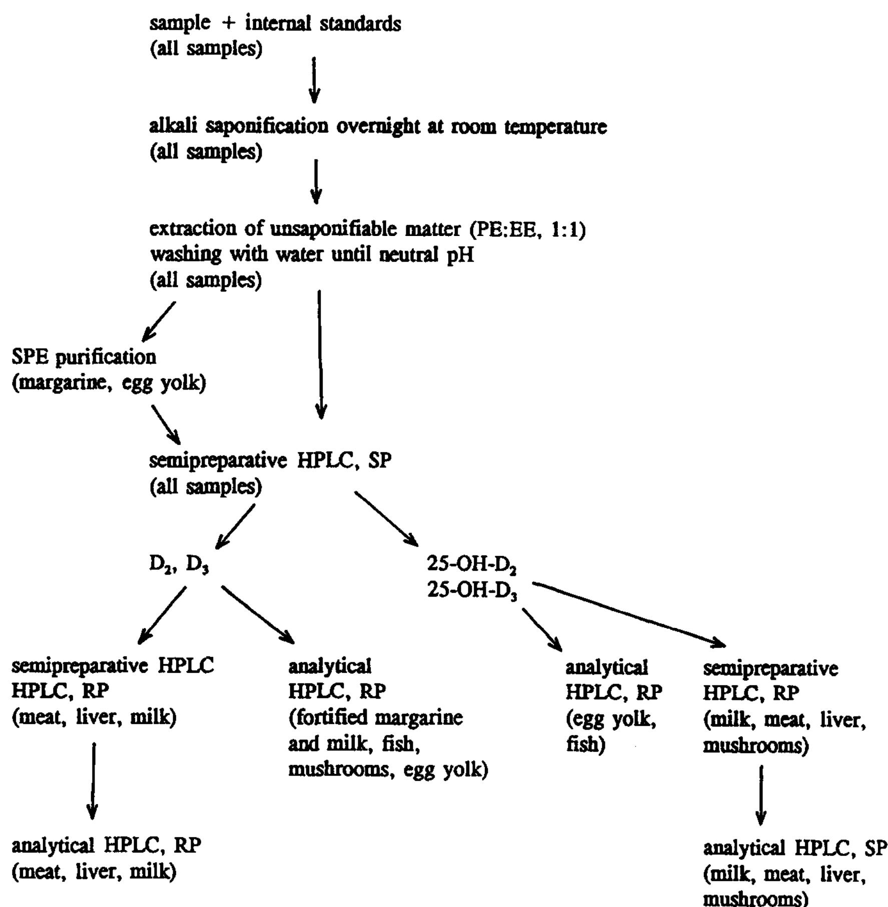

Fig. 1. Procedures for the determination of vitamin D compounds in foods.

MATERIALS AND METHODS

The procedure used is presented in Fig. 1. The sample sizes were 50–100 g for milk and milk products, 1 g for lyophilized mushroom and 20 g for fish, egg yolk, meat, margarine and liver. Following Mattila *et al.* (1992) samples were saponified at room temperature overnight. The following morning the saponified samples were extracted twice with a mixture of diethyl ether and petroleum ether in the ratio 1:1. SPE (solid phase extraction) clean-up was carried out for egg yolk and margarine using silica SPE columns (2 g for egg yolk and 0.5 g for margarine) and solvent systems of *n*-hexane–2-propanol, according to Mattila *et al.* (1992, 1993, 1995*a*), respectively. Semi-preparative HPLC purification was carried out for all samples; both isocratic and gradient elutions were used. Isocratic purification (straight-phase mode) was applied if the fractions of vitamin D had already been separated by SPE (Mattila *et al.*, 1992, 1993) or if the analysis was to cover only cholecalciferol (Mattila *et al.*, 1995*a*). On the other hand, gradient elution (straight-phase mode) was used when more than a single fraction of vitamin D compounds had to be collected during the semi-preparative HPLC (Mattila *et al.*, 1994, 1995*b*,*c*). In the case of meat and milk products and edible mushrooms, a second semi-preparative step (reverse-phase type) was needed for the 25-hydroxycholecalciferol + 25-hydroxyergocalciferol fraction (Mattila *et al.*, 1994, 1995*c*). A second semi-preparative purification (reverse-phase type) was also needed for the ergocalciferol + cholecalciferol fractions in most milk and meat products (Mattila *et al.*, 1995*c*).

The concentrations of chole- or ergocalciferol and their 25-hydroxylated metabolites were determined using reverse-phase and either reverse- or straight-phase chromatography, respectively, with diode array or UV detection at 264 nm (Mattila *et al.*, 1992, 1993, 1994, 1995*a*,*b*,*c*). Internal standard methods were mostly used for quantification; cholecalciferol and 25-hydroxycholecalciferol were internal standards for ergocalciferol and 25-hydroxyergocalciferol (edible mushrooms), while ergocalciferol and 25-hydroxyergocalciferol were internal standards for cholecalciferol and 25-hydroxycholecalciferol (other samples). If the samples contained the compounds to be used as internal standards, these compounds were quantified using an external standard method with recovery corrections (Mattila *et al.*, 1995c).

The reliability of the methods was tested by recovery and repeatability tests, by running in-house reference samples (randomly chosen food items in the food groups under study), running blank tests with no added internal standard and participating in the EC MAT (European Community, Measurement and Testing) certification study on fortified margarine and milk powder

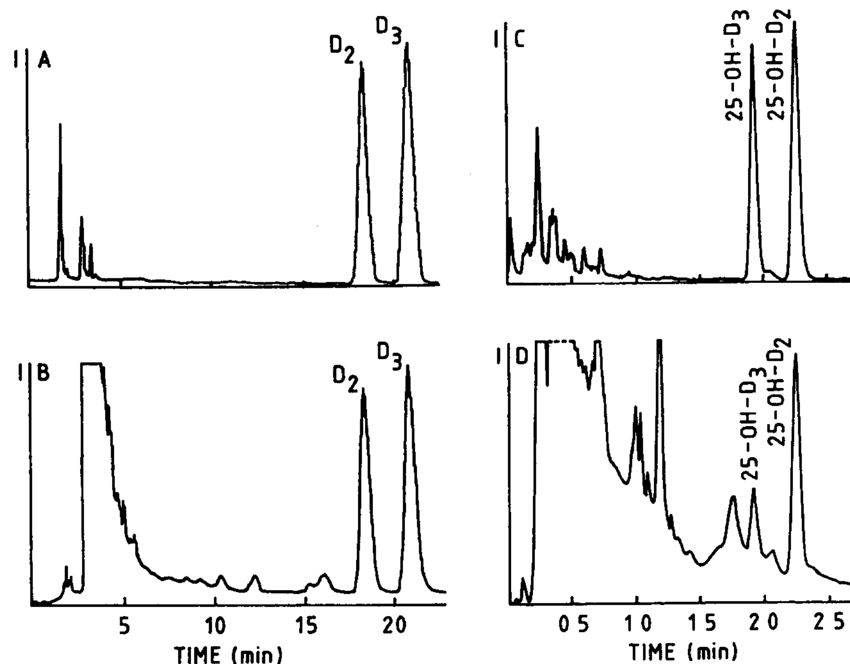

Fig. 2. Analytical HPLC chromatograms of: (A) a standard mixture of ergocalciferol and cholecalciferol; (B) ergocalciferol (IS) and cholecalciferol in a pike sample; (C) a standard mixture of 25-hydroxycholecalciferol and 25-hydroxyergocalciferol; and (D) 25-hydroxyergocalciferol (IS) and 25-hydroxycholecalciferol in a whitefish sample.

(Finglas *et al.*, 1994). In addition, the purity and identity of the peaks of vitamin D compounds were confirmed by diode array detection and by improving the separating capacity with the help of a tandem-column system. The detection limits and linearity of the detection were also determined for each analytical HPLC modification used (Mattila *et al.*, 1992, 1993, 1994, 1995*a*,*b*,*c*).

RESULTS AND DISCUSSION

The aim of the above procedure was to be able to quantify the amounts of chole- and/or ergocalciferol and their 25-hydroxylated metabolites contained in the same extract. As a result of the differences in polarity between chole- and ergocalciferol, on the one hand, and their 25-hydroxylated metabolites, on the other, the simultaneous quantification of these compounds during the same chromatographic run was not practical. Therefore, it was found reasonable to separate the fractions containing cholecalciferol + ergocalciferol and 25-hydroxycholecalciferol + 25-hydroxyergocalciferol during the SPE or the semi-preparative HPLC steps, and to quantify these fractions separately.

The number of purification steps was optimized individually for each food group. The lower the vitamin D compound content or the more complex the matrix, the more purification steps were required before quantification. To optimize the efficiency of purification, reverse- and straight-phase types of chromatography were used in turn. Quantification using internal standard methods was preferred to external standard methods because of possible losses caused by the many purification steps. In the present study, to our knowledge, the use of 25-hydroxyergocalciferol as the internal standard for 25-hydroxycholecalciferol and vice versa is presented for the first time. Since these compounds are chemically very similar, as are chole- and ergocalciferol, they proved to be excellent internal standards for each other. This was demonstrated in the study by determining overall recoveries for the internal standards and compounds examined. Although only 50% for some high-fat foods, the overall recoveries of chole- and ergocalciferol were practically the same for every recovery test sample, and also for the 25-hydroxymetabolites.

The methods developed were well suited to determining the contents of ergo- and cholecalciferol and their 25-hydroxylated metabolites in food. Some milk and meat products contained, however, indeterminable amounts of the vitamin D compounds. The chole- and ergocalciferol and the 25-hydroxymetabolite peaks separated well from each other and from the matrix (Fig. 2 and Fig. 3) (Mattila *et al.*, 1992, 1993, 1994, 1995*a*,*b*,*c*). Monitoring the peaks using a diode array detector confirmed their identity and purity. In most samples interfering compounds did not elute at the same retention times as the internal standards. The repeatability of the methods was good, considering the low concentration of the vitamin D compounds present in the samples; coefficients of variation (CV%) for the triplicated samples were $<10\%$ for chole- or ergocalciferol and $<20\%$ for their 25-hydroxymetabolites. The CV% of the reference sample was mainly $<5\%$. In addition, good recoveries (90–110% for chole- or ergocalciferol and 90–100% for 25-hydroxychole- or 25-hydroxyergocalciferol) were obtained when calculated using the internal standards. The cholecalciferol results

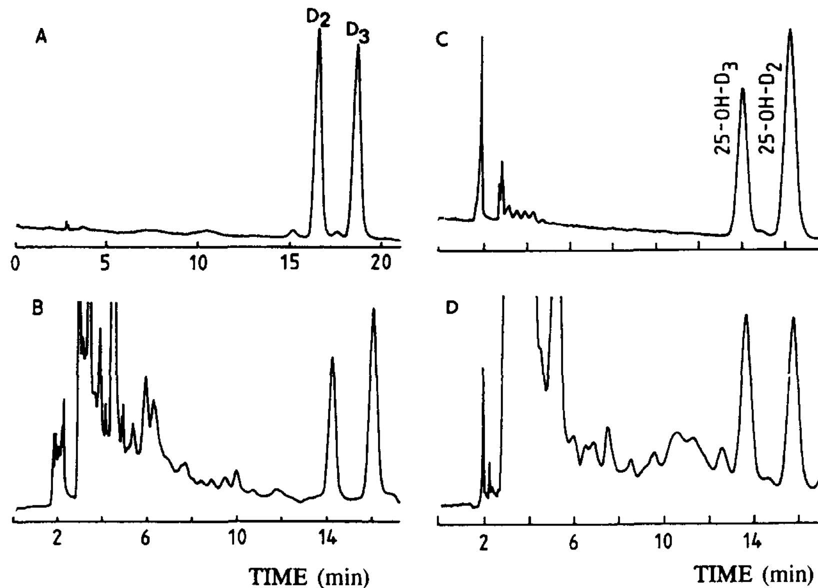

Fig. 3. Analytical HPLC chromatograms of: (A) a standard mixture of ergocalciferol and cholecalciferol; (B) ergocalciferol (IS) and cholecalciferol in an egg yolk sample; (C) a standard mixture of 25-hydroxycholecalciferol and 25-hydroxyergocalciferol; and (D) 25-hydroxyergocalciferol (IS) and 25-hydroxycholecalciferol in an egg yolk sample.

obtained in our laboratory were very similar to the mean values obtained from 11 laboratories in the EU MAT certification study for fortified margarine and milk (Finglas *et al.*, 1994). The detector responses were linear over the ranges tested (2–5 to 300–700 ng/injection), the coefficient of correlation being >0.999. The detection limits of the diode array and conventional UV detector were 1.5–2 and 0.5–0.7 ng/injection, respectively, for the compounds determined.

It is assumed that the concentrations of vitamin D metabolites other than 25-hydroxylated are so low in food that they are of no practical significance (Hollis *et al.*, 1981; Takeuchi *et al.*, 1988; Koskinen & Valtonen, 1985; Mawer & Gomes, 1994). Thus, by determining only the amounts of chole- and ergocalciferol and their 25-hydroxylated metabolites, it is possible to derive quite good estimates of vitamin D activity in foods. Based on the present study, the predominant compounds in fish and mushrooms were cholecalciferol and ergocalciferol, respectively, and quite a good estimate of the vitamin D activity of these foods can be derived by analysing only the above compounds. On the other hand, cholecalciferol and 25-hydroxycholecalciferol should be determined from egg yolk, and meat and milk products.

REFERENCES

Finglas, P. M., van den Berg, H., Lumley, I. D. & Faure, U. (1994). The certification of the mass fractions on vitamins in a margarine, a milk powder, and a lyophilized brussels sprouts powder reference material. Preliminary report. Commission of the European Communities, Community Bureau of Reference.

Hollis, B. W., Roos, B. A., Draper, H. H. & Lambert, P. W. (1981). Vitamin D and its metabolites in human and bovine milk. *J. Nutr.*, **111**, 1240–1248.

Homberg, E., von (1993). Vitamin D-Bestimmung in margarine. *Fat Sci. Technol.*, **95**, 181–185.

Johnsson, H., Halen, B., Hessel, H., Nyman, A. & Thorzell, K. (1989). Determination of vitamin D_3 in margarines, oils and other supplemented food products using HPLC. *Int. J. Vit. Nutr. Res.*, **59**, 262–268.

Koskinen, T. & Valtonen, P. (1985). Identification and analysis of vitamin D_3 forms in chicken eggs. In *Vitamin D. A Chemical, Biochemical and Clinical Update. Proceedings of the Sixth Workshop on Vitamin D, Merano, Italy, March, 1985*, eds A. W. Norman, K. Schaefer, H.-G. Grigoleit & D. W. Herrath. Walter de Gruyter, New York, pp. 588–589.

Kunz, C., Niesen, M., Lilienfield-Toal, H.v & Burmeister, W. (1984). Vitamin D, 25-hydroxy-vitamin D and 1,25-dihydroxy-vitamin D in cow's milk, infant formulas and breast milk during different stages of lactation. *Int. J. Vit. Nutr. Res.*, **54**, 141–148.

Kurman, A. & Indyk, H. (1994). The endogenous vitamin D content of bovine milk: influence of season. *Food. Chem.*, **50**, 75–81.

McDermott, C. M., Beitz, G. S., Chandraratna, R. A. S., Okamura, W. H., Kruse, J. R., Popjak, G., Bishop, L. E. & Norman, A. W. (1983). Effects of dietary vitamin D_3 on concentrations of vitamin D and its metabolites in blood plasma and milk of dairy cows. *J. Dairy Sci.*, **68**, 1959–1967.

Mattila, P., Piironen, V., Bäckman, C., Uusi-Rauva, E. & Koivistoinen, P. (1992). Determination of vitamin D_3 in egg yolk by high-performance liquid chromatography (HPLC) with diode array detection. *J. Food Comp. Anal.*, **5**, 281–290.

Mattila, P., Piironen, V., Lehikoinen, K., Bäckman, C., Uusi-Rauva, E. & Koivistoinen, P. (1995*a*). Cholecalciferol contents in fortified margarine and milk. *Finnish Journal of Dairy Science*, **51**, 37–46.

Mattila, P., Piironen, V., Uusi-Rauva, E. & Koivistoinen, P. (1993). Determination of 25-hydroxycholecalciferol content in egg yolk by HPLC. *J. Food Comp. Anal.*, **6**, 250–255.

Mattila, P., Piironen, V., Uusi-Rauva, E. & Koivistoinen, P. (1994). Vitamin D contents in edible mushrooms. *J. Agric. Food Chem.*, **42**, 2449–2453.

Mattila, P., Piironen, V., Uusi-Rauva, E. & Koivistoinen, P. (1995*b*). Cholecalciferol and 25-hydroxycholecalciferol contents in fish and fish products. *J. Food Comp. Anal.*, **8**, 232–243.

Mattila, P., Piironen, V., Uusi-Rauva, E. & Koivistoinen, P. (1995c). The contents of cholecalciferol, ergocalciferol and their 25-hydroxylated metabolites in meat and milk products as determined by HPLC. *J. Agric. Food Chem.*, **43**, 2394–2399.

Mawer, E. B. & Gomes, U. C. S. (1994). Estimation of vitamin D and its metabolites in meat. In *Vitamin D, A Pluripotent Steroid Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications. Proceedings of the Ninth Workshop on Vitamin D, Orlando, Florida (USA), May 28–June 2 1994*, eds A. W. Norman, R. Bouillon & M. Thomasset. Walter de Gruyter, New York, pp. 775–776.

Parviainen, M. T., Koskinen, T., Ala-Houhala, M. & Visakorpi, L. K. (1984). A method for routine estimation of vitamin D activity in human and bovine milk. *Acta Vitaminol. Enzymol.*, **6**, 211–219.

Reeve, L. E., Jorgensen, N. A. & DeLuca, H. F. (1982). Vitamin D compounds in cow's milk. *J. Nutr.*, **112**, 667–672.

Rychener, M. & Walter, P. (1985). Vereinfachte und verbesserte Bestimmung von Vitamin D in Fett, Öl und Margarine mit HPLC. *Mitt. Gebiete Lebensm. Hyg.*, **76**, 112–124.

Sliva, M. G., Green, A. E., Sanders, L. K., Euber, J. R. & Saucerman, J. R. (1992). Reversed-phase liquid chromatographic determination of vitamin D in infant formulas and enteral nutritionals. *J. Ass. Off. Analyt. Chem.*, **75**, 566–571.

Takeuchi, A., Okano, T., Tsugawa, N., Katayama, M., Mimura, Y. & Kobayashi, T. (1988). Determination of vitamin D and its metabolites in human and cow's milk. *J. Micronutr. Anal.*, **4**, 193–208.

van Niekerk, P. V. & Smit, S. C. C. (1980). The determination of vitamin D in margarine by high performance liquid chromatography. *J. Am. Oil. Chem. Soc.*, **57**, 417–421.