Isolation and Properties of Saponins from *Madhuca butyracea* Seeds

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The chemical composition of defatted seed flour of *Madhuca butyracea* was studied. The flour contains carbohydrates (45.7%), proteins (27.4%), and saponins (10.4%) as major constituents. The saponins were extracted with 80% (v/v) ethanol and isolated by a modified Wall's procedure. They were resolved into five compounds on thin-layer chromatography. Acid hydrolysis and soil bacterial hydrolysis yielded two different genins as evidenced by gas chromatography and the sugars xylose, arabinose, glucose, and rhamnose as identified by paper chromatography. Resolution of the total saponins on silica gel column using increasing ratios of methanol in chloroform resulted in the isolation of two compounds, saponins A and B. The LD_{50} and LD_{90} values to guppy fish (*Lebistes reticulatus*) were 11 and 14 ppm, respectively. The results suggest the potential usefulness of these saponins in piscicidal preparations and also in shampoos and other toiletry preparations.

Madhuca butyracea syn. *Bassia butyracea* syn. *Diploknema butyracea* (family Sapotaceae), commonly known as the Indian Butter Tree, is a forest tree found in the sub-Himalayan tract and outer Himalayas up to an altitude of 5000 ft. The seeds are the source of the commercial Phulwara fat (60–67%, on kernel basis), which is used in soaps and cosmetics. It is also used by the natives for cooking (*Wealth of India—Raw Materials*, 1952). The fat is also found to be suitable for use in confectionary (*Annual Report*, 1984). Though figures are not available for *M. butyracea*, it is known that a substantial quantity of *Madhuca* seeds is processed for the fat. The cake goes mainly as a low-grade fertilizer (Vimal and Naphade, 1980). Its use in food or livestock feeds is limited by the presence of bitter and toxic components, viz. the saponins (*Wealth of India—Raw Materials*, 1952).

Saponins are glycosides with triterpenoid or steroid ring structure to which a number of sugar moieties are attached. They are characterized by common properties like profuse foaming, hemolysis of red blood cells, toxicity to fish, insects, fungi, etc. Saponins from a variety of plant sources have been well studied (Tschesche and Wulff, 1973; Birk and Peri, 1980).

Not much work has been reported on the saponins of *M. butyracea*. In view of these various biological properties it was thought that saponins may be obtained as a useful byproduct from the defatted cake. The present study describes the isolation of saponins from the seed cake of this plant and some of their characteristic properties.

MATERIALS AND METHODS

M. butyracea seed kernels obtained from the National Botanical Research Institute, Lucknow, India, were crushed in a Hander crusher, and the resulting cake was repeatedly extracted with hexane at room temperature by percolation until the residual fat content was ~0.1%. The cake was dried in air to remove the residual solvent and powdered in a micropulverizer to 30 mesh.

Analytical Methods. Moisture, ash, crude fiber, tannins (AOAC, 1970), proteins (micro-Kjeldahl method), total soluble sugars (Lane and Eynon, 1923), reducing sugars (Somogyi, 1945), starch (Southgate, 1976), and phosphorus (Fiske and Subba Row, 1925) were estimated according to standard methods. Calcium and magnesium were determined by atomic absorption spectrometry at (Instrumentation Laboratory, Model aa/ae-751) 422.7 and 285.2 nm, respectively. Trypsin inhibitor activity was

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measured in 0.01 N NaOH extract of the flour with benzoyl-DL-arginine *p*-nitroanilide (Sigma Chemical Co.) as substrate. (Kakade et al., 1974). Hemagglutinin activity was measured in 0.9% saline extract of the flour on rabbit blood cells (Liener and Hill, 1953).

Isolation of Saponins. The saponins were extracted and isolated by a slight modification of the method of Wall et al. (1952). The defatted flour (1 kg) was extracted with 80% (v/v) ethanol (3 L) in a Soxhlet apparatus, and the extract was concentrated under reduced pressure at 40–50 °C into a syrupy liquid. This was extracted several times with diethyl ether in a separating funnel, until the coloring matter was removed. The syrupy mass was diluted to about 500 mL with water and extracted four to five times with butanol in the ratio of (v/v) 5:1. The pooled butanol extracts were evaporated under reduced pressure and dried over anhydrous phosphorus pentoxide. A solution of the powder thus obtained taken in 50–100 mL of dry methanol was poured into large excess quantities of ether (500–600 mL), and the precipitated saponins were centrifuged out and dried over P_2O_5 to constant weight.

Resolution of the Saponins by Thin-Layer Chromatography (TLC). Thin-layer chromatography (TLC) was performed on silica gel G layers (0.25 mm thick; 20 × 10 cm), which were activated for 1 h at 110 °C and cooled for 1 h at room temperature. The following solvent systems were tried: (1) butanol–ethanol–water (6:2:3, 7:2:5); (2) ethyl acetate–methanol–water (100:16.5:13.5); (3) chloroform–methanol–propanol–water (9:10:1:8, upper layer or 9:12:1:8); (4) chloroform–methanol–water mixture prepared according to Khanna et al. (1975), with slight modification (to a mixture of chloroform and water in the ratio 10:1 was added methanol dropwise until the mixture became clear). About 10–20 μg of the saponins was loaded. Chromatograms were developed up to a height of 18 cm, and the spots were visualized by spraying with 10% (v/v) sulphuric acid and heating at 110 °C. For densitometric scanning, 50% (v/v) sulfuric acid was sprayed and the chromatograms were developed at 120 °C for 10–15 min. The chromatograms were scanned in a CAMAG TLC densitometric scanner under the following conditions: lamp, 110–852; primary filter, 7–60; secondary filter, 110–823; range selector, 10.

Separation of Saponins on the Silica Gel Column. A glass column (60 × 1.5 cm) was packed with a slurry of silica gel (60–120 mesh; 180 g) in chloroform. The saponin mixture was loaded and eluted with increasing proportions of methanol in chloroform. Fractions of 250-mL volume were collected, concentrated by distillation, and monitored by TLC in solvent system 4. Fractions giving similar TLC

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Table I. Separation of Saponins on Silica Gel

fraction no.	% (v/v) MeOH–CHCl₃	no. spots obsd on TLC	fraction no.	% (v/v) MeOH–CHCl₃	no. spots obsd on TLC
1–5	0–10.0	1	22	16.0	1
6	10.0	1	23	16.0	2
7	10.0	2	24	16.0	1
8	15.0	2	25	16.0	2
9	15.0	1	26	16.0	1
10	15.0	1	27	16.0	2
11	15.0	2	28	16.0	2
12	15.0	4	29	16.0	3
13	15.0	3	30	17.5	1
14	15.0	2	31	17.5	2
15	15.0	2	32	17.5	1
16	15.0	2	33	17.5	1
17	15.0	2	34	17.5	1
18	15.0	3	35	17.5	1
19	15.0	2	36, 37	17.5	series
20	16.0	1	38	17.5	1
21	16.0	4			

Table II. Chemical Composition of Defatted *M. butyracea* Seed Flour

chemical constituent	g/100 g[a]
moisture	8.0
crude fiber	4.0
ash	6.6
crude protein (N × 6.25)	27.5
nonprotein nitrogen	0.4
total soluble sugars	4.7
reducing sugars	1.1
starch	6.3
saponins	10.4
carbohydrates (by difference)	42.3
tannins	0.8
trypsin inhibitors	nil
hemagglutinins	nil
phosphorus	1.53
calcium	0.56
magnesium	0.03

[a] Values are averages of three independent estimations.

patterns were pooled. The progress of chromatography is shown in Table I.

Optical rotations of pure saponins obtained above were recorded in methanol, on a Perkin-Elmer 243, digital polarimeter. Melting points were recorded in a Gallenkamp melting point apparatus, Design 889 339.

Preliminary Analysis of the Saponin Mixture. The saponin mixture was hydrolyzed under different conditions to the component genin and sugars.

(a) *Hydrolysis with 7% (w/w) Sulfuric Acid.* A sample of saponin mixture (100 mg) was refluxed with 25 mL of 7% (w/v) sulfuric acid in 80% (v/v) aqueous methanol for 8 h. The genin was precipitated by adding excess water and filtered out. The filtrate was neutralized with BaCO₃ and qualitatively tested for sugars by paper chromatography.

(b) *Hydrolysis with Trifluoroacetic Acid* (TFA). The saponin mixture (100 mg) was hydrolyzed with 5 mL of 2 M TFA by heating over a boiling water bath for 12 h. The hydrolysate was extracted several times with ether, and the pooled extracts were washed with water, dried over anhydrous Na₂SO₄, and concentrated.

(c) *Hydrolysis with Kiliani's Mixture (Kiliani, 1930).* A sample of saponin mixture (5 mg) was hydrolyzed with 1 mL of Kiliani's mixture (HCl–HOAc–H₂O, 5:7:10 (v/v)) on a boiling water bath in a sealed tube for 4 h. The genin was extracted with chloroform (5 mL × 5) in a separating funnel. The extracts were pooled and dried. The aqueous portion was tested for sugars by paper chromatography.

(d) *Soil Bacterial Hydrolysis.* The soil bacterial strain required for the hydrolysis was isolated in a synthetic medium containing saponins (3 g/L) as the sole carbon source (Yosioka et al., 1966). Conical flasks of 1-L capacity, containing 250 mL of the same medium, were inoculated with the bacterial culture and incubated statically for 36 days at 31 ± 0.5 °C. At the end of this period, the culture broth was repeatedly extracted with ether (200 mL × 5). The pooled ether extracts were washed, dried, and concentrated at low pressure.

Paper Chromatography of Sugars. The aqueous portions obtained after extracting genins from the hydrolysate were analyzed by descending paper chromatography using butanol–pyridine–water (6:4:3). The sugars were visualized by spraying with aniline hydrogen phthalate reagent (Partridge, 1949) and heating at 100 °C for 10 min. The sugars were identified by direct comparison with authentic sugars.

Gas–Liquid Chromatography (GLC) of Genins. The gas chromatograms of the genins were run in a Hewlett-Packard Model 5730-A gas chromatograph equipped with a flame ionization detector. A column (3 mm (i.d) × 2 m) of OV-101 was used at 300 °C under isothermal conditions (carrier gas N₂ at 30 mL min⁻¹).

Properties of *M. butyracea* Saponins. (a) *Hemolytic Property.* The hemolytic property was measured by the hemolytic index (HI) against rat and rabbit red blood cells (Mulky, 1976). To a series of dilutions of the saponins in 0.9% saline (1 mL) was added 1 mL of RBC suspension (with an optical density of 1.5 at 540 nm) and the resultant mixture left at room temperature for 30 min. The reciprocal of the minimum concentration (in grams) at which complete hemolysis occurred was taken as the HI.

(b) *Piscicidal Property.* The piscicidal action of the *M. butyracea* saponins was assessed by calculating the LD_{50} and LD_{90} doses to the guppy fish, *Lebistes reticulatus*. To a series of saponin concentrations (0–15 ppm) in 10 L of tap water were introduced the fish with a length of 3.75 ± 0.13 cm and weighing 0.66 ± 0.02 g. The experiments were conducted in triplicate with 10 fishes/replicate. The mortalities were noted at 12-h intervals up to 96 h. The total number of deaths of 96 h were converted to percent mortalities, and the LD_{50} and LD_{90} concentrations were calculated by the method of Probit analysis (Finney, 1971).

(c) *Foaming Property.* Foaming characteristics such as foam capacity (FC) and foam stability (FS) were determined on 0.02% aqueous solution of saponins at different pH conditions (Huffman et al., 1975).

RESULTS AND DISCUSSION

Chemical analysis of the defatted flour of *M. butyracea* (Table II) showed that it contained 10.4% saponin and 0.84% tannin as antinutritional principles. Thus, in spite of the considerable amount of protein (27.5%) and carbohydrate (42.7%) the defatted flour cannot be used in feed preparations. The saponin content of this species is higher than that reported for other species of *Madhuca*, viz. *Madhuca latifolia* and *Madhuca longifolia* where 6–8% saponin content has been reported (Mitra and Aswathi, 1962; Chand and Mahapatra, 1974; Mulky, 1976).

The saponin content of the flour was determined gravimetrically after isolation of the saponins by the procedure shown in Figure 1. Since this involves a lengthy procedure, a TLC–densitometric alternative using crude alcohol extract was attempted. However, due to the accompanying impurities, the saponins resolved very poorly, leading to inconsistent results. Hence, the gravimetric method was adopted.

Among the solvent systems used to resolve the saponins present in the mixture, the best separation was obtained
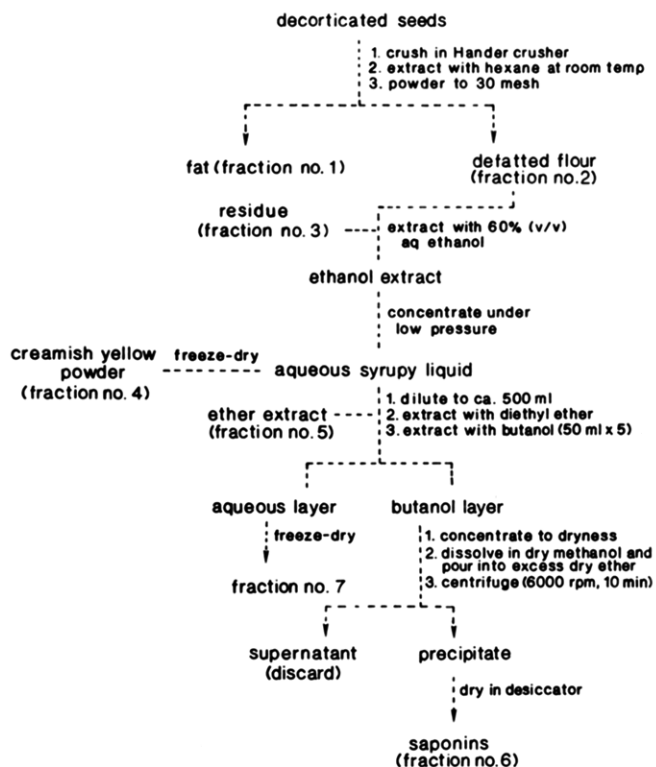
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Figure 1. Scheme for isolation of saponins from *M. butyracea* seeds.
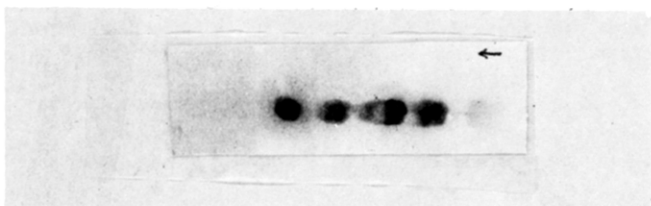

Figure 2. Thin-layer chromatogram of *M. butyracea* saponins in chloroform–methanol water (solvent system 4), showing 5 components. ← indicates the direction of the run.

Table III. Retention Times (RT) of Peaks Obtained by GC (OV-101) of Genin Mixture from *M. butyracea* Saponins

| | area, % | | |
RT, min	Kiliani's hydrolysis	TFA hydrolysis	bacterial hydrolysis
0.72	23.59	39.36	80.05
0.95	9.28	33.51	
1.39	1.96	0.77	
1.99	7.89	0.70	19.95
5.65	3.20		
9.33	53.59	25.66	

Table IV. Hemolytic Index (HI) of *M. butyracea* Saponins and Saponin-Containing Extracts

| | hemolytic index | |
test material	rat RBC	rabbit RBC
Madhuca saponin (ether precipitate)	28 570	50 000
butanol extract	25 000	40 000
ethanol extract	20 000	25 000
aqueous extract	20 000	25 000
Merck saponin	40 000	100 000

I), most of them heterogeneous mixtures of two to three compounds. Elution with 16% methanol in chloroform resulted in four homogeneous fractions, numbered 20, 22, 24, and 26. The fractions numbered 24 and 26 were obtained in sufficient yields and were designated as saponins A and B, respectively. Other compounds were obtained in low yields. The R_f values of the various saponins obtained as mixtures were very close, and hence preparative separation of these compounds by TLC was not successful.

The pure compounds saponin A [mp 220.5–225.0 °C dec; $[\alpha]^{20}_D$ −32.0° (methanol)] and saponin B [mp 213.5–220.5 °C dec; $[\alpha]^{20}_D$ −37.2° (methanol)] were further purified by crystallization from methanol. The structure of these two compounds will be dealt with in a separate paper.

A preliminary analysis of the saponin mixture was conducted to determine the number and type of sugars and the number of genins present. The hydrolysis was conducted under both strong, mild, and optimal conditions using soil bacteria in order to identify any artifacts that may arise as the result of hydrolytic conditions. On sulfuric acid or Kiliani's hydrolysis, the saponins yielded xylose, rhamnose, arabinose, and glucose and a 10–16% of a mixture of genins. The GLC examination of the genins obtained by three hydrolytic methods, viz. Kiliani's hydrolysis, trifluoroacetic acid hydrolysis, and soil bacterial hydrolysis, showed a predominant peak with retention time of 0.72. The acid hydrolysates showed an extra peak with retention time 9.33, while soil bacterial hydrolysis did not show the corresponding peak (Table III). Thus, the peak corresponding to retention time 9.33 may be due to an artifact arising out of acid-rearranged products, while the peak corresponding to retention time 0.72 may be due to the genuine aglycon. Such artifacts have been known and soil bacterial hydrolysis has been successfully used to distinguish the genuine aglycon, protobassic acid from the artifact bassic acid in the case of *M. longifolia* saponins (Kitagawa et al., 1972; Komori et al., 1974). However, the genins obtained from the hydrolysis of *M. butyracea* saponins did not compare with an authentic sample of bassic acid when tested by TLC.

Properties of *M. butyracea* Saponins. The hemolytic index (HI) of *M. butyracea* saponins against rat and rabbit RBC was compared with that of Merck saponins and also with crude preparations containing saponins at various stages of purification (Table IV). The HI values of the *M. butyracea* saponins were in general lower than those of Merck saponins. The HI increased with successive
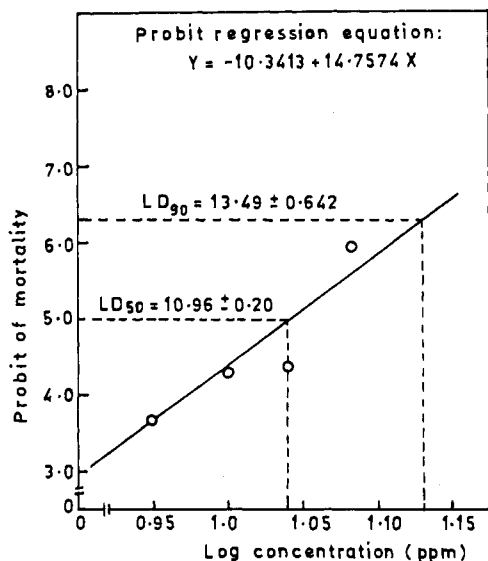
in solvent system 4, followed by solvent system 3. The other two solvent systems did not resolve the saponins. In solvent system 4, the mixture separated into five compounds (Figure 2). However, on the silica gel column the saponin mixture resolved into a series of compounds (Table

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Figure 3. Dose–response curve of guppy fish (*L. reticulatus*) treated with *M. butyracea* saponins.
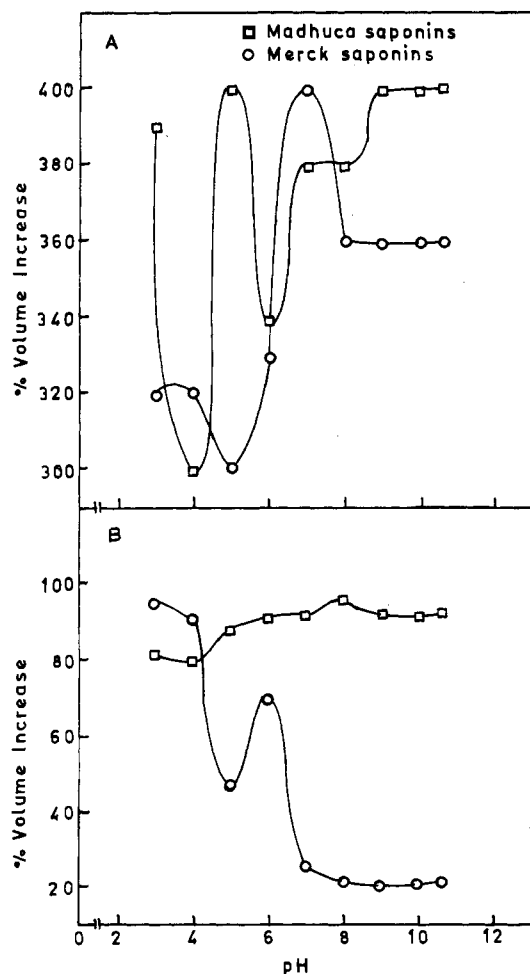

Figure 4. Foaming characteristics of *M. butyracea* saponins: A, foam capacity; B, foam stability.

steps of purification and can thus serve as an index of purity of these saponins. However, the HI of the pure compounds A and B isolated from the total saponins by column chromatography were only 17 000 and 10 000 units, respectively, against 28 000 units of the total saponins. This may be due to the synergistic action of individual saponins and/or the accompanying impurities.

The LD_{50} and LD_{90} concentrations of *M. butyracea* saponin to guppy fish were 11 and 14 ppm, respectively

(Figure 3). In a similar experiment conducted in our laboratory, common carp (*Cyprinus cardio*) were not killed at concentrations of saponins up to 25 ppm. These results suggest that these saponins can be useful in selectivity eliminating the weed fish guppies without harming the edible fish common carps. The commercial seed cake of *Madhuca spp.* has been traditionally used to control predatory fishes in ponds (Vimal and Naphade, 1980), and this can be replaced by saponins.

The foam capacity (FC) of *M. butyracea* saponins was comparable to that of Merck saponins with minimum FC of 300% and maximum of 400% in both the cases (Figure 4A). However, the foam stability (FS) of these saponins is superior to that of Merck saponins. While the FS remained constant between 80 and 96% for *M. butyracea* saponins, that of Merck saponins decreased over the range pH 4–10 (Figure 4B). These properties of saponins can be utilized in shampoos, detergents, and toiletry preparations provided their safety is established.

These saponins were found to have antifungal and insect-deterrent properties to be dealt with in separate papers. Thus, saponins can be obtained as useful byproducts from the oil industry. It has been shown that after these saponins are extracted by a two-stage alcohol extraction, the resulting cake of *M. butyracea* can be fed to rats without any serious ill effects (Shanmugasundaram and Venkataraman, 1985) and thus can be utilized as a feed component.

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Cis/Trans Isomerization of Retinyl Palmitate in Foods

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Heat treatment (120–170 °C) of *all-trans*-retinyl palmitate for various time intervals showed an increase in the ratio of 13-cis/all-trans. The increase followed first-order kinetics, and an apparent Arrhenius activation energy was calculated as 12 kcal/mol. Analysis of selected food products showed 13-cis/trans ratios ranging from 0.15 to 0.63. Pasteurization and ultrahigh-temperature processing of skim milk supplemented with retinyl palmitate produced no changes in isomeric composition. Commercial retinyl palmitate samples used to prepare supplementation and fortification mixes had variable amounts of 13-cis isomer. Exposure of samples to indirect sunlight induced primarily the formation of 9-*cis*-retinyl palmitate. Retinyl palmitate isomers found in food products may in part be attributed to the composition of the added supplemental vitamin as well as effects of food-processing treatments, particularly heating.

Synthetic retinyl palmitate is a major source of vitamin A in the diet of many individuals (Parrish, 1977). Retinyl palmitate is commonly added to foods supplemented or fortified with vitamin A. Ester hydrolysis to retinol occurs readily during digestion (Goodman and Blaner, 1984).

The all-trans form of retinol and retinyl palmitate have the greatest biological activity relative to other isomeric forms. The 13-cis and 9-cis isomers of retinyl palmitate have biological activities of 75% and 26%, respectively (Ames et al., 1960). Prompted by these findings, a number of laboratories have reported analysis of cis–trans retinoid isomers in foods and feeds (Steuerle, 1985; Sivell et al., 1984; Wiggins et al., 1982; Stancher and Zonta, 1982; Egberg et al., 1977). These studies have found that 13-*cis*-retinol was the most common of the cis isomers in foods and detected in varying amounts. If the isomeric composition of retinoids is not accounted for, inaccurate nutritional estimates may result. Generally, for determination of vitamin A in foods with added retinyl palmitate, interfering lipids must be removed by saponification. Hydrolysis of palmitate ester occurs and analyses are quantified as retinol.

In two recent studied (Landers and Olson, 1986; Mulry et al., 1983), it was reported that upon photolysis of *all-trans*-retinyl palmitate, the 13-cis isomer was formed as a minor component. The 9-cis isomer predominated and varied in content depending upon the solvent used during light exposure. There are no reports that have detected measurable levels of 9-*cis*-retinyl palmitate in foods. Furthermore, reasons for the presence of appreciable amounts of 13-*cis*-retinyl palmitate in food samples remains unclear.

The objectives of this study were to investigate the mechanism for the formation of 13-*cis*-retinyl palmitate in foods and to determine whether isomerization of *all-trans*-retinyl palmitate can be induced by heat treatments,

such as those used in commercial food-processing facilities.

MATERIALS AND METHODS

Source of Materials. *all-trans*-Retinal, retinol, retinyl palmitate, and 9-*cis*-retinal were purchased from Sigma Chemical Co. (St. Louis, MO), and 13-*cis*-retinal was from Eastman Kodak Co. (Rochester, NY). Retinyl palmitate fortification samples (corn oil as carrier) and skim milk (0.2% fat) for processing were provided by North Carolina State University Dairy Processing Plant. Food products were purchased from local sources. Infant formula powders were reconstituted following package directions. All other chemicals and solvents were reagent grade.

HPLC Conditions. The HPLC system consisted of a Model 510 solvent delivery system with U6K injector, both from Waters Associates (Milford, MA), a Du Pont guard column packed with μ-Porasil (10 μm) a Du Pont Zorbax Sil normal-phase column (4.6 mm × 25 cm), 5-μm particle size (Wilmington, DE), a Chira Tech UV-106 HPLC detector (Fort Collins, CO) equipped with a 340-nm filter, and a Fisher dual-pen Series 5000 recorder (Fisher Scientific, Raleigh, NC). Spectral scans were obtained with a linear UV-203 HPLC detector (Reno, NV) in the stopped-flow mode.

Solvent systems employed were ethyl acetate/methylene chloride/hexane (0.4/1.0/98.6, v/v/v) to separate retinyl palmitate isomers and ethyl acetate/methylene chloride/hexane (6.2/7.7/86.1) to resolve retinol isomers (Tsukida et al., 1977b). Injections of 25–50 μL were made, and all chromatograms were run at ambient temperature at a flow rate of 2 mL/min. Absorbance units full scale varied from 0.01 to 1.0. All ratios reported were determined from peak heights and calculated from duplicate experiments.

Assignment of Peaks. Retinoid isomers were assigned by comparison to standards when available. The 9-*cis*- and 13-*cis*-retinol standards were prepared by reduction of 9-*cis*- and 13-*cis*-retinals following the method described by Egberg et al. (1977). Mixtures of retinol isomers were obtained by photochemical catalysis of *all-trans*-retinol and by iodine-catalyzed isomerization followed by NaBH$_4$

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