The Determination of Vitamin D in Margarine by High Performance Liquid Chromatography

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ABSTRACT

Margarine manufactured in South Africa contains vitamin D added at a level of 1-3 international units per gram. Because of the high ratio of lipid to vitamin D, it is difficult to determine the vitamin content for control purposes. A high performance liquid chromatographic method for the determination of vitamin D in margarine is proposed. The unsaponifiable fraction of a margarine sample is chromatographed twice on an adsorption system. The vitamin D fraction collected from this column is finally injected onto a reverse phase system where vitamins D_2 and D_3 are separated and quantitated with an ultraviolet detector. Vitamin D_2 is used as an internal standard for vitamin D_3 or vice-versa, depending on the form of the vitamin added to margarine. The recovery of 100 ng/g vitamin D_3 added to a margarine mix was 100.1% (coefficient of variation 13.2% for 12 replicates) as calculated from the ratio of peak areas. The detection limit is 1 ng vitamin D. Results for 3 samples of margarine and one sample of butter are given.

INTRODUCTION

Margarine manufactured in South Africa is fortified with vitamin D at a level of 1-3 international units (IU) per gram. Because of the high ratio of lipid to vitamin D, it is difficult to determine the vitamin content for control purposes.

Biological methods (1), which take ca. 1 month to complete, have for a long time been the only means of determining vitamin D in low potency samples. Direct colorimetric methods (2) can only be applied to oily samples containing more than 100,000 IU/g. A series of purification steps (3) such as saponification, column chromatography and thin layer chromatography (TLC) are necessary before colorimetry can be applied to low potency samples. Gas chromatographic methods (4) similarly need extensive sample preparation and can be applied to fortified samples only when a flame ionization detector is used.

Simple high performance liquid chromatographic (HPLC) methods (5,6) have been reported for high potency samples, but for foods low in vitamin D, more extensive sample preparation is necessary (7,8). Mankel (9) described an HPLC method for the determination of vitamin D which was applied to margarine samples but did not give any quantitative results, and stated that when the separation of vitamin D from interfering substances was insufficient—further purification and concentration of the sample with column chromatography would be necessary.

We shall report a method for the determination of vitamin D in margarine using HPLC for sample preparation and quantitation.

EXPERIMENTAL PROCEDURES

Apparatus

The HPLC equipment consisted of a Varian 5000 pump, Valco inlet valve and a Varichrom UV-Vis variable wavelength detector. A model 1203 UV monitor from Laboratory Data Control with a fixed wavelength of 254 nm also was used. Peak retention times and areas were determined with a Hewlett-Packard 3352B laboratory data system. Stainless steel columns (25 × 0.4 cm) were slurry packed (10) with 5 μm Lichrosorb SI 60 (E. Merck) for adsorption chromatography or 5 μm Nucleosil 5 C_{18} (Machery-Nagel) for reverse phase chromatography. Both columns gave ca. 4,000 theoretical plates for the vitamin D_2 peak at 2 ml/min for the adsorption column and 1 ml/min for the reverse phase column.

Reagents

Dioxane, *n*-hexane, anhydrous sodium sulfate, isopropanol, vitamin D_2, vitamin D_3 and α-tocopherol were chemically pure reagents and chloroform (containing ca. 1% ethanol), potassium hydroxide and methanol were analytical reagents from E. Merck. γ-Tocopherol was obtained from Distillation Products Industries. Ethanol (96%) was purchased from National Chemical Products.

The water content of the *n*-hexane was adjusted by mixing one part water-saturated *n*-hexane with 2 parts *n*-hexane dried over anhydrous sodium sulfate. The ethanol content of the chloroform was adjusted by washing it with water and drying over anhydrous sodium sulfate. Four parts of the washed chloroform were mixed with one part of unwashed chloroform. All solvents used for chromatography were filtered through Schleicher and Schüll regenerated cellulose membrane filters of 0.6 μm pore size.

Methods

100 μg α-tocopherol as antioxidant and 1 μg vitamin D_2 or D_3 as internal standard were added to 10 g margarine, which was saponified in 100 ml alcohol (96%) with 10 ml of a 50% (by wt) potassium hydroxide solution. The alkali was added after the alcohol had been boiling for 1 min and boiling under reflux was maintained for 30 minutes. The saponification flask was cooled in running water, 100 ml water was added and the unsaponifiable material was extracted with 3 50-ml portions of *n*-hexane. The combined *n*-hexane extracts were washed 3 times with water and then dried with anhydrous sodium sulfate. The solution was concentrated under vacuum with a rotary evaporator to 1 ml before filtering it through a 0.6 μm membrane filter. γ-Tocopherol (60 μg) was added to the filtrate and the solvent was removed by heating on a water bath at 40-50 C under a stream of nitrogen.

The residue was taken up in 100 μl chloroform of which 10 μl was used for chromatography on the adsorption column with a mobile phase consisting of chloroform and *n*-hexane (1 + 1 by vol) pumped at 2 ml/min. The detector was set at 295 nm and 1 absorbance unit full scale (AUFS).

γ-Tocopherol was used as a tracer in order to determine the exact elution time for vitamins D_2 and D_3 (which elute together on this column). The relative retention α ($\alpha = [t_{R2} - t_o]/[t_{R1} - t_o]$ where t_o, t_{R1} and t_{R2} are the retention times of a nonretarded peak—γ-tocopherol and vitamin D, respectively) for vitamin D and γ-tocopherol was determined with the aid of standards. This value of α was used to calculate the retention time of vitamin D (ca. 8 min) from the retention time of γ-tocopherol (ca. 5 min) during each

run. A portion of the eluate, corresponding to the elution time of vitamin D, was collected, evaporated to dryness in a stream of nitrogen and dissolved in 50 µl chloroform. Before injecting the next sample, the column was cleaned by increasing the flow rate to 4 ml/min and running a gradient from 50% chloroform to 100% chloroform in 2 min. It was held at 100% for 5 min before a reverse gradient to 50% chloroform was run (2 min). After a further 4 min, the flow was returned to 2 ml/min and the system equilibrated for 2 min.

The fraction collected during the first run was re-injected on the same system with the detector set at 264 nm and 0.1 AUFS and the center portion of the vitamin D peak was carefully collected (by collecting for an equal length of time before and after the peak maximum) after its exact retention time had been established by cochromatography with a standard. The use of a gradient was unnecessary at this stage.

The vitamin D fraction was evaporated to dryness in a stream of nitrogen and dissolved in 50 µl methanol for chromatography on the reverse phase system with a mobile phase consisting of methanol and water (95 + 5 by vol), pumped at a flow rate of 1 ml/min. Either the fixed wavelength detector at 254 nm and 0.016 AUFS or the variable wavelength detector at 264 nm and 0.02 AUFS was used. Vitamin D_2 was separated from vitamin D_3 and their peak heights and areas were determined. Vitamin D_2 was used as an internal standard for vitamin D_3 or vice-versa, depending on the form of the vitamin added to the margarine. Calibration curves (ratio of the mass of vitamin D_3 and D_2 injected against the ratio of the peak areas or peak heights of vitamins D_3 and D_2) were obtained by injecting 3 µl of solutions in methanol containing vitamins D_3 and D_2 in different ratios.

Recovery determinations were conducted by adding 50 ng/g or 100 ng/g vitamin D_3 to a margarine mix which was prepared without the addition of vitamins.

Vitamin A was determined by the method of reference (11) with the following changes: a stainless steel column (25 x 0.4 cm) packed with 5 µm Lichrosorb SI 60 was used with a mobile phase of dioxane and *n*-hexane (8 + 92 by vol) pumped at 3 ml/min. Calculations were based on peak areas.

RESULTS AND DISCUSSION

At the start of our work, a mobile phase consisting of dioxane and *n*-hexane (4 + 96 by vol) was used with the adsorption column and the vitamin D fraction was collected. The residue after evaporation of the solvent, however, contained a white waxy material which was difficult to dissolve in methanol. Subsequent chromatography of the fraction, on the reverse phase system, revealed a fairly large peak at the retention time of vitamin D_2 even when a margarine mix, known to not contain vitamin D, was used. Similar problems also were encountered later when the Egaas and Lambertsen method (12), developed for fish oils, was applied to margarine.

By chromatographing the unsaponifiable material with a mobile phase of chloroform and *n*-hexane on the adsorption column prior to the chromatography with the dioxane system, the problems with the insoluble material and interfering peak could be eliminated. In later experiments, the dioxane-hexane system was successfully replaced by injecting the vitamin D fraction on the first (chloroform-hexane) adsorption system for a second time. The reason for injecting a sample twice on the same system is that the unsaponifiable material, which is injected on the adsorption column, overloads the column to a certain extent. This slight overloading, which is necessitated by the low concentration of vitamin D in the samples, causes the retention times to vary slightly from one injection to another. In order to ensure that the vitamin D fraction is adequately collected, the collection period is extended on both sides of the retention time of vitamin D to allow for possible variations. With the second injection, the amount of sample placed on the column is much lower and the retention times consequently are more consistent, so that the vitamin

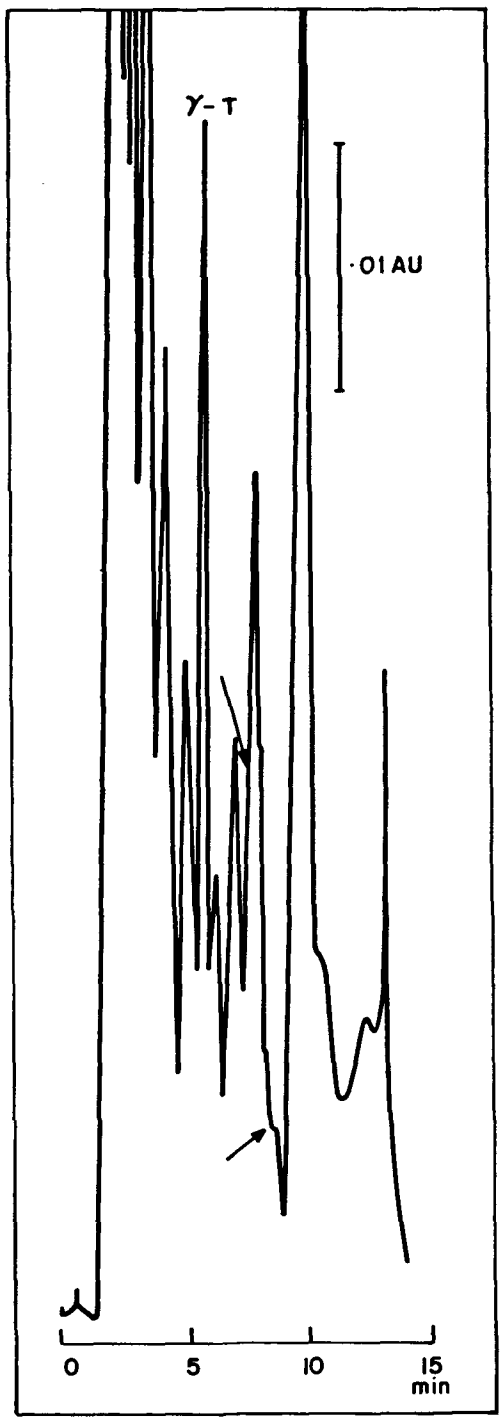

FIG. 1. Chromatogram of the unsaponifiable material from a margarine sample. Steel column (25 X 0.4 cm id) slurry-packed with Li-Chrosorb SI 60 (5 µm) with chloroform and *n*-hexane (1 + 1) as mobile phase (2 ml/min) and a UV detector at 264 nm and 0.1 AUFS. The vitamin D fraction is indicated by the arrows and γ-T is γ-tocopherol peak.

could be collected with more accuracy.

A chromatogram of the unsaponfiable material from a margarine sample on the adsorption column, monitored at 264 nm and 0.1 AUFS, is shown in Figure 1. From this it is clear that, because of the low concentration, the vitamin D peak cannot be recognized.

Figure 2 is a chromatogram of the same sample on the adsorption column with the detector set at 295 nm and 1 AUFS. Here the γ-tocopherol peak (capacity factor $k' = 2.7$) which is used as a tracer for the vitamin D, can be clearly seen. Figure 3 shows a chromatogram of the collected vitamin D fraction when rechromatographed on the adsorption column with the detector set at 264 nm and 0.1 AUFS. The fraction to be collected (at $k' = 4.4$) is indicated by the 2 arrows. A chromatogram of the purified vitamin D fraction on the reverse phase column, showing the separation of vitamins D_2 and D_3 with a resolution $R_s = 0.8$ ($k' = 5.0$ for vitamin D_2 and $k' = 5.3$ for vitamin D_3), is depicted in Figure 4.

The calibration curve of the ratio of the mass of vitamin D_3 to vitamin D_2 against the ratio of the peak heights of vitamins D_3 and D_2, as shown in Figure 5, was not linear and a curve ($y = 0.04822\ x^3 + 0.00557\ x^2 + 1.0744\ x - 0.0184$) was fitted to the data by the method of least squares. A similar calibration curve ($y = -0.0309\ x^3 + 0.3100\ x^2 + 1.0548\ x - 0.0081$) for the ratio of peak areas was obtained.

Vitamin D in solution isomerizes to pre-vitamin D and forms an equilibrium mixture with a composition which is

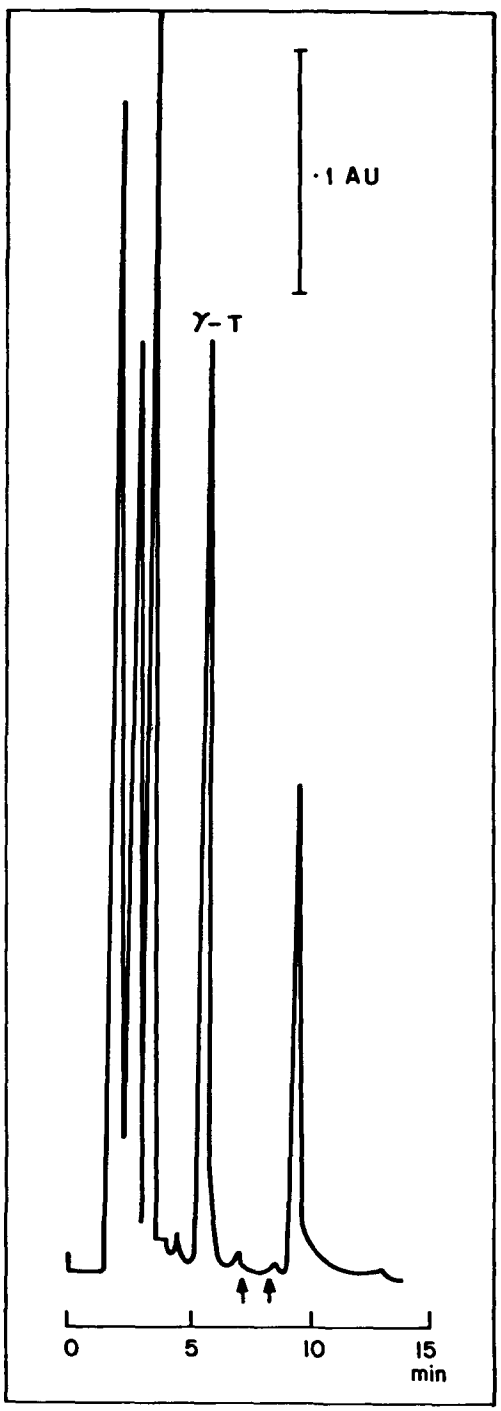

FIG. 2. Same sample, column and flow rate as in Fig. 1. UV detector at 295 nm and 1 AUFS. The vitamin D fraction is indicated by the arrows and γ-T is the γ-tocopherol peak.

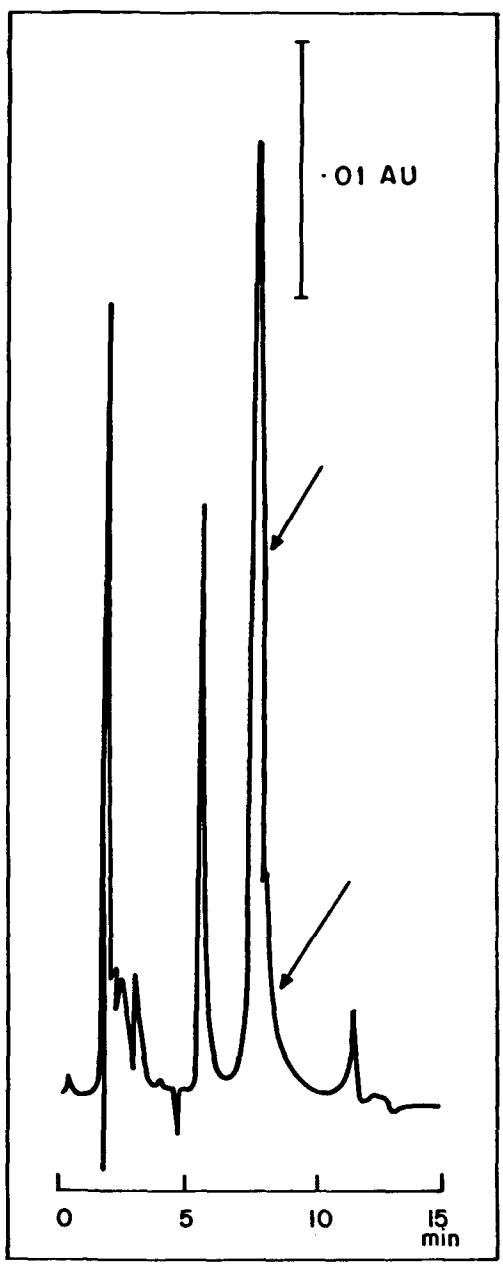

FIG. 3. Chromatogram of the collected vitamin D fraction. Conditions are the same as for Fig. 1. The fraction to be collected is indicated by the arrows.

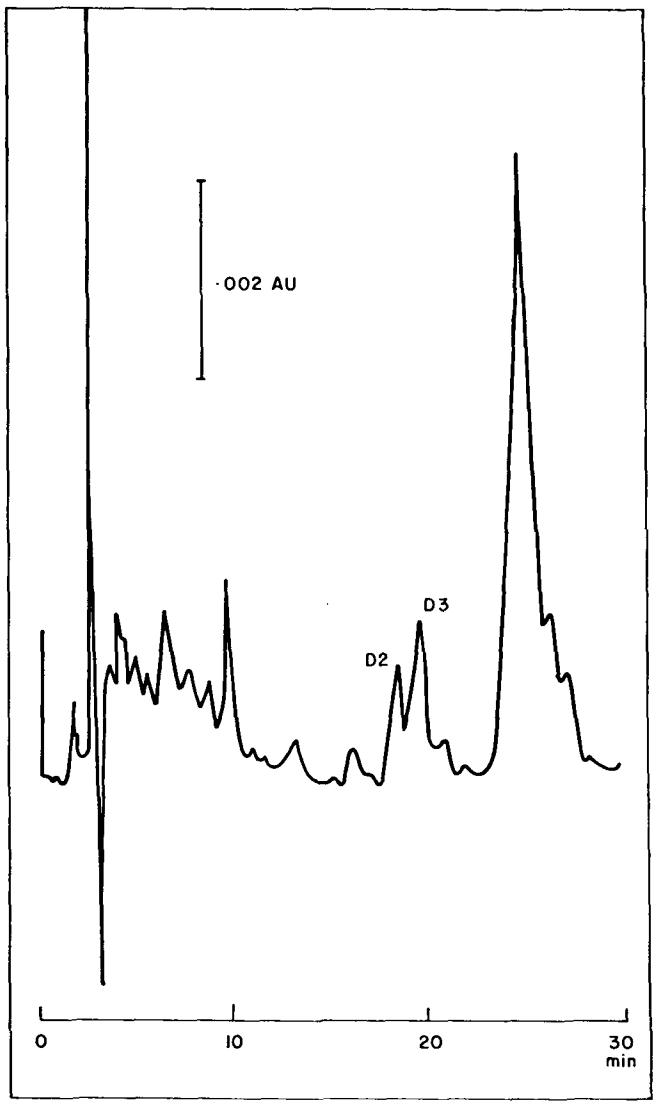

FIG. 4. Chromatogram of the vitamin D fraction collected from the adsorption system. Steel column (25 × 0.4 cm id) slurry-packed with 5 μm Nucleosil 5 C_{18} with methanol and water (95 + 5) as mobile phase (1 ml/min) and a UV detector at 264 nm and 0.02 AUFS. D2 is vitamin D_2 and D3 is vitamin D_3.

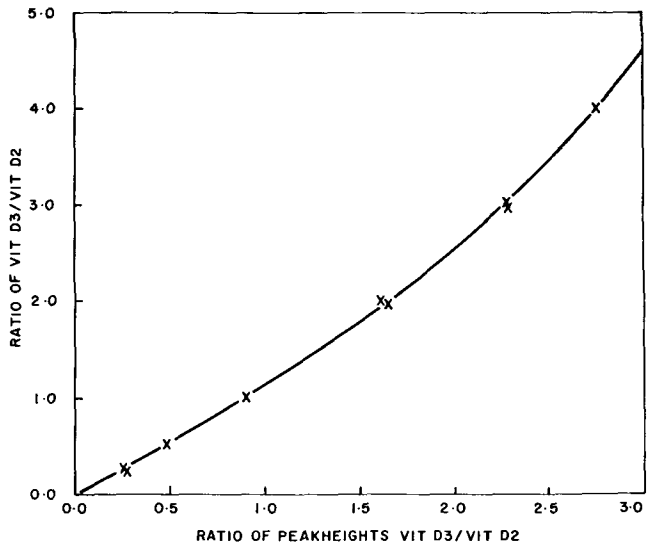

FIG. 5. Calibration curve of the ratio of the mass of vitamin D_3 to vitamin D_2 against the ratio of the peak heights of vitamins D_3 and D_2.

temperature-dependent. The internal standard was added to the margarine before saponification so that it was subjected to the same temperature fluctuations as the vitamin D in the sample. Because the isomerization rates of vitamins D_2 and D_3 are equal (13), the "potential vitamin D" (pre-vitamin D plus vitamin D) content of the sample was determined.

The recovery of 100 ng/g vitamin D_3 added to a margarine mix was 93.3% (coefficient of variation 11.9% for 12 replicates) as calculated from the ratio of peak heights and 100.1% (coefficient of variation 13.2% for 12 replicates) when calculated from the ratio of peak areas. When 50 ng/g vitamin D_3 was added, the recovery was 83% (coefficient of variation 18.5% for 7 replicates) as calculated from peak height ratios. The precision at this level could be improved to a coefficient of variation of 6.2% (4 replicates) by combining the fractions, obtained from 2 replicate injections on the adsorption column, before chromatography on the reverse phase column, and this procedure was followed for all subsequent analyses. The detection limit for vitamins D_2 and D_3 was 5 ng with the variable wavelength detector and 1 ng with the fixed wavelength detector, which gave a more stable baseline.

The method was applied to a number of margarine samples and their vitamin D and vitamin A content is given in Table I. The vitamin A content is included because it is added with the vitamin D to the margarine in a set ratio. Because of the difficult nature of vitamin D determinations, manufacturers in South Africa have used only the vitamin A content of their margarines as a quality control measure to ensure correct dosage of both vitamins. From the table it is clear that both vitamins are added in excess of the label claims. The fact that no definite ratio of vitamin A to vitamin D emerges probably results from different stabilities of the 2 vitamins during processing and storage. The result for the butter sample lay within the range given by reference (14). The variance ratio test (15) was applied to the variances calculated from the coefficients of variation in Table I. None of the variances differed significantly from each other at the 0.05 level of probability.

TABLE I

Vitamin D and Vitamin A Content of Margarine and Butter Samples

Sample	Vitamin D_3 IU/g	Coefficient of variation (4 replicates)	Vitamin D label claim IU/g	Vitamin A IU/g	Vitamin A label claim IU/g
Margarine 1	2.2	6.1%	1	33.0	20
Margarine 2	3.9	9.8%	3	25.7	20
Margarine 3	2.6	13.2%	1	26.7	20
Butter	0.5	11.5%	(0.4-0.6)		

The method was found to be practical and gave reliable results with sufficient precision (considering the low levels involved) if enough attention is given to detail.

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✂*Betula platyphylla* var. *japonica* Seed Oil: A Rich Source of Linoleic Acid

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ABSTRACT

Component fatty acids of the oil extracted from *Betula platyphylla* Sukatchev var. *japonica* Hara (Betulaceae) seeds were analyzed by gas liquid chromatography. The predominant fatty acid was linoleic acid (87%), and together with oleic and linolenic acids the 18-carbon unsaturated acids amounted to 97% of the total acids.

INTRODUCTION

Betula platyphylla Sukatchev var. *japonica* Hara, a member of the family Betulaceae, is a deciduous tree with white bark and is distributed from the middle to northern regions of Japan. No information is available on the fatty acid composition of the seed oil.

In this investigation, oils were extracted from 2 seed samples of different origins and examined for their characteristics and fatty acid composition.

EXPERIMENTAL PROCEDURES

Material

Two seed samples were used in the investigation. One sample was collected in September 1978 from trees grown at Kawagoe-shi, Saitama-ken and the other was collected in the same season from trees grown at Sapporo-shi by the Hokkai-do Forestry Cooperative Association, Sapporo-shi, Hokkai-do, Japan.

Extraction of Oil

Winged nutlets separated from each air-dried sample were dissected into seed and wing fractions by abrasion between the hands. The seed fraction (nutlets) was sorted to remove the loosened wings and trash by a combination of winnowing, sieving and hand-picking. The cleaned seeds were crushed in a mortar and placed in a Soxhlet extractor for reflux with ethyl ether solvent. The ether-extract was treated with hexane in the manner previously reported (1), and the resulting hexane-soluble oil was analyzed for its characteristics and fatty acid composition.

Preparation and Gas Liquid Chromatography (GLC) of Methyl Esters

Each sample oil was saponified in the usual manner. After removal of the unsaponifiable matter, the mixed fatty acids were refluxed with H_2SO_4/CH_3OH to give methyl esters.

The methyl esters were analyzed using a Hitachi 163 gas chromatograph equipped with a hydrogen flame ionization detector and a Takeda Riken TR-2217 automatic integrator. A 4 m x 3 mm stainless steel column packed with 5% diethylene glycol succinate polyester coated on 60/80 mesh Chromosorb G AW was used under the conditions: flow rate of nitrogen 20 ml/min; temperature of column, 200 C; temperature of injection port and detector, 300 C. Identification of each component was made by comparing its retention time with that of a reference sample.

RESULTS AND DISCUSSION

Sample seeds grown in Kawagoe (sample A, 51.3 g) and in Sapporo (sample B, 28.1 g) yielded 9.8g (19.1%) and 7.9g (28.1%) of greenish yellow oils, respectively. Geographical location influenced the oil content. Characteristics of the oils are listed in Table I.

The UV and IR spectra of the oils gave no definite evi-

TABLE I

Properties of Oils and Their Mixed Fatty Acids

	Samples	
	A	B
Oil		
Sp grav (20 C/20 C)	0.9268	0.9241
Saponification value	191.2	192.2
Iodine value (Wijs)	158.3	159.1
Unsaponifiable matter (%)	3.54	1.93
Mixed fatty acids		
Neutralization value	199.0	198.9
Iodine value	167.1	168.1