High-Performance Liquid Chromatographic Determination of Vitamin D in Foods, Feeds and Pharmaceuticals by Successive Use of Reversed-Phase and Straight-Phase Columns

Atsuko TAKEUCHI, Toshio OKANO, Sumiko TERAOKA, Yumiko MURAKAMI, and Tadashi KOBAYASHI1*

Department of Hygienic Sciences, Kobe Women's College of Pharmacy, Higashinada-ku, Kobe 658, Japan

(Received August 15, 1983)

Summary A simplified and accurate method for the determination of vitamin D in foods, feeds and pharmaceuticals was established by high-performance liquid chromatography (HPLC) using successively reversed-phase and straight-phase columns. About 1–2 g of a sample was accurately weighed and directly saponified. The extracted unsaponifiable matter was subjected to preparative HPLC using a reversed-phase column and a vitamin D fraction was collected. The fraction was subsequently subjected to analytical HPLC using a straight-phase column and vitamin D was assayed by estimating the peak height. The proposed method was applied to various kinds of samples, e.g., fishery products, fish meals, mixed feeds for fish farming and chicken farming, egg yolk, milk products, cattle liver, Shiitake, fortified foods and multivitamin preparations. The results showed that the proposed method was useful for the determination of vitamin D in such samples, because the peak of vitamin D in the profile of the second HPLC was always clearly separated from other concomitants and good recovery was obtained. Therefore, we think that the method is useful as a routine one for the determination of vitamin D in foods, feeds and pharmaceuticals.

Key Words determination of vitamin D, foods, feeds, high-performance liquid chromatography, pharmaceuticals, vitamin D, vitamin D₂, vitamin D₃

1 To whom correspondence should be addressed.

Abbreviations: 25-OH-D₂/D₃, 25-hydroxyvitamin D₂/D₃ (25-hydroxy-ergocalciferol/-cholecalciferol); HPLC, high-performance liquid chromatography; AUFS, absorbance unit as full scale; UV, ultraviolet; M, mean; SD, standard deviation; CV, coefficient of variation.
Physico-chemical determination of vitamin D ('vitamin D' is used as a general name for vitamin D$_2$ and D$_3$) in foods, feeds, pharmaceuticals and other biological materials has been very difficult until recently for the following reasons: (a) Since the biological potency of vitamin D is so high (1 I.U. = 25 ng) and the content of the vitamin is usually very low, micro-determination is always required; (b) Since neither a specific chemical reaction nor a specific detector useful for vitamin D assay was found, the removal of interfering substances, e.g., vitamin A, E, sterols and others, is always required. Colorimetric determination using Nield's reagent (1) (SbCl$_3$ chloroform solution including acetyl chloride) was used for some time (2, 3). This method required complete elimination of interfering substances, but the clean-up procedures were too complicated to be used as routine methods. Although gas-liquid chromatographic methods were also investigated by several groups (4–6), the clean-up procedures were still complicated and the sensitivity using a hydrogen flame ionizing detector was not enough to enable determination of the extremely low levels of vitamin D in foods or feeds.

Recently, significant progress has been made in the field of HPLC analysis because of the development of microparticle columns showing good separation and of a UV detector having extremely high sensitivity. Such progress in HPLC has permitted the micro-determination of vitamin D and its metabolites. The technique of HPLC has been used for the determination of vitamin D in vitamin D concentrates (7), feeds (8, 9) and foods including milks and infant formulas (10–15). In a previous report (16), we established an HPLC method for assaying vitamin D$_2$ in fortified dried milk by using two different kinds of columns. The first preparative HPLC using a reversed-phase column was for the purpose of clean-up and the second analytical HPLC using a straight-phase column was for assay of vitamin D. This idea was derived from our method for the determination of 25-OH-D$_2$ and 25-OH-D$_3$ in plasma (17). The same method as described in a previous paper (16) was confirmed to be useful for various kinds of samples of foods, feeds and pharmaceuticals. In this paper, the results are described.

**EXPERIMENTAL**

1. Materials and reagents

   1) Vitamin D$_2$ and D$_3$. Commercial grades of vitamin D$_2$ and D$_3$ (Philips-Duphar Co., The Netherlands) were used as the respective standard compounds.

   2) Aldehyde-free ethanol. After adding 5 ml of 50% KOH solution and 5 g of zinc powder to each 1,000 ml of ethanol, the resultant solution was refluxed for 2 h and then distilled.

   3) Benzene, n-hexane and acetonitrile. Guaranteed reagents were distilled before use.

   4) Isopropanol. A guaranteed reagent was used.

   5) Methanol. A guaranteed reagent for HPLC was used.

   6) Silica cartridge. Silica cartridge for rapid preparation was selected from
commercial grades of SEP-PAK (Part no. 51900) from Waters Assoc. Inc. (USA).

7) Reversed-phase column for HPLC. A stainless column (7.5 mm i.d. x 300 mm) packed with Nucleosi15C18 (Nagel Co., West Germany) was used.

8) Straight-phase column for HPLC. A stainless column (4.6 mm i.d. x 250 mm) packed with Zorbax SIL (DuPont Co., USA) was used.

Other guaranteed reagents were used.

2. Samples for assay

1) Fishery products. Fresh and dried Japanese pilchard, fresh skipjack, Katsuobushi (dried skipjack), Shiokara (viscera of skipjack pickled with salt), tuna fatty meat, fresh eel and Satsumaage (fried ground fish and flour meal) were purchased from various markets.

2) Fish meals. Fish meals are usually used as materials of mixed feeds for fish farming. One kind of pollack meal and two kinds of Japanese pilchard meals were used. These were kindly supplied by Tokai Regional Fisheries Research Laboratory.

3) Mixed feeds for fish farming. Three kinds of mixed feeds for carp were used. These are used for fingerlings of carp weighing 1–3 g and 7–12 g and for raising fish weighing 12–50 g, respectively. One kind of mixed feed for fingerlings of rainbow trout was also used. These feeds were prepared by mixing fish meals, flour and soybean cake and supplemented by vitamin D3. The feeds were kindly supplied by Tokai Regional Fisheries Research Laboratory.

4) Mixed feeds for chicken. A commercial mixed feed for chicken was used. This was made by mixing fish meals, yellow corn powder, kaoliang powder, soybean cake and others and supplemented by vitamin D3 (guaranteed value: 200 I.U./100 g).

5) Vitamin D-fortified foods. Commercial caramel and biscuit containing cream fortified by vitamin D2 were used. Both guaranteed values of vitamin D2 were 100 I.U./100 g.

6) Egg and milk products. Egg (egg yolk and albumen), mayonnaise, cheese (Gouda), butter and margarine were purchased from various markets.

7) Cattle liver. Cattle liver was purchased from various markets.

8) Shiitake (Lentinus edodes). Raw and dried Shiitake were purchased from various markets.

9) Multivitamin preparations. Commercial multivitamin preparations were purchased from drug stores. Guaranteed vitamin D2, A and E in them are shown in Table 1.

3. Instrumentation

1) First preparative HPLC. The first preparative HPLC to isolate the vitamin D fraction from the unsaponifiable matter of a sample was performed as described in a previous report (16), the outline being as follows:

   Apparatus: Shimadzu-DuPont 841 high-performance liquid chromatograph
Table 1. Guaranteed contents of vitamin D₂, A and E in samples of commercial multivitamin preparations.

| Multivitamin preparation | Guaranteed contents (I.U. or mg/tablet or capsule) and I.U. or weight ratio to vitamin D₂ |
|--------------------------|----------------------------------------------------------------------------------------------|
|                          | Vitamin D₂ (I.U.) | Vitamin A (I.U.) | I.U. ratio (A/D₂) | Vitamin E (mg) | Weight ratio (E/D₂) |
| Tablet No. 1             | 200               | 2,000            | 10                | —              | —                   |
| Tablet No. 2             | 200               | 2,000            | 10                | 3              | 60                  |
| Capsule                  | 300               | 3,000            | 10                | 100            | 13,333              |

equipped with a Shimadzu SPD-2 detector (265 nm, AUFS 0.005).
Column: Nucleosil 5C₁₈ (7.5 i.d. x 300 mm).
Mobile phase: 50% Methanol in acetonitrile.
Flow rate: 2.0 ml/min (55 kg/cm²).
Vitamin D (vitamin D₂ and D₃) fraction: 2,500–2,900 drops (collection time 16–19 min).

2) Second analytical HPLC. The second analytical HPLC was also performed according to a previous report (16), the outline being as follows:
Apparatus: Shimadzu LC-3A high-performance liquid chromatograph equipped with a Shimadzu UVD-2 detector (254 nm, AUFS 0.001).
Column: Zorbax SIL (4.6 i.d. x 250 mm).
Mobile phase: 0.4% isopropanol in n-hexane.
Flow rate: 1.8 ml/min (60 kg/cm²).
Retention time: 21.1 min for vitamin D₂ and D₃.

4. Procedure for the determination of vitamin D in foods, feeds and pharmaceuticals
The procedure proposed for the HPLC determination of vitamin D in foods, feeds and pharmaceuticals is summarized in Fig. 1. This is nearly the same as that proposed for fortified dried milk in a previous paper (16) except for the samples which need a clean-up procedure using SEP-PAK silica cartridge.

1) Saponification and extraction of unsaponifiable matter. When necessary, a sample was homogenized with an electric mixer or a Polytron homogenizer before sampling. About 0.5–2 g of a sample containing not less than 1 I.U. of vitamin D was accurately weighed in a saponification flask. Saponification and extraction of unsaponifiable matter were then performed according to a previous report (16). Exactly 80.0 ml of the benzene extracts was evaporated under reduced pressure at below 40°C.

2) Clean-up with SEP-PAK silica cartridge. This procedure was applied to some samples of fishery products, e.g., eel and tuna fatty meat, and egg yolk, whereas it could be omitted for the other samples because these did not include such concomitants as observed in fishery products and eggs. When the procedure
Fig. 1. Procedure for the determination of vitamin D in foods, feeds and pharmaceuticals.

was necessary, the residue obtained above was treated as follows. The residue was dissolved in 5.0 ml of n-hexane. Exactly 4.5 ml of the solution was placed in a test tube and evaporated under reduced pressure. The resulting residue was dissolved in 500 μl of 0.4% isopropanol in n-hexane and exactly 400 μl of the solution was applied to SEP-PAK silica cartridge previously permeated by 0.4% isopropanol in n-hexane. Elution was performed using 0.4% isopropanol in n-hexane with the use of a syringe. After throwing off the first 10 ml of the eluate, the following 20 ml was collected and evaporated under reduced pressure.

3) First preparative and second analytical HPLC. The residue obtained in 1) or 2) was dissolved in 5.0 ml of n-hexane. In accordance with a previous report (16), the first preparative and the second analytical HPLC were performed and the content of a sample was determined.

Since commercial multivitamin preparations, fortified foods and Shiitake contain vitamin D₂, this vitamin was used as the reference standard compound. On the other hand, vitamin D₃ was used as the reference standard compound for foods and feeds with the exception of Shiitake, because these samples contain vitamin D₃.

RESULTS AND DISCUSSION

1. Identification of vitamin D₂ or D₃ in samples

The separation of vitamin D₂ and D₃ could not be achieved by the second
analytical HPLC, the two vitamins giving the same retention time on the chromatogram. Therefore, when identification of vitamin D₂ or D₃ was performed on the samples, the eluate corresponding to the vitamins on the second analytical HPLC was collected and further applied to the HPLC using a reversed-phase column (the same conditions as with the first preparative HPLC) which made possible the separation of the vitamins from one another. Co-chromatography to identify the vitamins was also performed by mixing the eluate with the respective standard compounds. The results show that fishery products, fish meals, mixed feeds for fish farming and chickens, egg yolk and milk products contained vitamin D₃, whereas vitamin D₂ was identified in Shiitake, fortified foods and multivitamin preparations.

2. Calibration curves and recovery tests

Authentic vitamin D₂ and D₃ were dissolved in 0.4% isopropanol in n-hexane to concentrations of 50, 100, 200, 300, 400, 500 and 1,000 ng/ml. Each 100 μl aliquot of the solutions was applied to the second analytical HPLC to obtain calibration curves. The results showed that straight lines passing through the origin were observable between the peak heights and the amounts of vitamin D in both cases of vitamin D₂ and D₃.

Each 1–2 g sample of a fish meal, a mixed feed for chicken or a multivitamin preparation (tablet) was treated according to the whole procedure with or without addition of authentic vitamin D₂ or D₃ in order to perform recovery tests. As shown

| Sample                      | Trials | Sample weights (g) | Added value | Recovery (%) (M ± SD) | CV (%)  |
|-----------------------------|--------|--------------------|-------------|------------------------|---------|
| Fish meal (pollack)         | 5      | 1.0                | 30 ng       | 94.3 ± 11.5            | 12.2    |
| Mixed feed for chicken farming | 6     | 1.0                | 100 ng      | 93.1 ± 17.9            | 19.2    |
| Multivitamin preparation    | 6      | 0.5                | 5 μg        | 99.7 ± 7.5             | 7.5     |

Note: Vitamin D₃ was added to the samples of fish meal and mixed feed for chickens, whereas vitamin D₂ was added to the multivitamin preparation sample.

Table 3. Comparison between the assayed values of vitamin D₂ in a multivitamin tablet, obtained by two laboratory workers.

| Laboratory worker | Trials | Assayed value of vitamin D₂ (M ± SD) | CV (%)  |
|-------------------|--------|-------------------------------------|---------|
| A                 | 6      | 255 ± 141 U. /tab                   | 5.7     |
| B                 | 6      | 255 ± 81 U. /tab                    | 3.0     |

*J. Nutr. Sci. Vitaminol.*
in Table 2, the results of overall recovery were higher than 93%, which were satisfactory.

When vitamin D₂ in a multivitamin tablet was repeatedly determined by two laboratory workers, the results as shown in Table 3 were obtained. Since there was little difference between the two data and the CV values were lower than 10%, the
experimental error by use of the proposed method was thought to be very little.

3. **Elimination of unknown concomitant by the use of SEP-PAK silica cartridge**

As shown in Fig. 2, a big peak due to large amounts of unknown concomitant was observed after elution of the vitamin D fraction, in the profiles of the first preparative HPLC on eel and egg yolk. Since the peak was clearly separated from that of the vitamin D fraction, it did not disturb the determination of vitamin D itself. However, when repeated use of the first preparative HPLC is carried out, continuous elution for about 60 min after vitamin D fraction should be done to eliminate the peak of the unknown concomitant. The procedure was found to be time-consuming and a malfunction of an expensive reversed-phase column might soon occur by overloading with such large amounts of concomitants. Therefore, we decided to apply the clean-up procedure with SEP-PAK silica cartridge before the first preparative HPLC to eliminate such concomitants in a sample. As shown in Fig. 3, a big peak due to such concomitants was observed in the profile of the first

![Profiles of the second analytical HPLC on fishery products. The vitamin D fractions obtained from samples were applied to the second analytical HPLC according to EXPERIMENTAL.](image)
preparative HPLC on a fresh eel sample, but it could be clearly eliminated by applying the clean-up procedure to the sample. It took about 15 min to perform the clean-up procedure for a sample and therefore the application of the procedure was time-saving. This procedure was necessary for some samples, e.g., eel, tuna fatty meat and egg yolk, whereas it could be omitted for the other samples because these did not contain such concomitants as observed in the samples mentioned above.

4. Determination of vitamin D₃ in fishery products

Determination of vitamin D₃ in fishery products was performed according to the proposed procedure. Figure 4 shows the second analytical HPLC chromatograms on fishery products. All the peaks due to vitamin D₃ in the chromatograms were clearly separated from interfering substances, showing that the proposed method was very useful for the determination of vitamin D₃ in fishery products. The assayed values are shown in Table 4. When the values were compared with the respective values described in the Standard Table of Food Composition in Japan (18), those for fresh Japanese pilchard and Shiokara were found to be very similar to the respective data in the Table. However, the values in fresh eel and Satsumaage were rather different from those in the Table. Since the data described in the Table were determined using a colorimetric method about 20 years ago (19), accuracy might be uncertain on such samples containing an extremely low level of vitamin D and large amounts of interfering substances. Therefore, we think the data in the Table should be soon revised according to the values arrived at by HPLC methods.

5. Determination of vitamin D₃ in fish meals and mixed feeds for fish farming

Determination of vitamin D₃ in fish meals and mixed feeds for fish farming was performed using the proposed method without the clean-up procedure with SEP-PAK silica cartridge. Figure 5(a) shows the profiles of the first preparative and the

| Sample                  | Assayed value (I) (I.U./100 g) | Value in Table* (II) (I.U./100 g) | Ratio (I/II) |
|-------------------------|---------------------------------|-----------------------------------|--------------|
| Fresh Japanese pilchard | 544                             | 530                               | 1.03         |
| Dried Japanese pilchard | 736                             | 160                               | 4.60         |
| Fresh skipjack          | 749                             | 420                               | 1.78         |
| Katsuobushi             | 273                             | 430                               | 0.63         |
| Shiokara                | 1,423                           | 1,700                             | 0.84         |
| Tuna fatty meat         | 146                             | —                                 | —            |
| Fresh eel               | 1,070                           | 150                               | 7.13         |
| Satsumaage              | 21                              | 450                               | 0.05         |

*The values are taken from the Standard Table of Food Composition in Japan (The third revised edition, 1963) (18).
Fig. 5. Profiles of the first preparative HPLC and second analytical HPLC on the following samples: (a) pollack meal, (b) butter, (c) cattle liver, (d) dried Shiitake. The samples were applied to the whole procedure except the clean-up with use of SEP-PAK silica cartridge according to EXPERIMENTAL.

Table 5. Assayed values of vitamin D₃ in fish meals and mixed feeds for fish farming.

| Sample                        | Trials | Assayed value* (I.U./100 g) | CV (%) |
|-------------------------------|--------|-----------------------------|--------|
| Pollack meal                  | 5      | 128 ± 10                    | 7.9    |
| Japanese pilchard meal No. 1  | 6      | trace                      | —      |
| Japanese pilchard meal No. 2  | 6      | 75 ± 5                      | 6.5    |
| Mixed feed for fish farming   |        |                             |        |
| Fingerling of carp (1–3 g)    | 6      | 568 ± 27                    | 4.8    |
| Fingerling of carp (7–12 g)   | 6      | 437 ± 50                    | 11.5   |
| Raising carp (12–50 g)        | 6      | 164 ± 22                    | 13.6   |
| Fingerling of rainbow trout   | 6      | 477 ± 45                    | 9.5    |

*a The values are shown as M ± SD.  
*b Trace < 20 I.U./100 g.
6. Determination of vitamin D3 in a mixed feed for chickens

Determination of vitamin D3 in a mixed feed for chickens was performed according to the proposed method without the clean-up procedure with SEP-PAK silica cartridge. When vitamin D3 in the original sample bought from a market was repeatedly determined, the assayed values showed a big variation as shown in Table 6(a). We thought that this variation might be due to the existence of unhomogeneous vitamin D3 in the sample. Therefore, a homogeneous sample was made by dissolving the original sample in n-hexane, mixing and then evaporating to dryness under reduced pressure. Since the variation of the assayed values of vitamin D3 in the homogeneous sample became smaller than the previous data as shown in Table 6(b), we concluded that the big variation observed on the original sample was due to the existence of unhomogeneous vitamin D3.

7. Determination of vitamin D3 in egg yolk, milk products and cattle liver

Determination of vitamin D3 in egg yolk, albumen, mayonnaise, Gouda cheese, butter, margarine and cattle liver was performed. The whole procedure was applied for egg yolk to eliminate large amounts of concomitants, while the clean-up procedure with SEP-PAK silica cartridge could be omitted for the other samples. The profiles of the first and second HPLC on butter and cattle liver in the samples are shown in Fig. 5(b) and 5(c). The assayed value of vitamin D3 in butter was 29

| Trials | Assayed value of vitamin D3 (I.U./100 g) | Trials | Assayed value of vitamin D3 (I.U./100 g) |
|--------|----------------------------------------|--------|----------------------------------------|
| 1      | 348.0                                  | 1      | 519.6                                  |
| 2      | 172.4                                  | 2      | 238.4                                  |
| 3      | 1,435.2                                | 3      | 349.2                                  |
| 4      | 1,130.4                                | 4      | 438.4                                  |
| 5      | 557.2                                  | 5      | 242.4                                  |
| 6      | 257.6                                  | 6      | 338.4                                  |
| 7      | 234.0                                  |        |                                        |
| 8      | 512.8                                  |        |                                        |

M ± SD: 354.4 ± 110.1

Note: The mean value (354.4 I.U./100 g) was 177.2% for the guaranteed value (200 I.U./100 g)

Table 6. Variation of vitamin D3 in a mixed feed for chickens before and after homogenization.

(a) Original sample (before homogenization)

(b) Homogeneous sample (after homogenization)

Vol. 30, No. 1, 1984
Table 7. Assayed values of vitamin D$_3$ in egg yolk, milk products and cattle liver.

| Sample         | Assayed value (I) (I.U./100 g) | Values in Table* (II) (I.U./100 g) | Ratio (I/II) |
|----------------|--------------------------------|-----------------------------------|--------------|
| Egg yolk       | 155                            | 30                                | 5.17         |
| Albumen        | ND                             | 0                                 | —            |
| Mayonnaise     | trace$^b$                       | 0                                 | —            |
| Gouda cheese   | ND$^c$                          | —                                 | —            |
| Butter         | 29                             | —                                 | —            |
| Margarine      | ND                             | —                                 | —            |
| Cattle liver   | ND                             | —                                 | —            |

$^a$The values are taken from the Standard Table of Food Composition in Japan (The third revised edition, 1963)(18). $^b$Trace <20 I.U./100 g. $^c$ND, not detected.

I.U./100 g which was the lowest limit for obtaining an accurate value. The peak due to vitamin D$_3$ was clearly observed and separated from the other peaks as shown in Fig. 5(b). On the other hand, an extremely small peak which might be due to vitamin D$_3$ was observed in the profile of the second HPLC on cattle liver as shown in Fig. 5(c). However, since it was too small to recognize as a peak due to vitamin D$_3$, we decided that vitamin D$_3$ in the sample was undetectable. The assayed values in egg yolk, milk products and cattle liver are shown in Table 7. The contents of vitamin D$_3$ in the samples were generally not very high.

8. Determination of vitamin D$_2$ in Shiitake

Determination of vitamin D$_2$ in raw and dried Shiitake (Lentinus edodes) was performed according to the proposed method without the use of SEP-PAK silica cartridge. As shown in Fig. 5(d), the peak due to vitamin D$_2$ was clearly separated from many kinds of large peaks due to interfering substances in the second analytical HPLC chromatogram. The assayed values are shown in Table 8. Although comparatively higher values were obtained from our results, the data described in the Standard Table of Food Composition in Japan (18) were zero for raw and dried Shiitake. The reason why the zero values were given in the Table was...
9. Determination of vitamin D\textsubscript{2} in vitamin D\textsubscript{2}-fortified foods

Determination of vitamin D\textsubscript{2} in vitamin D\textsubscript{2}-fortified caramel and biscuit including cream was performed according to the proposed method without use of SEP-PAK silica cartridge. The profiles of HPLC on the samples are not shown, because clear separation of vitamin D\textsubscript{2} from interfering substances was obtained. The assayed values are shown in Table 9.

10. Determination of vitamin D\textsubscript{2} in multivitamin preparations

Determination of vitamin D\textsubscript{2} in commercial multivitamin preparations was performed according to the proposed method without use of SEP-PAK silica cartridge. The profiles of HPLC on the samples are not shown, because clear separation of vitamin D\textsubscript{2} from interfering substances was obtained. The assayed values are shown in Table 10. When the determination was performed repeatedly, good reproducibility was obtained and the CV values on the 3 samples were within 7\%. These results showed that the proposed method was useful for not only foods or feeds but multivitamin preparations also.

The authors wish to thank Dr. T. Yamakawa, Dr. Y. Fujii, Dr. T. Kinumaki, Dr. K. Vol. 30, No. 1, 1984

---

### Table 9. Assayed values of vitamin D\textsubscript{2} in vitamin D\textsubscript{2}-fortified foods.

| Sample  | Guaranteed value of vitamin D\textsubscript{2} (I.U./100g) | Assayed value of vitamin D\textsubscript{2} (I.U./100g) | Percent for guaranteed value (%) |
|---------|-----------------------------------------------------------|---------------------------------------------------|---------------------------------|
| Caramel | 100                                                       | 173                                               | 173.0                           |
| Biscuit | 100                                                       | 60\textsuperscript{a}                               | 60.0                            |

\textsuperscript{a}The value was calculated from the assayed value of vitamin D\textsubscript{2} in the cream (430 I.U./100g) which was contained in the biscuit.

### Table 10. Assayed values of vitamin D\textsubscript{2} in multivitamin preparations.

| Multivitamin preparation | Guaranteed value of vitamin D\textsubscript{2} (I.U./tab.) | Assayed value of vitamin D\textsubscript{2} (I.U./tab.)\textsuperscript{a} | Percent for guaranteed value\textsuperscript{a} (%) |
|-------------------------|----------------------------------------------------------|-------------------------------------------------------------|---------------------------------------------------|
| Tablet No. 1            | 200                                                      | 255 ± 14                                                   | 128 ± 7                                          |
| Tablet No. 2            | 200                                                      | 298 ± 20                                                   | 149 ± 10                                         |
| Capsule                 | 300\textsuperscript{b}                                   | 251 ± 6\textsuperscript{b}                                 | 84 ± 2                                           |

\textsuperscript{a}The values are shown as M ± SD. \textsuperscript{b}I.U./capsule.
Sugii and Dr. M. Takeuchi of Tokai Regional Fisheries Research Laboratory for their helpful discussion and for donating the samples of fish meals and mixed feeds for fish farming.

REFERENCES

1) Nield, C. H., Russell, W. C., and Zimmerli, A. (1940): The spectrophotometric determination of vitamin D₂ and D₃. *J. Biol. Chem.*, 136, 73–79.
2) Mulder, F. J., and De Vries, E. (1974): Analysis of fat-soluble vitamins. XIII. Chemical vitamin D assay in vitamin D and multivitamin preparations. *J. Assoc. Offic. Anal. Chem.*, 57, 1349–1356.
3) Ueda, F., Makino, T., Kazama, A., and Watanabe, K. (1971): Separation of fat-soluble vitamins by gel filtration. VII. Determination of vitamin D in the presence of vitamin A and α-tocopherol. *J. Vitaminol.*, 17, 142–147.
4) Kobayashi, T., and Adachi, A. (1976): Gas-liquid chromatographic determination of vitamin D in pharmaceutical preparations. *J. Nutr. Sci. Vitaminol.*, 22, 41–51.
5) Touw, H. D. M., Krose, B. M. C., and Molenaar, H. M. (1972): Gas-liquid chromatographic determination of vitamins D₂ and D₃ in infant formulas and feeding preparations. *J. Assoc. Offic. Anal. Chem.*, 55, 622–624.
6) Edlund, D. O., Filippini, F. A., and Datson, J. K. (1974): Gas-liquid chromatographic determination of vitamin D₂ in multiple vitamin tablets containing minerals and vitamin E acetate. *J. Assoc. Offic. Anal. Chem.*, 57, 1089–1091.
7) De Vries, E. J., Zeeman, J., Esser, R. J. E., Borsje, B., and Mulder, F. J. (1979): Analysis of fat-soluble vitamins. XXI. High pressure liquid chromatographic assay methods for vitamin D in vitamin D concentrates. *J. Assoc. Offic. Anal. Chem.*, 62, 129–135.
8) Cohen, H., and Lapointe, M. (1980): Determination of low levels of vitamin D₃ in animal feeds, using Sephadex LH-20 and normal phase high pressure liquid chromatography. *J. Assoc. Offic. Anal. Chem.*, 63, 1158–1162.
9) Cohen, H., and Lapointe, M. (1979): Quantitative analysis of vitamin D₃ in a feed using normal phase high pressure liquid chromatography. *J. Chromatogr. Sci.*, 17, 510–513.
10) Egaas, E., and Lambertsen, G. (1979): Naturally occurring vitamin D₃ in fish products analysed by HPLC, using vitamin D₂ as an international standard. *Int. J. Vit. Nutr. Res.*, 49, 35–42.
11) Barnett, S. A., Frick, L. W., and Baine, H. M. (1980): Simultaneous determination of vitamins A, D₂ or D₃, E and K₁ in infant formulas and dairy products by reversed-phase liquid chromatography. *Anal. Chem.*, 52, 610–614.
12) Thompson, J. N., Hatina, G., Maxwell, W. B., and Duval, S. (1982): High performance liquid chromatographic determination of vitamin D in fortified milks, margarine, and infants formulas. *J. Assoc. Offic. Anal. Chem.*, 65, 624–631.
13) Muniz, J. F., Wehr, C. T., and Wehr, H. M. (1982): Reversed phase liquid chromatographic determination of vitamin D₂ and D₃ in milk. *J. Assoc. Offic. Anal. Chem.*, 65, 791–797.
14) Jackson, P. A., Shelton, C. J., and Frier, P. J. (1982): High-performance liquid chromatographic determination of vitamin D₃ in foods with particular reference to eggs. *Analyst*, 107, 1363–1369.
15) Muller-Mulot, W., and Rohrer, G. (1982): Quantitative Bestimmung der Vitamine D₂ und D₃ in Margarine mittels HPLC. *Fette Seifen Anstrichchem.*, 84, 354–358.
16) Okano, T., Takeuchi, A., and Kobayashi, T. (1981): Simplified assay of vitamin D₂ in *J. Nutr. Sci. Vitaminol.*
fortified dried milk by using two steps of high-performance liquid chromatography. J. Nutr. Sci. Vitaminol., 27, 539-550.

17) Okano, T., Mizuno, N., Shida, S., Takahashi, N., Kobayashi, T., Kuroda, E., Kodama, S., and Matsuo, T. (1981): A method for simultaneous determination of 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ in human plasma by using two steps of high-performance liquid chromatography. J. Nutr. Sci. Vitaminol., 27, 43–54.

18) Resources Council, Science and Technology Agency of Japan (1963): The Standard Table of Food Composition in Japan, the 3rd revised edition.