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FOOD ADDITIVE SAFETY

November 23, 2005

Via Hand Delivery

Laura Tarantino, Ph.D.
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety, HFS-200
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for Tomato Lycopene

Dear Dr. Tarantino:

Pursuant to proposed 21 C.F.R. § 170.36(c), on behalf of our client, San-Ei Gen F.F.I., Inc., we hereby notify the Agency of San-Ei Gen's determination, on the basis of scientific procedures in accordance with 21 C.F.R. § 170.30, that use of its tomato lycopene (from tomato oleoresin concentrate) as an ingredient in various non-alcoholic beverages is generally recognized as safe (GRAS) and therefore exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act. The reasons for San-Ei Gen's GRAS conclusion regarding its tomato lycopene product are discussed in detail in the company's GRAS Notification, which is enclosed in triplicate.

We trust you will find the enclosed notification acceptable. Should any questions arise during the review process, please do not hesitate to contact us, preferably by telephone, so that we may respond as quickly as possible.

Very truly yours,

Melvin S. Drozen
John F. Foley

Enclosures
cc: Shimmo Hayashi Ph.D., San-Ei Gen F.F.I., Inc.

Before the
FOOD AND DRUG ADMINISTRATION
Department of Health and Human Services
Washington, D.C.

GRAS NOTIFICATION

Name of Notifier:	San-Ei Gen F.F.I., Inc.
Post Office Address:	All communications on this matter are to be sent in care of Counsel for the Notifier: Melvin S. Drozen John F. Foley Keller and Heckman LLP 1001 G Street, N.W., Suite 500 West, Washington, D.C. 20001. Telephone: (202) 434-4222, (202) 434-4186
Name of Substance and Intended Use:	Tomato lycopene for use in beverages as a food ingredient with an antioxidant activity
Dated:	November 23, 2005

Melvin S. Drozen
John F. Foley
Counsel for San-Ei Gen

Claim of GRAS Status

A. Name and Address of Notifier:

San-Ei Gen F.F.I., Inc.

1-1-11 Sanwa-cho

Toyonaka, Osaka 561-8588 JAPAN

Telephone: +81-6-6333-0521

All communications on this matter are to be sent in care of counsel for the Notifier:

Melvin S. Drozen

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1001 G Street, N.W., Suite 500 West

Washington, D.C. 20001.

Telephone: (202) 434-4222, (202) 434-4186

B. Common or Usual Name of the Notified Substance:

Tomato lycopene (synonym "Tomato oleoresin extract").

C. Applicable Conditions of Use:

Tomato lycopene is intended to be used as a food ingredient with an antioxidant activity in non-alcoholic beverages.

D. Basis for GRAS Determination:

The described use of tomato lycopene is generally recognized as safe (GRAS) based on scientific procedures, in accordance with 21 C.F.R. § 170.30, as discussed more fully in the accompanying summary of the basis for the GRAS determination.

E. Statement of Availability of Data:

The data and information that are the basis for the GRAS determination are available for the Food and Drug Administration's review and copying or will be sent to FDA upon request.

* * * * *

The foregoing and attached information considered, it is respectfully submitted that the use of tomato lycopene in various beverages as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because it is generally recognized as safe.

Respectfully submitted,

San-Ei Gen F.F.I., Inc.

By:_____

Melvin S. Drozen

John F. Foley

Keller and Heckman LLP

COUNSEL FOR THE NOTIFIER

I. Identity of the Notified Substance

Tomato Lycopene (synonym Tomato oleoresin extract)

A. Name: Tomato lycopene

Chemical Name: (all-*trans*)-Lycopene

Chemical Abstract Name: ψ,ψ-Carotene

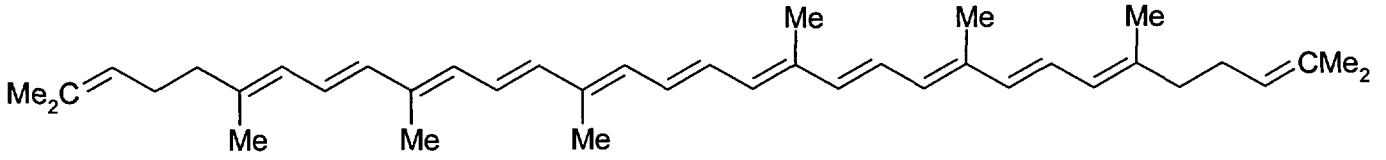
CAS Reg. No. for Tomato Lycopene: 502-65-8

B. Chemical Formula of Tomato Lycopene: $C_{40}H_{56}$

C. Structure of Tomato Lycopene:

Tomato lycopene is a mixture of lycopene and other tomato-derived oleoresins.

The structure of lycopene is:



D. Physical Properties of Tomato Lycopene:

The principal component of tomato lycopene (synonym Tomato oleoresin extract) is lycopene, which is a carotenoid with a melting point of 172-173°C. Carotenoids are the natural, fat-soluble color pigments that contribute to the deep red color of tomatoes and other fruits and vegetables. Lycopene's color and antioxidant properties are derived from its unsaturation.

Lycopene is insoluble in water and soluble in fat and most organic solvents. The octanol-water coefficient is 17.64. Its molecular weight is 536.87, absorption max of the *trans*-isomer (petroleum ether) are 446, 472, 505nm ($E^{1\%}_{1cm}$ 2250, 3450, 3150).(Merck Index, 12th Ed.,

1996).

II. Stability of Tomato Lycopene

Pure lycopene is sensitive to air and light, but is stable when stored in an inert atmosphere in the dark. San-Ei Gen's tomato lycopene is formulated with ascorbic acid and we provide data demonstrating that lycopene in beverages containing 100 parts per million (ppm) and 150 ppm ascorbic acid are stable at 40°C through 3 and 7 months, respectively. *See* Attachment 1.

III. Manufacturing Process

San-Ei Gen's tomato lycopene is prepared from LycoRed Natural Products Industries, Ltd.'s "Tomato oleoresin," trade name "Lyc-O-Mato®." Lyc-O-Mato® is obtained from tomatoes by crushing the tomatoes, subjecting them to heat treatment, and extracting the crushed tomatoes with ethyl acetate. The resulting oleoresin contains about 6-8% lycopene. San-Ei Gen extracts LycoRed's tomato oleoresin with ethanol to form a concentrated product containing at least 90% lycopene, which it calls "tomato lycopene." Trace residual solvent levels for tomato lycopene are provided in Attachment 2. Both solvents are widely used for this purpose by the food industry. San-Ei Gen's manufacturing process also results in an increase in lycopene (*cis+trans*) and a decrease in *beta*-carotene. *See* Attachment 3.

Comparative LC/MS chromatograms and MS spectra of four lycopenes (lycopene extracted directly from tomato, LycoRed's tomato oleoresin, BASF's synthetic lycopene, and San-Ei Gen's tomato lycopene) are provided in Attachment 4.

IV. Specifications for Tomato Lycopene

Specifications for San-Ei Gen's tomato lycopene are shown in the following table:

Assay (as total carotenoids)	>96.0%
Lead	< 2 μg/g
Arsenic	< 0.5 μg/g, as As_2O_3
Heavy Metals (as lead)	< 5 μg/g
Residue on Ignition (Ash)	<0.2%

Lot analyses and supporting analytical methods are provided in Attachments 5a and 5b.

V. Level of Use and Intended Technical Effect

San-Ei Gen's formulated products containing tomato lycopene with antioxidant activity are intended to be consumed as food ingredients in soft drinks, including carbonated beverages. Levels of use, as lycopene, may vary from about 1 ppm in the beverage to 20 ppm, with 5 ppm being the typical value. Although some of these levels might produce an incidental coloring effect in an uncolored beverage, the tomato lycopene is not intended to impart color. When added to colored beverages, there should be no noticeable change in the color of the beverage.

VI. Methodology for Analysis of the Added Substance in Food

Lycopene is analyzed in beverages by extraction with hexane and measurement of absorbance of the resulting solution at 470 nm. A more detailed analytical method is provided in Attachment 1.

VII. Consumer Exposure

As stated above, San-Ei Gen's liquid or powdered tomato lycopene commercial products will typically be added to soft drinks at levels that provide 5 ppm lycopene. The maximum level will be 20 ppm, as lycopene.

We understand that FDA has estimated that the mean *users only* intake of carbonated beverages is 502.5 grams per person per day (g/p/d) and the 90th percentile users only intake is 1016.1 g/p/d.[1] The intake was obtained from the 1994-96 U.S. Department of Agriculture (USDA) Continuing Survey of Food Intakes (CSFII) study, 2 days. Although the reported intake is for carbonated beverages, a subset of soft drinks, mean and 90th percentile intake from "soft drinks" will be lower than for carbonated beverages, which represents the worst case exposure.[2] Using these estimates, the 90th percentile users intake of lycopene from the proposed use typically will be 5.1 mg/p/d (i.e., when lycopene is present at 5 ppm), and the maximum intake level will be 20.3 mg/p/d (when the lycopene concentration is 20 ppm).

Current estimated intakes of lycopene due to its natural presence in food vary widely. BASF, in GRN 119, estimates from NHANES III that the mean intake of lycopene was 10.9 mg, and 90th percentile intake is 27.8 mg/day. However, mean lycopene intake from users of select foods containing processed tomato products, such as catsup, pizza, tomato juice, and spaghetti sauce, ranges from 4.7 to 29.2 mg/day; intake at the 90th percentile ranges from 11.6 to 60.9 mg/day. Jonker *et al.* (2003) report that mean lycopene intake is between 0.5 mg/p/d and 27 mg/p/d, based on a literature survey.[3] McLain and Bausch (2003) estimate that the mean total

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1 Communication from Dr. Paul Kuznesof, Senior Chemist at the Office of Food Safety, to Michael T. Flood, Keller and Heckman LLP, November 16, 2005.

2 For example, the 1977-78 food intake survey of USDA, reported that the 90th percentile users intake of carbonated soft drinks was 556 g/p/d, while the corresponding intake for noncarbonated soft drinks was 413 g/p/d. [Foods Commonly Eaten by Individuals: Amount per Day and per Eating Occasion, E.M. Pao, *et al.*, Consumer Nutrition Center, Human Nutrition Information Service, USDA. Home Economics Research Report No. 44, March 1982.

3 Jonker, D., Kuper, C.F., Fraile, N., Estrella, A. and Otero, C.R., 2003. Ninety-day oral toxicity study of lycopene from *Blakeslea trispora* in rats. *Reg. Toxicol. & Pharmacol.* **37,** 396-406.

population intake is 10.9 mg/p/d, the mean users intake is 12.2 mg/p/d, and the 90th percentile total population intake is 32.1 g/p/d, based on the CSFII, 1998 intake study.[4] Matulka *et al.* (2004) estimate that the mean users intake of lycopene is 8.2 mg/p/d, with the 90th percentile intake 15.7 mg/p/d.[5] The authors of this latter report considered 26 food commodities and estimated lycopene intake using the USDA 1994-96 survey with USDA's carotenoid database. Based on the typical value of 5 ppm in beverages, the 90th percentile users intake from San-Ei Gen's proposed use—5.1 mg/p/d—is well within estimated lycopene intakes, and even the 90th percentile intake based on the maximum use level of 20 ppm—20.3 mg/p/d—is lower than that of regular consumers of tomato-based products.

VIII. Detailed Summary of Basis for Notifier's GRAS Determination

Published animal feeding studies in the scientific literature, as well as corroborative unpublished studies, support the safety of tomato lycopene. With regard to the lycopene that makes up more than 90% of tomato lycopene, a published subchronic rat feeding study establishes a no-observed-adverse-effect level (NOAEL) of 601 mg/kg/day for lycopene, and, hence (applying a 1000-fold safety factor), an acceptable daily intake (ADI) of 0.6 mg/kg bw/day, which is equivalent to 36 mg/kg for a 60 kg person. This ADI is more than six times the 90th percentile estimated daily intake (EDI) for lycopene from typical use levels of San-Ei Gen's tomato lycopene commercial product in beverages (5 ppm as lycopene), and also substantially higher than the 90th percentile daily intake for lycopene from beverage use of the extract at

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[4] McLain, R.M. and Bausch, J., 2003. Summary of safety studies conducted with synthetic lycopene. *Reg. Toxicol. & Pharmacol.* **37,** 274-285.

[5] Matulka, R.A., Hood, A.M. and Griffiths, J.C., 2004. Safety evaluation of a natural tomato oleoresin extract derived from food-processing tomatoes. *Reg. Toxicol. & Pharmacol.* **39,** 390-402.

maximum levels (20 ppm as lycopene). The Notifier has concluded that these studies, which are discussed in more detail below, establish the GRAS status of the substance for San-Ei Gen's intended use.

The GRAS status of the lycopene in San-Ei-Gen's tomato lycopene is also supported by analysis of lycopene in the tomato lycopene, which demonstrates San-Ei Gen's lycopene is essentially the same as the lycopene in tomatoes, which have been safely consumed in substantial amounts for centuries. Based on the entirety of the available information, the Notifier has concluded that its tomato lycopene is generally recognized as safe, based on scientific procedures, for the intended use described here.

A. Safety of Tomato Lycopene—Scientific Studies Supporting the Safety of Lycopene

The discussion that follows focuses on the safety of lycopene since, as noted above, San-Ei Gen's tomato lycopene contains at least 90% lycopene, including all-*trans*-lycopene and various *cis*-isomers. The only other carotenoid present is all-*trans*-beta carotene. *See* Attachment 3. All components, except for residual solvents, are components of tomatoes.

Although lycopene has been ingested for hundreds of years in foods with no harmful health effects, independent safety assessments based on animal studies have been performed on lycopene or lycopene products during the last three years. The studies—many of which have been published in peer-reviewed journals—uniformly show that ingestion of lycopene in laboratory animals is safe. Specifically, there have been 4 published independent safety assessments on various lycopene products, and 4 independent 90-day rat studies have been

performed. In none of these studies was the NOAEL lower than the highest administered dose.[6] Because no adverse effects were observed in any study, use of the highest NOAEL is justified for estimating an ADI.

B. Synthetic Lycopene (BASF)

The data generated by BASF Corporation formed the basis of GRN No. 119, to which FDA had no objections. BASF's synthetic lycopene is not obtained from a natural product, so it can be expected to have a different impurity profile than San-Ei Gen's tomato lycopene. Nevertheless, the toxicity studies on BASF's lycopene are quite relevant to other lycopene products, even if the impurities are not the same. BASF's synthetic lycopene consists of about 98% lycopene (*cis* and *trans* isomers, with greater than 80% *trans*). Commercial products are formulated to contain about 10% synthetic lycopene.

1. 13-Week Gavage Studies in Rats (Mellert, W. *et al.* (2002).)[7]

Thirteen-week studies were carried out on two products: Lycopene 10 CWD® and Lyco Vit® 10%. Lycopene 10 CWD® was administered to male and female Wistar rats (10 rats/sex/group) at doses of 0, 500, 1500, or 3000 mg/kg bw/day. Lyco Vit® was administered at doses of 0 or 3000 mg/kg bw/day. Red discoloration was seen in the jejunum and cecum of the treatment groups, but there were no histopathological changes. The NOAEL for both products was 3000 mg/kg bw/day, equivalent to **324 mg synthetic lycopene/kg bw/day**.

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[6] Coloration was observed in the feces, which is not surprising when large quantities of highly colored substances are ingested. Lycopene may also accumulate in the liver and, to a lesser extent, in the spleen. However, there are no histopathological changes, and the accumulation is reversible.

[7] Mellert, W., Deckardt, K. *et al.* (2002). Thirteen-week toxicity study of synthetic lycopene products in rats. *Food and Chemical Toxicology* **40,** 1581-1588.

2. **Developmental Toxicity Studies in Rats and Rabbits** (Christian, M.S. *et al.* (2003).)[8]

Developmental toxicity studies were carried out on female rats [Crl CD® (SD) IGS BR-COBS-VAF®] (23 rats/group) and female rabbits [New Zealand White, KBL-NZW, SPF] (29-34 rabbits/group). Lycopene 10 CWD® was administered by intubation at 0, 500, 1500, and 3000 mg/kg bw/day (rats) or 2000 mg/kg bw/day (rabbits). Lyco Vit ® 10% was administered at 0 and 3000 mg/kg bw/day (rats) or 2000 mg/kg bw/day (rabbits). The high dosages were at the maximum achievable concentrations and dosage volumes (15 and 10 mL/kg for rats and rabbits, respectively). Dosages were administered on gestation days 6 through 19 (rats) or 6 through 28 (rabbits). Treatment with the maximum feasible dosages did not adversely affect embryo-fetal viability, gender body weight, or external, soft tissue, or skeletal morphology. No maternal toxicity was observed. The NOAEL was 3000 mg/kg bw/day (rats) and 2000 mg/kg bw/day (rabbits). Expressed as lycopene, the NOAEL's are **324 mg/kg bw/day** and **216 mg/kg bw/day**, respectively.

3. **Genotoxicity Studies**

As reported in GRN 119, a genotoxicity battery (Ames assay, *in vivo* micronucleus assay, *in vitro* chromosome aberration assay, and *in vitro/in vivo* unscheduled DNA assays) indicated no concern for genotoxicity of products formulated with synthetic crystalline lycopene.

C. Synthetic Lycopene (Roche Vitamins Ltd.)

Safety studies are described by McClain, R.M. and Bausch, J. (2003).[9] Roche's synthetic lycopene is prepared by the Wittig reaction. The crystalline product contains 96% pure

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[8] Christian, M.S., Schulte, S., and Hellwig, J. (2003). Developmental (embryo-fetal toxicity/teratogenicity) toxicity studies of synthetic crystalline lycopene in rats and rabbits. *Food and Chemical Toxicology* **41,** 773-783.

lycopene, most of which is the *trans* isomer. The authors note that conventional reversed phase HPLC tends to overestimate the *trans* isomer. When analyzed using high resolution normal phase silica columns, synthetic lycopene is found to contain 76% of the *trans* isomer; however, when analyzed by reversed phase C18 columns, the percentage rises to 96%.

1. 14-Week Feeding Study in Rats

In a 14-week study, Han-Ibm Wistar rats (26 rats/sex/group) were administered Lycopene 10% WS beadlet formulation in their diets at 0, 50, 150, or 500 mg/kg bw/day, as lycopene. Lycopene was found to induce pigmentation in liver cells (heptocytes), but the effect was reversible, and there were no associated histopathological changes. The NOAEL was **500 mg/kg bw/day**, as lycopene.

2. 4-Week Feeding Study in Rats

In a subsequent four-week study, the same strain of rats (6/sex) received 1000 mg/kg bw/day lycopene in the same lycopene formulation. The only treatment related changes were a red discoloration of the feces and a brown-orange discoloration of the liver. Histologically, the discoloration of the liver correlated with deposits of brown-yellow fine granulated pigment in hepatocytes, but there were no associated histopathological alterations. Interestingly, analysis of plasma samples collected on the day of necropsy showed group mean concentrations of lycopene to be similar to those from the previous 14-week study, indicating that saturation in plasma was attained with oral doses of ≤ 500 mg/kg bw/day, the high dose in the 14-week study.

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[2] McClain, R.M. and Bausch, J. (2003). Summary of safety studies conducted with synthetic lycopene. *Reg. Toxicol. & Pharmacol.* **37,** 274-285.

3. Teratology Study in Rats

Fourteen mated Wistar female rats were administered 1000 mg/kg bw lycopene in their diets during days 6 to 18 of gestation. A second group received the same dose of lycopene but with an added impurity specific to the manufacturing process. No signs of maternal toxicity or teratogenic effects were observed. Reproductive and developmental parameters were normal except for a very small increase in the incidence of extra-thoracic (14th) ribs observed in the two lycopene groups as compared to the controls. The incidence was 14.7% in the control group, 26.1% in lycopene group, and 16.8% in the lycopene + impurity group. Such effects were not observed in the studies by BASF and may have been due to normal variation.

4. Genotoxicity Studies

Lycopene 10% WS beadlet formulation was tested in a number of genotoxicity assays. No increase in the mutant frequency was observed at the tk locus in mouse lymphoma cells, with or without S9 metabolic activation. When tested for structural and numerical chromosome aberrations in human peripheral blood lymphocytes, there was no increased incidence of cells having chromosome aberrations, with or without metabolic activation.

In *in vivo* assays, neither fresh nor aged formulations showed increased DNA repair activity in the hepatocytes of rats dosed orally with the formulation (UDS assay). (The aged material was stored for 8 weeks at 45°C with oxygen, then at 4°C for 8 days, protected from light and under nitrogen.) Neither fresh nor aged lycopene beadlet formulations tested negative in the mouse micronucleus test in bone marrow. The route of administration was not reported, but was probably a single intraperitoneal injection. In a second test, fresh (50 ppm) and aged (25-50 ppm) soft drink preparations were administered to mice for 14 days in drinking water. Tomato juice containing natural lycopene was also tested. Under the test conditions, the lycopene-

containing soft drinks did not induce clastogenic or spindle-damaging effects in the peripheral blood of treated mice.

A series of Ames assays were performed on different preparations of lycopene. A mutagenic effect at high doses was observed in *S. typhimurium* strains TA100 and TA97 using unformulated crystalline lycopene. However, freshly prepared crystalline lycopene tested negative in 7 tester strains, which implied that an impurity was the source of the activity. Subsequently, pure crystalline lycopene was found to develop mutagenicity when exposed to air and light. Lycopene in a beadlet formulation did not form mutagenic breakdown products. The formulated products are stabilized with DL-α-tocopherol and ascorbyl palimitate.

5. Hepatic Uptake Studies

Because of liver coloration observed in 13- and 4-week oral studies, a number of uptake studies were performed. In one experiment, female Wistar rats were fed lycopene at doses of 20 mg/kg bw/day as tomato concentrate, mixed with feed, for 4, 8, and 13 weeks, and compared to animals fed 5 mg/kg bw/day lycopene beadlet formulation in feed for 4 and 8 weeks. The concentrations of lycopene in the liver and the presence of birefringent deposits were similar for both formulated lycopene and the tomato concentrate groups after 4 and 8 weeks of treatment. Depletion was studied in the animals fed the tomato concentrate. During the depletion period, lycopene concentration decreased, but birefringent deposits remained after 12 weeks of depletion.

In a second experiment, Wistar rats were fed lycopene at 5 or 10 mg/kg bw/day for 2 and 4 weeks as the beadlet formulation. After 4 weeks of treatment, rats in both dose groups accumulated lycopene in the liver and spleen, and birefringent deposits were seen in the liver. In

a subsequent recovery period of up to 21 weeks, a marked depletion in liver and spleen was observed, and deposits in the liver were no longer observed after 13 weeks of depletion.

Female Wistar rats were then fed lycopene at 10 mg/kg bw/day in form of the beadlet formulation, and the concentration of lycopene was monitored in the liver, spleen and plasma in rats sacrificed at various times. The liver was found to have the highest concentration, about 0.25 mg/g liver after 64 weeks of treatment, at which time a steady state was reached. The spleen showed levels of 0.10 mg/g after 64 weeks, and a steady state was reached after about 14 weeks. Despite significant accumulation of pigment in liver cells, there was no evidence of lycopene-related liver damage after 64 weeks of exposure, when the livers were examined histopathologically. Minimal to slight deposition of pigment was also observed in the spleen.

A group of female rats was followed through 104 weeks of treatment. The concentration of lycopene in the liver decreased during the second year of treatment. There remained no histopathological indication of liver damage. Yellow to orange discoloration of adipose tissue was also observed at the end of the administration period.

D. Ninety-day Dietary Study of Lycopene from *Blakeslea trispora* (Vitatene SAU) Jonker, D. *et al.* (2003)[10]

The lycopene used in this 90-day study was obtained from the fungus *B. trispora*. As described in the application to the European Union (EU), the fungus is obtained from a fermentation process. Lycopene is obtained by crystallization from an isobutyl acetate extract of the dried fungus.[11] Crystalline lycopene is dissolved in high oleic acid sunflower oil containing

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[10] Jonker, D., Kuper, C.F., Fraile, N., Esterella, A., and Otero, C.R. (2003). Ninety-day oral toxicity study of lycopene from *Blakeslea trispora* in rats. *Reg. Toxicol. & Pharmacol.* **37,** 396-406.

[11] *See:* http://www.gr.nl/pdf.php?ID=1034

tocopherol and diluted to specification (20% lycopene). The lycopene formulation was added to the diet of male and female Wistar rats (20 rats/sex/group) at dietary levels of 0, 0.25, 0.5, or 1.0% lycopene. Although rats from all treated groups showed reddish discoloration of the contents of the gastrointestinal tract, discoloration of organs, including gut tissue and liver, was not observed in this study. The NOAEL is 1.0%, equivalent to about **586 mg/kg bw/day for males** and **616 mg/kg bw for females (average 601 mg/kg bw/day)**.

Also reported in the E.U. application are results from genotoxicity studies on a 20% cold water dispersion of lycopene. The product tested negative in the Ames assay and *in vitro* chromosome aberration assay. No details are provided.

E. Tomato Oleoresin (Lyco-Red Natural Product Industries)

Safety studies are summarized in Matulka *et al.* (2004).[12] These studies are of particular relevance because LycoRed's tomato oleoresin product is the precursor to San-Ei Gen's tomato lycopene. Therefore, the impurity profile should be similar. In contrast to the lycopene products discussed above, LycoRed's product contains small amounts of other carotenoids, such as phytoene, phytofluene, and beta-carotene. The product used in the toxicity studies was obtained by crushing food-processing tomatoes to form a pulp, followed by extraction with ethyl acetate. After evaporation of the solvent, the product was standardized by batch mixing to provide about 5.8% lycopene. The commercial product is known as Lyc-O-Mato®. FDA had no questions concerning a GRAS Notification (GRN No. 156) for this substance, submitted to FDA by LycoRed under the name "Tomato lycopene extract."

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[12] Matulka, R.A., Hood, A.M., and Griffiths, J.C. (2004). Safety evaluation of natural tomato oleoresin extract derived from food-processing tomatoes. *Reg. Toxicol. & Pharmacol.* **39,** 390-402.

1. Thirteen-Week Study

In a 13-week study, male and female CD rats (Charles River) (20 rats/sex/group) received by gavage 0, 45, 450, or 4500 mg/kg bw/day of a tomato extract containing an average of 5.8% lycopene, as described above. Other than an increased incidence of orange-stained feces, there were no treatment-related effects. Analysis of blood for lycopene concentrations after 13 weeks of treatment found that plasma levels of lycopene were similar in males given 450 or 4500 mg/kg bw/day, although a lower level was found in males given 45 mg/kg bw/day. These results imply that saturation occurs at a level between 45 mg/kg bw/day and 450 mg/kg bw/day. Under the test conditions, the NOAEL was 4500 mg/kg bw/day, **270 mg/kg bw/day as lycopene**.

2. Bacterial Gene Mutation

LycoRed's tomato oleoresin did not increase the numbers of revertant colonies of *S. typhimurium* strains TA100, TA1535, TA98, TA1537, and TA1538, and *E. coli* strain WP2uvrA- with or without metabolic activation at levels up to 5000 μg/plate.

3. Acute Oral Toxicity

The LD_{50} of the product in Sprague Dawley rats was >5 g/kg bw.

4. Dermal Studies

The acute dermal LD_{50} in Sprague Dawley rats was > 2 g/kg bw. Six separate dermal irritation studies were performed on New Zealand White rabbits. A product containing 5% lycopene was found to be irritating to the skin in several of the studies. It is thought that these results were due to partial fermentation of the tomato oleoresin, which was eliminated in later products by raising the pH. Similar results were obtained in Dunkin-Hartley guinea pigs in the skin maximization test. A product manufactured before the change in manufacturing process did show sensitization, whereas a product manufactured after the change was not sensitizing.

F. ADI from Rat Feeding Studies

Application of a safety factor of 1000 to the highest NOAEL of 601 mg/kg bw/day, gives an ADI of 0.6 mg/kg bw/day. This is equivalent to **36 mg/day** for a 60 kg person.

G. Human Studies

A number of clinical studies have been performed on lycopene or lycopene-containing products, including Lyc-O-Mato®. The objective of many of these studies was to examine the effect of lycopene on prostate cancer. Lycopene levels varied up to 120 mg/p/day. The only adverse effects observed were occasional gastrointestinal side effects that may not have been treatment related.

Studies have been summarized in Appendix B of LycoRed's EU application.[13] In one as yet unpublished study (Hall *et al.*, 2004), 36 prostate cancer patients were treated with escalating doses of lycopene (as tomato oleoresin, probably Lyco-O-Mato®) for 12 months. Doses began at 15 mg lycopene and were increased to 120 mg lycopene. Attainable serum levels were not significantly different among patients over the dose range, consistent with animal studies and other human studies which show lycopene saturation. One patient was taken off the study due to diarrhea. Otherwise, there were no adverse effects.

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The foregoing information considered, it is respectfully submitted that, under the intended conditions of use, San-Ei Gen's tomato lycopene is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because it is generally recognized as safe.

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[13] http://www.food.gov.uk/multimedia/pdfs/lycotox.pdf

ATTACHMENTS

1. Stability of Lycopene as a "Lycopene Base No. 35198" Formulation Effected by Vitamin C in Beverages (December 2, 2004).

2. Anaylsis of Remaining Solvent in Tomato Lycopene (March 23, 2005).

3. Confirmation on *Trans-/Cis-* Lycopene Shifts (February 21, 2002).

4. Comparison Analysis of Four (4) Lycopene Product Compositions (June 25, 2004).

5a. Analysis of Specifications on Lycopene (June 10, 2004).

5b. Nutritional Analysis of Lycopene (July 12, 2004).

ARTICLES

Christian, M.S., Schulte, Stefan, and Hellwig, Jürgen (2003). Developmental (embryo-fetal toxicity/teratogenicity) toxicity studies of synthetic crystalline lycopene in rats and rabbits. *Food and Chem. Toxicology.* **41**, 773-783.

Jonker, D., Kuper, C.F., Fraile, N., Estrella, A., and Otero, C. Rodriguez (2002). Ninety-day oral toxicity study of lycopene from Blakeslea trispora in rats. *Reg. Toxicology and Pharmacol.* **37**, 396-406.

Matulka, R.A., Hood, A.M., and Griffiths, J.C. (2004). Safety evaluation of a natural tomato oleoresin extract derived from food-processing tomatoes. *Reg. Toxicology and Pharmacol.* **39**, 390-402.

McClain, R. Michael and Bausch, Jochen (2002). Summary of safety studies conducted with synthetic lycopene. *Reg. Toxicology and Pharmacol.* **37**, 274-285.

Mellert, W., Deckardt, K., Gembardt, C., Schulte, S., Van Ravenzwaay, B., and Slesinski, R.S. (2002). Thirteen-week oral toxicity study of synthetic lycopene products in rats. *Food and Chem. Toxicology.* **40**, 1581-1588.

Study Report LY009
December 2, 2004

Stability of lycopene as a "Lycopene Base No. 35198" formulation effected by vitamin C in beverages.

Dec. 2, 2004

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Phone : 81 6 6333 0521

Laboratory:

Name : Emulsion Laboratory Division
San-Ei Gen F.F.I.,Inc.

Address : 1-1-11,Sanwa-cho, Toyonaka, Osaka 561-8588

Study Period : April 18, 2004 to November 22, 2004

Study Director : Norihiko Inada
Emulsion Laboratory Division.

Study Coordinator : Ryoko Irifune
Emulsion Laboratory Division.

Study Title:

Stability of lycopene as a "Lycopene Base No.35198" formulation effected by vitamin C in a beverage.

1. Materials and Methods

1-1. Materials

Lycopene Base NO.35198 (lycopene content 2.5%) LOT 020122 was used.

Compositions:

Tomato lycopene	2.65 % (lycopene purity: 94 %)
Glycerol	45 %
High fructose corn syrup	14.5 %
Glycerol esters of fatty acids	4 %
Sucrose esters of fatty acids	2 %
Ethanol	0.3 %
Water	31.55 %

1-2. Method

Five (5) carbonated beverages were prepared according to the following formulation and the beverages were stored under given conditions and its lycopene content was analyzed in certain intervals.

1-2-1. Five (5) beverage recipes: (w/w)

		VC 0	VC 50ppm	VC 100ppm	VC 150ppm	VC 200ppm
①	High fructose corn syrup	14.0	14.0	14.0	14.0	14.0
	Citric Acid	0.12	0.12	0.12	0.12	0.12
	Ascorbic Acid	0	0.005	0.01	0.015	0.02
	Water	10.855	10.85	10.845	10.84	10.835
②	Lycopene base NO35198	0.025	0.025	0.025	0.025	0.025
③	Carbonated water (3.5kg/cm^2)	75	75	75	75	75
	Total	100	100	100	100	100

1-2-2. Preparation of beverages:

Preparation was made initially by dissolving ①, and then adding ②, and the solutions were mixed for 20 min on a magnetic stirrer at 1000rpm. Then, the solutions were heated with stirring in a stainless steel container, and cooled in water until the temperature reached 95℃. After adding distilled water to the solution to make 25 g weight, this 25g solution was placed in a 100ml-glass bottle and 3.5Kg/cm$_2$ of carbonated water was added to the solutions to make a 100 g beverage.

1-2-3. Storage of beverages: 104

Beverages prepared above were stored at 40℃, 25℃, and 5℃ for 3 or 7 months, and evaluated on lycopene content. Other beverages were irradiated with Fade Meter immediately after preparation in the 3 or 7 months storage and evaluated on lycopene content. The irradiation conditions were explained at the next section.

1-3. Conditions of Fade Meter irradiation:

Sample:	Beverage immediately after preparation was used as a sample.
Device:	Xenon long-life Fade Meter XWL-75R (SUGA Test Instruments)
Irradiation intensity:	600 W/m^2
Irradiation temperature:	20℃
Irradiation time:	20 hours
Accumulated illuminance:	1056 Langley (ultraviolet + visible light)(300 - 700nm)

1-4. Analysis of lycopene content:

A 200g of beverage was weighed accurately and a given amount of salt and acetone were added, then lycopene was extracted with n-hexane. The n-hexane layer was collected, washed and dehydrated. Further, n-hexane was added to fill up to 100ml and then the solution was used as a test

solution (1).

Absorbance (A) of the test solution (1) at 470nm was determined by referring n-hexane as a control, and lycopene content in the beverage was calculated according to the following formula.

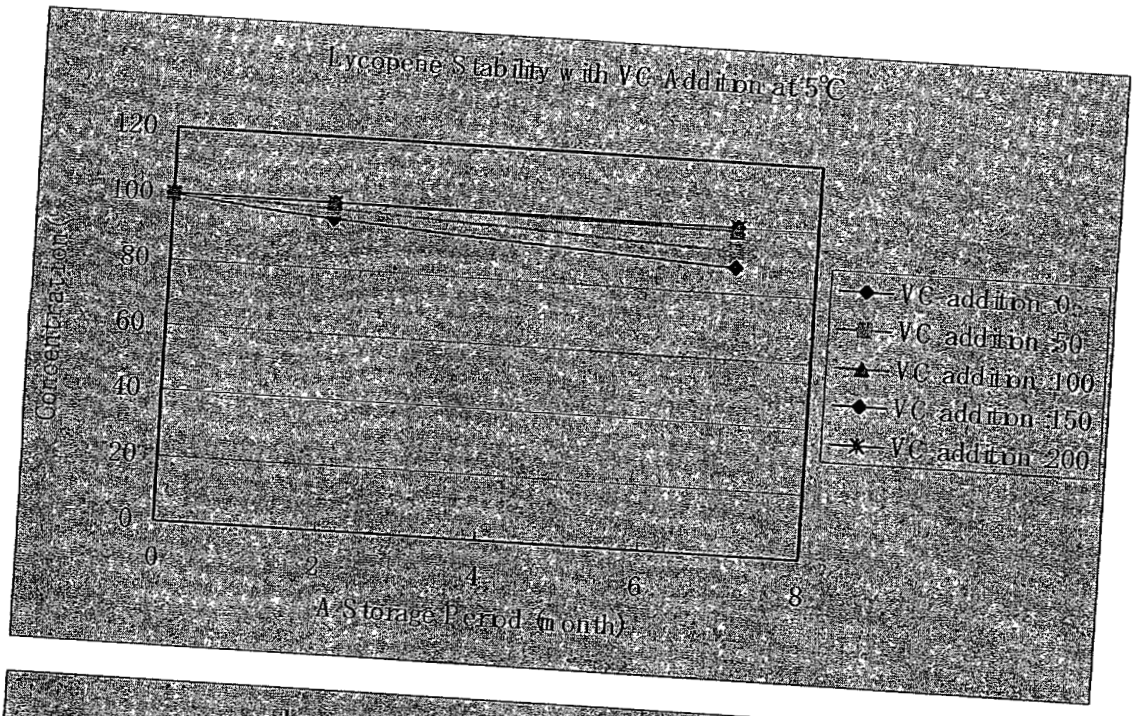
Lycopene content in beverage(μ g/g) = (Absorbance(A) $\times$ 100 $\times$ 10^4) / (3450 $\times$ weighed sample(g))

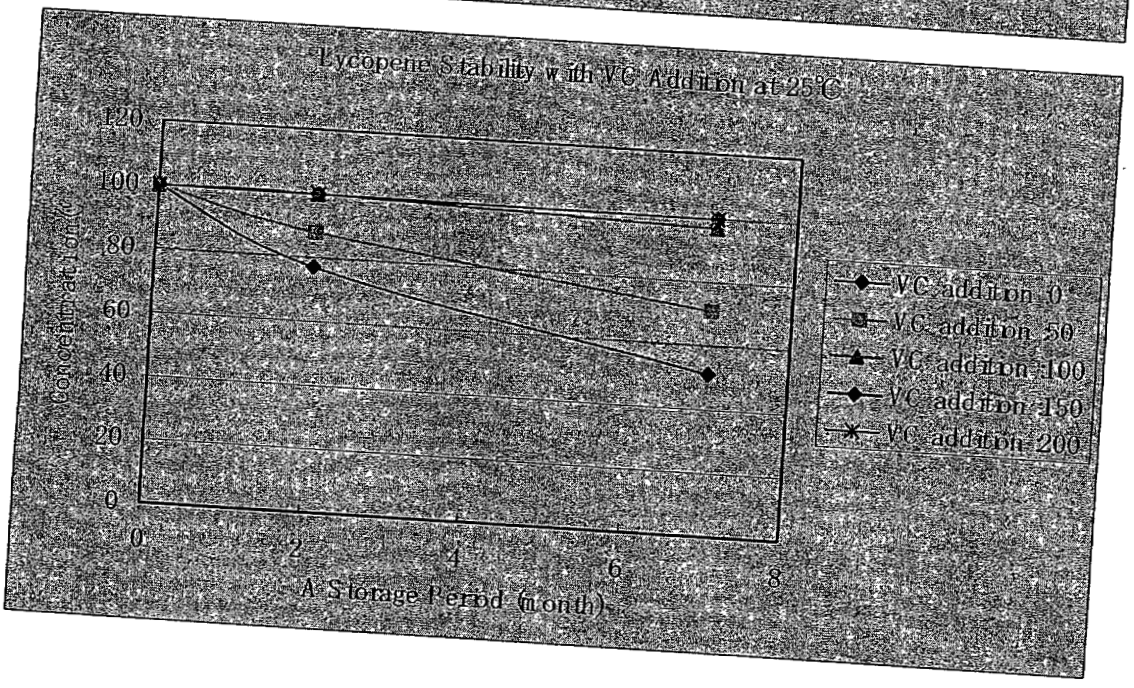
2. Results

2-1. Lycopene stabilities in the Lycopene Base NO.35198 with addition of Vitamin C in a beverage.

VC Addition	Storage Temperature	Lycopene Concentration (ppm)				
		0	3 months	Stability	7 months	Stability
0	5 ℃	6.25	5.94	95%	5.50	88%
	25 ℃		4.81	77%	3.19	51%
	40 ℃		2.75	44%	1.44	23%
50ppm	5 ℃	6.24	6.12	98%	5.80	93%
	25 ℃		5.49	88%	4.43	71%
	40 ℃		4.06	65%	2.93	47%
100ppm	5 ℃	6.25	6.25	100%	6.19	99%
	25 ℃		6.26	100%	6.06	97%
	40 ℃		6.13	98%	4.38	70%
150ppm	5 ℃	6.25	6.26	100%	6.25	100%
	25 ℃		6.24	100%	6.26	100%
	40 ℃		6.24	100%	6.25	100%
200ppm	5 ℃	6.24	6.25	100%	6.24	100%
	25 ℃		6.25	100%	6.25	100%
	40 ℃		6.24	100%	6.25	100%

* Stability (%) = (Lycopene Conc. (ppm) after storage／Lycopene Conc. (ppm) at preparation) $\times$100





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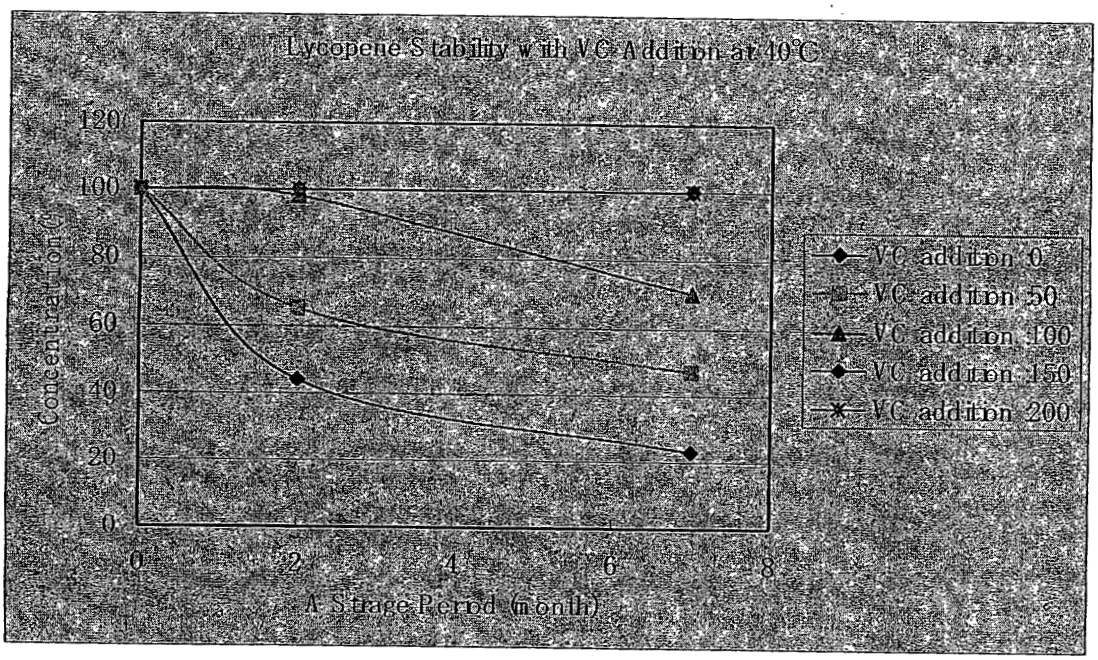

2-2. Lycopene stabilities in the Lycopene Base NO.35198 with addition of Vitamin C in a beverage under Fade Meter irradiation.

VC Addition	Lycopene Conc. (ppm)	Stability (%)
0	1.50	24
50ppm	3.69	59
100ppm	5.81	93
150ppm	6.26	100
200ppm	6.25	100

＊ Stability (%) ＝ (Lycopene Conc. (ppm) after iiradiation／Lycopene Conc. (ppm) at preparation) ×100

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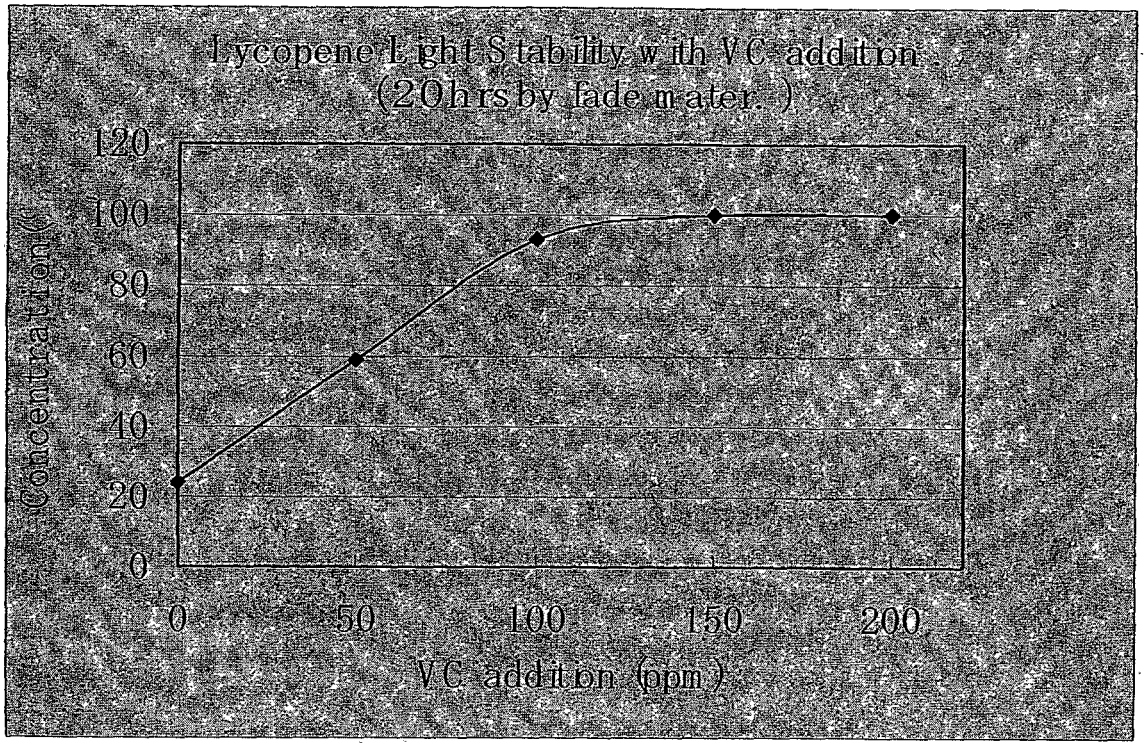

3. Considerations

It was observed that the lycopene contents in beverages declined during its storage and the addition of vitamin C would make lycopene more stable, the higher temperature would make lycopene the less stable and lighting would make lycopene less stable. The addition of vitamin C with 150ppm or more kept the lycopene content at 100 % under the conditions of 5, 25 and 40 ℃, and with light for 7 months. It is concluded that the addition of vitamin C with 150 ppm or more in a formulation is necessary to keep lycopene stable for 7 months at least.

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2

Study Report

Analysis of remaining solvent in Tomato Lycopene

March 23, 2005

San-Ei Gen F.F.I., Inc.

Emulsion Laboratory

000031

Study Purpose : Analysis test was conducted to confirm the content of the remaining solvent (ethyl acetate and ethanol) in Tomato Lycopene

Study Location :

San-Ei Gen F.F.I., Inc.
Emulsion Laboratory and Quality Assurance Laboratory
1-1-11, Sanwa-cho, Toyonaka, Osaka

Date:

Study Period : March 10, 2005 to March 23, 2005
Analysis Period : March 15, 2005 to March 16, 2005
Reporting date March 23, 2005

Study coordinator :

Name : Norihiko Inada
Emulsion Laboratory San-Ei Gen F.F.I., Inc.

Study Operator :

Name : Norihiko Inada
Emulsion Laboratory San-Ei Gen F.F.I., Inc.

Name : Shinichiro Suko
Quality Assurance Laboratory San-Ei Gen F.F.I., Inc.

Tomato Lycopene	Lot 040823
Tomato Lycopene	Lot 041109
Tomato Lycopene	Lot 050307

2. method

2-1 Measurement method of the Lycopene content

Lycopene content (%) in the materials was measured according to the following procedure. Measurement was conducted three times for the same materials, and the mean value of measurements was calculated

Weigh exactly 0.1g of the Tomato Lycopene and dissolve it by adding a small amount of chloroform, and then fill up to 50ml with additional chloroform.
Withdraw 2ml of this liquid and fill up to a 50ml solution by adding n- hexane.
Withdraw further 1ml of this solution and fill up to 50ml by adding n-hexane.
Measure the absorbance of this solution as a test solution at the maximum absorption wavelength around 470nm at the depth of 10nm solution layer by regarding n-hexane as a reference, and calculate the Lycopene content according to the following formula.

$$\text{Lycopene content (\%)} = \frac{\text{absorbance} \times \text{dilution magnification}}{3450^{\text{note 1)}} \times \text{weighed material (g)}}$$

Note 1) The value of 3450 is an absorbance coefficient of Lycopene in n-hexane(10%E).

Equipment: Ultraviolet and Visible Spectrophotometer V-550-DS (JASCO International Co, Ltd)

2-2 Measurement method of remaining solvent

Gas chromatography analysis (GC) was performed on the remaining solvent(ethanol and ethyl acetate) in the Tomato Lycopene by using a flame ionization detector(FID) according to the following method.

2-2-1 Preparation of standard solution of ethanol and ethyl acetate

(1) Weigh exactly about 0.5g of tetrahydrofuran (THF), and add toluene to fill up to a 100ml solution(5,000ppm).
(2) Weigh exactly 2ml of this solution and fill up to 100ml by adding toluene (internal standard solution.(100ppm)).
(3) Weigh exactly about 0.5g of ethanol and ethyl acetate, and fill up to a 100ml solution with toluene (standard concentrate solution 1 (5,000ppm)).
(4) Weigh exactly 2ml of this solution, and fill up to a 100ml solution by adding toluene (standard concentrate solution 2(100ppm)).
(5) Weigh exactly 2ml of standard concentrate solution 1 and 2.5ml of internal standard solution, and fill up to a 50ml solution by adding toluene(standard solution 200ppm).
(6) Weigh exactly 1ml of standard concentrate solution1and 2.5ml of internal standard solution, and fill up to a 50ml solution with toluene (standard solution 100ppm).
(7) Weigh exactly 10ml of standard concentrate solution2 and 2.5ml of internal standard solution, and fill up to a 50ml solution with toluene(standard solution 20ppm).
(8) Weigh exactly 2.5ml of standard concentrate solution 2 and 2.5ml of internal standard

(9) Weigh exactly 1ml of standard concentrate solution2 and 2.5ml of internal standard solution, and fill up to a 50ml solution with toluene (standard solution 2ppm).

2-2-2 Preparation of test solution

(1) Weigh exactly about 5g of Tomato Lycopene and pour it into a recovery flask with several boiling stones added, and then add 100ml of toluene and mix it well.
(2) Connect the recovery flask to a distillation apparatus.
(3) Weigh exactly 2.5ml of internal standard solution and pour it into a volumetric flask.
(4) Heat the recovery flask, and transfer the distilled liquid into the volumetric flask(3).
(5) Discontinue distillation when about 45ml of the distilled liquid is obtained, and fill up to a 50ml solution with toluene and use it as a test solution.
(6) Analyze each standard solution and test solution by the following conditions.

2-2-3 GC analysis condition

GC and FID	: 6890N(Agilent)
Column	: HP-PLOT Q (inside diameter 0.53mm, length 30m, film thickness 40 μ m, Agilent)
Injection volume	: 1 μ l
Inlet temperature	: 250℃
Column temperature	: 150℃ - 3℃/min - 185℃
Injection method	: Direct injection
Detector temperature	: 250℃
Carrier gas	: He
Flow rate	: Flow rate was adjusted to ensure the retention time of internal standard solution to be about 4 to 5 minutes.

2-2-4 Quantitative calculation

Calibration curve was made by calculating the ratio of peak area of ethanol and ethyl acetate of each standard solution to that of THF. Contents of ethanol and acetic ether were obtained according to the calibration curve.

3. Result

3-1 Lycopene content

Material	Lot	Lycopene content(%)				
		First	Second	Third	Mean value	Standard deviation
Tomato Lycopene	40823	95.54	95.29	95.2	95.34	0.176
	41109	97.22	96.92	97.31	97.15	0.204
	50307	96.91	96.79	96.67	96.79	0.120

Excel(Microsoft Excel 2000) was used for all data processing.
Four significant digits were described in table. The mean value was rounded to the nearest four significant digits, and the standard deviation was rounded to the nearest thousandth.
The mean value of three lots of the material was 96.4%.

3-2 Remaining solvent

material	Solvent	Lot	Concentration of remaining solvent(μg / g)				
			First	Second	Third	Mean value	Standard deviation
Tomato Lycopene	Ethanol	040823	231	233	226	230	3.61
		041109	252	267	249	256	9.64
		050307	388	402	371	387	15.52
	Ethyl acetate	040823	1395	1347	1307	1350	44.06
		041109	1317	1296	1337	1317	20.50
		050307	1918	1801	1900	1873	63.00

Excel(Microsoft Excel 2000) was used for all data processing.
In table, the mean value was rounded to the nearest whole number, and the standard deviation was rounded to the nearest hundredth.
The mean value of three lots of the sample was 291 μg/g for ethanol and 1513 μg/g for ethyl acetate.

000034.001

3

February 21, 2002

Confirmation on *trans*-/*cis*- Lycopene Shifts

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Laboratory:

Name: Emulsion Laboratory, San-Ei Gen F.F.I., Inc.

Address: 1-1-11, Sanwa-cho, Toyonaka, Osaka

Study period:

February 21, 2002

Analysis date:

February 21, 2002

Study coordinator:

Name: Yasushi Sasaki

Emulsion Laboratory, San-Ei Gen F.F.I., Inc.

Address: 1-1-11, Sanwa-cho, Toyonaka, Osaka

Study operator:

Name: Miki Fujii

Emulsion Laboratory, San-Ei Gen F.F.I., Inc.

1. Study title:

Confirmation on *trans-/cis-* Lycopene Shifts

2. Study purpose:

Tomato processing, e.g., heating, is known to increase *cis-* lycopene content. Tomato lycopene was manufactured from Tomat-O-Red (tomato oleoresin) by ethanol washing. Accordingly, *trans-/cis-* lycopene shifts by ethanol and heat treatments was checked by HPLC.

3. Tested sample:

1) Tomato lycopene prepared from Tomat-O-Red (tomato oleoresin)
2) Tomat-O-Red (tomato oleoresin) purchased from LycoRed
3) Tomat-O-Red (tomato oleoresin) that was heated at 140 degree C for one minute

4. Analysis

• HPLC Analysis

About 0.15 g of sample was weighed accurately and added with chloroform to make a 100 ml solution exactly. The solution was sonicated for 5 min, filtered with 0.45 nm membrane PTFE filter, then used.

Column: YMC Carotenoid S5 diameter 4.6 X 250 mm

Column Temperature: 20 degree C

Flow Rate: 1.0 ml/min

Detection Wave Length: UV 470 nm

Injection Volume: 40 micro liter

Mobile Phase: A methanol/t-butylmethylether/water = 81/15/4

B methanol/t-butylmethylether/water = 10/86/4

Gradient:

Time (min)	Mobile Phase	
	A (%)	B (%)
0	100	0
63	30	70
117	0	100
140	0	100

5. Results

1) Content by Absorbance

Carotenoides	Rt (min)	Composition (%)		
		Tomato oleoresin	Tomato lycopene	Tomato oleoresin heated
Not identified	37	0.82	-	1.59
All *trans*-beta-Carotene	44	4.9	1.3	3.70
Not identified	46	0.7	-	1.42
cis-Lycopene	64 – 65	1.6	1.7	13.63
cis-Lycopene	74 – 75	4.9	-	21.74
all-*trans*-Lycopene	86	80	94	30.13
cis-Lycopene	87	7.1	3.1	9.51

As shown in the above table, it was observed that ethanol washing tends to increase a ratio of *trans*-lycopene to *cis*-lycopene and heat treatment should facilitate a shift from *trans*-lycopene to *cis*-lycopene. A LC chart of tomato oleoresin is Fig. 1., that of tomato lycopene, Fig. 2., and that of tomato oleoresin heated at 140 degree C for one minute, Fig. 3. Accordingly, *trans*-lycopene content is the highest in tomato lycopene (San-Ei Gen) and it shows the simplest pattern.

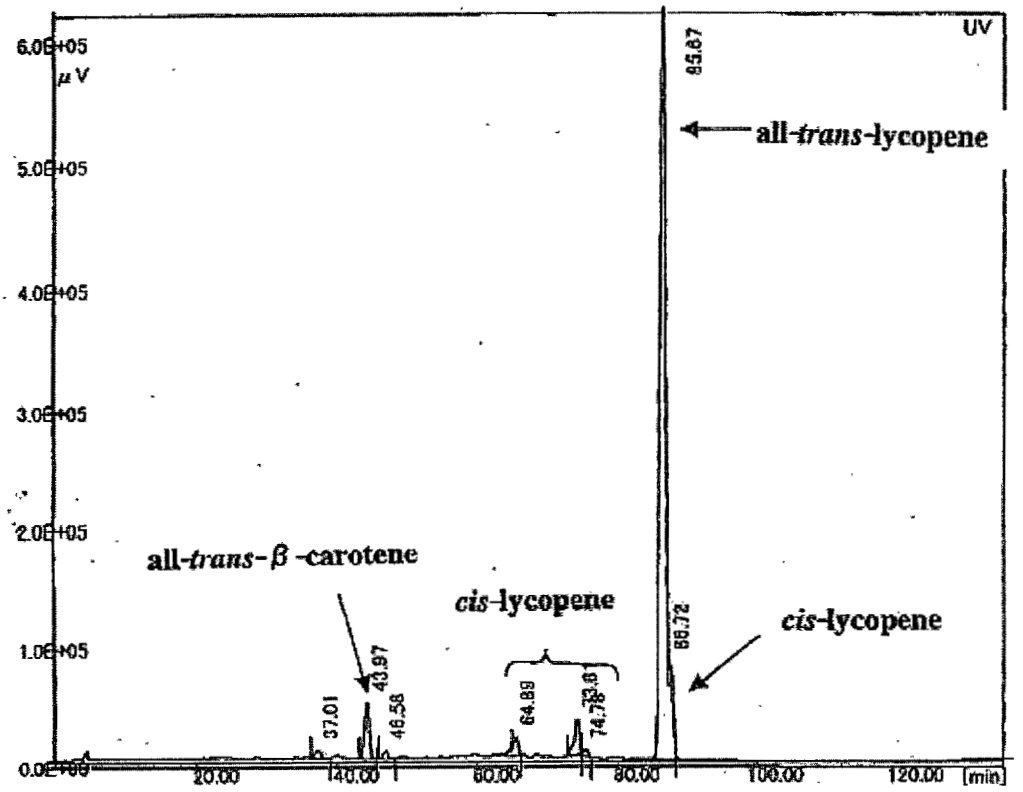

Fig. 1. Tomato oleoresin (LycoRed) LC Chart

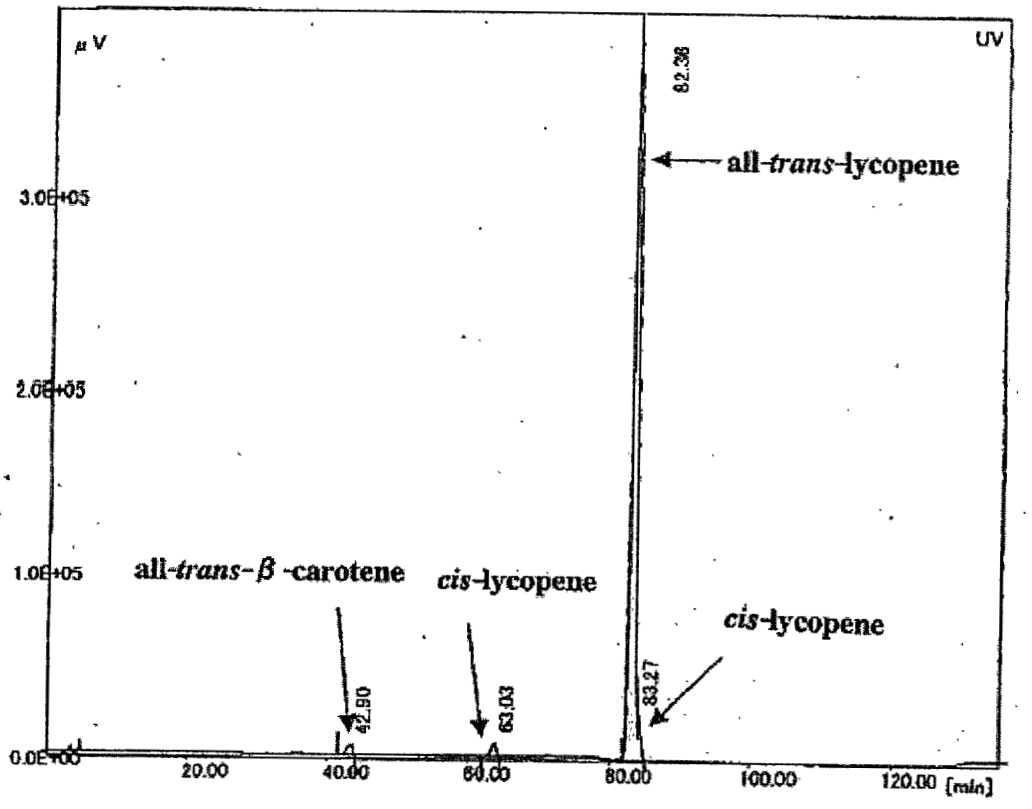

Fig. 2. Tomato lycopene (San-Ei Gen) LC Chart

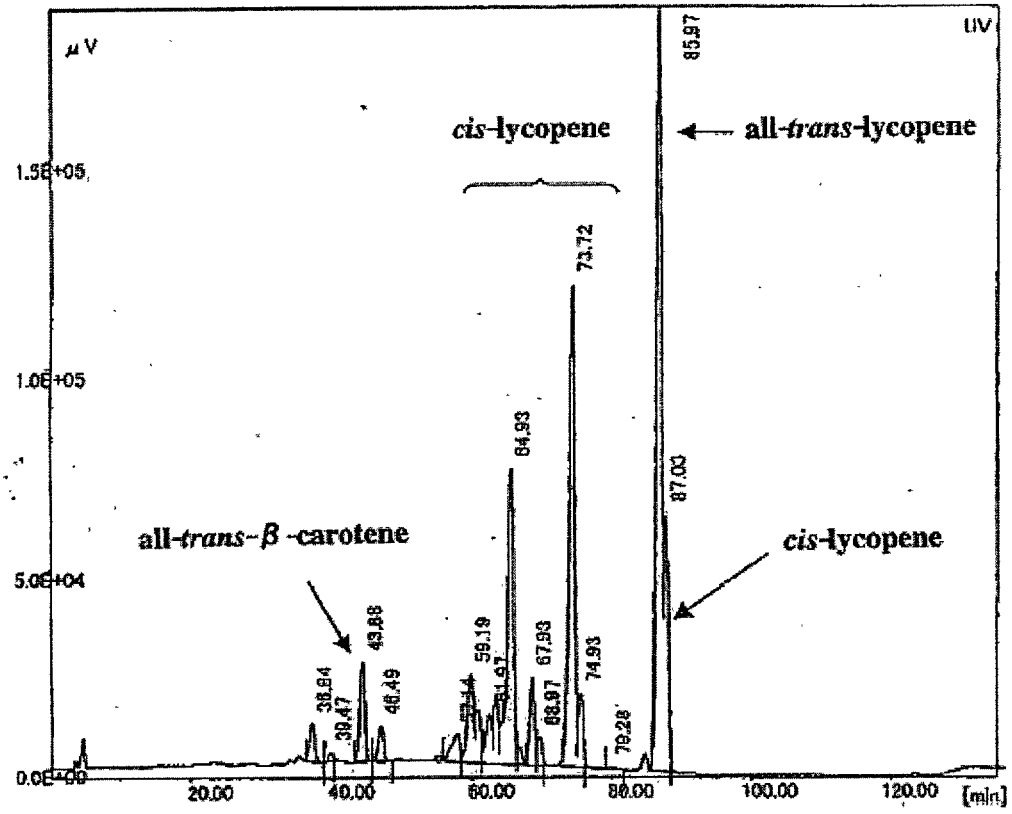

Fig. 3.

Tomato oleoresin (LycoRed; heated at 140 ℃ for one minute) LC Chart

4

Comparison Analysis of Four (4) Lycopene Product Compositions

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Laboratory:

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Address: 1-1-11, Sanwa-cho, Toyonaka, Osaka

Study period:

May 13 to June 19, 2004

Analysis date:

June 16 to June 19, 2004

Study coordinator:

Name: Norihiko Inada

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Address: 1-1-11, Sanwa-cho, Toyonaka, Osaka

Study operator:

Name: Takeshi Miuchi

Emulsion Laboratory, San-Ei Gen F.F.I., Inc.

Study operator:

Name: Shin-ichirou Suko

Quality Assurance Laboratory, San-Ei Gen F.F.I., Inc.

1. Study title:

Comparison Analysis of Four (4) Lycopene Product Compositions

2. Study purpose:

Our tomato lycopene is manufactured from Tomat-O-Red (tomato oleoresin) by ethanol washing (extraction). Compositions of four lycopenes were analyzed with LC/MS and compared, which were tomato lycopene (San-Ei Gen), tomato oleoresin (LycoRed), synthetic lycopene (BASF) and lycopene directly extracted from tomato by n-hexane.

3. Tested sample:

1) Tomato lycopene (San-Ei Gen)
2) Tomato oleoresin (Tomat-O-Red: LycoRed)
3) Synthetic lycopene (Lycopene 10% CWD: BASF)
4) Lycopene extracted from tomato (Raw tomato)

4. Analysis

4.1 Sample preparation

1) Lycopene extracted from tomato (Raw tomato)

Tomato was cut and ground into juice by a mixer and filtrated with a gauze patch. The filtrate was extracted with n-hexane, washed with water and adequately diluted with mobile phase solvent to make a sample solution.

2) Tomato oleoresin (Tomat-O-Red: LycoRed)

0.5 g of the sample was accurately weighed and added with dichloromethane to make a 25 ml solution. One (1) ml of the solution was added with n-hexane to make a 50 ml solution. The solution was adequately diluted with mobile phase solvent to make a sample solution.

3) Synthetic lycopene (Lycopene 10% CWD: BASF)

0.5 g of the sample was accurately weighed and added with water to make a 200 ml solution. 2 ml of the solution was extracted with n-hexane. The extraction was washed with water and adequately diluted with mobile phase solvent to make a sample solution.

4) Tomato lycopene (San-Ei Gen)

0.05 g of the sample was accurately weighed and added with dichloromethane to make a 25 ml solution. One (1) ml of the solution was added with n-hexane to make a 50 ml solution. The solution was adequately diluted with mobile phase solvent to make a sample solution.

4.2 LC-APCI-MS Analysis

Column: Develosil C30-UG-5 (2.0 mm diameter 4.6 X 150 mm length)

Mobile Phase: A methanol/t-butylmethylether/water = 81/15/4

B methanol/t-butylmethylether/water = 10/86/4 (upper layer)

Gradient:

Time (min)	Mobile Phase	
	A (%)	B (%)
0	80	20
40	60	40
50	60	40
51	80	20
60	80	20

Flow Rate: 0.2 ml/min

Temperature: 30 degree C

Detection Time: 60 min

Injection Volume: 20 micro liter

Mode: APCI negative

Corona: 3.5 kV
Cone: 40V
Source Temperature: 150 degree C
Prove Temperature: 400 degree C
Desovation Gas Flow: 500 l/hr
Cone Gas Flow: 50 l/hr
MS Span: m/z 400-600
PDA: 200-600

5. Results

LC/MS chromatograms and MS spectrums on the four lycopenes were shown in Figures. A LC chart of lycopene extracted with toluene directly from tomato is shown in Fig. 1. and a MS chart, in Fig. 2. A LC chart of tomato oleoresin (LycoRed) is shown in Fig. 3. and a MS chart, in Fig. 4. A LC chart of synthetic lycopene (BASF) is shown in Fig. 5. and a MS chart, in Fig. 6. A LC chart of tomato lycopene (San-Ei Gen) is shown in Fig. 7. and a MS chart, in Fig. 8. Four spectrum charts at UV 470 nm on lycopene extracted from tomato, tomato oleoresin (LycoRed), syntheticlycopene (BASF) and tomato lycopene (San-Ei Gen) are shown in Fig. 9.

Generally speaking, all lycopene's charts are almost similar and, around RT 32.80, a main all-trans lycopene peak appears and, at RT 24, 25 and 28, there are isomer or derivative peaks because all the recognized peaks are calculated to be 536 to 537 molecular weight compounds. The tomato lycopene (San-Ei Gen) shows the most simple pattern and it means the purity increased by the ethanol washing and cis-lycopene and beta-carotene are eliminated.

Fig. 1. Lycopene extracted from tomato LC Chart

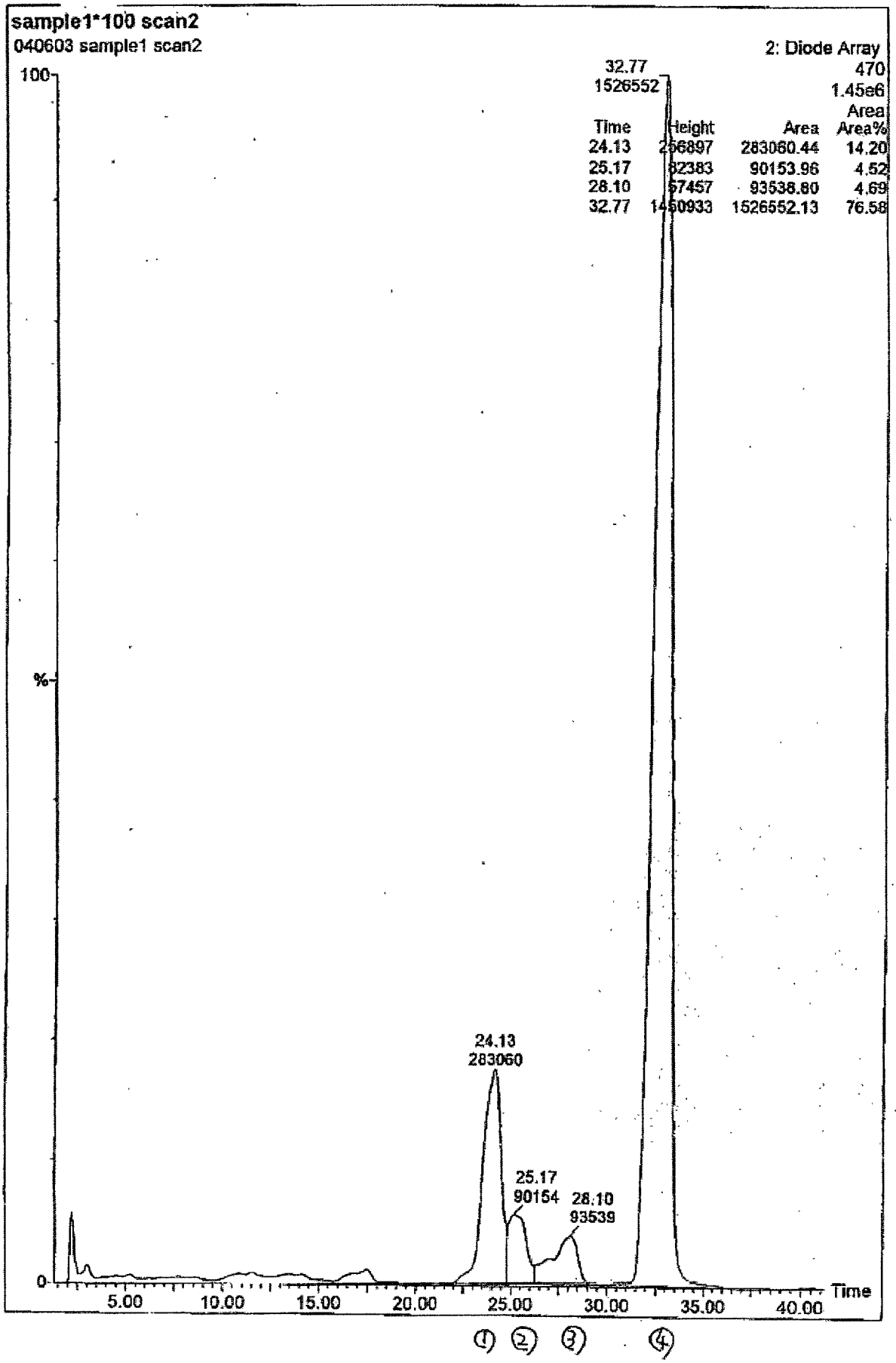


Fig. 2. Lycopene extracted from tomato MS Chart

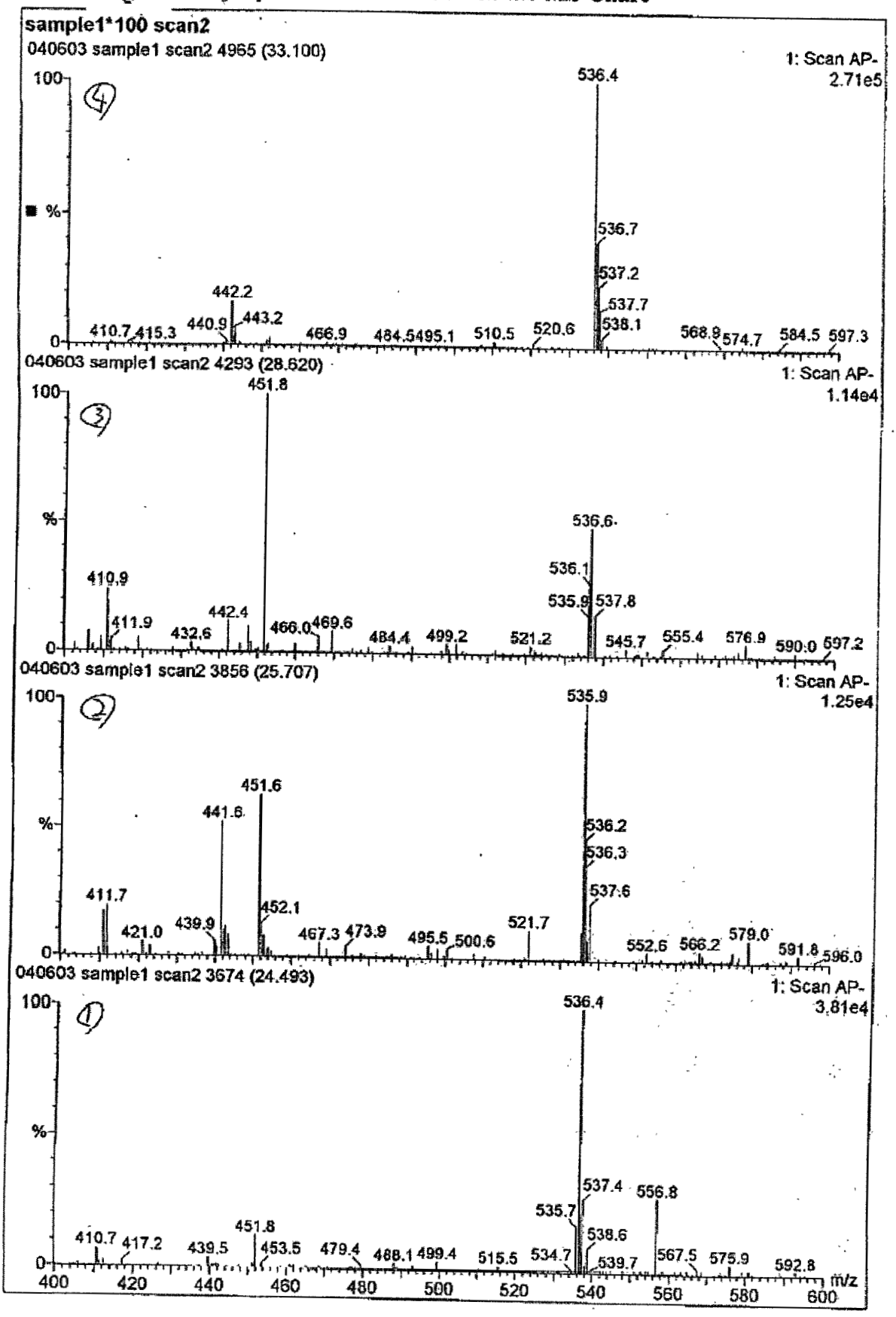


Fig. 3. Tomato oleoresin (Lycored) LC Chart

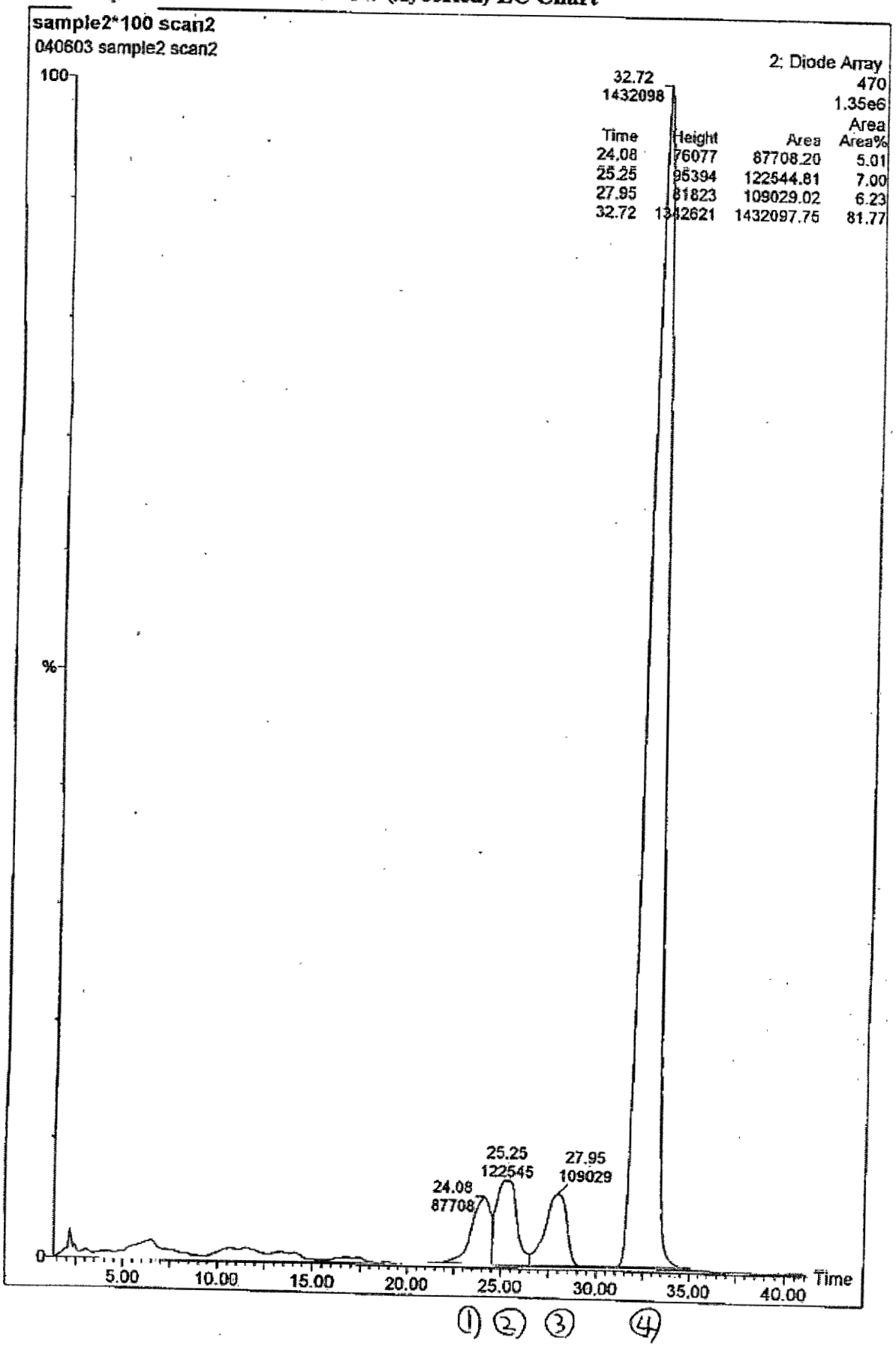


Fig. 4. Iodipamide diatrizoate (Hypaque) MS Chart

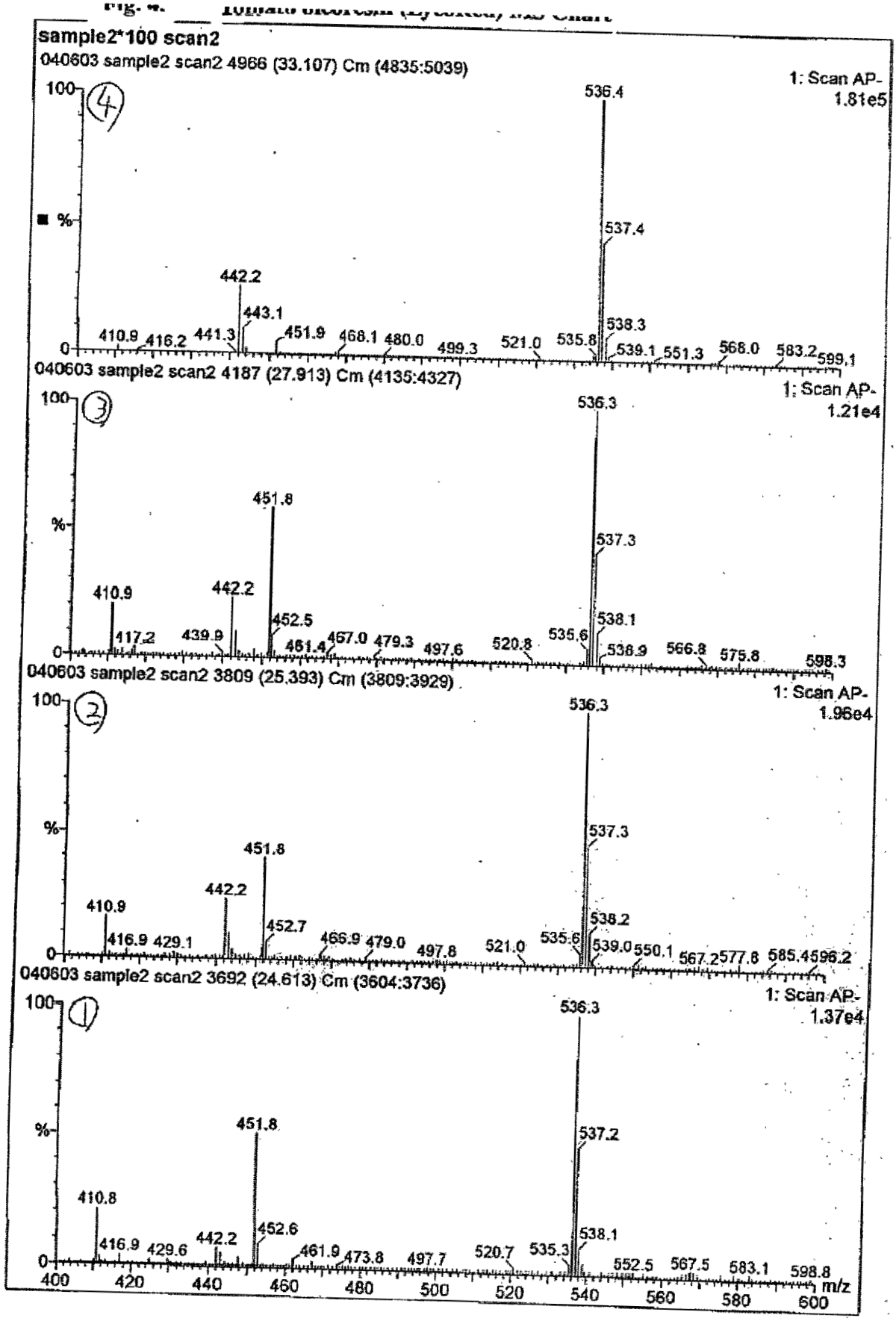


Fig. 3. Synthetic lycopene (BASF) LC Chart

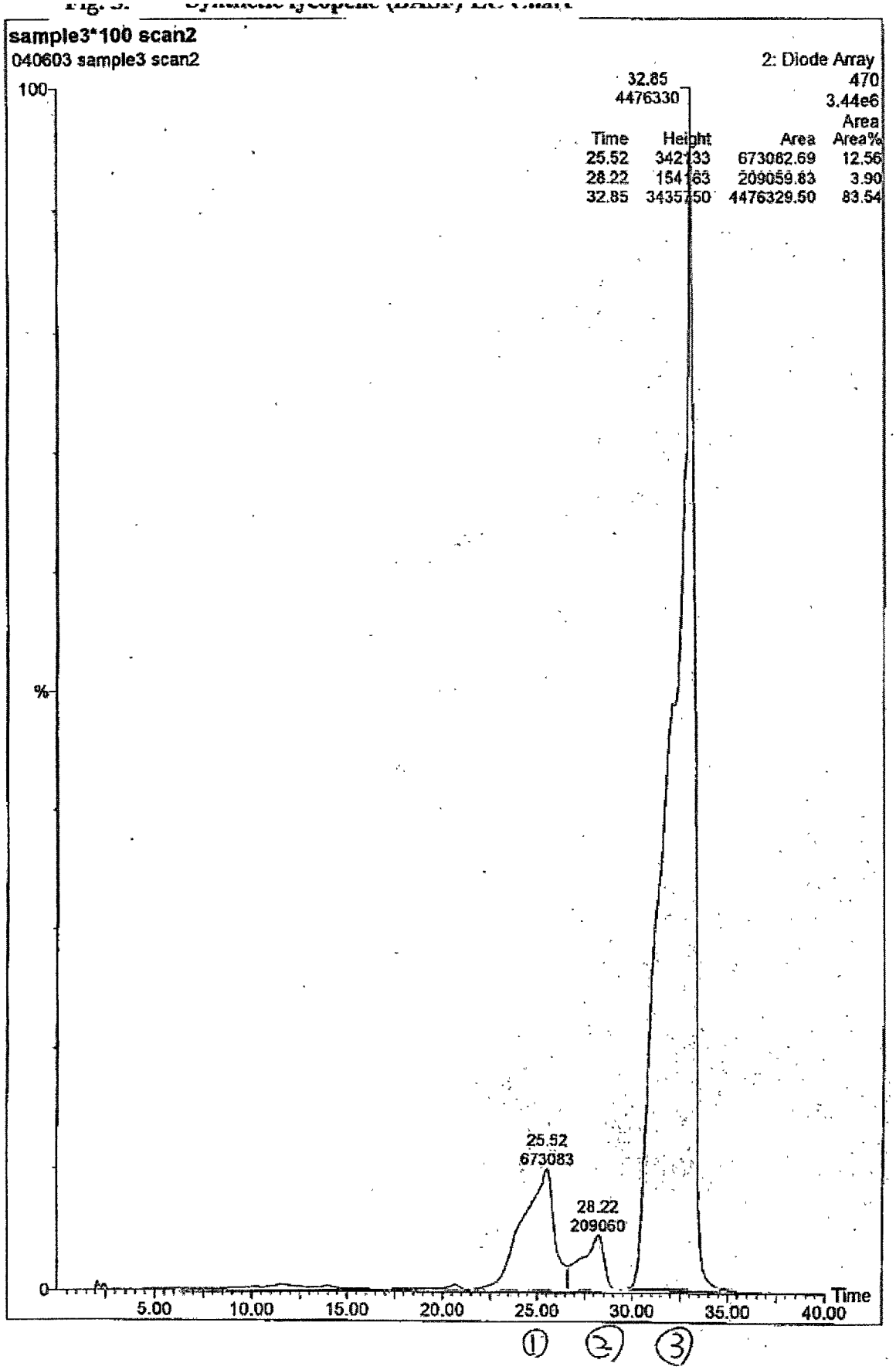


Fig. 6. Synthetic lycopene (BASF) MS Chart

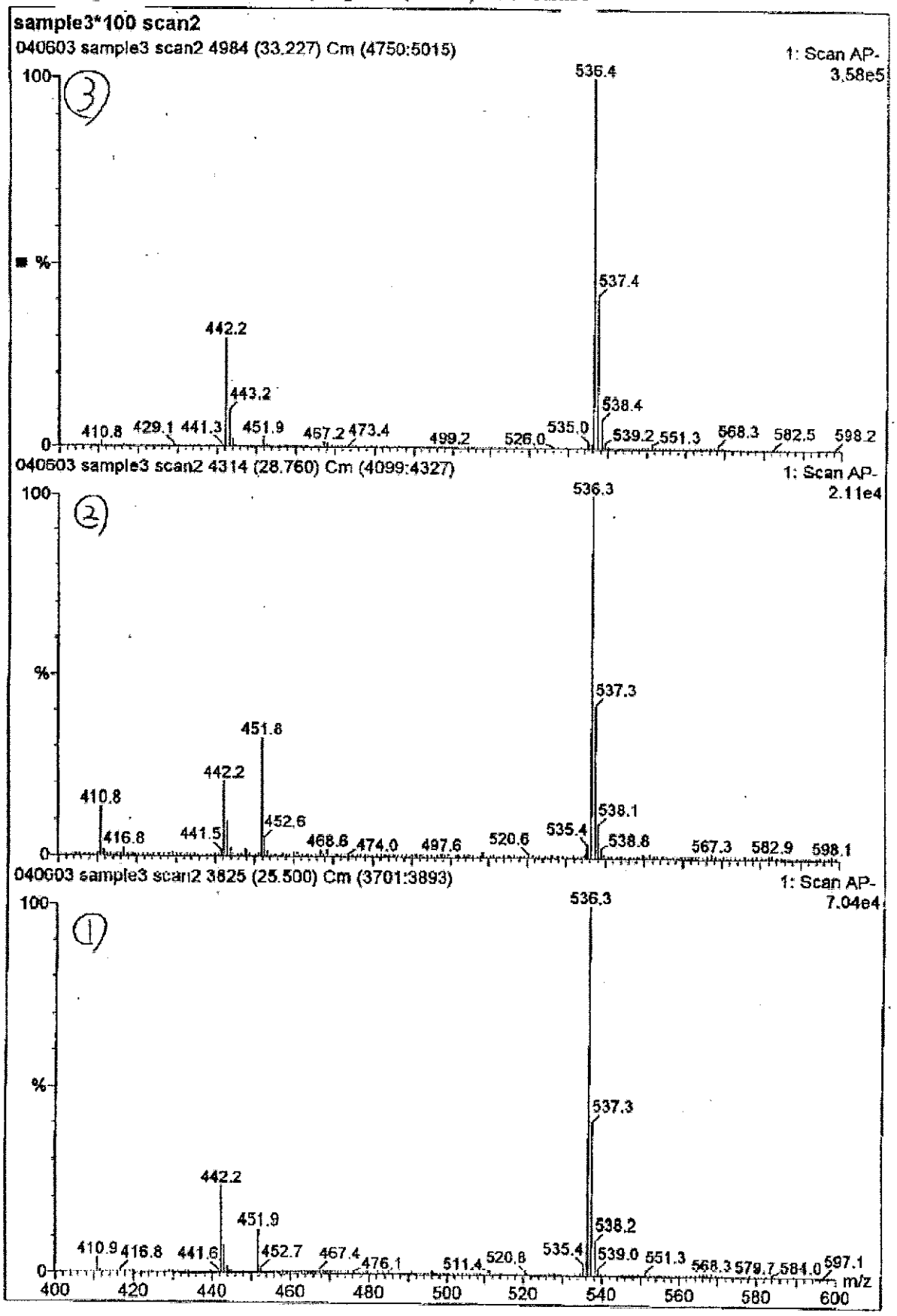


Fig. 7. Tomato lycopene (San-Ei Gen) LC Chart

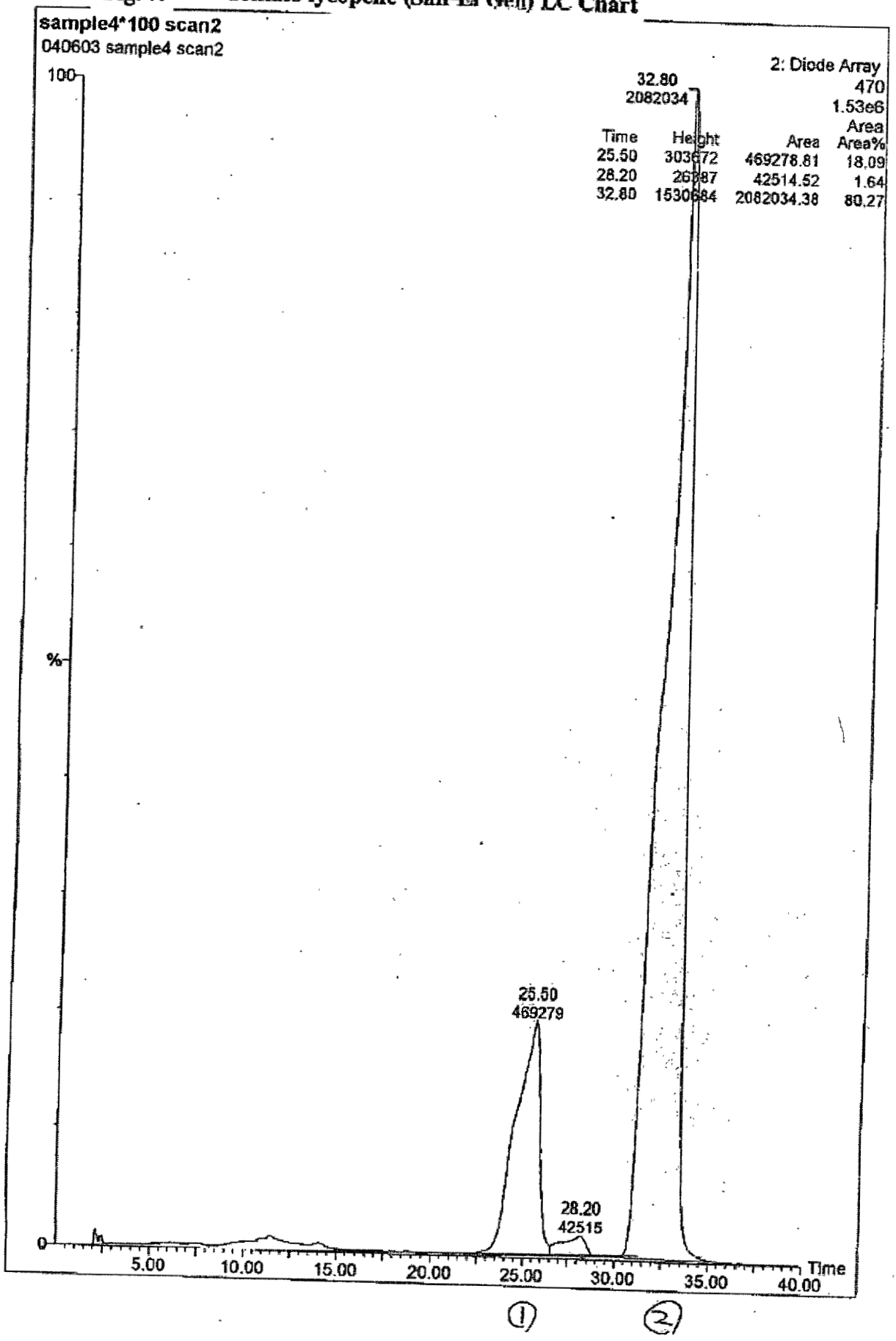
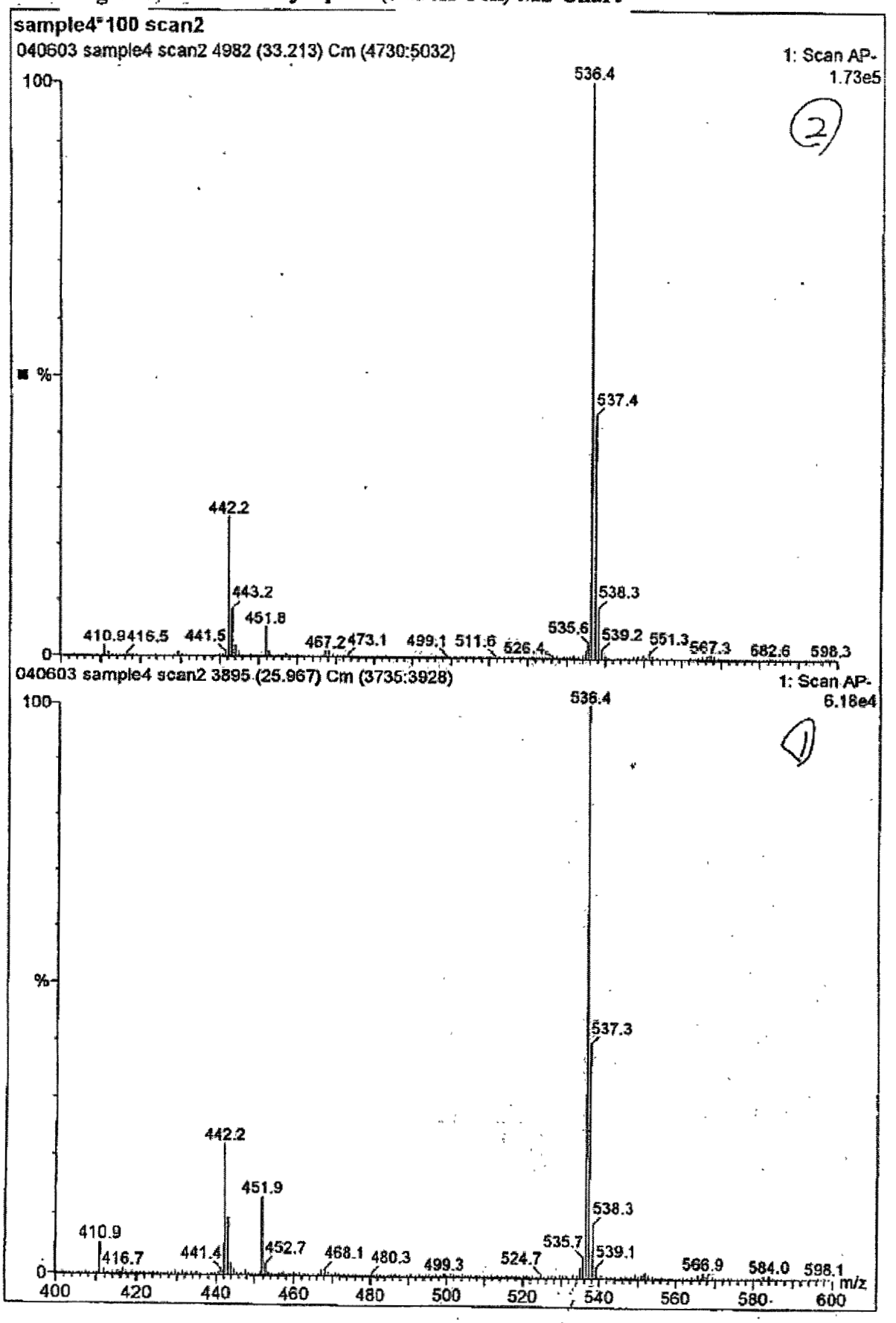
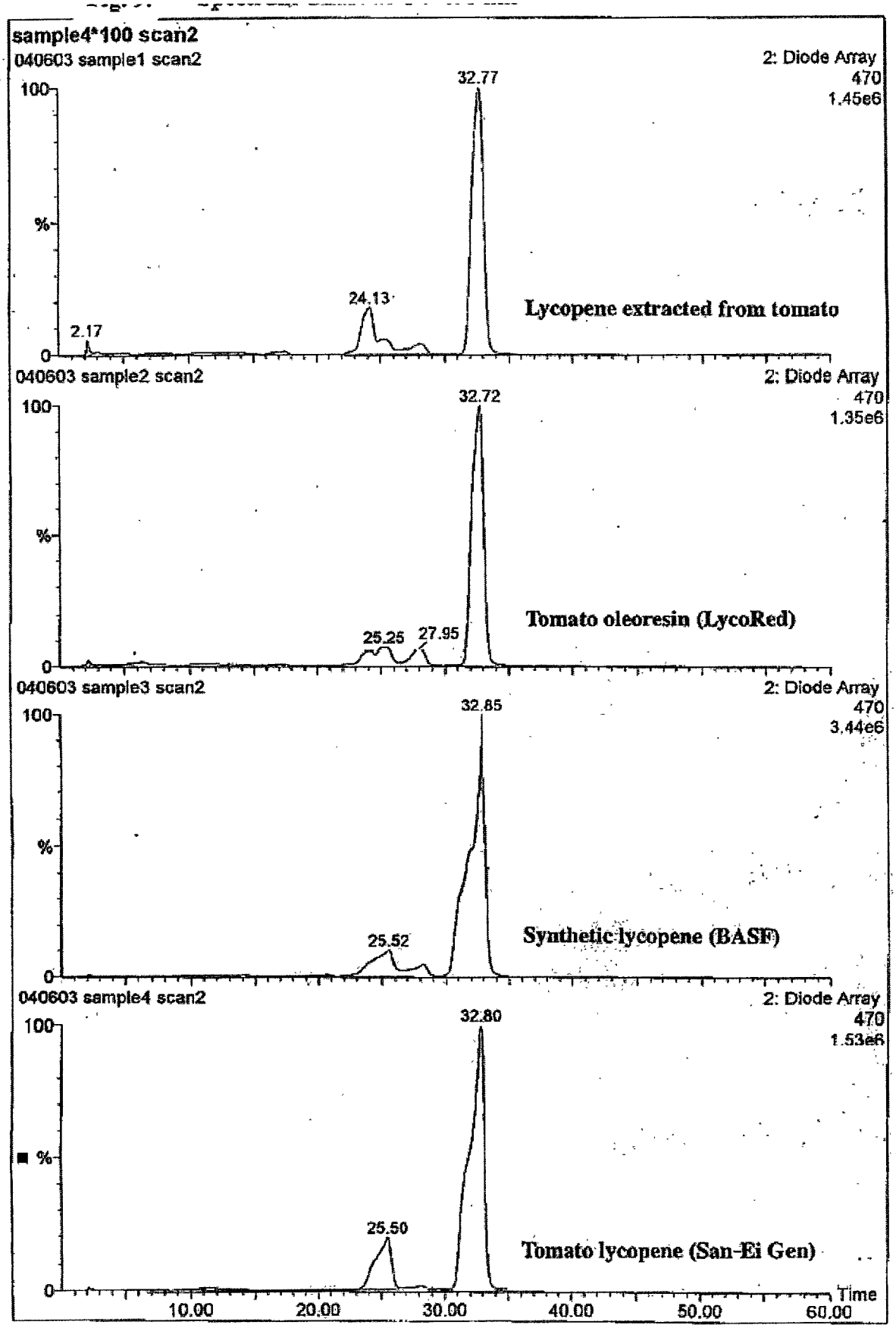


Fig. 8. Tomato lycopene (San-Ei Gen) MS Chart




sample4*100 scan2
040603 sample1 scan2
2: Diode Array
470
1.45e6
32.77
24.13
2.17
Lycopene extracted from tomato
040603 sample2 scan2
2: Diode Array
470
1.35e6
32.72
25.25
27.95
Tomato oleoresin (LycoRed)
040603 sample3 scan2
2: Diode Array
470
3.44e6
32.85
25.52
Synthetic lycopene (BASF)
040603 sample4 scan2
2: Diode Array
470
1.53e6
32.80
25.50
Tomato lycopene (San-Ei Gen)
100
%
0
Time
10.00
20.00
30.00
40.00
50.00
60.00

5

A

June 10, 2004

Analysis of Specifications on Lycopene

Emulsion Laboratory
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Laboratory:

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Address: 1-1-11, Sanwa-cho, Toyonaka, Osaka

Study period:

April 9, 2004 to May 10, 2004

Analysis date:

April 9 to May 11, 2004

Study coordinator:

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Study operator:

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Emulsion Laboratory, San-Ei Gen F.F.I., Inc.

Name: Yasuko Kimura

Emulsion Laboratory, San-Ei Gen F.F.I., Inc.

1. Study Title

Analysis of Specifications on Lycopene

2. Study Purpose

To confirm the accuracy and reproducibility of analysis methods on some specifications designated for lycopene products.

3. Materials

Tomato Lycopene Lot 040506-01

Lot 040506-02

Lot 040510-01

Lot 040510-02

Lot 040510-03

4. Mehods

Every 5 lots were analyzed for the following items in triplicate.

4.1 Assay

About 0.05 g sample was accurately weighed and added with dichloromethane to make a 25 ml solution exactly.

1 ml of the solution above was measured by a volumetric pipette and added with n-hexane to make a

50 ml solution exactly.

2 ml of the solution was measured by a volumetric pipette and added with n-hexane to make a 50 ml solution exactly. It was the sample solution.

The absorbance strength (A) was measured at 470 nm with 10 mm liquid layer length to a control n-hexane solution. The lycopene content was calculated with the following equation:

Content (Lycopene) % = Absorbance strength (A) × 25 × 50 × 25 / (3450 × Sample Weight (g))

4.2 Purity

4.2.1 Lead (Method 1)

Test solution. Weigh the specified quantity of the sample, place into a platinum or quartz crucible, moisten with a small amount of sulfuric acid, and ignite slowly at a temperature as low as possible until the sample is almost incinerated.

Cool, add 1 ml of sulfuric acid, heat slowly, and ignite at 450 degree C to 550 degree C to incinerate.

Dissolve the residue in a small amount of diluted nitric acid (1 -> 150), add diluted nitric acid (1 -> 150) to make 10 ml.

Control solution. Measure 1.0 ml of Lead Standard Solution, add diluted nitric acid (1 -> 150) to make 10 ml.

Test. Measure the atomic absorbances in the flame type method for the test solution and the control solution under the conditions given below. The absorbance of the test solution does not exceed that of control soluiton.

Operating Conditions

Light source: Lead hollow cathode lamp

Analytical line wavelength: 283.3 nm

Supporting gas: Air

Inflammable gas: Acetylene

4.2.2 Arsenic (Method 3, Apparatus C)

Weigh the specified amount of the sample, and place it in a crucible of platinum, quartz, or porcelain.

Add 10 ml of a solution of magnesium nitrate in ethanol (1 -> 50), ignite ethanol, and heat gradually at 450 degree C to 550 degree C to incinerate.

If the carbonized material still remains, moisten with a small quantity of a solution of magnesium nitrate in ethanol (1 -> 50), and ignite again at 450 degree C to 550 degree C to incinerate.

After coolong, add 3 ml of hydrochloric acid, heat on a water bath to dissolve the residue, and use it as the test solution.

To 4 ml of the test solution, add 1 ml of hydrochloric acid and 1 ml of potassium iodide solution (1 -> 10), heat on a water bath at 70 degree C for 4 minutes, and add water to make 20 ml.

Flowing argon gas through the apparatus, introduce the test sample, an appropriate concentration of hydrochloric acid (1 - 6 mol/l), and sodium tetrahydroborate TS into the apparatus continuously at flow

rate of 1 - 10 ml/min with pump A and mix successively in the apparatus to form arsenic hydride.

The method that potassium iodide solution (1 -> 10) is also introduced continuously into the apparatus, introduce the test solution (if necessary, after dilution with water), an appropriate concentration of hydrochloric acid (1 - 6 mol/l), potassium iodide solution (1 -> 10), and sodium tetrahydroborate TS successively into the apparatus in the same procedure as described above.

Arsenic hydride formed is separated from the waste liquid by gas liquid separator F, and gas containing arsenic hydride is introduced into an atomic absorption spectrophotometer (flame type) quipped with an absorption cell.

Measure the atomic absorbance of the test solution at 193.7 nm.

The absorbance of the test solution does not exceed that of the following control solution.

Preparation of the control solution is carried out at the same time as the test solution.

Prepare the control in the same manner as for the test solution, using the specified Arsenic Standard Solution.

4.2.3 Heavy metals (Method 2)

Test solution. Weigh the specified amount of the sample, place inot a quartz or porcelain crucible, cover it loosely, and carbonize by gently heating.

Cool, add 2 ml of nitric acid and 5 drops of sulfuric acid, heat until no white fumes are any longer evolved, and ignite at 450 degree C to 550 degree C to incinerate.

Cool, add 2 ml of hydrochloric acid, evaporate to dryness on a water bath, add 3 drops of hydrochloric acid to the residue, add 10 ml of boiling water, and warm for 2 minutes.

Cool, add 1 drop of phenolphtalein TS, and add ammonia TS until the solution becomes slightly red.

Then, transfer it quantitatively into a Nessler tube using water, add 2 ml of diluted acetic acid (1 -> 20) and water to make 50 ml.

Control solution. Place 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid into a crucible of the same quality as used for the sample, heat to evaporate to dryness, and add 3 drops of hydrochloric acid to the residue.

Then, proceed as directed in the preparation for the test solution, transfer it quantitatively into another Nessler tube, add the specified amount of Lead Standard Solution, 2 ml of diluted acetic acid (1 ->20) and water to make 50 ml.

If the test solution is not clear, filter the both the test solution and control solution under the same conditions.

4.3 Identification

(1) Weigh accurately 0.01g of the sample, dissolve it with 1000 ml ethylacetate. The solution shows orange.

(2) n-Hexane solution of the sample has a maximum absorption at the wavelength of 438 to 450 nm, of 465 to 475 nm and of 495 to 505 nm.

(3) Weigh accurately 0.01 g of the sample, dissolve it with 1000 ml ethylacetate. It is the sample

solution. Take a 5 μl sample solution, develop a thin layer chromatography with a developing solvent of n-hexane (7)/ acetone (3). When the solvent climbs about 10 cm from the baseline, stop the development. Then, yellowish red spot appears around the Rf 0.7 to 0.8. When it is air-dried, sprayed with 5 % sodium nitrite then with 0.5 mol/l sulfuric acid, the spot disappears. The layer whose carrier is a silica gel dried at 100 degree C for one hour is used.

5. Results

5.1 Assay (Lycopene content)

Lot	Lycopene content (%)			
	1	2	3	Averaged
040506-01	96.8	97.7	96.5	97.1
040506-02	96.8	96.8	96.5	96.8
040510-01	96.2	96.5	96.8	96.5
040510-02	97.1	97.1	97.1	97.1
040510-03	97.7)	97.7	97.7	97.7

5.2 Purity

5.2.1 Lead

Lot	Lead (μg/g)		
	1	2	3
040506-01	2 >	2 >	2 >
040506-02	2 >	2 >	2 >
040510-01	2 >	2 >	2 >
040510-02	2 >	2 >	2 >
040510-03	2 >	2 >	2 >

5.2.2 Arsenic

Lot	As_2O_3 (μg/g)		
	1	2	3
040506-01	0.5 >	0.5 >	0.5 >
040506-02	0.5 >	0.5 >	0.5 >
040510-01	0.5 >	0.5 >	0.5 >
040510-02	0.5 >	0.5 >	0.5 >
040510-03	0.5 >	0.5 >	0.5 >

5.2.3 Heavy Metal

Lot	Heavy metal (μg/g)		
	1	2	3
040506-01	5 >	5 >	5 >
040506-02	5 >	5 >	5 >
040510-01	5 >	5 >	5 >
040510-02	5 >	5 >	5 >
040510-03	5 >	5 >	5 >

5.3 Identity

5.3.1 Identity (1)

Lot	Solution Color		
	1	2	3
040506-01	Pass	Pass	Pass
040506-02	Pass	Pass	Pass
040510-01	Pass	Pass	Pass
040510-02	Pass	Pass	Pass
040510-03	Pass	Pass	Pass

5.3.2 Identity (2)

Lot	Maximum absorption wavelength		
	1	2	3
040506-01	444nm, 471nm, 502nm	444nm, 471nm, 502nm	444nm, 471nm, 502nm
040506-02	444nm, 471nm, 502nm	444nm, 471nm, 502nm	444nm, 471nm, 502nm
040510-01	444nm, 471nm, 502nm	444nm, 471nm, 502nm	444nm, 471nm, 502nm
040510-02	444nm, 471nm, 502nm	444nm, 471nm, 502nm	444nm, 471nm, 502nm
040510-03	444nm, 471nm, 502nm	444nm, 471nm, 502nm	444nm, 471nm, 502nm

5.3.2 Identity (3)

Lot	Identity (3)		
	1	2	3
040506-01	Pass	Pass	Pass
040506-02	Pass	Pass	Pass
040510-01	Pass	Pass	Pass
040510-02	Pass	Pass	Pass
040510-03	Pass	Pass	Pass

B

Nutritional Analysis of Lycopene

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Study period:

June 29 to July 7, 2004

Analysis date:

June 29 to July 7, 2004

Study coordinator:

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Study operator:

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1. Study title

Nutrition analysis study of tomato lycopene

2. Purpose

To analyze nutrition of tomato lycopene

3. Sample for analysis

Tomato lycopene Lot 040506-01

Lot 040510-01

Lot 040510-02

4. Method

We measured the following items.

4-1. Water content

Water content was determined according to the Karl Fisher Method in the JAPAN'S SPECIFICATIONS AND STANDARDS FOR FOOD ADDITIVES SEVEN EDITION (JSFA-Ⅶ).

4-2. Ash

Ignite a crucible of porcelain in electric furnace until a constant weight at 600 ℃, allow to cool it in a desiccator, and weigh it accurately. Place 1 g of sample into the crucible and weigh it accurately. Heat it gently first, then raise the temperature gradually, ignite it in electric furnace until a constant weight at 600 ℃, allow to cool it in a desiccator, and weigh it accurately again.

Calculate as below.

$$\frac{\text{Weight of Ash and crucible after ignition} - \text{Weight of crucible}}{\text{Sample weight}} \times 100 = \text{Ash (\%)}$$

4-3. Protein (Nitrogen Determination)

Nitrogen was at first determined by Kjeldhal method. Multiply 6.25 to obtain protein content since the Nitrogen Determination Test is designed to quantify ammonia in ammonium sulfate obtained by decomposing organic substances containing nitrogen with sulfuric acid.

Kjeldahl Method

Apparatus Use the apparatus illustrated in the figure below. Ground-glass surfaces may be used for joints.

Procedure Unless otherwise specified, proceed as directed below.

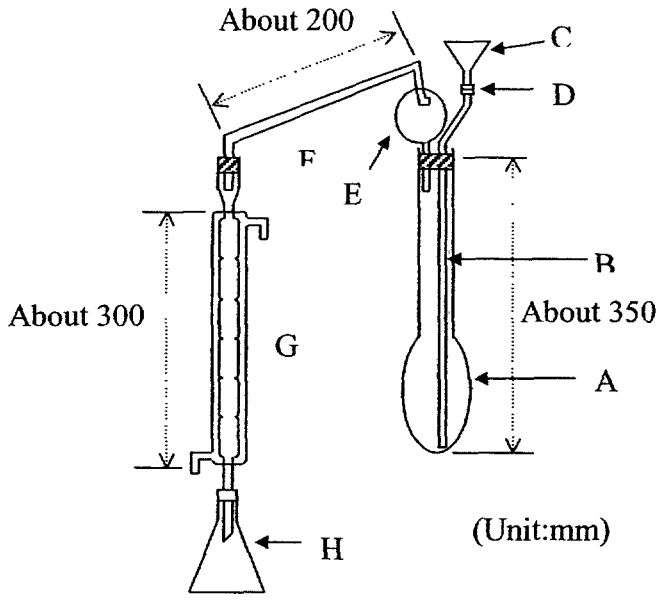
Weigh accurately a quantity of the sample corresponding to about 20 to 30 mg of nitrogen, place it into Kjeldahl flask A, and add 5g of powdered potassium sulfate, 0.5g of cupric sulfate, and 20ml of sulfuric acid. Tilt A at about 45° , heat gently until effervescence almost stops, and raise the temperature to boil. After the contents become a clear, blue solution, heat for another 1 to 2 hours. Cool, add gradually 150 ml of water, and cool again. Add 2 or 3 granules of boiling tips or granulated zinc, and assemble the apparatus.

Measure exactly 25 ml of 0.05mol/l sulfuric acid, transfer into absorption flask H, add about 50 ml of water, and immerse the lower end of condenser G into this solution. Add gradually 85 ml of sodium hydroxide solution(2→5) through funnel C, wash with a small quantity of water, close the pinch cock on rubber tube D, mix the contents by lightly shaking A, and heat gently. When it starts to boil, turn up the heat, and distill until about two thirds of the contents are distilled. Remove the lower end of G from the solution in H, continue

the distillation for a short time, wash the lower end of G with a small quantity of water, and titrate the excess acid in the solution in H with 0.1 mol/l sodium hydroxide solution. The endpoint is usually confirmed using a potentiometer.

When an indicator (3 drops of bromocresol green-methyl red mixture TS) is used, the endpoint is the time when the color of solution changes from red-purple through pale grayish yellow to pale grayish green. Perform a blank test and make any necessary correction.

1 ml of 0.05 mol/l sulfuric acid= 1.4007 mg of N



A: Kjeldahl flask (Made of hard grass. Capacity: about 300 ml)

B: Glass tube

C: Funnel for addition of alkaline solution

D: Rubber tube(Coupling of B and C. With pinch cock)

E: Spray trap

F: Delivery tube

G: Condenser

H: Absorption flask(Capacity: about 300 ml)

4-4. Dietary fiber

Dietary fiber was determined according to AOAC Official Method 985.29 Total Dietary Fiber in Foods.

4-5. Sodium

Apparatus

Atomic absorption spectrometer

Hollow cathode lamp: Sodium

Acetylene: Use dissolved acetylene in bomb

Compressor: Supply air of auxiliary gas. Use a filter between an atomic absorption spectrometer and a compressor.

Shaker

Reagents

1% hydrochloric acid solution: Dilute 20% hydrochloric acid for an atomic absorption analysis or for a precise analysis with ion-exchange water(above 10MΩ in electrical resistance).

Sodium standard solution: Dilute commercially available standard solution for an atomic absorption analysis with 1% hydrochloric acid, and prepare a standard solution in concentration of 0.5 to 3.0 μg/ml to create an calibration curve. Store it in a polyethylene or polypropylene bottle before used.

Preparation of test solution

Ⅷ-1. Test solution was prepared according to dilute acid extraction method.

Ⅷ-2. For dry ashing, use platinum evaporating dish or quartz glass beaker as an ashing container and also plastic instruments for pretreatment except for seaweed with high in salt and processed foods added with salt.

Ⅷ-3. Do not adopt wet digestion method expect for seaweed with high in salt and processed foods added with salt because of possible contamination of acid and decomposition vessel.

Procedure

Determine absorbance of the test solution for measurement using an atomic absorption spectrometer and obtain the concentration of test solution (A) based on the prepared calibration curve.

Set the wavelength at 589.0nm. Basically, use 1% hydrochloric acid as a measuring test solution without diluting. Spray it directly using a nebulizer and install acetylene - air frame.

If the concentration of sodium is too high, lower the sensitivity by rotating a burner head

of an atomic absorption spectrometer or by setting wavelength at 330.3nm(standard solution to construct calibration curve is from 30 to 150 μ g/ml), which has a lower sensitivity.

Calculation

$$\text{Sodium (mg/100g)} = \frac{A \times V \times d}{\times 1000} \times 100$$

A: Sodium concentration in the test solution for measurement according to the calibration curve (μ g/ml)

V: Quantity of test solution (ml)

d: Dilution factor

W: Collected quantity of sample (g)

4-6. Lipid

Apparatus

Electric thermostat: temperature range from 50 to 80℃

Electric constant temperature drying oven: temperature range from 80 to 80℃

Soxhlet extractor: Use an extractor (made of grass) and select the quantity of extraction tube and Soxhlet flask depending on sample volume.

Cylindrical filter paper: Select an appropriate filter paper based on an extraction tube.

Reagents

Diethyl ether: high grade

Procedure

Weight sample (W) and put it in a cylindrical filter paper. Fill the cylindrical filter paper with absorbent cotton on the sample slightly, and then dry it using an electric constant temperature drying oven ranging from 100 to 150℃ for 1 to 2 hours.

After drying, put the cylindrical filter paper in an extraction tube and connect to a cooling tube. Fill two thirds of the Soxhlet flask in constant weight (W_0) with diethyl ether, connect to the extraction tube, and distill it using an electric thermostat for about 8 to 16 hours.

After distillation, dislodge the extraction tube and remove the cylindrical filter paper using a pair of tweezers. Connect to the extraction tube again, warm it in an electric thermostat. When almost all diethyl ether in the Soxhlet flask transfer to the extraction tube, dislodge the Soxhlet flask.

Remove completely the remaining ether by either warming the tilted the flask or by spraying clean air using a narrow glass tube. Wipe outside of the Soxhlet flask with gauze

or a towel.

Dry it using a electric constant temperature drying oven ranging from 100 to 150℃ for an hour, place it in a desiccator to cool for one hour, and weigh it. (W_1)

Calculation

$$\text{Lipid (g/100g)} = \frac{W_1 - W_0}{W} \times 100$$

W_0: Weight of Soxhlet flask in constant weight (g)

W_1: Weight of dried Soxhlet flask after extraction of lipid (g)

W: Collected quantity of sample (g)

5. Rresults and consideration

Lot	Water content (%)	Ash (%)	Protein (g/100g)	Dietary fiber (g/100g)	Sodium (mg/100g)
040506-01	0.47	0.19	0.2	0	38.7
040510-01	0.29	0.18	0.2	0	38.2
040510-02	0.27	0.19	0.2	0	39.7

Lipid data is omitted in the table because lycopene is dissolved in diethyl ether and the figures are exaggerated.

Water content ranges from 0.3 to 0.5%, ash and protein do around 0.2%

Articles

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000128

Reference List for Industry Submission, GRN 000185

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
000073 - 000083	Christian, M.S.; Schulte, Stefan; Hellwig, Jurgen	Developmental (embryo-fetal toxcity/teratogenicity) toxicity studies of synthetic crystalline lycopene in rats and rabbits	2003	Food and Chemical Toxicology	Volume 41, pgs 773-783
000084 - 000094	Jonker, D.; Kuper, C.F.; Fraile, N.; Estrella, A.; Otero, C. Rodriguez	Ninety-day oral toxicity study of lycopene from Blakeslea trispora in rats	2003	Regulatory Toxicology and Pharmacology	Volume 37, pgs 396-406
000095 - 000107	Matulka, R.A.; Hood, A.M.; Griffiths, J.C.	Safety evaluation of a natural tomato oleoresin extract derived from food-processing tomatoes	2004	Regulatory Toxicology and Pharmacology	Volume 39, pgs 390-402
000108 - 000119	McClain, R. Michael; Bausch, Jochen	Summary of safety studies conducted with synthetic lycopene	2003	Regulatory Toxicology and Pharmacology	Volume 37, pgs 274-285
000120 - 000127	Mellert, W.; Deckardt, K.; Gembardt, C.; Schulte, S.; Van Ravenzwaay, B.; Slesinski, R.S.	Thirteen-week oral toxicity study of synthetic lycopene products in rats	2002	Food and Chemical Toxicology	Volume 40, pgs 1581-1588

NA- Not applicable