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Original Article

Wide Range of Biotin (Vitamin H) Content in Foodstuffs and Powdered Milks as Assessed by High-performance Affinity Chromatography

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Abstract. The biotin (vitamin H) contents of various foodstuffs were determined by using a newly developed high-performance affinity chromatography with a trypsin-treated avidin-bound column. Biotin was derivatized with 9-anthryldiazomethane (ADAM) to fluorescent biotin-ADAM ester. A wide range of biotin contents were found in various foodstuffs depending upon the species (strain), season, organ (of plants and animals), geography, freshness, preparation method and storage method. Among the foodstuffs and fermented foods tested, it was found that wide distributions of biotin content were observed in powdered milk, natto, sake (rice wine), beer, edible oil and sea weed. Since powdered milk is important for child health and development, 14 kinds of powdered and special milks for use in children's diseases were intensively measured. We found that several special milk powders for children with allergies contained low levels of free biotin. Use of these powdered milks caused skin diseases and alopecia in some patients possessing thermolabile serum biotinidase, and administration of free biotin improved their symptoms dramatically. Therefore, it is essential to estimate the total and free biotin contents on each foodstuff in order to improve effective biotin intake and support better health and quality of life for people.

Key words: total biotin, free biotin, wide distribution, foodstuffs, powdered milk

Introduction

Determination of biotin, especially free-form biotin, in foodstuffs is important, because appropriate biotin intake is beneficial in attaining a good quality of life (QOL), better health and development of children and adults, improved physical mechanisms that combat aging and disease and efficient mental capacity.

Recently, we developed a new high-performance affinity chromatographic (HPAC) determination method for biotin using a trypsin-

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Dedication: In memory of the kind encouragement of my beloved daughter, Reiko Hayakawa (21 November 1979 – 1 February 2007).

treated avidin-bound column (1). In this new method, biotin is derivatized by 9-anthryldiazomethane (ADAM) to an ester of fluorimetric biotin-ADAM and detected fluorimetrically at an excitation wavelength of 365 nm and emission wavelength of 412 nm (1–3). This is a simple chromatographic method using the affinity of avidin for biotin. We recently found that avidin is a bifunctional binding protein; i.e., avidin (a well-known biotin-binding protein) can also strongly recognize lipoic acid (4). However, biotin and lipoic acid can be separated and measured safely using this new chromatographic technology. It is a rapid (analysis requires one day per sample), reliable and sensitive fluorometric detection method that makes use of the linear calibration line through the origin. Furthermore, other nutrients and antibiotics do not interfere with this chemical method; i.e., other biological biotin assay methods are sensitive to nutrients and antibiotics in serum samples.

Herein, we describe the wide ranges of biotin contents detected among various foodstuffs depending on the species (strain), season, organ (of plants and of animals), geographical area, freshness and preparation and storage methods. The importance of the free biotin content in powdered milk in relation to babies, who have unstable biotinidase and exhibit biotin deficiency symptoms, is also discussed.

Materials and Methods

Chemicals and reagents

Highly pure form of methanol, acetonitrile, ethanol and ethyl acetate (>99.8%), D-biotin, activated charcoal (acid washed; for column chromatography; P/N 035-18081), 2-propanol (HPLC grade), ethylene glycol (amino acid analysis grade), 25% ammonia water (metal analysis grade), sulfuric acid, sodium chloride, lithium chloride (anhydrous; amino acid analysis grade; >97%) and sodium dihydrogen phosphate dihydrate were purchased from Wako Pure Chemical Industries (Osaka, Japan). D-Desthiobiotin (5-methyl-2-oxo-4-imidazolidine hexanoic acid; D 1411), biocytin (ε-N-biotinyl-L-lysine, Mr 372.5; B 4261) and biotin methyl ester (B 7883) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 9-anthryldiazomethane (ADAM) was purchased from Funakoshi Pharmaceutical (Tokyo, Japan). A 0.25% (w/v) trypsin-EDTA solution was purchased from Invitrogen Corporation (Grand Island, NY, USA).

A light-intercepting microtube with a cap (2 ml; P/N 72.693.018) and a microtube with cap (2 mL; P/N 72.694.007) were obtained from Sarstedt Aktiengesellschaft & Co. (Nümbrecht, Germany). Microcentrifuge tubes (1.5 mL, polypropylene, lock-cap; P/N 96.8668.9.01) were obtained from Treff AG (Degersheim, Switzerland). Ekicrodisc 13 CR (0.2 μm; PTFE; P/N E135), Ekicrodisc 13 (0.2 μm; Versapor; P/N E134) and Ekicrodisc 25 membrane filters (0.2 μm; Versapor; P/N E254) were obtained from Nihon Pall Ltd. (Tokyo, Japan). Paper pH indicator (pH 6.4–8.0, narrow range) were obtained from Whatman Ltd. (Maidstone, Kent, England). Blades and disposable scalpels were obtained from Feather Safety Razor Co. (Osaka, Japan).

An affinity column, Bioptic AV-1 (250 × 4.6 mm I.D.; with chicken egg-white avidin bound to a 5 μm diameter silica gel), was purchased from GL Sciences Inc. (Tokyo, Japan). The contents of the column were removed using an HPLC pump. Bioptic AV-1 affinity gels (5 μm diameter silica gel) are now available (1 g and/ or 10 g) from GL Sciences Inc.

Trypsin-treated avidin-bound gel was prepared as described previously (1). A trypsin-treated avidin-bound column (33 × 4.6 mm I.D.) was then prepared.

Ten types of natto (a Japanese food made from fermented soybeans), thirteen sakes (rice wines), ten beers, four coffees, three red wines, four breads, four cheeses, three vinegars, four bananas (three from the Philippines and one from Formosa), two peanut butters, four edible oils (salad oils including soybean and rapeseed, rice bran, olive, and sesame oils), seven sea weeds, ("Aosa [*Ulva pertusa*], Me-hijiki [the sporophylls of Hijiki seaweed [*Sargassum fujifome*], Hijiki [*Sargassum fujifome*], Kinu-mozuku [*Nemacystis decipiens*], Ne-Kombu [root of the Sea Tangle; *Laminaria japonica*], Ao-nori [green laver; *Enteromorpha compressa*], and Nori [laver; *Porphyra tenera*], three bovine milks (purchased in February [winter] and May [summer]), four flours (buckwheat, potato and weak and strong wheat flours), five root crops (onion, carrot, scallion, bamboo shoot and garlic), sauerkraut (Hengstenberg, Esslingen, Germany), shiitake (mushroom), soy sauce (Kikkoman Corporation, Noda City, Chiba, Japan), miso (soybean paste), chicken eggs, sujiko (salmon roe), sea urchin roe, black pepper, rice bran, Yakult (purchased in February and May, Yakult Honsha Co. Ltd., Tokyo, Japan), peanuts (parched), soybeans (parched), soy milk, pickles, Nukamiso-zuke (vegetables pickled in fermented rice bran, Lactobacillus and yeast), tofu (bean curd), honey, komatsuna (*Brassica rapa* var. *pervidis*), spinach, Japanese pepper (*Zanthoxylum piperitum*), pork (thigh), corned beef and chocolate were purchased from grocery stores. Dried yeast (The Japan Pharmacopoeia; Ebios; Tanabe Pharmaceutical Co., Osaka, Japan) was purchased from a drugstore. Royal jellies were purchased from apiaries (Yamada Apiary Corp., Kagamino-cho, Okayama, Japan; Bushu Apirary Co., Kumagaya, Saitama, Japan; and San Ken Co., Tokyo, Japan). Bee pollen (imported from Spain) was purchased from Kano Apiary Co., Yame, Fukuoka, Japan. An anemone flower (*Anemone coronaria*) was purchased from a flower shop.

Human breast milk, milk powders and special milk powders for diseases were kindly donated by our institution. Human serum and urine were kindly donated by volunteers. LEW rats (9 wk of age; male) were purchased from Sankyo Labo Service Corporation (Tokyo, Japan).

Lactobacillus casei (*Shirota*) and *Bacillus natto* cells were prepared as described in a previous study (5).

High-performance liquid chromatography

The HPLC system used was as described previously (1). A six-bored high-pressure valve (GL Sciences Inc.) was used with a 0.1 mL sample-loading loop. Biotin-ADAM was detected with a fluorescence detector (Shimadzu Model RF-10Axl with a Cell Temperature Controller) at an excitation wavelength of 365 nm, emission wavelength of 412 nm and flow-through cell temperature of 28°C. The parameters used for the fluorescence detector were gain of 1, sensitivity of 1 and range of 4. One analysis cycle took 80 min using the program shown in Table 1.

Determination of total biotin

Hydrolysis treatment was performed as follows. First, 0.35 mL of sample solution (dispersed in distilled water) and 0.05 mL of concentrated sulphuric acid were mixed together (final concentration of sulphuric acid of 2.25 M). The mixture was placed in a light-intercepting microtube with a cap and autoclaved at 120°C for 1 h (1). Normally, 0.05–0.2 mL of liquid samples were adjusted to 0.35 mL with distilled water, and 5–50 mg of powdered or wet solid samples were dispersed in 0.35 mL of distilled water; the resulting samples were then hydrolyzed after adding 0.05 mL of concentrated sulphuric acid. After hydrolysis, the samples were treated and derivatized as described previously (1).

Determination of free biotin

Free-form biotin was measured as follows. The samples (0.2 mL of milk, 0.1 mL of serum and 10–100 mg of powdered dry and/or minced wet foodstuffs were suspended in 95% methanol and ultrasonicated for 5 min. After filtration through Ekicrodisc 13CR or 25 fiter, the filtrate was dried under a stream of nitrogen gas. The dried methanol extract was dissolved in 1 mL of

Table 1 Typical elution program for the trypsin-treated avidin-affinity column used for the biotin analysis with an analysis time of 80 min*

Time (min)	Function	Value (%)	Value (mL/min)
0.01	B conc	8.0	
0.01	T flow		0.38
0.01	B conc	8.0	
1.0	T flow		0.38
16.0	T flow		0.38
16.01	T flow		1.0
31.99	T flow		1.0
32.0	T flow		0.38
32.0	B conc	8.0	
32.0	B curv	0	
32.0	T flow		0.38
66.0	T flow		0.38
66.0	B conc	47.0	
66.01	B conc	99.0	
66.01	T flow		1.40
72.00	B conc	99.0	
72.00	T flow		1.40
72.01	B conc	8.0	
74.00	B conc	8.0	
74.00	T flow		1.40
74.01	T flow		0.38
74.02	Stop (of programme)		

*A Shimadzu LC-10AD two-pump system (system controller: SCL-10A) was used. The injector was a high-pressure 6-bored valve with a sample loop of 0.10 mL (internal volume). The column temperature was 17°C. The initial conditions were a flow rate of 0.38 mL/min and an 8.0% concentration of solvent B. B conc: concentration (%) of solvent B. B curve: curve mode, 0 was linear. T flow: flow rate (mL/min). Stop: end of program. One analysis cycle takes 80 min. The other conditions are as described in the Materials and Methods section.

distilled water, and 0.015 mL of 2.25 M NaOH was added to the dissolved free biotin in water. After 0.06 mL of 1.40 M phosphoric acid was added and mixed with the extract (pH of approximately 5.4), activated charcoal (ca. 4 mg) was added to adsorb the biotin onto the surface of activated charcoal. The charcoal was then washed by centrifugation with PBS (phosphate-buffered saline) and distilled water 3 times each. The free biotin adsorbed by the charcoal was extracted with 1 mL of 5% ammonia-ethanol, and the extracted solution was dried under a stream of nitrogen gas. The dried free biotin was dissolved by adding 0.1 mL of methanol and was derivatized by adding 0.08 mL of 0.1% ADAM solution as described previously (1).

Statistics

Since the numbers of foodstuff and biological samples were insufficient for estimating the distribution pattern, non-parametric analysis was applied in this text. Therefore, values are indicated as the median and range.

Results and Discussion

Commercially available foodstuffs in Tokyo were analyzed using a short column (3.3 cm long). Using the improved timetable shown in Table 1, the time required for analysis of biotin was reduced to 80 min from the previously reported analysis time of 92 min (1). A representative example of analysis of foodstuffs (carrot, onion and natural cheese) is shown in Fig. 1.

The distributions of total biotin in foodstuffs are summarized in Table 2 in descending order. The biotin concentration, as assessed by the median value, was highest in the royal jelly product from Okayama. The difference in the values for royal jelly may be due to the production methods of the different prducers. Natto (a Japanese food made from soybeans fermented with *Bacillus natto*) also possessed a high median value of biotin and a wide range in distribution. This wide range may be due to strain differences in *Bacillus natto*. Plant species may be important, since the ranges of peanut butter, root crops, banana and coffee were relatively narrow. Edible seeweeds, cheese and bread also showed wide ranges in biotin content. Geographical differences may also be observed; i.e., biotin content is higher

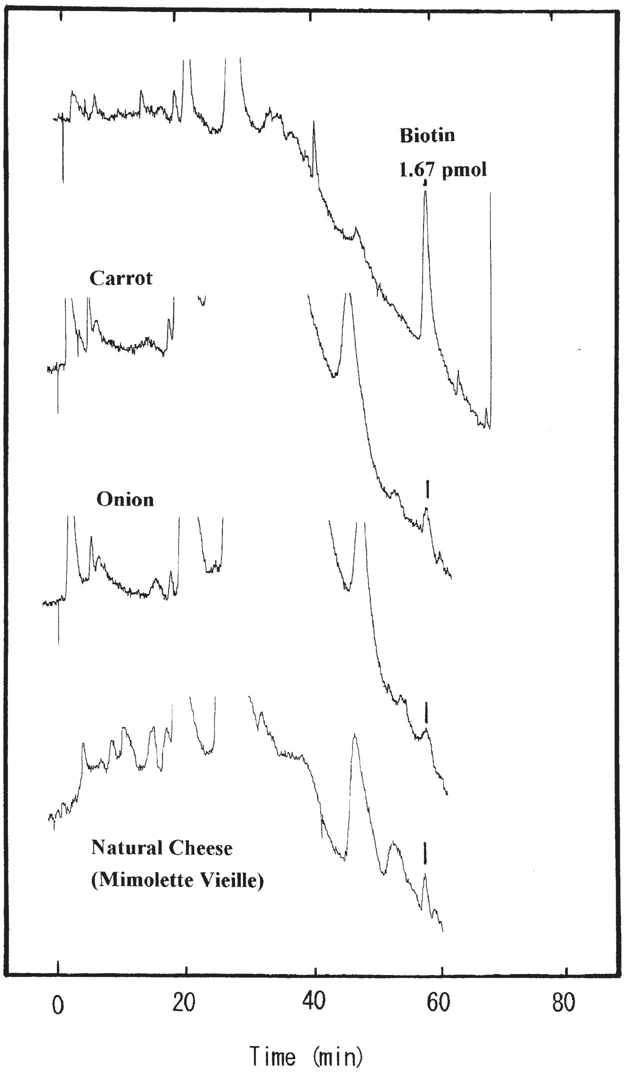

Fig. 1 Typical examples of total biotin analyses of foodstuffs. Upper chromatogram: standard biotin (1.67 pmol). Second chromatogram from the top: 0.002 mL of 10-fold diluted carrot sample (from 34.4 mg wet weight) was injected. The total biotin content of the carrot was 4.03 μg/g wet weight. Third chromatogram from the top: 0.002 mL of 10-fold diluted onion sample (from 33.3 mg wet weight) was injected. The total biotin content of the onion was 3.60 μg/g wet weight. Bottom chromatogram: 0.002 mL of 10-fold diluted natural cheese sample (from 5.1 mg of weight) was injected. The total biotin content of the natural cheese (Mimolette Vieille) was 28.8 μg/g.

Table 2 Total biotin distributions in various foodstuffs available in Tokyo*

Foodstuff	Total biotin (μg/g)**	
	Median	Range
Royal jelly[###, 1] (n=3)	180	(1120–20.6)
Natto[#, 2] (n=4)	40.3	(558–22.4)
Natto[##, 3] (n=9)	13.2	(49.7–4.80)
Coffee[#, 4] (n=4)	13.1	(37.5–10.4)
Peanut butter[###, 5] (n=2)	12.7	(13.5–9.95)
See weed[##, 6] (n=7)	9.27	(12.2–1.52)
Cheese[###, 7] (n=4)	9.16	(28.8–1.71)
Bread[###, 8] (n=4)	3.52	(12.3–1.31)
Yogurt[##, 9] (n=3)	2.86	(9.60–2.10)
Root crops[##, 10] (n=5)	2.50	(4.03–2.19)
Meat[###, 11] (n=3)	1.81	(3.45–0.0827)
Flour[#, 12] (n=4)	1.62	(2.00–0.398)
Banana[###, 13] (n=4)	1.18	(1.27–0.511)

*Median, distribution, and the top product name and producer are indicated. ** [#]: Value per gram dry weight. [##]: Value per g wet weight. [###]: Foodstuff was weighed as is. [1]Ounyuu-no-hana (Yamada Apiary Corp., Kagamino-cho, Okayama, Japan), [2]Okame Hikiwari Natto (Takano Foods Co. Ltd., Omitama, Ibaragi, Japan), [3]Kizami Natto (Yamada Foods Co. Ltd., Misato-cho, Akita, Japan), [4]Instant coffee; Saty Cafe (Brazil) (Ryoennet Co., Fukutsu-city, Fukuoka, Japan), [5]Skippy (Unilever, Englewood Cliffs, NJ, USA), [6]Aosa (Ulva pertusa) (Ohmoriya Co. Ltd., Osaka, Japan), [7]Mimolette Vieille (Nihon Maisera Co., Kanagawa, Japan; imported from France), [8]Pain Traditionnel (Yamazaki Baking Co. Ltd., Tokyo, Japan), [9]Bulgaria Yogurt (Meiji Milk Products Co. Ltd., Tokyo, Japan), [10]Carrot (Daucus carota), [11]Nozaki Corned Beef (Kawasho Foods Corp., Tokyo, Japan), [12]Buckwheat flour, [13]Mindanao (product of highland), Philippines.

in the Philippines than in Formosa for bananas and is higher in Brazil than in Indonesia and Columbia for coffee. The distributions of biotin in various drinks and beverages are summarized in Table 3. It is apparent that fermented and fermented drinks have wider ranges in their distributions (Tables 2 and 3). This may be due to differences in the microbe strains used in their fermentation and production methods. In our

Table 3 Total biotin distributions in various drinks available in Tokyo*

Drink	Total biotin (μg/mL)** Median	Range
Vinegar[1] (n=3)	1.40	(2.33–0.977)
Edible oil[2] (n=4)	1.14	(1.58–0.422)
Wine[3] (n=3)	0.428	(1.36–0.285)
Sake[4] (rice wine; n=13)	0.406	(1.07–0.129)
Bovine milk[5] (n=3)**	0.364	(0.624–0.164)
Beer[6] (n=10)	0.318	(1.99–0.076)
Milk powder (ordinary)[7] (n=3)	0.301	(0.730–0.233)
Peptide milk for allergy[8] (n=3)	0.532	(0.743–0.492)

*Median, distribution, and top product name and producer are indicated. Milk powder and milk for people with allergies were dissolved in hot water in accordance with the procedures of the suppliers. **Presence of seasonal difference; i.e., the summer product value is higher than winter product value. [1]Pure Rice Vinegar (Japan Livelihood Cooperative Association, Tokyo, Japan), [2]Oil for salad (soybean and rapeseed) (Nisshin Oillio Group, Ltd., Tokyo, Japan), [3]Red wine (Shinshu Concord Co., Shijiri, Nagano, Japan), [4]Seishu Senkoma (Senkoma Brewery Co. Ltd., Shirakawa, Fukushima, Japan), [5]Hokkaido Tokachi low-fat milk (Japan Livelihood Cooperative Association), [6]Kirin Ichiban Shibori (Kirin Holdings Co. Ltd, Tokyo, Japan), [7]Hagukumi for 0–9 months (Morinaga Milk Industry Co. Ltd., Tokyo, Japan), [8]Morinaga MA-mi (Morinaga Milk Industry Co. Ltd., Tokyo, Japan).

previous study (1), we confirmed the seasonal differences in biotin content of bovine milk (first reported by Dr. Umetaro Suzuki); i.e., milk in summer contains a biotin concentration that is approximately 3 times that in winter. A similar range in total biotin content was also observed for ordinary milk powder (Table 3). This may due to the seasons in which the raw materials (bovine milk) were obtained by the producers.

Free biotin is the important nutrition, since free biotin should be liberated and obtained from the usual bound-form biotin via hydrolysis by the amidase (biotinidase) in animals and in some bacteria and fungi, which are not able to synthesize biotin. Typical foodstuffs that contain high ratio of free biotin are summarized in Table 4. Sera from healthy humans, microbes and plant vegetable cells usually contain less than 10% free biotn (lower part of Table 4). Foodstuffs containing high amounts of free biotin included good nutritional materials such as natto, chicken egg-yolk and milk. These foodstuffs seem to have their own mechanisms against invasion of microbes (upper part of Table 4).

Milk and milk powder are very important foodstuffs for infants, who receive nutrition for several months after birth from only milk. The biotin contents of milk and milk powders are summarized in Table 5. We found that all the tested milk and milk powders, except for GSD (glycogen storage disease) formulas, contained sufficient amounts of biotin (Table 5). Milk powder C (follow-up milk) contains relatively lower amounts of total and free biotin; however,

Table 4 Typical examples of high free-biotin containing foodstuffs and possible protection mechanisms against potential pathogens (bacteria, fungi, etc)*

Foodstuff	Total biotin (μg/g)	Free biotin (μg/g)	Ratio of free biotin (%)	Protection method (Potential)
Hikiwari Natto (Okame; dried)	558	514	92.1	poly(gamma-glutamic acid) (pH 6.5)
Chicken egg-yolk	35.2	31.7	90.1	Avidin, lysozyme (egg-white) and pH 5.3 (egg-yolk)
Sake (rice wine)** (Tatsuizumi)	0.334	0.251	75.0	Ethanol (12%)
Bee pollen	10.7	6.41	59.9	Bacteriocin peptide***
Milk powders (ordinary; n=3)	0.301	0.172	57.1	Dried powder
Pollen (Anemone)	23.9	10.5	43.9	Cell wall of cellulose
Bovine milk** (product of February)	0.164	0.0669	40.8	Immunoglobulin A Lactoferrin
Beer** (Kirin Ichiban Shibori)	1.99	0.736	37.0	Ethanol (6%)
Royal jelly A	1120	376	33.6	10-Hydroxydecanoic acid
Royal jelly B	180	37.6	20.9	
Royal jelly C	20.6	6.95	33.7	
Rice bran	30.4	6.02	19.8	Cell wall of cellulose
Human serum**	1.80	0.122	6.8	
Mozuku (dried)	11.7	0.411	3.5	
Dried yeast (Ebios)	15.5	1.29	8.3	
Dried Bacillus natto	49.7	3.42	6.9	

*Total and free biotin were measured as described in the Materials and Mehthods section. **Expressed as μg/mL. ***Refer to (8).

infants who are 9 mo of age or older can consume nutrients from ordinary foodstuffs. Although soy milk is a foodstuff intended for adults, both soy milk and soy baby formula contained a high concentrations of total biotin (Table 5). Furthermore, soy formula also contained a high concentration of free biotin (Table 5). Soy formula may be a good milk for babies.

Biotin deficiency may occur in babies consuming milks with low levels of free biotin and low free biotin ratios. In fact, two babies receiving Milfy (Meiji) and one baby receiving New MA-1 (Morinaga) were found to be biotin deficient at our hospital (6). Elemental formula (Meiji) may also cause biotin deficiency in babies with normal biotinidase (7). One 3-yr-old female GSD patient (apparently normal biotinidase with heat labile *K*m; Michaelis constant) receiving GSD formulas D and N produced by Meiji has also been found to be biotin deficient (Dr. Kenji Ihara, personal communication). Babies and adults exhibiting unstable (heat labile) biotinidase may have a tendency to become biotin deficient (Table 6). The two biotin deficient patients showed increased *K*m values (decreased affinity) compared with fresh controls after treatment at 37°C for 4 h. The biotin-deficient baby received Milfy (Meiji) and was biotin deficient. This baby was then administerd 10 mg/day of biotin for 13 wk, and his biotin status improved (serum total biotin 3.89 μg/mL, free biotin 0.052 μg/mL) and dermatitis disappeared. The biotin-deficient adult in Table 6 took Ebios (dried yeast) and Yakult (*Lactobacillus casei, Shirota*) for 4 mo, and her biotin status improved slightly (serum total biotin 2.04 μg/mL, free biotin 0.077 μg/mL). This patient seems to have improved slightly; however, the precise neurological tests may be necessary to be studied on this patient.

In conclusion, precise knowledge of the biotin

Table 5 Summary of the biotin contents of milk, milk powders and special milk powders for milk-related conditions*

Milk	Total biotin (μg/mL)	Free biotin (μg/mL)	Ratio of free biotin (%)	Appearance of dermatitis patient**
Human breast milk[1]	0.376	**0.209**	55.6	
Bovine milk[2]	0.364	0.248	68.1	
Ordinary milk powders				
Milk powder A[3]	0.730	0.338	46.3	–
Milk powder B[4]	0.301	**0.172**	57.1	–
Milk powder C[5]	**0.233**	**0.162**	69.5	–
Special milk powders (only milk peptides are present; for milk allergies)				
MA-mi (Morinaga)	0.743	0.494	66.5	–
New MA-1 (Morinaga)	0.532	**0.164**	**30.8**	+ (1 case)
Milfy (Meiji)	0.492	**0.056**	**11.4**	++ (2 cases)
Pepdiet[6] (Beanstalk-Snow)	1.18	0.792	67.1	–
MCT formula[7] (Meiji)	1.15	0.909	79.0	–
MCT formula[8] (Meiji)	1.25	0.754	60.3	–
Elemental formula[9] (Meiji)	1.73	**0.251**	**14.5**	?
Lactoless[10] (Meiji)	1.18	**0.094**	**7.97**	?
GSD formula Day[11] (Meiji)	**0**	**0**	**+**	
GSD formula Night[11] (Meiji)	**0**	**0**	**+**	
Soy milk[12]	2.98	1.99	66.8	
Soy formula[13]	1.36	0.899	66.1	–

*Total and free biotin were measured as described in the Materials and Methods section. Milk powders were dissolved as indicated by the suppliers. [1] 9-mo-old baby, [2] product of October; Glyco Co., Yoji-yuryo-gyunyu, [3] for 0–9 mo; Morinaga Hagukumi, [4] for 0–9 mo; Yukijirushi Pure, [5] for 9 mo – 3 y; follow-up milk; Yukijirushi Tacchi, [6] plus a small amount of milk proteins, lecithin and edible oils; for lactose intolerance, [7] defatted milk and lecithin, [8] defatted milk, lecithin and added essential fatty acids, [9] amino acids+ edible oils; for lactose intolerance and galactosemia, [10] Casein and edible oils; for lactose intolerance and galactosemia, [11] Casein milk, for liver-type glycogen storage disease; GSD, [12] Marusan-Ai Co. Ltd., [13] Bonlacto I; Wakodo.

contents of foodstuffs is expected to be useful in improving the health and development of babies and adults.

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References

1. Hayakawa K, Katsumata N, Hirano M, Yoshikawa K, Ogata T, Tanaka T, *et al.* Determination of biotin (vitamin H) by the high-performance affinity chromatography with a trypsin-treated avidin-bound column. J Chromatogr B 2008;869:93–100.
2. Nimura N, Kinoshita T. Fluorescent labelling of fatty acids with 9-anthryldiazomethane (ADAM) by high performance liquid chromatography. Analytical Letters 1980;13:191–202.
3. Hayakawa K, Oizumi J. Determination of free biotin in plasma by liquid chromatography with fluorimetric detection. J Chromatogr

Table 6 Thermal instability of serum biotinidase in biotin-deficient patients*

Serum	*A*mo (L/mol × 1/s)	*V* (pmol per min per mg)	*K*m (μmol/L)	*K*ip (μmol/L)	*R*ep (mol/L × 1/s × 10^{-3})	*C*ap (1/s)
Healthy baby (1 yr, female)						
(Biotin status: Total biotin 3.21 μg/mL, free biotin 0.180 μg/mL)						
Fresh	22.2	156	8.91	494	97.6	46.5
Treated	18.1#	131#	9.03#	485#	80.6#	38.5#
Biotin-deficient baby (4 mo, male; dermatitis and alopecia)**						
(Biotin status: Total biotin 2.31 μg/mL, free biotin 0.535 μg/mL)						
Fresh	25.5	182	9.02	365	83.9	46.3
Treated	8.63	122	**17.9**	271#	41.8	19.0
Healthy adult (male)						
(Biotin status: Total biotin 1.80 μg/mL, free biotin 0.122 μg/mL)						
Fresh	27.9	131	5.94	359	59.3	40.7
Treated	20.6#	109#	6.71#	501	69.3#	37.8#
Biotin-deficient adult (32 yr, female; optical atropy, polyneuropathy [sensory dominant])						
(Biotin status: Total biotin 1.14 μg/mL, free biotin 0.041 μg/mL)						
Fresh	18.4	63.6	4.45	342	27.7	22.4
Treated	6.11	89.3	**18.5**	530	59.9	19.1#

*The thermal instability test was performed as follows; serum was diluted 11-fold with serum dilution buffer containing 1 mM EDTA and 10% glycerol (5). The diluted serum was then incubated at 37°C for 4 h. After 10-fold dilution of this treated serum, the biotinidase activity was measured as described previously (5). *A*mo; affinity for substrate. *V*; specific activity. *K*ip; competitive inhibition constant by the product biotin. *R*ep; repulsion. *C*ap; enzyme capacity. Fresh refers to is non-heat treated controls. #: Parameters that fluctuated by no more than 30%, were considered to be stable. **This patient received Milfy (Meiji; Table 5) and experienced biotin deficiency.

1987;413:247–50.
4. Hayakawa K, Katsumata N, Yoshikawa K, Hirano M, Ogata T, Tanaka T, *et al.* Determination of lipoic (thioctic) acid by high-performance affinity chromatography with a trypsin-treated avidin-bound column. Trends Chromatogr 2007;3:31–42.
5. Hayakawa K, Guo L, Terentyeva EA, Li XK, Kimura H, Hirano M, *et al.* Determinations of specific activities and kinetic constants of biotinidase and lipoamidase in LEW rat and *Lactobacillus casei* (*Shirota*). J Chromatogr B 2006;844:240–50.
6. Abe K, Yoshii K, Ayabe T, Naiki Y, Horikawa R, Nagai A, *et al.* One case with biotin deficiency which is due to the long intake of powdered milk for allergy. Nihon-shounika-gakkai-shi 2008;112:320 (Abstract, in Japanese).
7. Higuchi R, Noda E, Koyama Y, Shirai T, Horino A, Juri T, *et al.* Biotin deficiency in an infant fed with amino acid formula and hypoallergenic rice. Acta Paediatr 1996;85:872–4.
8. Audisio MC, Terzolo HR, Apella MC. Bacteriocin from honeybee beebread *Enterococcus avium*, active against *Listeria monocytogenes*. Appl Environ Microbiol 2005;71:3373–5.