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Analysis of Acrylamide in Green Tea by Gas Chromatography−Mass Spectrometry

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Optimization of the solid-phase extraction cleanup procedure enabled the GC-MS analysis of acrylamide in tea samples without the interference of bromination by tea catechins. Although polyvinylpolypyrrolidone (PVPP) is available for removing tea catechins from tea extract, the peaks derived from PVPP had the same retention time as brominated acrylamide in mass chromatograms obtained by GC-MS. A considerable amount of acrylamide was formed at roasting temperatures of ≥120 °C; the highest acrylamide level was observed when tea samples were roasted at 180 °C for 10 min. Higher temperatures and longer processing times caused a decrease in the acrylamide content. Furthermore, an analysis of 82 tea samples showed that rather than the reducing sugar content, the asparagine content in tea leaves was a significant factor related to acrylamide formation in roasted products. The acrylamide level in roasted tea products was controlled by asparagine in the presence of reducing sugars.

KEYWORDS: Acrylamide; green tea; roasting; polyvinylpolypyrrolidone; GC-MS

INTRODUCTION

The discovery of acrylamide in heated foods consumed by humans (*1*) prompted us to investigate the effects of the components of tea leaves (*Camellia sinensis* L.) and the processing conditions on acrylamide formation in tea. This was because acrylamide was found to be carcinogenic in rodents (*2*) and has been classified as a probable human carcinogen by the International Agency for Research on Cancer (*3*).

Tea is one of the most popular beverages in the world owing to its attractive aroma, taste, and health benefits. Approximately 3.0 million tons of tea is now produced and consumed per year around the world. Several types of teas are available; these are generally classified into the following three major categories: nonfermented green tea, partially fermented oolong tea, and the fully fermented black or Pu-erh tea. Of these, green tea is a more popular beverage; approximately 0.6 million tons of it is consumed per year, mainly in Asian countries. Green tea is prepared by steaming or pan-firing fresh leaves to inactivate polyphenol oxidase prior to the drying steps, which are performed at temperatures below 80 °C. It is finally manufactured by roasting the leaves from 120 to 140 °C for approximately 20 min; roasted green tea such as Houjicha is processed from 170 to 200 °C for approximately 10 min. In contrast, black and oolong teas are manufactured by drying the leaves at temperatures below 100 °C to preserve their attractive flavors.

It is now well-established that amino acids, mainly asparagine, and reducing sugars are important precursors for acrylamide in heated foods (*4*−*6*) and that processing conditions such as time, temperature, and matrix influence its formation and degradation (*6*−*14*). Acrylamide formation was found to occur during the browning process or the Maillard reaction at temperatures above 120 °C (*4*−*6*, *10*). Roasted materials used in beverages, such as coffee and roasted barley grains, were reported to contain considerable amounts of acrylamide (*15*−*19*). The preliminary analysis of tea showed that acrylamide is present in a wide variety of commercial tea products (*15*, *17*, *19*, *20*). In the course of the analysis by gas chromatography−mass spectrometry (GC-MS), epigallocatechin in the tea samples inhibited the bromination of the acrylamide. Therefore, the solid-phase extraction (SPE) process was improved to avoid contamination of epigallocatechin (*20*). Polyvinylpolypyrrolidone (PVPP) is available to remove tea catechins and other polyphenols from the tea extract. However, preliminary studies (*20*) did not examine the sample cleanup procedure using PVPP for acrylamide analysis. Moreover, this study demonstrated the effects of roasting

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Table 1. Acrylamide Content in Locally Purchased Tea and Infusion

		acrylamide		
type of tea	sample	product (ng/g)	infusion (ng/mL)	conditions of infusion[a]
green	1	110	2.1	10 g, 90 °C, 430 mL, 1 min
	2	77	1.4	
	3	70	1.6	
	4	68	1.6	
	5	67	1.4	
	6	54	1.3	
	7	53	1.3	
	8	42	0.8	
	9	38	0.8	
	10	35	1.1	
	11	31	0.6	
	12	27	0.7	
roasted green	1	1880	41.2	15 g, 90 °C, 650 mL, 0.5 min
	2	784	17.1	
	3	778	16.9	
	4	774	16.5	
	5	678	15.9	
	6	641	15.1	
	7	637	14.7	
	8	556	11.5	
	9	544	10.8	
	10	512	11.1	
	11	414	8.6	
	12	411	9.1	
	13	247	4.8	
oolong	1	85	2.1	15 g, 90 °C, 650 mL, 0.5 min
	2	55	0.9	
	3	31	4.8	
black	1	25	ND[b]	5 g, 90 °C, 360 mL, 4 min
	2	20	ND	
	3	18	ND	

[a] Tea infusion was prepared according to the standardized method established by the Japan Science and Technology Agency. [b] Not detected (below the detection limit of 0.2 ng/mL).

conditions on the acrylamide formation in green tea without repeated roasting (*20*). This information is indispensable for the reduction of acrylamide in green tea; therefore, we investigated the effects of roasting conditions on acrylamide formation in detail. The main precursor of acrylamide in heated foods is asparagine, as mentioned above. However, the effects of the components of tea leaves on the acrylamide formation are unclear. Thus, fresh leaves from various cultivars in Japan were plucked in 2004, and the amino acids and sugars present in them were analyzed before roasting to elucidate the relationship between these components and the formation of acrylamide in roasted green tea. This paper would contribute to the improvement of the process for the reduction of acrylamide in green tea.

MATERIALS AND METHODS

Materials. Green tea, roasted green tea, oolong tea, and black tea products were purchased from local supermarkets and tea stores in Shizuoka prefecture, Japan, from December 2003 to August 2004. Their infusions were prepared according to standardized methods, which are shown in **Table 1** (*21*), established by the Japan Science and Technology Agency.

For the experiment to assess the effects of the roasting conditions, tea leaves of the cultivar 'Yabukita' were plucked on May 1, 2004, in Shizuoka prefecture, Japan. The tea leaves were processed by drying them at temperatures below 80 °C, and the weight of the product obtained was approximately 3.5 kg. The moisture content of the tea leaves was 5.05% wet basis (wb).

To analyze the effects of the sugar and amino acid contents on the formation of acrylamide, we selected 82 samples that varied widely in these contents. The fresh leaves of several cultivars of tea plants, such as 'Yabukita', 'Meiryoku', 'Fushun', 'Saemidori', 'Okumidori', and 'Benifuki', were mechanically plucked from late April to late June in 2004, in Shizuoka prefecture, Japan. The experimental materials included young shoots and moderately mature and excessively mature leaves. After plucking, the leaves (2.0 kg) were processed by drying at temperatures below 80 °C; they were then stored in a refrigerator at 5 °C until use. The moisture content of the tea samples varied from 4.05 to 6.20% wb.

Roasting Condition Test of Green Tea Leaves. Tea leaves weighing 100 g were placed on an aluminum plate (310 mm × 180 mm) and roasted at temperatures of 100, 120, 140, 160, 180, 200, and 220 °C. At each of these temperatures, roasting was carried out for 10, 20, and 30 min in a laboratory oven (DK-660; Yamato Scientific Co., Ltd., Tokyo, Japan). The roasted product was then placed in a desiccator with 300 mL of silica gel to cool the sample to room temperature (20−25 °C). For acrylamide extraction, the roasted product was pulverized using a Cyclone sample mill (3010-018; Udy Corp., Fort Collins, CO) and then stored in a refrigerator at 5 °C. The roasting treatment was repeated in triplicate.

Analysis of Amino Acids. To extract amino acids and sugars prior to the roasting treatment, 100 g of the tea leaves was pulverized using a Cyclone sample mill. The amino acids in the tea sample were analyzed according to a previous report (*22*). A finely ground tea sample weighing 1 g was spiked with 1 mg of homoserine as an internal standard prior to extraction. PVPP (0.1 g) (Sigma-Aldrich, St. Louis, MO) was added to the tea sample to remove catechins and other polyphenols from the tea extract, and the extraction was performed using 100 mL of hot water for 30 min at 80 °C. The mixture was centrifuged at 20000 rpm for 20 min at 20 °C. The supernatant was filtered through a 0.45-μm filter disk (Advantec, Tokyo, Japan). Aspartic acid, glutamic acid, asparagine, serine, glutamine, arginine, alanine, and theanine were analyzed by a high-performance liquid chromatography (HPLC) system (L-6000; Hitachi, Tokyo, Japan) that employed precolumn derivatization using *o*-phthalaldehyde at pH 9.0. To separate each amino acid derivative in the filtrate (10 μL), an octadecylsilane column (150 mm, 4.6 mm i.d., 5 μm) (Develosil ODS-HG; Nomura Chemical, Seto, Japan) and a multistep linear gradient of acetonitrile in citrate buffer were used (*22*). The derived amino acids were detected by a fluorescence detector (F-1080; Hitachi, Tokyo, Japan) at 340/450 nm. The amino acid contents were analyzed in triplicate, and the average value of the three measurements was calculated.

Analysis of Sugars. The sugars in the tea sample were analyzed according to Anan's method (*23*) with a few modifications. A finely ground tea sample weighing 2.5 g was spiked with 0.1 g of sorbitol as an internal standard and extracted using 50 mL of hot water for 15 min at 80 °C. After centrifugation, the supernatant (2 mL) was passed through 2 mL of IRA-45 and 2 mL of IR-120B ion-exchange resins (Rohm and Haas, Philadelphia, PA). The resins had been equilibrated with water for >24 h prior to use. The eluate was filtered through a 0.45-μm filter disk (Advantec). The concentrations of sucrose, glucose, and fructose in the filtrate were determined by HPLC. Sugars in the filtrate (10 μL) were separated on a Sugar SC1011 column (300 mm, 8.0 mm i.d., 6 μm) (Shodex, Tokyo, Japan) at 80 °C and under isocratic conditions by using water (resistance > 17.5 MΩ/cm) as an eluate. The peaks were detected by a differential refractometer (YRD-880; Shimamura, Tokyo, Japan).

Analysis of Acrylamide in Tea Samples. Acrylamide was analyzed according to the method described by Ono et al. (*18*), in which the analyte was detected as a dibromo derivative (2,3-dibromopropionamide) by GC-MS with the following modifications. A finely ground tea sample weighing 5 g was spiked with [1,2,3-$^{13}C_3$]acrylamide (isotopic purity, 99%; Cambridge Isotope Labs, Andover, MA) as an internal standard prior to extraction and then mixed with 100 mL of water. The mixture was shaken for 1 min and then centrifuged at 20000 rpm for 20 min at 20 °C. For the analysis of the tea infusion, [1,2,3-$^{13}C_3$]acrylamide was added to 100 mL of the infusion in a volumetric flask. The supernatant (2 mL) was applied to an SPE cartridge (Isolute

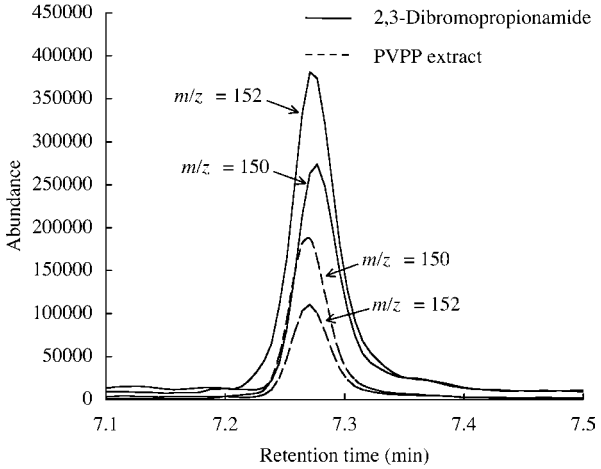

Figure 1. Mass chromatograms for the brominated acrylamide and the interference derived from the PVPP extract at *m/z* 150 and 152 obtained using GC-MS. PVPP (5 g) without [1,2,3-$^{13}C_3$]acrylamide was mixed with 100 mL of water. We next followed the procedure as described under Materials and Methods.

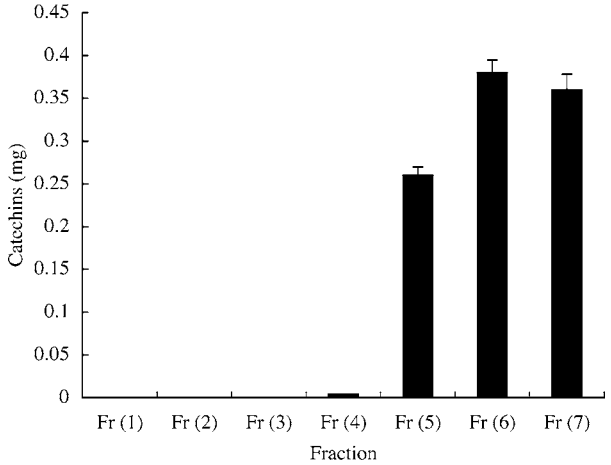

Figure 2. Total catechins in the fractions of the SPE eluate. The supernatant (2 mL) of the tea extract was applied to the SPE cartridge, and then the eluate was collected in the fraction of 0.5 mL.

Multimode, 500 mg; International Sorbent Technology, Hengoed, Mid Glamorgan, U.K.) that had been conditioned with methanol (1 mL) and water (2 mL). The characteristic features of the Multimode sorbent include hydrophobic interactions (presence of C_{18} functional groups) and strong cationic and anionic exchanges. The SPE eluate was cooled in an ice bath, and a bromination reagent (KBr, 15.2 g; HBr, 0.8 mL; bromine water, 5 mL; water, 60 mL) (*19*) was added. Over an hour later, excess bromine was reduced to a colorless solution by adding a sodium thiosulfate solution (1 mol/L); subsequently, ethyl acetate (4 mL) was added for extraction. The extract was dried over anhydrous sodium sulfate, and most of the solvent was removed by centrifugal evaporation under reduced pressure at 30 °C. The brominated extract (1 μL) in ethyl acetate was injected into the GC-MS system by using the splitless injection method. The gas chromatography system used was an HP5890 series II unit (Hewlett-Packard, Palo Alto, CA) equipped with a DB-17 MS capillary column (30 m × 0.25 mm i.d.; film thickness, 0.15 μm; Agilent, Palo Alto, CA). The injector temperature was 120 °C, and the temperature program used was as follows: isothermal for 1 min at 85 °C, temperature increased by 25 °C/min to 175 °C, isothermal for 6 min, temperature increased by 40 °C/min to 250 °C, and isothermal for 7.5 min. Helium was used as the carrier gas at a column head pressure of 100 kPa. The mass spectrometer (SX102H; JEOL, Tokyo, Japan) was operated in the selected ion monitoring mode. Electron impact mass spectra were obtained at 70 eV. Two ions (*m/z* 150 and 152) were used to characterize 2,3-dibromopropionamide derived from acrylamide, and two other ions (*m/z* 153 and 155) were used to characterize those derived from [1,2,3-$^{13}C_3$]-acrylamide. This process was repeated in triplicate, and the average of the three measurements was calculated.

RESULTS AND DISCUSSION

Optimization of the Cleanup Procedure for Acrylamide Analysis in Tea. Tea catechins, mainly epigallocatechin, inhibit the formation of the brominated derivative (*20*). Therefore, tea catechins should be removed from the tea extract for acrylamide analysis by GC-MS. PVPP has often been used to remove tea catechins and other polyphenols from the tea extract during the analyses of tea components, such as the amino acid analysis described under Materials and Methods. Preliminarily, we conducted a blank test by using PVPP. PVPP (5 g) without [1,2,3-$^{13}C_3$]acrylamide was mixed with water (100 mL), and the mixture was shaken for 1 min. Later procedure was followed as described under Materials and Methods. The peaks derived from the PVPP extract had the same retention time as the peaks of 2,3-dibromopropionamide at *m/z* 150 and 152 (**Figure 1**). Therefore, after the bromination, PVPP was unavailable for acrylamide analysis in the tea sample performed by GC-MS.

We examined the optimization of the SPE cleanup procedure. In this experiment, we used the tea sample [epigallocatechin, 86.6 mg/g dry basis (db); epigallocatechin-3-gallate, 95.7 mg/g db; epicatechin, 19.3 mg/g db; epicatechin-3-gallate, 26.6 mg/g db] that contained the highest amount of tea catechins among the samples we tested. The tea sample (5 g) was mixed with water (100 mL), and the mixture was shaken for 1 min and then centrifuged. The supernatant (2 mL) of the tea extract was applied to the SPE cartridge, and the eluate was collected in the fraction of 0.5 mL. The later fractions were found to contain mainly epigallocatechin and lower amounts of epigallocatechin-3-gallate and epicatechin when analyzed by using a previously reported method (*24*) (**Figure 2**). For the bromination, the preliminary study (*20*) used only a 1.5-mL fraction after discarding the first 1 mL of the eluate, and we confirmed that >2 mL of the bromination reagent was consumed for the bromination. However, we discarded the first 0.5 mL [fraction (Fr) 1] of the SPE eluate and used Fr 2−4, which contained small amounts of catechins; this was done because approximately 0.2 mL of the bromination reagent enabled the bromination in the fractions of the SPE eluate.

Our developed method seems to be available for acrylamide analysis in not only green tea but also other food materials containing catechins. Takatsuki et al. (*15*) succeeded in acrylamide determination in green tea and roasted green tea by LC-MS using column switching, and they used three types of the SPE cartridge (Bound Elute C18, ACCUCAT, and PSA; Varian Inc., Lake Forest, CA) for sample cleanup. Nemono et al. (*19*) analyzed acrylamide in roasted green tea by GC-MS after converting 2,3-dibromopropionamide to 2-bromopropenamide by triethylamine. Our method appears to be relatively simple because the acrylamide determination in green tea was achieved using one type of the SPE cartridge, and the conversion of 2,3-dibromopropionamide to 2-bromopropenamide was not required.

Validation of the Method Used for the Acrylamide Analysis in Tea. We next performed experiments for the validation of the method developed by us. For the calibration test, the measurements were performed in triplicate by using eight acrylamide standard solutions with different concentrations (1.6, 3.1, 12.5, 31, 62, 250, 640, and 1280 ng/mL). The linear range of the calibration curve extended from 1.6 to 1280 ng/mL when the areas of the peaks at *m/z* 150 and 155 were used for quantification ($r = 0.9999$). The linearity was better than

the following three patterns: $r = 0.9997$ for m/z 150 and 153, $r = 0.9892$ for m/z 152 and 153, and $r = 0.9921$ for m/z 152 and 155. Therefore, the areas of the peaks at m/z 150 and 155 were selected for the determination of the acrylamide content. The detection and quantification limits were evaluated according to the ICH guidelines (*25*). These values were calculated to be 0.2 and 0.6 ng/mL, respectively, from the values obtained after performance of the analysis using seven standard solutions in triplicate. The concentrations of the acrylamide in these solutions were 0.43, 1.2, 2.5, 4.3, 6.0, 9.7, and 11.2 ng/mL. The relative standard deviations calculated for the triplicate measurements of three tea powder samples with acrylamide concentrations of 14, 255, and 514 ng/g db were <8.0%. To verify the accuracy, we carried out a recovery test by adding known amounts of standard acrylamide (10, 20, 60, 120, 500, and 700 ng) to the finely ground tea powder that had been shown to contain 14 ng/g db of acrylamide. The overall recovery rates evaluated by averaging the three measurements ranged from 94 to 108%. The results thus obtained confirmed the validity of this analytical method.

Acrylamide Levels in Teas. The content of acrylamide in various tea products and their infusions was investigated (**Table 1**). The level of acrylamide in green tea ranged from 27 to 110 ng/g db, and that in roasted green teas such as Houjicha ranged from 247 to 1880 ng/g db. The level of acrylamide in the black tea and oolong tea samples analyzed in this study was lower than that in the green tea samples. However, slightly higher levels of acrylamide were detected in some of the oolong tea samples (*15*). The acrylamide levels in the tea samples, except for roasted green tea, were generally lower than those in coffee (*15*−*17*). This is because teas are manufactured at lower temperatures to preserve their flavors. Furthermore, roasted green tea was reported to have a high acrylamide content in a meeting of the Joint FAO/WHO Expert Committee on Food Additives in 2005 (*17*). Because large amounts of green tea, including products roasted at high temperatures, and coffee are consumed in Asian countries, we investigated the factors that affect the acrylamide formation in green tea.

Effects of Roasting Conditions on Acrylamide Formation in Green Tea. Acrylamide was absent (below the detection limit) in the tea leaf sample that was prepared below 80 °C and in the product that was roasted at 100 °C. This finding is in accordance with previous studies, which suggest that acrylamide formation requires temperatures of >120 °C (*4*, *5*, *10*). Acrylamide formation was detected at temperatures from 120 °C onward; its effects on roasting conditions are illustrated in **Figure 3**. At temperatures of <160 °C, the acrylamide concentration in the product increases with an increase in the roasting time. However, roasting at temperatures of >160 °C for 30 min decreased the acrylamide level. The maximum level of acrylamide was detected at 180 °C for the roasting times of 10 and 20 min; the acrylamide levels decreased at higher temperatures. The highest acrylamide level was observed in the product roasted at 180 °C for 10 min.

Similar patterns of time- and temperature-dependent acrylamide formation were observed in a model system of a mixture of asparagine and sugar (*4*). The maximum acrylamide yield in this mixture was at a temperature of 175 °C, beyond which the yield decreased (*4*). Additionally, high temperatures and long processing times resulted in a decrease in the acrylamide content of roasted coffee beans (*12*), grated potatoes (*7*), potato strips (*8*, *10*), gingerbread (*11*), breads (*9*), and almonds (*14*). This is considered to be associated with the physical properties of acrylamide, which decomposes and polymerizes on melting at

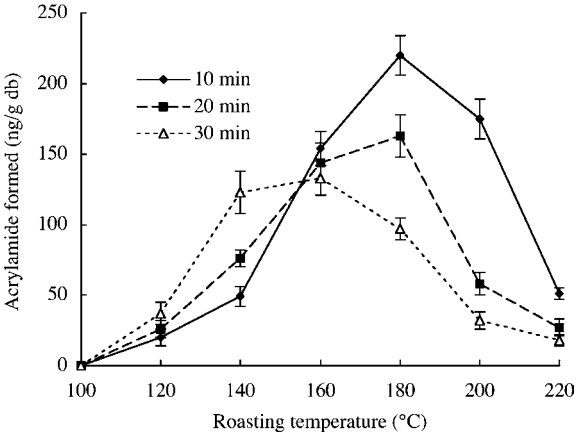

Figure 3. Effects of temperature and roasting time on acrylamide formation in roasted products. Tea leaves weighing 100 g were placed on an aluminum plate and roasted in a laboratory oven. The roasting treatment and acrylamide extraction process were repeated in triplicate. Each value is presented as the mean ± SD ($n = 9$).

temperatures of >175 °C (*10*). The decrease in the acrylamide content at temperatures below its melting point was assumed to be predominately due to degradation rather than polymerization (*10*). As shown in these experiments, the reaction time and temperature were covariant parameters for controlling the acrylamide levels in roasted green tea.

Correlation between Amino Acid and Sugar Contents in Tea Leaves and the Acrylamide in Roasted Products. The effects of the components of tea leaves on acrylamide formation in the roasted products were investigated by using the tea leaves of six cultivars that were roasted at 180 °C for 10 min. The roasting treatment was repeated in triplicate. Acrylamide was detected in all roasted products, and its concentration significantly varied from 49 to 584 ng/g db. The individual data in this experiment (**Table 2**) were used for multiple linear regression (MLR) analysis of the response surface model using Microsoft Excel. The amino acid and sugar contents of the tea leaves prior to roasting at 180 °C were used as variables. The variance ratio and probability (P) value were calculated using this model. MLR results provide a large amount of information by using this model. In particular, the parameter with a higher variance ratio is considered to be more significant than that with a lower variance ratio and low P value.

Table 3 lists the variance ratio and P value obtained by using the MLR model. Asparagine, with a variance ratio of 178 ($P < 0.001$), had the highest value among the parameters investigated in this experiment. Additionally, aspartic acid, glutamic acid, and theanine also showed statistically significant values calculated using the model. The variance ratios were 6 ($P < 0.05$) for aspartic acid, 13 ($P < 0.01$) for glutamic acid, and 5 ($P < 0.05$) for theanine. The concentrations of sugars and other amino acids had no significant correlation with the acrylamide level.

Model studies on acrylamide formation conducted using asparagine, aspartic acid, and glutamic acid revealed that a substantial amount of acrylamide was formed from asparagine in the presence of reducing sugars (*4*, *26*, *27*). The levels of aspartic acid and glutamic acid are strongly related to the level of asparagine in tea leaves ($r = 0.84$ for aspartic acid; $r = 0.71$ for glutamic acid); this may be the reason for considering them to be significant parameters in the result of the statistical analysis (**Table 3**). In contrast, the level of theanine, the most abundant amino acid in tea samples (*21*), did not correlate with the asparagine level ($r = 0.48$), although it was demonstrated to be a significant parameter for acrylamide formation. Next, we

Table 2. Acrylamide Formed in the Roasted Tea Product and the Amino Acids and Sugars in the Tea Leaves

	amino acids and sugars (mg/g, db)										
acrylamide formed (ng/g db)	Asp	Glu	Asn	Ser	Gln	Arg	Ala	Thea	sucrose	glucose	fructose
584	2.51	2.05	0.57	1.08	1.62	1.02	0.08	10.52	10.57	0.81	1.05
571	3.34	3.63	0.58	0.68	1.71	0.92	0.14	7.74	17.37	1.94	3.24
551	4.38	3.49	0.58	2.07	2.53	3.15	0.17	11.74	26.04	3.24	4.49
476	5.21	6.32	0.61	3.35	3.01	4.50	0.15	15.11	20.11	2.01	2.51
448	4.19	3.93	0.56	2.38	2.87	4.30	0.19	13.69	23.22	2.21	3.16
431	3.57	3.22	0.40	1.74	2.14	2.24	0.15	9.02	26.87	3.89	4.76
420	2.01	2.23	0.34	0.43	0.48	1.66	Tr[a]	2.98	21.33	10.87	7.08
352	3.13	2.72	0.39	0.63	1.69	2.60	0.09	12.43	23.43	2.15	3.54
350	1.88	1.91	0.13	0.26	0.29	–[b]	Tr	1.54	16.37	4.71	3.51
341	1.42	1.95	0.19	0.42	0.31	–	0.16	2.49	25.35	11.70	11.82
312	2.48	2.73	0.19	0.31	0.85	0.54	0.09	8.11	30.33	6.16	6.79
287	0.88	1.04	0.08	0.18	0.14	–	0.08	1.39	22.05	17.22	6.86
264	1.83	3.36	0.25	0.57	2.87	2.40	0.19	15.78	20.38	7.82	2.32
264	1.83	1.80	0.21	0.49	0.68	0.04	0.14	2.59	41.14	7.63	4.94
261	1.97	2.27	0.11	0.26	0.60	0.62	0.05	4.87	38.61	2.72	5.51
261	1.72	1.75	0.13	0.45	0.26	–	0.11	2.36	16.67	5.41	3.78
259	1.20	1.56	0.09	0.37	0.32	–	0.02	3.08	10.49	1.48	4.58
254	0.96	1.14	0.06	0.21	0.14	–	0.07	4.14	17.44	8.39	13.62
245	1.50	1.84	0.16	0.43	0.30	–	0.04	3.91	15.86	2.74	2.86
241	1.69	2.01	0.07	0.28	0.35	–	0.04	6.29	32.40	7.62	13.26
240	2.12	2.59	0.15	0.45	1.53	1.32	0.12	12.93	26.70	5.88	4.00
234	1.85	2.13	0.19	0.51	1.98	0.06	0.23	12.57	36.18	5.12	7.45
230	2.33	3.41	0.22	0.66	3.44	4.68	0.08	21.85	55.23	4.96	8.37
229	1.98	2.29	0.12	0.30	1.73	1.23	0.05	9.02	16.38	2.15	1.96
228	1.58	1.97	0.06	0.20	0.49	Tr	–	4.18	30.29	9.91	9.25
226	2.21	2.75	0.20	0.58	1.87	1.83	0.14	15.25	30.95	5.01	5.19
220	3.27	4.44	0.23	0.90	4.99	5.84	0.15	22.04	36.57	3.08	7.66
220	1.94	2.72	0.20	0.73	2.60	3.00	0.25	12.35	35.55	3.80	5.27
220	1.85	2.05	0.20	0.25	0.14	Tr	0.01	1.27	26.11	8.15	7.02
220	1.78	2.29	0.16	0.55	0.71	0.26	0.09	5.27	16.79	1.52	2.17
218	3.50	4.36	0.30	0.67	5.81	5.41	0.09	23.64	17.35	0.87	1.82
205	3.24	4.07	0.12	0.69	4.26	0.67	0.14	13.17	29.70	4.62	4.06
204	2.58	3.34	0.17	0.86	3.94	3.51	0.23	16.63	34.58	2.62	4.04
201	0.92	1.03	0.04	0.34	0.17	–	0.01	5.29	16.06	6.03	3.14
200	1.64	2.41	0.11	0.25	0.88	–	–	4.46	21.11	5.23	4.50
196	2.80	3.38	0.21	0.58	2.45	5.46	0.07	13.84	27.65	3.00	4.12
195	1.83	2.78	0.17	0.59	1.96	1.21	0.22	8.11	23.14	2.11	3.09
194	1.37	2.06	0.05	0.45	0.51	2.47	0.05	4.50	33.48	5.64	5.56
194	1.44	1.87	0.07	0.40	1.55	0.25	0.06	7.00	36.97	7.74	7.07
194	1.95	2.52	0.10	0.41	0.81	–	0.04	6.39	25.70	8.24	7.04
191	2.72	3.24	0.19	0.83	3.22	3.26	0.17	17.07	28.63	1.46	3.63
190	1.74	2.43	0.11	0.70	2.23	1.80	0.18	12.37	30.61	3.76	5.07
186	1.16	1.63	0.05	0.27	0.19	–	0.02	4.12	22.79	10.89	3.96
186	2.34	2.69	0.13	2.48	2.98	1.38	0.13	10.87	19.66	3.89	4.44
184	2.45	3.19	0.16	0.57	2.23	1.51	0.17	14.59	24.73	3.11	3.62
180	1.55	2.35	–	0.59	0.65	0.41	0.08	8.01	25.43	4.15	4.66
179	0.78	1.01	–	0.26	0.16	–	0.02	0.82	30.78	14.30	6.89
174	1.76	2.12	0.09	0.27	0.41	0.45	0.01	3.31	23.69	13.00	5.28
170	2.36	3.23	0.19	0.68	0.97	1.91	0.14	18.13	14.43	2.22	2.08
170	1.56	1.81	0.05	0.19	0.42	–	–	3.69	23.45	7.80	5.65
170	1.06	1.48	0.07	0.22	0.18	–	0.04	3.77	24.72	12.85	3.55
168	1.06	1.62	0.08	0.42	0.76	–	–	6.78	30.96	4.85	5.28
168	1.29	1.46	0.04	0.25	0.61	Tr	0.01	4.92	24.24	9.67	7.44
168	0.75	1.12	–	0.15	0.17	–	0.03	1.63	24.49	12.03	6.19
167	1.63	1.90	0.08	0.33	0.41	–	0.01	4.72	22.99	4.85	5.25
164	1.50	1.93	0.09	0.39	0.57	0.30	0.06	4.89	12.88	1.92	2.29
156	1.06	1.48	0.03	0.14	0.21	–	–	3.65	25.29	10.60	3.88
152	0.93	Tr	–	0.12	0.18	–	Tr	3.48	23.50	16.08	4.60
148	1.39	1.84	0.08	1.69	0.65	0.04	0.11	2.70	43.03	8.97	4.36
141	1.06	1.66	–	0.15	0.27	–	0.06	2.43	21.19	6.50	5.57
140	4.02	2.43	0.36	0.87	6.08	6.23	0.26	19.50	26.12	8.32	7.07
139	2.80	1.63	0.16	0.68	1.43	2.38	0.19	10.93	14.45	1.70	1.73
134	1.63	1.55	0.16	0.64	5.10	0.37	0.22	9.28	20.90	5.10	5.03
132	3.73	2.54	0.30	0.90	6.50	5.90	0.23	19.83	23.79	5.77	5.93
128	2.59	1.61	0.14	0.81	1.65	1.90	0.20	8.61	25.10	3.40	3.26
128	2.28	1.82	0.12	0.59	3.98	4.02	0.18	15.03	37.59	7.34	7.42
127	1.21	1.64	Tr	0.13	0.26	0.26	0.07	2.63	19.22	5.33	4.35

Table 2. (Continued)

acrylamide formed (ng/g db)	amino acids and sugars (mg/g, db)										
	Asp	Glu	Asn	Ser	Gln	Arg	Ala	Thea	sucrose	glucose	fructose
118	0.86	1.12	–	0.28	0.16	–	0.02	2.90	21.39	11.59	10.50
116	1.31	1.90	0.07	0.45	1.88	0.06	0.15	10.14	43.05	11.83	10.35
113	2.92	2.22	0.18	0.73	2.63	2.10	0.24	10.69	19.45	3.10	2.94
108	0.96	1.31	0.06	0.25	0.19	–	Tr	4.19	22.82	11.55	7.67
103	1.62	1.50	0.07	0.50	3.14	0.49	0.14	9.21	27.61	6.76	6.73
102	1.70	1.40	0.08	0.50	3.64	0.26	0.15	8.80	25.88	6.89	6.42
98	1.59	1.69	0.06	0.52	2.25	1.17	0.29	9.10	22.52	2.90	3.82
88	1.83	1.73	0.07	0.37	1.45	0.21	0.11	10.84	20.30	4.76	5.72
84	1.77	1.34	0.08	0.52	2.07	0.29	0.13	8.80	18.63	2.93	2.92
82	1.95	2.26	0.12	0.73	1.56	0.93	0.27	6.92	18.70	3.62	3.59
79	1.42	1.24	0.04	0.40	0.91	0.13	0.07	4.84	28.72	3.96	4.08
71	2.11	1.51	0.07	0.49	0.83	0.29	0.07	6.28	20.42	4.93	4.87
53	1.09	0.98	ND	0.26	0.51	ND	0.06	7.24	39.16	8.91	7.11
49	0.86	0.86	0.02	0.30	0.88	ND	0.07	9.63	14.40	2.55	2.92
49	1.40	1.13	0.01	0.25	0.73	0.09	0.05	6.81	27.21	8.19	6.85

[a] Trace level, 0.003 mg/g db ≤ Tr < 0.009 mg/g db. [b] Not detected, <0.003 mg/g db. The values for amino acids and sugars are the means of the triplicate measurements in tea leaves. The value for the acrylamide level in the roasted product is the mean of nine replicates.

Table 3. Variance Ratio and *P* Value for the Amino Acids and Sugars in the Tea Leaves Containing Acrylamide in Their Roasted Products[a]

parameter	variance ratio	*P* value
Asp	6	<0.05
Glu	13	<0.01
Asn	178	<0.001
Ser	1	NS[b]
Gln	1	NS
Arg	<1	NS
Ala	4	NS
Thea	5	<0.05
sucrose	<1	NS
glucose	<1	NS
fructose	3	NS

[a] Values were obtained by multiple linear regression analysis of the response surface model. [b] Not significant.

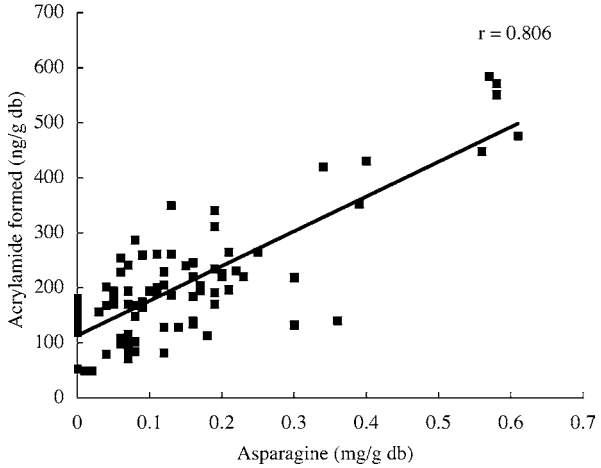

Figure 4. Correlation between the asparagine level in tea leaves and the acrylamide formed in the product roasted at 180 °C for 10 min. Each value is presented as the mean ($n = 9$ for acrylamide; $n = 3$ for asparagine).

performed a model reaction study to estimate the potential of theanine for acrylamide formation by using a previously reported method (*4*) with a few modifications. Significant quantities (1.2 μmol) of acrylamide were detected when an equimolar (0.1 mmol) mixture of asparagine and fructose was added to a 20-mL glass tube and reacted at 180 °C for 20 min in 0.5 M phosphate buffer (1 mL, pH 5.5) (*4*). Under the same conditions, a mixture of theanine and fructose formed a small quantity (0.6 nmol) of acrylamide. Therefore, it was considered that theanine was not a significant precursor in acrylamide formation, and a substantial amount of acrylamide in roasted products was mainly formed from asparagine in the presence of reducing sugars.

A correlation was observed between the acrylamide level in the roasted product and the asparagine level in the tea leaves ($r = 0.806$) (**Figure 4**). In contrast, no correlation was observed between acrylamide and glucose ($r = -0.196$) or between acrylamide and fructose ($r = -0.114$) (**Figure 5**). The same result was reported for roasted almonds (*14*). However, this result differs from that obtained for potatoes in which the reducing sugar level generally determines the acrylamide formation, and the asparagine level does not correlate with the acrylamide level (*28*−*30*). In our experiments on tea samples, the level of asparagine was <0.61 mg/g db, and the levels of fructose and glucose varied from 0.81 to 17.22 mg/g db (**Table 2**). Thus, on a molecular basis, the asparagine content was lower than the total reducing sugar content. Therefore, the asparagine content in the leaves determined the acrylamide level in the tea products. In contrast, the asparagine content is generally more constant and substantially higher than the reducing sugar content in potato tubers (*28*, *29*). Thus, the reducing sugar levels determine acrylamide formation in heated potatoes (*28*−*30*).

Conclusions. By using the 1.5-mL fraction of the SPE eluate for the bromination after discarding the first 0.5 mL of the eluate, we successfully analyzed the acrylamide in tea samples by GC-MS without the interference of tea catechins. Although PVPP can remove tea catechins from the tea extract, the peaks derived from PVPP had the same retention time as brominated acrylamide in mass chromatograms. The roasting temperature and time are important determining factors for acrylamide formation in tea. A considerable amount of acrylamide was formed at roasting temperatures of ≥120 °C. This could be responsible for the roasted green tea containing acrylamide at levels comparable to those in coffee, whereas other types of tea contained lower levels of acrylamide. Maximum acrylamide formation was observed when the tea leaves were roasted at 180 °C for 10 min. Higher temperatures and longer processing times were associated with a decrease in the acrylamide content. Asparagine, aspartic acid, glutamic acid, and theanine in tea leaves

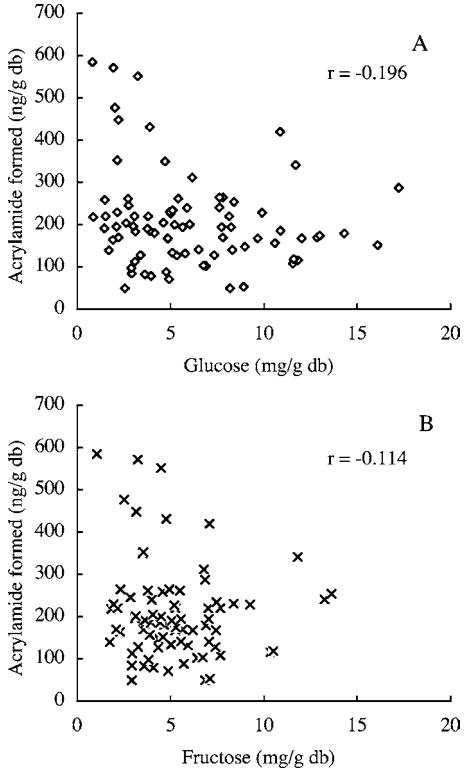

Figure 5. Correlation between the sugar (**A**, glucose; **B**, fructose) in tea leaves and the acrylamide formed in the product roasted at 180 °C for 10 min. Each value is presented as the mean ($n = 9$ for acrylamide; $n = 3$ for sugar).

are also statistically significant parameters for analyzing the acrylamide formation in roasted products. However, the model reaction demonstrated that a substantial amount of acrylamide in green tea was mainly formed from asparagine in the presence of reducing sugars, and the level of acrylamide in roasted green tea strongly correlates with the level of asparagine in the tea leaves. The results obtained in this study would be helpful in the selection of materials and for the improvement of processing conditions for the reduction of acrylamide formation in green tea.

ABBREVIATIONS USED

GC-MS, gas chromatography−mass spectrometry; wb, wet basis; db, dry basis; PVPP, polyvinylpolypyrrolidone; HPLC, high-performance liquid chromatography; SPE, solid-phase extraction; Fr, fraction; MLR, multiple linear regression; *P*, probability.

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