Simultaneous Determination of *S*-Methylmethionine, Vitamin U and Free Amino Acids in Extracts of Green Tea with an HPLC-Amino Acid Analyzer

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Free amino acids and *S*-methylmethionine (MMS, an anti-ulcer factor, Vitamin U) in green tea extracts were simultaneously determined with an HPLC-amino acid analyzer using lithium citrate buffers.

Rapid analysis of MMS and free amino acids was achieved with a high resolution column, MCI Gel CK-10U 0.46 ϕ $\times$ 15 cm, within 180 min. MMS in the column eluates was identified as dimethyl sulfide by a gas-chromatographic method with a flame photometric detector. The contents of MMS and free amino acids in various green teas were determined and compared with respect to the quality of commercial teas, blended teas and non-blended teas, and the place of cultivation of the tea. The described method is rapid and useful for checking the quality of green tea.

Various vegetables^{1~5)} and green tea^{4,6,7)} contain as much *S*-methylmethionine (MMS, an anti-ulcer factor, vitamin U) as free amino acids. MMS was also reported to have been detected in a soybean-meal hydrolyzate after neutralization.^{8,9)} In this case, MMS was thought to be an undesirable by-product and to be formed from methionine and pectin methyl ester during the acid-hydrolysis of the soybean meal.

In the previous paper,⁷⁾ a rapid analytical method for MMS in extracts of various green teas, oolong teas and black tea was reported, involving cation-exchange chromatography, and the conditions for extraction of MMS from green tea and the heat-stability of MMS in the green-tea extracts were also described.

We have also determined the quantity of dimethylsulfide (DMS), which is formed from MMS in shaded green teas (Ooi-cha), such as Gyokuro and high-grade Sen-cha,¹⁰⁾ by flame photometric detection (FPD) with a gas-chromatograph.¹¹⁾

This paper describes analyses of MMS as well as free amino acids in extracts of green teas on the same chromatogram or by the more rapid analytical method for MMS, using an HPLC-amino acid analyzer. Some analytical data for green teas of commercial sources and some kinds of non-blended green teas cultivated and processed in Kyoto Pref. Tea Res. Inst. or in other places are also presented.

MATERIALS AND METHODS

Materials. Green teas (various Sen-cha, #1~#5, at prices of ¥1,500~¥250 per 100 g) were purchased from a shop in Kyoto Pref. The other Sen-chas, #6 and #7, were purchased at prices of ¥600 and ¥1,000 from Gifu Pref. and Shimane Pref., respectively. These commercial teas are thought to be blended ones. Non-blended teas, Gyokuro and Sen-cha, #8~#11, were cultivated and processed at Kyoto Pref. Tea Res. Inst. The other non-blended teas, #12~#14, were cultivated and processed at farms in Kyoto Pref., Nara Pref. and Shizuoka Pref.

Authentic amino acids were obtained from Sigma (USA). MMS and the other chemicals were purchased from Nakarai Chem. Co. (Kyoto).

Extraction of MMS and free amino acids. Five hundred mg of green tea, powdered and filtered through a 0.5 mm mesh sieve, was suspended in 10 ml of distilled water at 60°C for 30 min with agitation. The suspension was then centrifuged at 3000 rpm for 10 min. The precipitate was re-extracted and then centrifuged again as above. After the centrifugation, the precipitate was washed with water at 80°C. The supernatants and washings were combined and then filtered through a Toyo membrane filter (Toyo-roshi Co. TM-2P, 0.45 μ m pore size) before injection into the amino acid analyzer.

Determination of MMS and free amino acids. Amino acid analyses of the extracts were carried out with a Shimadzu HPLC, LC-6A, modified for amino acid analysis of physiological-fluids, see Fig. 1, in our laboratory under the following conditions; a stainless steel column (0.46 ϕ $\times$ 15 cm) of Mitsubishi MCI Gel CK-10U (5 μ m particle size) at 40°C, and elution buffers of 0.15 N Li-citrate, pH 2.75, 0.25 N Li-citrate, pH 3.9, 0.8 N Li-citrate, pH 3.4, 1.0 N Li-citrate, pH 4.05, and 0.2 N LiOH at a flow rate of 0.5 ml/min, and the conventional ninhydrin solution⁽¹²⁾ at a flow rate of 0.25 ml/min. Calculation of the peak areas on the chromatograms was carried out with a Chromatopac C-R3A (Shimadzu). When MMS was to be determined by the other method or needed to be analyzed rapidly, another analytical method was used, see Fig. 3; a stainless steel column of MCI Gel CK-10S (0.46 ϕ $\times$ 10 cm) at 50°C with 0.35 N Na citrate, pH 6.5, and the ninhydrin solution at flow rates of 0.5 ml/min and 0.25 ml/min, respectively.

Identification of MMS with the HPLC-amino acid ana-

lyzer eluates. The detection and identification of MMS in the eluates was carried out in the same manner with a gas-chromatographic apparatus as in the previous study.⁽⁷⁾ DMS, which is one of the decomposition products of MMS on heat treatment in alkaline medium, was separated on a column of Shimalite TPA (60~70 mesh) coated with 10% 1,2,3-TCEP, 3 mm ϕ $\times$ 3 m, and then detected by FPD at 394 nm, which is a wavelength specific and sensitive for sulfur compounds.⁽¹¹⁾

RESULTS AND DISCUSSION

A flow diagram of the HPLC-amino acid analyzer used for the analysis of MMS and free amino acids is shown in Fig. 1. The specified inner volume of the Teflon tube in the reaction bath was the maximum, in order to maintain the high resolution ability of the column, though the increased volume improved the color-yield of the ninhydrin reaction.

Since a green-tea extract contains free, ordinary amino acids and specific amino acids such as theanine⁽¹³⁾ and γ -aminobutyric acid, which cannot be separated from other amino acids with the conventional column and Na-citrate buffer system, it is indispensable to use a Li-citrate buffer system.^(14~19)

In our previous study, MMS was easily

FIG. 1. Flow Diagram of the HPLC for the Analysis of Free Amino Acids and MMS.

(a) Buffer reservoirs: 1)~4) elution buffers; 5) 0.2 N LiOH. (b) Buffer selector, changed with a system controller, SCL-6A. (c) UV-VIS spectrophotometric detector, Shimadzu SPD-6AV. (d) Eluent pump, Shimadzu LC-6A. (e) Reaction bath, 0.5 mm ϕ $\times$ 5 m Teflon tube in boiling water. (f) Waste. (g) Ninhydrin pump, Kyowa Seimitsu KHD-16. (h) T-Shaped mixer. (i) Ninhydrin solution. (j) Nitrogen gas. (k) Separation column, Mitsubishi MCI Gel CK-10U, 0.46 ϕ $\times$ 15 cm, with a constant-temperature bath at 40°C. (l) System controller, Shimadzu SCL-6A. (m) Auto injector, Shimadzu Sil-6A. (n) Recording integrator, Shimadzu Chromatopac C-R3A.

FIG. 2. Chromatograms of Standard Amino Acids and Tea Extracts.

B₁, 0.15 N Li-citrate, pH 2.75; B₂, 0.25 N Li-citrate, pH 3.9; B₃, 0.8 N Li-citrate, pH 3.4; B₄, 1.0 N Li-citrate, pH 4.1. Abbreviations. hS, homoserine; Th, theanine; α-A, L-α-amino adipic acid; Ci, L-citrulline; α-Ab, L-α-amino butyric acid; β-A, β-alanine; β-Ai, DL-β-amino-iso-butyric acid; hC, L-homocystine; γ-Ab, γ-amino-*n*-butyric acid; hK, δ-hydroxy-lysine; Or, L-ornithine; 1-Me, 1-methyl-L-histidine; 3-Me, 3-methyl-L-histidine; aS, L-anserine; Ca, L-carnosine; Ordinary amino acids, abbreviated as one letter. (a) Standard amino acids and MMS. (b) Green-tea extract (Sen-cha).

FIG. 3. Chromatograms of Standard Amino Acids and a Tea Extract with a Rapid Analytical Method.

Column, Mitsubishi MCI Gel CK10S, 0.46φ × 10 cm, at 50°C; buffer, 0.35 N Na-citrate, pH 6.5. (a) Standard amino acids. (b) Green-tea extract (Sen-cha).

analyzed by lowering the temperature of the column, but it is time-consuming, taking about 4 hr per one sample, and it is a mono-purpose

FIG. 4. Gas-chromatograms of Headspace Vapor of the Column Eluates.

(a) Standard sulfur compounds: 1, hydrogen sulfide; 2, methylmercaptan; 3, dimethyl sulfide. Headspace vapor of the alkaline- and heat-treated column eluate fractions of MMS in Fig. 2a or Fig. 3a (b); and in Fig. 2b or Fig. 3b (c).

TABLE I. AMINO ACID AND MMS CONTENTS OF COMMERCIAL GREEN TEAS

	Sen-cha							
	#1	#2	#3	#4	#5	#6	#7 ^a	
Asp ^b	478	383	264	329	151	429	193	(mg%)
Thr	49	52	13	54	11	16	14	
Ser	113	114	80	90	50	62	50	
Asn	80	128	46	93	33	47	4	
Glu	440	341	404	448	184	370	209	
Gln	337	575	164	118	40	198	104	
Thea	2725	2357	1258	982	437	1153	830	
Pro	25	19	18	18	11	8	7	
Gly	6	11	2	4	6	4	4	
Ala	49	43	44	65	34	39	21	
Val	37	84	38	42	48	46	15	
Cys	30	25	20	18	15	7	1	
Met	9	2	1	tr	tr	2	tr	
Ile	24	22	10	23	9	34	11	
Leu	31	27	11	21	8	40	14	
Tyr	46	47	13	28	24	44	19	
Phe	43	53	81	24	27	49	21	
GABA	19	17	15	14	6	15	9	
NH ₃	32	37	58	72	3	14	20	
Orn	7	9	8	5	2	6	4	
Lys	37	44	16	22	13	37	14	
MMS	9	6	3	5	3	6	4	
His	24	72	34	12	8	21	11	
Trp	31	36	23	38	24	27	9	
Arg	464	336	194	149	30	334	155	
Total	5145	4840	2818	2674	1177	3008	1743	
Grade ^c	A	A	B	B	C	A	B	

^a Teas #1~#5 were manufactured and sold at a shop in Kyoto Pref. Teas #6 and #7 were sold in Gifu Pref. and Shimane Pref., respectively.

^b Abbreviations: Thea, theanine; GABA, γ -aminobutyric acid; Orn, ornithine; tr, trace.

^c A, high quality; B, medium quality; C, ordinary quality. Judged by organoleptic testing at the time when the teas were bought.

method, that is, other amino acids cannot be determined at the same time.⁷⁾

To improve these points, in this study, rapid analysis of MMS as well as other specific amino acid derivatives was attained by using an HPLC-column packed with the cation-exchange resin of smaller particle size, 5 μ m, and an HPLC apparatus, Shimadzu LC-6A.

A chromatogram of standard MMS and the amino acids which are found in physiological

fluids is presented in Fig. 2(a).

A proline peak was detected at 440 nm with an automatic wavelength changeable photometer, Shimadzu SPD-6AV. In Fig. 2(a), homoserine, which is one of the decomposition products of MMS, was also separated from glutamine. However, even when an excess amount of a green-tea extract, Sen-cha, was applied, as shown in Fig. 2(b), almost no homoserine was detected, that is, MMS was

TABLE II. AMINO ACID AND MMS CONTENTS OF NON-BLENDED GREEN TEAS

	Gyokuro		Sen-cha					
	#8	#9	#10	#11	#12	#13	#14 ^a	
Asp ^b	546	283	185	145	449	180	355	(mg%)
Thr	46	33	27	20	58	9	24	
Ser	117	88	51	54	92	51	64	
Asn	590	344	49	28	193	23	53	
Glu	511	275	293	203	564	193	330	
Gln	418	258	358	134	323	78	92	
Thea	2245	1926	1317	902	2327	905	994	
Pro	8	29	10	18	12	21	8	
Gly	7	3	2	4	4	2	3	
Ala	78	46	34	19	50	17	48	
Val	62	25	31	17	26	13	39	
Cys	29	16	20	11	2	18	8	
Met	tr	tr	2	1	1	2	2	
Ile	50	14	16	8	36	8	30	
Leu	43	15	8	9	21	9	28	
Tyr	48	15	18	9	16	15	30	
Phe	45	17	14	10	14	16	26	
GABA	10	12	13	16	11	12	23	
NH ₃	6	4	8	5	37	8	11	
Orn	15	6	5	3	5	4	3	
Lys	57	23	12	11	20	13	29	
MMS	9	4	4	12	6	8	11	
His	36	10	15	37	40	10	16	
Trp	55	24	14	4	30	7	19	
Arg	781	288	292	80	632	132	151	
Total	5812	3758	2798	1760	4969	1754	2397	
Grade ^c	A	B	A	B	A	C	B	

^a Teas #8~#11 were cultivated and processed at Kyoto Pref. Tea Inst. #12, #13 and #14 were manufactured in Kyoto Pref., Nara Pref. and Shizuoka Pref., respectively.

^{b, c} Abbreviations the same as in Table I.

scarcely decomposed in the green-tea extract.

Theanine was the most abundant amino acid in all the green-tea extracts, as shown in Fig. 2(b), and Tables I and II.

Aspartic acid, serine, asparagine, glutamic acid, glutamine and arginine were abundant in the extracts, amounting to about one-tenth of their total amino acid quantity, respectively, as shown in Tables I and II.

Other minor component amino acids were also detected, as can be seen in Fig. 2(b).

The results with another rapid MMS de-

termination method, with an analysis time of 30 min, are shown in Fig. 3. This is a modification of the former method, with a $0.6\phi \times 8.5$ cm column and Na-citrate buffer.⁷⁾

The pooled MMS fractions in Fig. 2(a) or Fig. 3(a) were heated at 100°C for 10 min in the alkaline medium⁷⁾ and then the headspace vapor was analyzed by gas-chromatography, as shown in Fig. 4(b). In the same manner, the pooled MMS fractions in Fig. 2(b) or Fig. 3(b) were treated and analyzed, as shown in Fig. 4(c). The results confirmed the presence of

MMS.

The amino acid contents of the commercial green teas are shown in Table I. The theanine contents of #1, #2, #3 and #7 comprised as much as about 50% of the total amino acids, respectively. The arginine contents varied with the sample; in high quality green teas #1, #2, #6, #8, #10 and #12, it amounted to about 10% of the total amino acids, respectively.

Commercial green teas are usually produced by blending of green teas from several sources, to improve the quality of the tea. To compare blended green teas with non-blended ones, the analytical data for the latter are summarized in Table II. Non-blended green teas also contained as much theanine, arginine, glutamic acid, glutamine and aspartic acid as the commercial ones, that is, the same tendency was observed. The theanine and arginine contents were reported to be influenced by ammonium fertilizer used during the cultivation of the green tea plants.¹⁸⁾ The data for the non-blended green teas, Table II, showed that the quality, such as the taste, reflected the amino acid content, which was determined by the cultivation conditions. Takeo¹⁸⁾ also reported that the spring shoots of tea plucked in India did not contain arginine, and he assumed that the reason was the lack of ammonium fertilizer. Tables I and II show that every green tea cultivated in Japan contained arginine to various extents, and this reflected the ammonium fertilizer application during cultivation.

Sen-chas #1, #11 and #14, and Gyokuro #8 contained more MMS than the others, and this amino acid was abundant in fresh, high-grade green tea, as reported previously.⁷⁾

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