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Rapid analysis of amino acids in Japanese green tea by microchip electrophoresis using plastic microchip and fluorescence detection

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Abstract

Microchip electrophoresis for the short-time analysis of amino acids in Japanese green tea was developed. The amino acids in Japanese green tea were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). The derivatives were filtered and directly analyzed by electrophoresis on a plastic microchip with a 31-mm long separation channel with fluorescence detection. Amino acid analysis of Japanese green tea was improved by removing polyphenols using a polyvinylpolypyrrolidone pretreatment. Elution profiles of NBD-amino acids were examined under different running buffer conditions, and the sodium dodecyl sulphate in the running buffer exhibited a dramatically high-separation efficiency of amino acids by inhibiting their adsorption on the channel walls. Under the optimized conditions (5 m*M* phosphate buffer (pH 5.5) containing 0.05 m*M* sodium dodecylsulfate as running buffer), the main amino acids contained in Japanese green tea were well separated within 2 min, and theanine (1475 mg/100 g tea leaf), Arg (408 mg/100 g tea leaf) and Gln (217 mg/100 g tea leaf) were detected in Japanese green tea.
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1. Introduction

Tea is the most widely consumed beverage in the world. Especially Japanese green tea has recently received much scientific attention because of its medical or psychological effectiveness caused by abundant useful components. Amino acids are among them and are very crucial for the taste and quality [1–5]. The major amino acid is theanine (γ-glutamylethylamide, Fig. 1) and it is present in as much as 50% of all the amino acids. Theanine has a sweet and umami (a brothy or savory) taste and shows the highest correlation to the quality of Japanese green tea [5]. Furthermore, it has been found that theanine has considerable biological impact. For instance, there was a report that theanine decreased the level of norepinephrine and serotonin in the brain [6]. Another study showed that its administration in a naturally hypertensive rat decreased blood pressure [7]. More recently, the co-operative effects of anti-cancer agents and theanine on cancer have been reported [8]. Consequently, the analysis of amino acids in green tea is of great importance.

The analysis of amino acids in green tea has been

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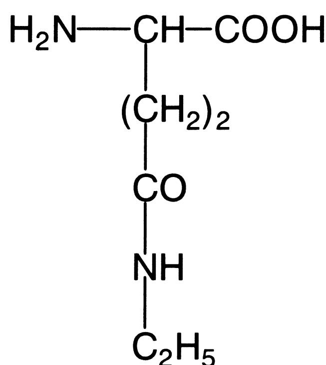

Fig. 1. Chemical structure of theanine.

mostly performed using reversed-phase HPLC after derivatizing the amino acids with *o*-phthalaldehyde, phenylthiocarbamoyl or 9-fluoromethoxycarbonylglycine chloride [9–12]. Capillary electrophoresis has also been adapted for the determination of theanine with UV detection to estimate the quality and taste of green tea [13].

In the past decade, miniaturization of conventional analytical instrumentation has been the focus of a great deal of attention [14,15]. Many liquid phase separation techniques including capillary zone electrophoresis have been successfully transferred to microscale [16,17]. The advantages inherent in miniaturizing analytical systems lie in improved efficiency with respect to sample size, response time, cost, analytical throughput and automation. Two types of substrates have been typically used for the fabrication of microfabricated devices. The first one is glass [16,17]. The excellent properties of glass with respect to electroosmotic flow (EOF) generation are very suitable for the separation channel. More recently, several plastic substrates have been used [18–23]. Among those, poly(dimethylsiloxane) (PDMS) [18,19] as a soft plastic and poly(methyl methacrylate) (PMMA) [20–23] as a hard plastic are the most popular. Because microfluidic devices using these substrates are less fragile and suitable for mass production, they are cost-effective.

Taking into account these advantages, we have developed analytical systems for amino acids in Japanese green tea using microchip electrophoresis aiming at a rapid and on-site analysis for quality checking at tea production sites. We have chosen PMMA as a substrate for our device. The microchip, which is commercially available and originally used for DNA separation, generated EOF and was applied to the determination of amino acid, peptides, and proteins with high separation efficiency [24].

2. Experimental section

2.1. Materials and chemicals

Theanine and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) were purchased from Tokyo Kasei (Tokyo, Japan). Polyvinylpolypyrrolidone (PVPP) and amino acids were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Sodium dodecylsulfate (SDS) was purchased from Wako (Osaka, Japan). Water was purified by Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.2. Apparatus

Separations were performed on a microfabricated PMMA chip, *i*-chip 3 DNA (Hitachi, Tokyo, Japan). The schematic layout is shown in Fig. 2. The microchip has dimensions of 85 mm×50 mm square with three simple cross-channels of 100 μm in width and 30 μm in depth. The distance between the sample reservoir (SR) and the sample waste (SW) was 10 mm, whereas the distance from the buffer

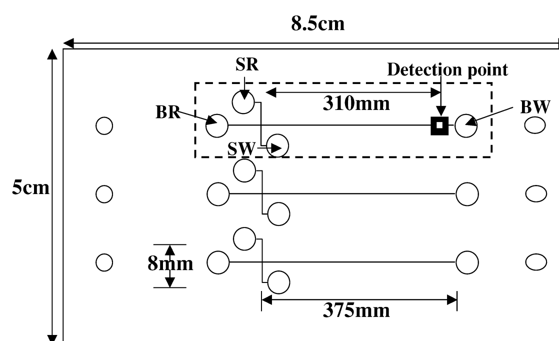

Fig. 2. Diagram of *i*-chip 3 DNA. The area within a dotted line is one cross-channel. SR, sample reservoir; SW, sample waste; BR, buffer reservoir; BW, buffer waste.

reservoir (BR) and the buffer waste (BW) was 44 mm. A small plastic tip was attached to each reservoir and each waste for sample or buffer introduction and removal, respectively. The microchip was placed on the stage of a fluorescence microscope, Model IX70 (Olympus, Tokyo, Japan) and could be set up for measurement without any difficulties. Light from a mercury lamp (Olympus) was introduced to the detection point of the microchip after filtering with a fluorescence cube Model U-MWIB (Olympus) that contains a 505-nm dichroic mirror and a 460–490-nm band-pass filter and is focused by a ×40 objective microscope lens. The fluorescence from the sample going back through the objective lens and the dichroic mirror was passed through a 515-nm interference filter and into a photomultiplier tube (Model H5784MOD) from Hamamatsu Photonics (Hamamatsu, Japan). The signal from the photomultiplier tube was amplified and recorded on an 833A data processor (Hitachi). The power supply for the microchip electrophoresis was a Model HOPP-3B1-L2 (Matsusada Precision Devices, Kusatsu, Japan), which was computer-controlled by the PC which was connected to a digital-to-analog converter, JJ Joker (Nippon Filcon, Inagi, Japan) through the RS-232C serial interface. The power supply was driven by a terminal emulation software named KTX (freeware). Sample introduction and separation were controlled through manipulation of the electric field strengths. Five m*M* phosphate buffer were used as the running buffer in all studies. The voltage settings were based on a previously published report [25]. In the experiments using phosphate buffer containing SDS as running buffer, the potentials of 100, −108, −500 V were applied to SR, BR and SW, respectively, for the 60-s sample introduction, while BW was grounded. This ensured minimal sample introduction bias in the injection cross-section. The voltage program for the separation of analytes was 630, 900, 630 V for SR, BR and SW, respectively, and BW was grounded. In the other studies, the positive and negative potentials were all reversed: −100, 108, 500, and 0 V were applied to SR, BR, SW, and BW, respectively, for introduction, and −630, −900, −630, and 0 V were applied to SR, BR, SW, and BW, respectively, for separation. After every run, each reservoir and channel was washed with fresh running buffer.

2.3. Pretreatment of Japanese green tea

The powdery tea sample (2.0 g, Kona Cha, Meiwa, Japan) was grounded and extracted with 50 ml of boiled water for 30 min. It was filtered through a filter paper. The filtrate was passed though the PVPP column, which was prepared by filling a Pasteur pipette with PVPP powder according to the previously published report [26]. The filtrate was used as the tea extract for analysis.

2.4. Derivatization of amino acids with NBD-F

Ten μl of 10 m*M* NBD-F in acetonitrile and 10 μl of 100 m*M* borate buffer (pH 8.5) were added to a 10 μl of 1 m*M* standard amino acids solution dissolved in 100 m*M* borate buffer (pH 8.5) or 10 μl of tea extract. The mixture was heated at 60 °C for 5 min and 100 μl of the running buffer was added, followed by the filtration by a 0.22-μm filter (Millipore). The filtrate was applied to SR.

3. Results and discussion

3.1. Effect of running buffer pH

In the analysis of charged analytes by CE, two major driving forces work to cause migration. One is EOF and the other is the electrophoretic mobility of the analyte. In a fused-silica capillary, EOF is generated under neutral and basic conditions, but it is negligible below pH 3. Under basic or neutral conditions, the channel surface of *i*-chip 3 DNA also generates EOF from BR to BW, due to the carboxy group of the channel surface [24]. Under these buffer conditions, the analytes are eluted in the order of basic, neutral, and acidic compounds by EOF as well as by their electrophoretic mobility. Because NBD-amino acids are negatively charged under those conditions, it is expected that the elution times will be longer than those of other compounds. Furthermore, it was assumed that many compounds other than amino acids could be eluted in this mode, which makes efficient separation difficult. On the other hand, under the acidic condition, where EOF is negligibly small, only negatively charged samples such as NBD-amino acids are selectively eluted by

reversing the applied voltage, such that BR is cathodic and BW is anodic, and selective separation of NBD-amino acids was expected. In consequence, the acidic condition was selected as the running buffer pH.

Fig. 3 shows the effect of running buffer pH on the migration times of the major amino acids contained in Japanese green tea [5]. At pH 2.5, separation between Asp and Asn, Glu and Gln was not achieved. Because the pK_a values of Asp and Glu are 1.88, 3.65 and 2.19, 4.25, respectively, they possess only one negatively charged carboxyl group in the running buffer at pH 2.5. Therefore, their characteristics concerning charge and molecular mass are almost the same as those of Asn and Gln, respectively, and separations were not achieved. Considering these results, a running buffer above pH 5.0 was examined.

At pH 5.0, Asp and Asn, Glu and Gln were separated from each other, and each amino acid was separated within 3 min. However, the peak shape of theanine was deteriorated. Migration times of amino acids other than Asp and Glu were largely affected by the buffer pH, and the migration times of amino acids increased with an increase in buffer pH. The velocity of an NBD-amino acid under acidic condition is described by:

$$v_{obs} = v_E + v_{eof} \quad (1)$$

where v_{obs} is the observed velocity of NBD-amino acid, v_E is the net electrophoretic velocity of NBD-amino acid, and v_{eof} is the velocity of the electroosmotic flow, which is in the opposite direction to v_E. Because the increase in the pH value increased the value of v_{eof}, the observed velocity of the NBD-amino acid decreased. Although separation of Ser, Asn, and Gln was improved at pH 5.5, theanine did not migrate within 4 min at the detection point. At pH 6.0, Ser, Gln, Asn, theanine did not migrate within 4 min. Therefore, pH 5.0 was the most appropriate.

3.2. Effect of acetonitrile

It is often observed that the addition of an organic modifier in a running buffer improves the peak shape and, as a result, the separation efficiency in electrophoresis [27,28]. To improve the peak shape of theanine, acetonitrile was added as an organic modifier to 5 mM phosphate buffer, pH 5.0. The addition of 2% (v/v) acetonitrile in the buffer sharpened the peak of theanine and decreased the migration times of the amino acids. Acetonitrile decreased the migration times of the amino acids, and this decrease resulted in poor separation of each amino acid. On the other hand, using acetonitrile in 5 mM phosphate buffer (pH 5.5) caused theanine to elute at 1.75 min (Fig. 4), which did not migrate without acetonitrile (Fig. 3). Other amino acids also migrated and separated better than those at pH 5.0. However, the

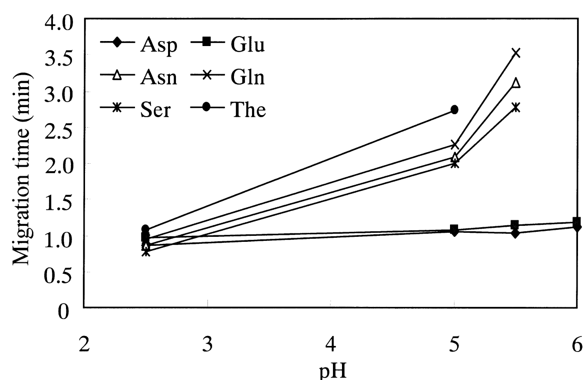

Fig. 3. Effect of running buffer pH on the migration times of major amino acids in Japanese green tea. Conditions: Sample, 1 mM amino acids; running buffer, 5 mM phosphate buffer at various pH values. Other conditions are in Section 2.

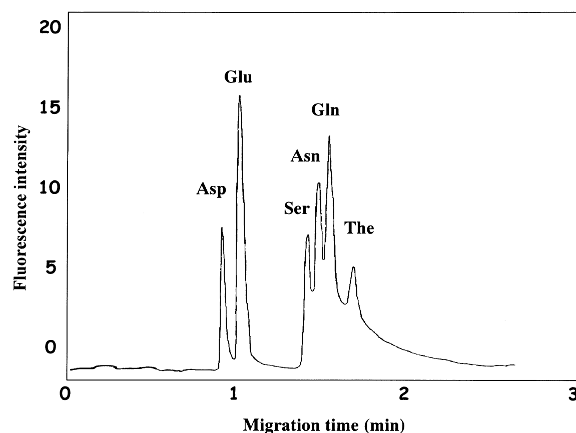

Fig. 4. Electropherogram of six standard amino acids. Conditions: sample, 1 mM amino acids; running buffer, 5 mM phosphate buffer (pH5.5) containing 2% acetonitrile. Other conditions are in Section 2.

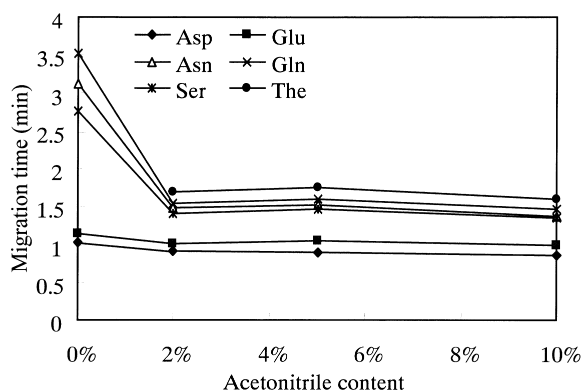

Fig. 5. Effect of acetonitrile content on the migration times of amino acids. Conditions: sample, 1 m*M* amino acids; running buffer, 5 m*M* phosphate buffer (pH 5.5) containing various contents of acetonitrile. Other conditions are in Section 2.

separations were not yet satisfactory (Fig. 4). Fig. 5 shows the effect of acetonitrile concentration on the migration times. Acetonitrile at 2, 5, and 10% was added to the phosphate buffer (pH 5.5). Within the pH values studied, acetonitrile concentration did not have much effect on the migration times, and the separations of the amino acids were not improved. The increase in the acetonitrile concentration could cause dissolution of the chip material, PMMA and adhesive; therefore, more than 10% acetonitrile was not appropriate as an additive.

3.3. Effect of SDS

Theanine, which is more hydrophobic than the other amino acids, was often adsorbed on the surface of the microchannel walls by hydrophobic–hydrophobic interaction. Although acetonitrile was efficient for inhibiting the absorption of theanine on the surface of the microchip, its use was limited to below 5% for the reason mentioned above, and the separation of amino acids was not sufficient. Because SDS is known to be adsorbed onto the inner surface due to hydrophobic–hydrophobic interaction and form a new dynamic layer on the surface of the plastic microchannel [29], it was assumed that SDS is effective in inhibiting the adsorption of theanine on the surface of the channels. In this case, SDS generated EOF from BR (anode) to BW (cathode) in 5 m*M* phosphate buffer (pH 5.5), and the elution profiles of the amino acids were changed from those mentioned above. That is, Asp and Glu did not migrate, whereas Arg migrated first and then the neutral amino acids, theanine, Gln, Asn, and Ser migrated in that order. SDS at the concentration of 20–0.01 m*M* in 5 m*M* phosphate buffer (pH 5.5) was used for the separation of the amino acids (Fig. 6). Below the critical micellar concentration (cmc: 8 m*M*) to 0.4 m*M* of SDS, the separation of theanine and Gln, the major amino acids in Japanese green tea, was insufficient, although adsorption of theanine on the channel surface was inhibited. The migration times were not changed depending on the SDS concentrations from 8 to 0.4 m*M* (Fig. 6) and buffer pH from pH 2.5 to 7.0 (Fig. 7). It may be assumed that SDS molecules which are adsorbed onto the inner surface form a new dynamic layer which causes the surface charge density to remain constant even when the buffer pH values change [29]. When SDS at a concentration of 20 m*M* was used, where the MEKC mode functioned, the migration times of theanine and Gln were decreased, but they were not separated from each other (Fig. 6). To improve the separation of theanine and Gln, the concentration of SDS was further decreased to reduce the EOF velocity. Using 0.05 m*M* SDS, the migration times of the amino acids increased, and their separations including theanine and Gln were satisfactorily obtained within 2 min (Figs. 6 and 8). This is presumably caused by the fact that the EOF velocity

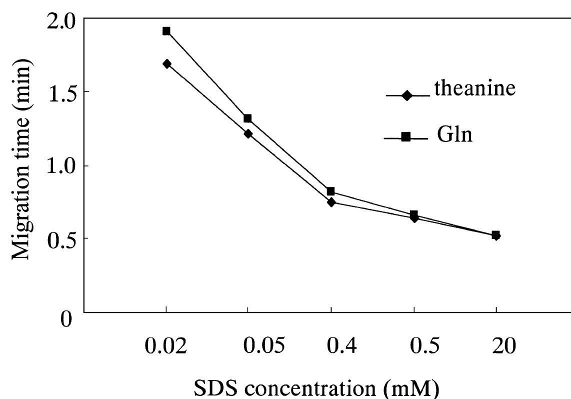

Fig. 6. Effect of SDS concentration on the migration times of theanine and Gln. Conditions: sample, 1 m*M* theanine and Gln; running buffer, 5 m*M* phosphate buffer (pH 5.5) containing various concentrations of SDS. Other conditions are in Section 2.

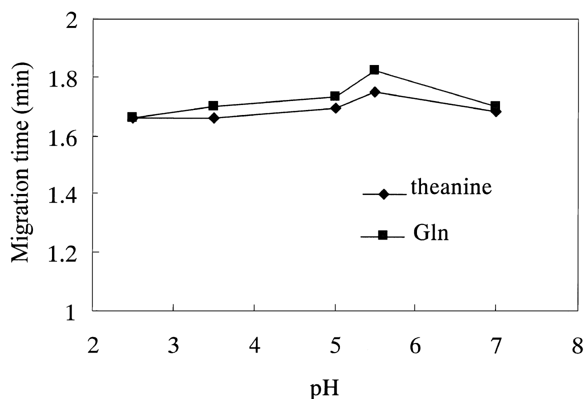

Fig. 7. Effect of buffer pH on the migration times of theanine and Gln. Conditions: sample, 1 m*M* theanine and Gln; running buffer, 5 m*M* phosphate buffer at various pH values containing 0.4 m*M* SDS. Other conditions are in Section 2.

decreased because of the reduction in the SDS molecules which were adsorbed onto the inner surface of the channel. Another reason is that the interaction between the amino acids and the plastic polymer of the inner surface of the channel was allowed to some extent due to the decrease in newly formed layer density of SDS, which contributed to the separation of the amino acids. Further reduction in SDS concentration (≤0.02 m*M*) resulted in an increase in the migration times and the reproducibility was poor.

3.4. Determination of amino acids in Japanese green tea

3.4.1. Pretreatment by PVPP

Japanese green tea contains a high amount of polyphenols (catechins). Catechins were also derivatized with NBD-F, thus inhibiting the derivatization procedures for amino acids. PVPP, a low-priced and excellent absorbent of catechins [30], was used to remove catechins for the analysis of amino acid in tea. By pretreatment with PVPP, amino acids in tea could be satisfactorily derivatized with NBD-F.

3.4.2. Analysis of Japanese green tea

Fig. 9 shows the electropherogram of amino acids in Japanese green tea, which were pretreated with PVPP, followed by derivatization with NBD-F. Five m*M* phosphate buffer (pH 5.5) containing 0.05 m*M* SDS was used as running buffer. Although Arg was not completely separated from the unknown peaks, the separation of theanine and Gln was good within

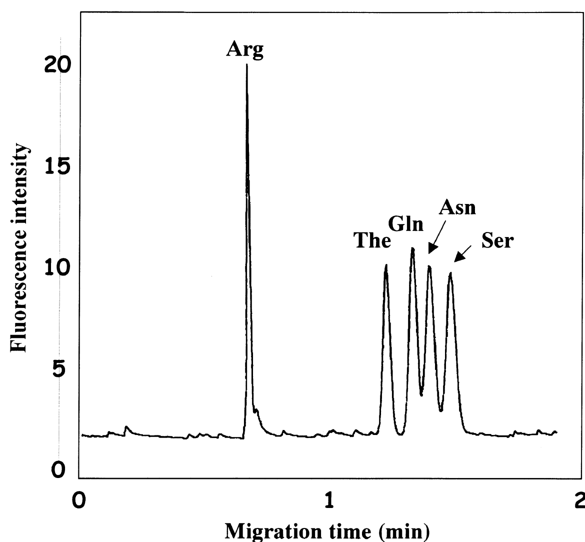

Fig. 8. Electropherogram of amino acids using the optimized buffer condition. Conditions: sample, 1 m*M* amino acids; running buffer, 5 m*M* phosphate buffer (pH 5.5) containing 0.05 m*M* SDS. Other conditions are in Section 2.

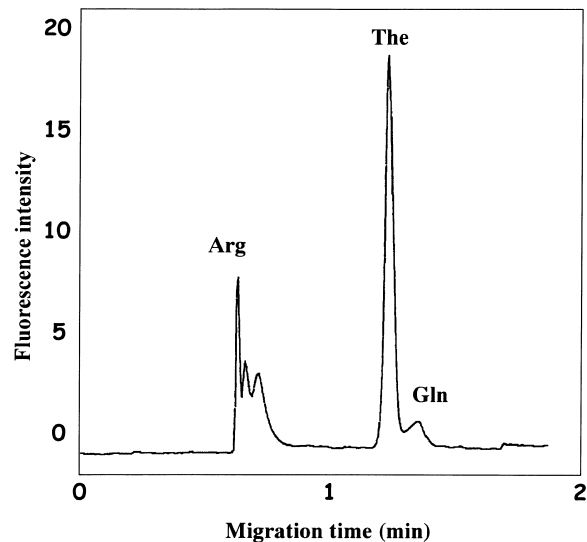

Fig. 9. Electropherogram of Japanese green tea using the optimized buffer condition. Conditions: sample, Japanese green tea; running buffer, 5 m*M* phosphate buffer (pH 5.5) containing 0.05 m*M* SDS. Other conditions are in Section 2.

2 min. The repeatability of the migration time of theanine by chip-to-chip analysis was good and the RSD was about 3% ($n=4$). Amino acid contents calculated from their calibration curves were 1475 (theanine), 408 (Arg), and 217 (Gln) mg/100 g tea leaf, respectively. These values coincided well with the values using the HPLC method and similar to those for other Japanese green teas reported previously [5]. Other amino acids such as Asn and Ser were not detected in this Japanese green tea.

4. Conclusions

We developed a rapid analytical system for amino acids in Japanese green tea by microchip electrophoresis. Addition of relatively low concentration of SDS in the running buffer created favorable conditions on the surface of microchip for good separation of the amino acids, where the adsorbed SDS molecules generated EOF forming a new dynamic layer on the surface of the microchannels, with a slight interaction remaining between the amino acids and the polymer materials on the channel surfaces which were not covered by SDS. This analytical system enabled the determination of amino acids in Japanese green tea within 2 min, which is a great improvement compared with other analytical systems such as HPLC or CE. Due to this high separation speed, the plastic microchip, which is commercially available at low cost, was found to be a suitable alternative as a novel platform for the analysis of Japanese green tea. On-site analysis of Japanese green tea for quality checking at production sites would be realized by the further improvement of complete automation of this analytical system.

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