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Identification and Detection of Genetically Modified Papaya Resistant to Papaya Ringspot Virus Strains in Thailand

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Many lines of genetically modified (GM) papaya (*Carica papaya* Linnaeus) have been developed worldwide to resist infection from various strains of papaya ringspot virus (PRSV). We found an unidentified and unauthorized GM papaya in imported processed papaya food. Transgenic vector construct that provides resistance to the PRSV strains isolated in Thailand was detected. An original and specific real-time polymerase chain reaction method was generated to qualitatively detect the PRSV-Thailand-resistant GM papaya.

Key words genetically modified organism; papaya; polymerase chain reaction; genomic DNA

Papaya (*Carica papaya* Linnaeus) is a widely grown fruit crop in tropical and subtropical areas.[1] A major constraint on papaya production has been infection by papaya ringspot virus (PRSV), which reduces papaya yields.[2] In response to this problem, genetically modified (GM) papaya that resists PRSV infection has been developed. Since the first successful generation of GM papaya in 1991,[3,4] many GM papaya lines, carrying different transgenic vector constructs, have been planted in various places, such as Hawaii, Florida, China, Jamaica, Taiwan, Thailand, Australia, Malaysia, the Philippines and Vietnam.[1]

Since Japan announced a mandatory safety assessment of GM foods, and processed foods containing GM ingredients, on April 1, 2001, foods on the Japanese market have been monitored for contamination with unauthorized GM products. We have developed qualitative detection methods for various GM crops, such as potato,[5,6] maize,[7–10] rice,[11,12] and flax,[13] using polymerase chain reaction (PCR) methods. For papaya, GM papaya line 55-1, a commercialized PRSV-resistant GM papaya developed in Hawaii, was the first authorized GM fruit allowed in Japan after a safety assessment by Food Safety Commission of Japan in 2009.[14] Subsequently, the use of any other GM papaya lines in foods has been prohibited. In 2011, an unauthorized GM papaya line, PRSV-YK, which was developed to resist the YK strain of PRSV in Taiwan, was found in some processed papaya products (papaya leaf tea, pickles and jam) on the Japanese market. The transgenic construct for PRSV-YK is similar to that of the GM papaya line 16-0-1/17-0-5 developed in Taiwan.[15] To monitor foods containing GM papaya on the Japanese market, we developed a qualitative detection method for GM papaya line 55-1 and for PRSV-YK using real-time PCR.[16–18] In addition, a histochemical assay[19] was developed to identify GM papaya line 55-1. Unauthorized GM papaya from Thailand has been found in Europe since 2012. Consequently, some papayas were rejected at the borders of some European countries.[20] Japan imports many papayas from overseas, thus it is required to monitor commercially processed products that include papaya as a major ingredient for contamination with the unauthorized GM papaya lines. In the present study, the presence of a new unauthorized GM papaya, PRSV-SC, was found in a processed papaya commodity (dried papaya) in Japan, and a new detection method for detecting PRSV-SC was developed using real-time PCR.

MATERIALS AND METHODS

Papaya Samples Processed papaya product (dried papaya) was purchased over the Internet in Japan. Hawaiian non-GM papaya (Sunset) fruit was purchased from a Japanese trade agency *via* the Hawaii Papaya Industry Association through the Consumer Affairs Agency, Government of Japan.

Purification of DNA Dried papaya was ground using a mixing mill. DNA was extracted and purified from 2 g of the samples using an ion-exchange resin-type DNA extraction and purification kit (Genomic-tip; Qiagen, Hilden, Germany) according to a previous report.[16] The purified DNA was quantified by measuring UV absorption at 260 nm using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.).

PCR Each PCR reaction mixture (50 μL) contained 5 μL 10× cloned *Pfu* reaction buffer (Agilent Technologies, Santa Clara, CA, U.S.A.), 0.18 mM deoxyribonucleotide triphosphate (dNTP) (Agilent Technologies), 0.3 μM forward and reverse primers, 2.5 U *Pfu Turbo* DNA polymerase (Agilent Technologies) and 50 ng template DNA. The PCR conditions were 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. A final terminal elongation occurred at 72°C for 10 min. PCR was performed using the GeneAmp PCR System 9700 (Life Technologies, Carlsbad, CA, U.S.A.). To determine the nucleotide sequence of the transgenic vector construct harbored in the GM papaya, DNA fragments were amplified by PCR using the following primer set:

Forward primer: 5′-GAC ATC TCC ACT GAC GTA AGG G-3′ (p324)
Reverse primer: 5′-CTATCRCTC TCT CCA GTT TTT G-3′ (p323)

DNA Sequencing PCR-amplified DNA fragments were extracted from the agarose gels and purified using a QIAquick PCR purification kit (Qiagen). The fragments were directly sequenced from both strands using forward and reverse primers with an ABI PRISM 3700 DNA analyzer and BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies), according to the manufacturer's instructions. Nucleotide sequences were analyzed using GENETYX software ver. 10.0.3 (GENETYX Corp., Tokyo, Japan).

Real-Time PCR Real-time PCR assays were performed using the ABI PRISM 7900 Sequence Detection System (Life Technologies). The 25 μL reaction mixture consisted of 2.5 μL sample DNA solution (25 ng), 12.5 μL Gene Expression Master Mix (Life Technologies), 0.8 μM forward and reverse primers, and 0.1 μM probe. The PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. GM papaya was detected using the following primers and probe:

Forward primer: 5′-CAT TTC ATT TGG AGA GAA CAC G-3′ (SC-F)
Reverse primer: 5′-ACC AGC ATC CAC AGC TTC-3′ (SC-R)
Probe: 5′-FAM-ACT CTA GAG GAT CCA TGT CCA A-TAMRA-3′ (SC-P)

To detect the papaya endogenous internal control gene, *chymopapain* (*Chy*; GenBank accession no. AY803756), the fol-

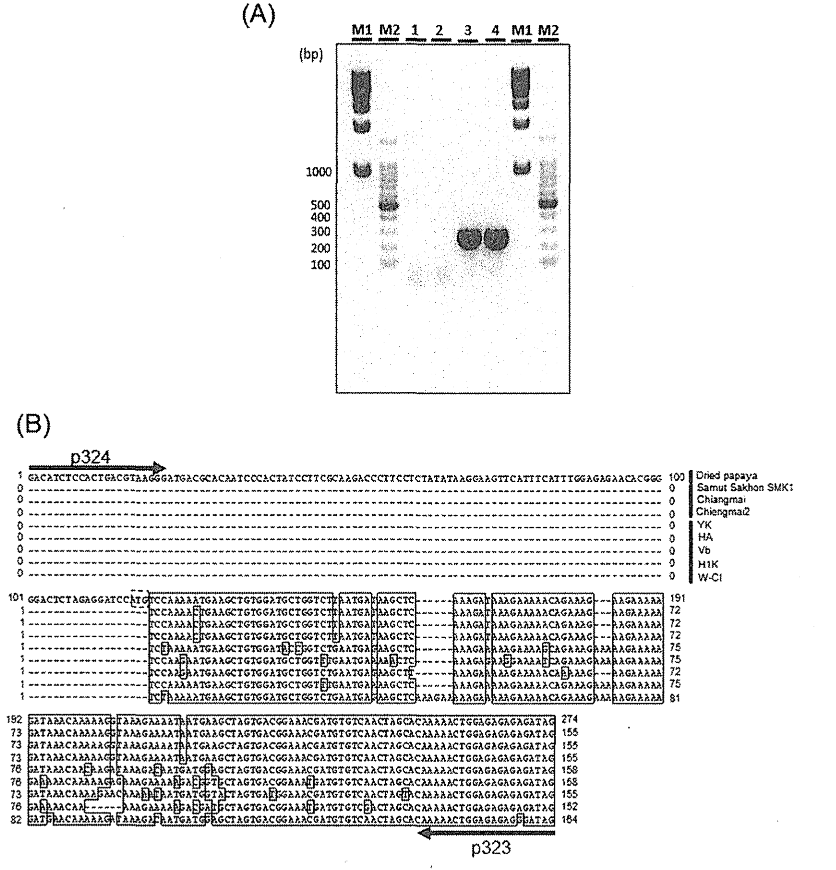

Fig. 1. PCR Targeting Construct-Specific Sequence of Papaya Ringspot Virus (PRSV)-Resistant Genetically Modified (GM) Papaya (*Carica papaya* Linnaeus)

(A) DNA templates (lane 1, no DNA was added; lane 2, non-GM papaya (Sunset); lanes 3 & 4, dried papaya product) were used for the PCR test using the primer set, p324 and p323, that hybridize to the cauliflower mosaic virus (CaMV) 35S promoter and the PRSV's coat protein (CP) gene, respectively. The PCR-amplified products were run on a 2% (w/v) agarose gel. Lane M1, 1-kbp DNA ladder marker, lane M2, 100-bp DNA ladder marker (B) Nucleotide sequence alignment of PRSV CP cloned from various isolates and strains (Samut Sakhon SMK1 [Thailand]; Chiangmai [Thailand]; Chiengmai2 [Thailand]; YK [Taiwan]; HA [Hawaii]; Vb [China]; H1K [Florida]; W-CI [Taiwan]) and the PCR products obtained using dried papaya product. Homologous sequences are boxed. The start codon for the GM papaya is boxed with a dashed line. Numerals beside the sequences indicate the number of nucleotides from the 5′ terminus. Arrows indicate the position of the primers p324 and p323.

The authors declare no conflict of interest.

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lowing primers and probe were used according to a previous report[17]:

Forward primer: 5′-CCA TGC GAT CCT CCC A-3′ (Q-Chy-1F2)

Reverse primer: 5′-CAT CGT AGC CAT TGT AAC ACT AGC TAA-3′ (Q-Chy-2R)

Probe: 5′-FAM-TTCCCTTCAT(BHQ1)CCA TTC CCA CTC TTG AGA-3′ (Q-Chy-P)

BHQ1 (black-hole quencher 1) was labeled in Q-Chy-P at the thymidine underlined in the nucleotide sequence.

All primers and probes were diluted with an appropriate volume of distilled water and stored at −20°C until used. Each real-time PCR reaction was tested in duplicate. Results were analyzed using SDS 2.1 sequence detection software (Life Technologies) for the ABI PRISM 7900 Sequence Detection System.

RESULTS AND DISCUSSION

Identification of Unauthorized GM Papaya To investigate the potential contamination of processed papaya products with a new unauthorized GM papaya, we used genomic DNA purified from the products as the PCR template. The primer set p323 and p324 was used as previously reported.[16] The forward primer (p324) was designed to hybridize to the sequence of the most common promoter, the cauliflower mosaic virus (CaMV) 35S promoter, which is used in the transformation of papaya to alter various papaya traits.[1] The reverse primer (p323) was designed to hybridize to the highly conserved sequence of the coat protein (CP) gene, which has been cloned from various isolates and strains of PRSV (GenBank accession nos. Samut Sakhon SMK1, DQ085864; Chiangmai, DQ085856; Chiengmai2, AY010720; YK, X97251; HA, S46722; Vb, AF243496.1; HIK, AF196839.1; W-CI, AY027810.2). When using DNA purified from a dried papaya product as the template, electrophoresis of the PCR products showed a single band of 200–300 bp (Fig. 1A). The DNA purified from the non-GM papaya (Sunset) as a control generated no PCR products of this length. A direct sequence analysis of the PCR product and a BLASTn analysis indicated that the 3′ end sequence was homologous to the CP gene in some Thai PRSV isolates (Samut Sakhon SMK1, Chiangmai, Chiengmai2), except that the fourth codon after the start codon had a single nucleotide polymorphism ("aat" in the dried papaya and "act" in Samut Sakhon SMK1, Chiangmai, and Chiengmai2) (Fig. 1B). The predicted amino acid sequences of the CP gene were identical to that of Thailand's PRSV isolates.[21] Furthermore, a multiple cloning site (containing restriction sites for *Xba*I and *Bam*HI) and the insertion of a start codon between the CaMV 35S promoter and the N-terminus of the CP gene were detected (Fig. 2A). The transgenic vector construct sequences of the CaMV 35S promoter and the CP gene in the GM papaya developed in Hawaii (line 55-1, GenBank accession no. FJ467933.1) and Taiwan (lines 16-0-1, 17-0-5)[15] showed no similarity (data not shown) to the sequence from the dried papaya product. In addition, our preliminary study using real-time PCR showed that the dried papaya product also contained high concentrations of two transgenic sequences, the *nopaline synthase* terminator, which is used in transgenic cassettes, and *neomycin phosphotransferase II*, which is used as a selectable marker (data not shown). These results suggest that the dried papaya product was contaminated with a new unauthorized GM papaya line, which we named PRSV-SC.

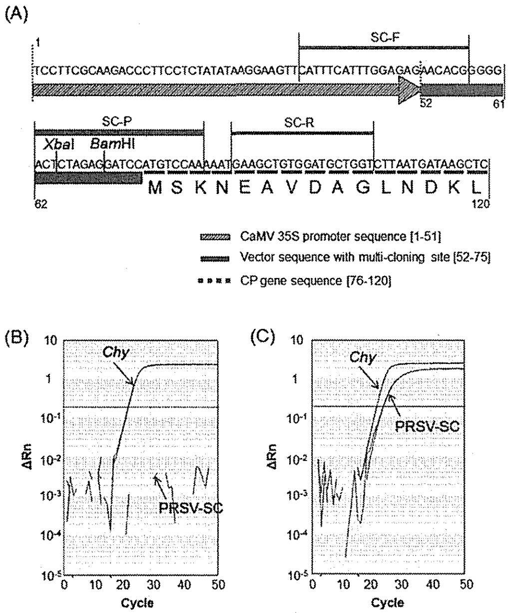

Fig. 2. Detection of a Papaya Ringspot Virus (PRSV)-Resistant Genetically Modified (GM) Papaya (*Carica papaya* LINNAEUS) Line, PRSV-SC, Using Real-Time PCR

(A) A fragment of the transgenic vector construct sequence from the line PRSV-SC was obtained; restriction sites are marked by vertical arrows. The primers (SC-F and SC-R) and probe (SC-P) designed for detecting the construct-specific sequence of PRSV-SC are indicated by lines above the sequence. Numerals indicate the number of nucleotides from the 5′ terminus. DNA purified from non-GM papaya (Sunset) (B) and the PRSV-SC contaminated dried papaya product (C) were tested using real-time PCR for endogenous *Chy* detection with primer set (Q-Chy-1F2 and Q-Chy-2R) and probe (Q-Chy-P) and for PRSV-SC detection with SC-F, SC-R and SC-P. The threshold value was set at 0.2. Positive amplification curves are designated by arrows.

Designing a Novel Construct-Specific Detection Method for PRSV-SC DNA fragmentation occurs during the manufacturing of processed foods.[22,23] To qualitatively detect PRSV-SC in processed foods, specific primers and a probe for a real-time PCR assay were designed to produce a short amplicon (70 bp) based on the detected transgenic construct sequence. Also, to prevent false-negative results using real-time PCR, the PRSV-SC detection method was designed to generate a target amplicon shorter than the endogenous papaya *Chy* detection method (amplicon size 72 bp). The primers were designed to amplify the region between the transgenic vector backbone and the CP gene sequence. The probe (SC-P) was designed to target the site of the CP gene's start codon (Fig. 2A).

The real-time PCR assay for PRSV-SC detection confirmed that the dried papaya was positive for PRSV-SC, producing Ct values of 23.48 and 23.34 with a threshold value of 0.2 in a duplicate test (Fig. 2C). Endogenous *Chy* detection was positive for all samples, with Ct values of 21.27 and 21.28 for the dried papaya and 20.77 and 20.87 for the non-GM papaya (Sunset), each with a threshold value of 0.2 in a duplicate test (Figs. 2B, C). No amplification signals were obtained from the non-template control, from genomic DNA derived from 14 other crops (maize, rice, soybean, flax, canola, chickpea, wheat, sugarbeet, cottonseed, potato, papaya, tomato, eggplant, and green pepper), or from other GM papaya lines, such as 55–1 (Hawaii) and PRSV-YK (Taiwan), using the developed PRSV-SC detection method (data not shown). These results indicated that the method is specific for detecting PRSV-SC.

In the present study, as a result of monitoring processed foods for contamination with unauthorized GM papaya, we found a dried papaya product containing a transgenic vector construct for the expression of PRSV's CP gene, which was cloned from isolates in Thailand. A novel construct-specific real-time PCR detection method was developed for detecting PRSV-SC. Because the genetic background of PRSV-SC was unknown, it was not possible to estimate the content level of PRSV-SC in the papaya product. Further studies are underway to determine the PRSV-SC detection limits of the real-time PCR, and whether the qualitative PCR method using the p323/p324 primer set can function as an initial screening for the presence of GM papaya in foods.

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Analytical Methods

Development and validation of a lateral flow assay for the detection of crustacean protein in processed foods

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ABSTRACT

We developed and validated a novel lateral flow assay for the detection of crustacean protein in processed foods. This assay had high sensitivity; the visual detection limit for shrimp protein extract was 25 μg/L, equivalent to 1 μg/g protein in a food sample, and results could be obtained within 20 min without sophisticated procedures or expensive equipment. Concordance between our assay and another validated quantitative enzyme-linked immunosorbent assay was 97% for commercially processed foods. This assay is rapid, simple, reliable, and highly correlated with validated enzyme-linked immunosorbent assays and is thus suitable for monitoring of food products, especially in food-processing facilities.

1. Introduction

In recent years, food allergies have become an important health problem in developed countries; it is estimated that more than 2–3% of people have some type of food allergy (Ebisawa, 2007; Rona et al., 2007). One of the most problematic aspects of food allergies is that effective therapies have not been established. Therefore, strict avoidance of the food allergen is currently the only means of preventing an adverse allergic reaction (Sampson, 2004; Wang & Sampson, 2011). However, various studies have shown that severe allergic reactions can be caused by accidental ingestion of food products containing allergenic materials (Furlong, DeSimone, & Sicherer, 2001; Yu et al., 2006), making it necessary to develop adequate and reliable information regarding potentially allergenic ingredients in processed foods.

Labelling system for allergenic ingredients in processed foods has been discussed by international organisations such as the Codex Alimentarius Commission of the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO). In 1999, the Joint FAO/WHO Codex Alimentary Commission Session agreed to recommend labelling of eight foods that contain ingredients known to be highly allergenic, including crustaceans (Hefle, Nordlee, & Taylor, 1996). In Japan, the Ministry of Health, Labour and Welfare (MLHW) has enforced a labelling system for allergenic food materials since April 2002 to provide information about these foods to allergic consumers. In this system, labelling is mandatory for seven food materials, including eggs, milk, wheat, buckwheat, peanut, shrimp, and crab, and labelling for an additional 18 food materials, such as soybean, is recommended (Akiyama, Imai, & Ebisawa, 2011).

An allergic reaction to crustacean products can be elicited by ingestion of trace amounts of crustacean protein, and anaphylaxis caused by exposure to such protein has been reported (Lopata, O'Hehir, & Lehrer, 2010; Musmand, Daul, & Lehrer, 1993). To protect allergic patients from exposure to hidden allergens, we developed a quantitative enzyme-linked immunosorbent assay (ELISA) for the detection of crustacean protein (Seiki et al., 2007). The method was acceptably accurate and suitable for quantitative measurement of crustacean protein in processed foods; however, the ELISA method is relatively time-consuming and requires expensive instrumentation and well-trained people. In contrast, the lateral flow assay is simple, rapid, and can be applied to the monitoring of processed foods (Röder, Vieths, & Holzhauser, 2009).

In this study, we developed and validated a novel lateral flow assay for the detection of crustacean protein in commercially processed foods as a useful tool for monitoring food products.

2. Materials and methods

2.1. Food samples

Ingredients and commercially processed foods were purchased at local grocery stores in Tsukuba, Japan. We procured commercially processed foods with and without crustacean labelling in the list of ingredients.

2.2. Preparation of shrimp protein extract and model processed foods

Shrimp protein extract was prepared from black tiger prawn (*Penaeus monodon*) (Seiki et al., 2007). Assay solutions were prepared by diluting the shrimp protein to 0.0, 12.5, 25.0, 50.0, 125.0, and 250.0 μg/L with extraction buffer containing PBS (8.1 mmol/L Na_2HPO_4, 1.47 mmol/L KH_2PO_4, 2.68 mmol/L KCl, and 137 mmol/L NaCl), 1% TritonX-100, and 0.009% NaN_3.

As a model processed food, tomato juice was prepared by adding freeze-dried black tiger prawn muscle powder at final concentrations of 0.0, 2.0, 5.0, or 10.0 μg/g; the liquid was thoroughly mixed for 60 min.

Fish-meat sausage and freeze-dried egg soup containing black tiger prawn muscle powder at final concentrations of 5.0 μg/g and 6.0 μg/g, respectively, were prepared as described (Seiki et al., 2007).

2.3. Preparation of assay solution

The samples were homogenised in an Ace AM-4 homogenizer (Nissei, Tokyo, Japan) three times for 30 s each. Extraction buffer (39 mL) was added to 1 g homogenised sample, which was then shaken using an Ace AM-4 homogenizer or VORTEX-GENIE2 (Scientific Industries Inc., New York, USA) three times for 30 s at room temperature. After extraction, the sample was centrifuged at 3,000×g for 20 min at 4 °C, and the supernatant was filtered through 5AB paper (Advantec, Tokyo, Japan).

2.4. Preparation of test strips

Test strips were prepared as described (Millipore Corporation). The monoclonal and polyclonal antibodies against black tiger prawn protein described in our previous ELISA study (Seiki et al., 2007) were used here. Monoclonal antibodies diluted in PBS (0.5 g/L) were applied to a nitrocellulose membrane in a test line, and anti-rabbit IgG antibodies diluted in PBS (2.5 g/L) were applied in a control line. The membrane was soaked in blocking solution containing 5 g/L casein and dried. Gold colloid solution (10 mL) was adjusted to pH 8.15 and 0.8 mL of 0.1 g/L polyclonal antibody was added. After 30-min incubation at room temperature, 1.2 mL of 100 g/L BSA was added. The mixture was centrifuged and the precipitate resuspended in 0.5 mL distilled water. Next, 190 μL distilled water was added to 10 μL polyclonal antibody–gold conjugate and 800 μL dilute solution; a glass fiber filter was soaked in the mixture and dried. The lateral flow devices were assembled with a mount, nitrocellulose membrane, glass fiber filter, filter paper, non-woven fabric, and cover (Fig. 1).

2.5. Evaluation of the lateral flow assay

Sample solution (2 mL) was placed in a plastic tube, and then a test strip was added for 3 s. The test strip was removed and placed on an aluminum sheet with the test line facing up for 20 min. The signal intensity of the test line was estimated visually; when at least 1 of 3 observers considered the results positive, it was judged positive and quantitated using an Immunochromato Reader C10066 system (Hamamatsu Photonics K.K., Shizuoka, Japan). To compare the reaction intensity, sample solutions of black tiger prawn, red king crab, pollicipes, skeleton shrimp, opossum shrimp, and euphausia were diluted 1:10,000 with extraction buffer and retested. Since black tiger prawn is comprised of approximately 18.4% protein (Ministry of Education, Culture, Sports, Science and Technology, 2010), the concentration of protein contained in diluted black tiger prawn solution was equivalent to approximately 20 μg/g in a food sample. ELISA was performed as described (Seiki et al., 2007).

2.6. Evaluation of assay variation

Intra- and inter-assay precision were measured as described (Anfossi et al., 2011). Model processed foods containing black tiger prawn protein at 0.0, 2.0, 5.0, 6.0 and 10.0 μg/g were used. To determine intra-assay precision, the mean coefficients of variation (CVs) were calculated from five replicates performed on the same day. Inter-assay variances were calculated from experiments performed on five different days.

3. Results

3.1. Optimisation of the lateral flow assay

Our first approach was to select for antibody combinations showing high immunoreactivity for shrimp protein. Shrimp protein extract at 100 μg/L was not detectable with the combination of gold-conjugated monoclonal antibodies and polyclonal antibodies in the test line; in contrast, sufficient reactivity was observed for 100 μg/L shrimp protein extract with a combination of gold-conjugated polyclonal antibodies and monoclonal antibodies in the test line.

We next examined the effect of reaction time on assay sensitivity. When the reaction time was less than 15 min, detection intensity for 25 μg/L shrimp protein was very low. However, reaction times over 20 min yielded sufficient reaction intensity with 25 μg/L shrimp protein extract for visual detection.

In lateral flow assays, a false-negative reaction known as the prozone phenomenon often occurs when a sample solution contains excessive amounts of the target protein (Burch, 2000). In this study, the prozone phenomenon was observed on the control line with the concentrated crab extract (data not shown). To solve this problem, we optimised the amount of antibody in the control line and found that the concentrated crab extract does not cause the prozone phenomenon when the antibody concentration is ⩾2.5 g/L.

3.2. Limit of detection for shrimp protein extract

The limit of detection (LOD) was determined for the shrimp protein extract. As shown in Table 1, reactivity was detected in samples at 12.5, 25.0, 50.0, 125.0, and 250.0 μg/L, and non-specific

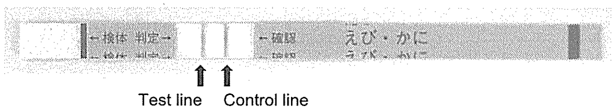

Fig. 1. The lateral flow device. When test and control lines appear, the sample is positive. If test line does not appear, the sample is negative.

Abbreviations: CV, coefficients of variation; mABS, milli absorbance; MS, mass spectrometry.

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reaction was not detected for the 0.0 µg/L negative control. Since the ratio of positive results/total from five replicates was 5/5 for ⩾25.0 µg/L, the visually determined detection limit of this assay was 25.0 µg/L, which is equivalent to 1 µg/g in a food sample.

3.3. Reactivity and specificity tests

The reactivity and specificity of the assay were tested using various crustaceans, molluscs, and other food ingredients. The assay was reactive for ten members of the Decapoda group and four other species of Crustacea, except acorn barnacle (Table 2). The mABS values of these reacted samples were >50.0. The diluted solution (1:10,000) of black tiger prawn and red king crab showed reactivity, but the diluted solution (1:10,000) of four other species of Crustacea did not (data not shown). No cross-reactivity was observed in 20 different molluscan samples and 59 other food ingredients (Supplementary Tables S1 and S2).

These results show that the assay has sufficient specificity for crustacean protein testing in processed foods.

3.4. Detection limits and reproducibility for model processed foods

To evaluate the applicability of the developed assay to processed foods, model tomato juice containing black tiger prawn protein was tested. The visual LOD for model tomato juice was estimated to be 1.0 µg/g. Intra- and inter-assay variations were assessed using the model tomato juice containing black tiger prawn protein (2.0, 5.0, and 10.0 µg/g), model fish-meat sausage (5.0 µg/g) and model freeze-dried egg soup (6.0 µg/g). As shown in Table 3, the method provided satisfactory precision and sensitivity.

3.5. Application to commercial food products

We tested 104 different commercially processed foods using the assay to evaluate its applicability to processed foods. The products with crustacean included in the list of ingredients were positive, except for spring roll. Products without crustacean in the list of ingredients yielded negative results. The level of crustacean protein in these foods was analysed using the validated quantitative ELISA. Since the limit of quantification (LOQ) was 2.25 µg/L (equivalent to 0.9 µg/g in samples) (Seiki et al., 2007), the samples containing over 1.0 µg/g of shrimp protein were tentatively considered to be positive in the ELISA. As shown in Table 4, concordance between the new assay and ELISA for products containing <1.0, 1.0–10.0, and >10.0 µg/g of crustacean protein was 100%, 88%, and 100%, respectively. Overall concordance was 97%. The ELISA detected 5.1, 1.5, and 6.4 µg/g crustacean protein in spring roll, kamaboko, and fried cake of ground fish, respectively, but the new assay did not show reactivity for these foods.

4. Discussion

We developed and validated a novel lateral flow assay for detection of crustacean protein in commercially processed foods. Other lateral flow assays for the determination of food allergens have been reported (Röder et al., 2009; Wen, Borejsza-Wysocki, DeCory, & Durst, 2005). However, there has been no scientific report of a lateral flow assay to detect crustacean protein and sufficient validation of the lateral flow assay to detect food allergens, although lateral flow assays for mycotoxin are well validated (Anfossi et al., 2011). We determined the sensitivity, specificity, reproducibility, and applicability of the assay in processed foods and demonstrated that the assay is a simple and reliable method for the detection of crustacean protein in food products.

Since the major allergen of Crustacea is tropomyosin (Shanti, Martin, Nagpal, Metcalfe, & Rao, 1993), we believed tropomyosin would be a target protein for the specific detection of crustacean protein. Detection methods for food allergens should have high specificity; however, the allergen of Mollusca is also tropomyosin, and the molluscan tropomyosin exhibits antigenic cross-reactivity to crustacean tropomyosin because of their homologous amino acid sequences (Shiomi, 2009). We previously prepared monoclonal and polyclonal antibodies with high specific reactivity to crustacean, not molluscan, protein and developed a validated ELISA method using these antibodies for detection of crustacean protein in processed foods (Sakai, Matsuda, et al., 2008; Seiki et al., 2007). In this study, we developed and validated a novel lateral flow method using the same antibodies used in the ELISA method. As expected, the lateral flow assay shows no reactivity for molluscan protein and the proteins of other major foods such as fish, meat, and crops, and the method has high specificity to crustacean protein. In the Japanese labelling system, the labelling of shrimp and crab in the Decapoda group of Crustacea is mandatory, but labelling of other groups of Crustacea, such as Mysidacea and Euphausiacea, is not (Akiyama et al., 2011). We confirmed that the new assay has high specificity to protein of the Decapoda group, similar to the ELISA method (Seiki et al., 2007). These results suggest no false-positive reactions derived from the protein of other groups of Crustacea would occur during testing of processed foods. Thus, the new assay is suitable for the specific detection of Decapoda protein in processed foods.

Table 2
Reactivity of various crustacean samples in the lateral flow assay.

Sample	Result
Decapoda group	
Black tiger prawn	+
Red spotted shrimp	+
Northern shrimp	+
Spotted shrimp	+
Freshwater prawn	+
Fan lobster	+
Caribbean spiny lobster	+
Red king crab	+
Swimming crab	+
Snow crab	+
Other species of Crustacea	
Acorn barnacle	–
Pollicipes mitella	+
Skeleton shrimp	+
Opossum shrimp	+
Euphausia	+

Table 1
Analysis of shrimp protein extract.[a]

Sample	Assay solution (µg/L)	Equivalent concentration in a food sample (µg/g)	Result (positive/total)	mABS (average)	% CV
Shrimp protein extract	250.0	10.0	5/5	50.3	6.8
	125.0	5.0	5/5	30.8	6.4
	50.0	2.0	5/5	15.0	5.2
	25.0	1.0	5/5	11.1	7.6
	12.5	0.5	4/5	6.5	12.5
	0.0	0.0	0/5	0.5	–

[a] mABS is the absorbance in the test line determined using an Immunochromato Reader. % CV is the coefficient of variation calculated from mABS.

Table 3
Intra- and inter-assay variances of the lateral flow assay using model processed foods[a].

Sample	Equivalent concentration in a food sample (µg/g)	Intra-assay		Inter-assay	
		mABS	% CV	mABS	% CV
Tomato juice	10.0	42.4	8.1	46.4	10.4
	5.0	26.0	9.4	28.5	10.8
	2.0	13.2	8.3	12.7	10.0
	0.0	0.7	–	0.9	–
Fish-meat sausage	5.0	15.8	8.8	16.3	12.6
Freeze-dried egg soup	6.0	21.6	6.9	22.0	13.5

[a] To determine intra-assay precision, mean coefficients of variation (CVs) were calculated from five replicates performed on the same day. Inter-assay variances were calculated from experiments performed on five different days.

Table 4
Lateral flow assay and ELISA in processed foods.

ELISA result	Result of lateral flow assay		Concordant results/total	Concordance (%)
	–	+		
<1.0 µg/g	48	0	48/48	100
1.0–10.0 µg/g	3	23	23/26	88
>10.0 µg/g	0	30	30/30	100
Total	51	53	101/104	97

Since the results of visual judgment correlated well with the value yielded by mABS (Table 1), we believed the visual judgment of three observers should be sufficient for estimating the visual detection limit in food samples. The visual detection limit for shrimp protein extract was 25 µg/L, which is equivalent to 1 µg/g protein in a food sample. Since analytical methods should detect food allergens at low concentrations (at the level of micrograms per gram of food weight) (Van Hengel, 2007), we sought to establish sufficient sensitivity for detecting food allergens in processed foods.

We prepared tomato juice as a model processed food to verify assay performance because tomato is commonly cooked with seafood (for example, soups and ketchup) and contains polyphenolic compounds (Aherne & O'Brien, 2002) that interfere with food protein–antibody reactions in immunological assays (Bignardi et al., 2012). Our new assay has sufficient sensitivity, equivalent to 1 µg/g protein in a food sample, and sufficient reproducibility for the detection of the black tiger prawn protein in the model tomato juice.

Mass spectrometry (MS)-based methods have been adopted in the field of food testing, and some MS-based methods for the detection of food allergens have been reported (Abdel Rahman, Lopata, Randell, & Helleur, 2010; Chassaigne, Nørgaard, & Hengel, 2007; Lutter, Parisod, & Weymuth, 2011). Although these methods should be highly specific and reliable, they require complex and expensive equipment, as well as technically qualified operators. Therefore, MS-based methods may be not appropriate for routine use. In contrast, our lateral flow assay is simple and requires no sophisticated procedures or expensive equipment; it is thus more suitable for routine use, especially in food-processing facilities.

Assay concordance with the validated ELISA was 97%. The ELISA showed reactivity to protein in extracts of kamaboko and fried cake of ground fish, although there was no crustacean labelling in the list of ingredients. We conclude these processed foods contain crustacean protein, because foods containing ground fish flesh often contain crustacean protein (Sakai, Adachi, et al., 2008). Since kamaboko and fried cake of ground fish are highly processed and the extraction time in the lateral flow assay (90 s) is shorter than in the ELISA (12 h), we believe the extraction time in the lateral flow assay is too brief to detect crustacean protein in these foods. Crustacean protein was not detected by the new assay in spring roll, although the labelling declared shrimp or crab in the list of ingredients and crustacean protein was detected using the ELISA method. Since crustacean extract, which is used as seasoning in processed foods, is generally processed by protease, this difference in reactivity may be due to structural differences in the crustacean protein and differences in antibody configuration between assays. Therefore, we speculate that highly processed or enzymatically digested crustacean protein might not be detectable by the lateral flow assay in some processed foods. It will be necessary to examine the reactivity of the assay to highly processed or enzymatically digested crustacean protein.

In conclusion, we developed and validated a novel lateral flow assay for the detection of crustacean protein. Since the new assay is rapid (20 min), simple, reliable, and highly consistent with the validated ELISA, this assay is suitable for monitoring food products, especially in food-processing facilities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.10.130.

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ORIGINAL RESEARCH

A simple and rapid chromatographic method to determine unauthorized basic colorants (rhodamine B, auramine O, and pararosaniline) in processed foods

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Keywords
Auramine O, HPLC, pararosaniline, rhodamine B

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Abstract

A simple and rapid high-performance liquid chromatography (HPLC) method to determine basic colorants such as pararosaniline (PA), auramine O (AO), and rhodamine B (RB) in various processed foods was developed. Linearity of the calibration curves ranged from 0.05 to 50 μg/mL for PA and 0.05–100 μg/mL for AO and RB. The detection and quantification limits (LOD and LOQ) of the basic colorants, which were evaluated as signal-to-noise ratios of 3 for LOD and 10 for LOQ, ranged from 0.0125 to 0.05 and 0.025 to 0.125 μg/g, respectively. The recoveries and relative standard deviations of three basic colorants in six processed foods, namely, chili sauce, curry paste, gochujang (hot pepper paste), tandoori chicken (roasted chicken prepared with yogurt and spices), powder soup, and shrimp powder ranged from 70.2% to 102.8% and 0.8% to 8.0%, respectively. The intraday precision of the recovery test ranged from 1.7% to 4.5%, whereas the interday precision ranged from 3.7% to 7.7%. The reported method has been successfully applied to basic colorant determination in various processed foods such as fat-based food matrices (curry paste and tandoori chicken), chili products (gochujang and chili sauce), and protein-based products (shrimp powder and powder soup). Thin layer chromatography and liquid chromatography/mass spectrometry methods for the determination of basic colorants in processed foods were also developed for rapid analysis and identification, respectively. These methods are very useful for monitoring unauthorized basic colorants in inspection centers or quarantine laboratories in many countries.

Introduction

Synthetic food colors are used worldwide to avoid the loss of original color in processed foods, as well as to make the products more attractive to consumers. Synthetic food colors are considered superior to natural food colors in terms of their color value, uniformity, and applicability in various processed foods. Synthetic food colors have been authorized and regulated for use in food additives in many countries (Ministry of Health, Labour and Welfare 1947; European Council 1994; U.S. Food and Drug Administration 2004).

While the use of water-soluble synthetic acid colors such as erythrosine, amaranth, or acid red is generally allowed worldwide, some basic colorants such as pararosaniline (PA), auramine O (AO), and rhodamine B (RB) are unauthorized food additives in Japan, the EU, and the United States because of their toxicity. PA and AO are possibly carcinogenic to humans and classified into Group 2B by the International Agency of Research on Cancer (2010). Rhodamine B is also proved to be carcinogenic and toxic to humans and animals (International Agency for Research on Cancer 1978) (Fig. 1).

However, these basic colorants have been detected in various processed foods. The use of RB and AO has been reported in several developing countries such as Malaysia (Food Safety Net 2010), the Philippines (Republic of the Philippines, Food and Drug Administration 2013), India (Dixit et al. 2011; Gresshma and Reject Paul 2012), Vietnam (Sai Gon Giai Phong 2012), Argentina (Alesso et al. 2012), and China (The Government of the Hong Kong Special Administrative Region, Centre for Food Safety 2011; SGS Hong Kong Limited 2012). In Japan, the use of RB and PA in imported processed foods has also been reported (Suzuki et al. 2007; Ministry of Health, Labour and Welfare 2005). Thus, effective monitoring of basic color contaminants in processed foods is necessary to ensure food safety.

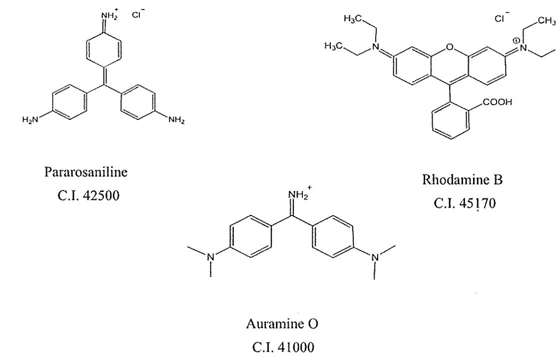

Figure 1. Chemical structures of PA, AO, and RB.

Although various analytical methods have been developed for the detection and determination of basic colorants in processed foods (Botek et al. 2007; Dixit et al. 2011; Alesso et al. 2012; Gresshma and Reject Paul 2012), such methods have several drawbacks, including time-consuming steps, a lack of application data to various processed foods, or unavailable data for the determination of low levels of basic colors. Many of them were determination methods of only RB (Alesso et al. 2012; Gresshma and Reject Paul 2012); nevertheless PA and AO were detected in processed foods as contaminants (Suzuki et al. 2007; Dixit et al. 2011), there is no simultaneous determination method of PA, AO, and RB. Therefore, it is necessary to develop a simultaneous determination method for PA, AO, and RB in various processed foods.

In this study, we developed a simple and rapid extraction and determination method to detect low levels of basic colorants (0.5 μg/g) by high-performance liquid chromatography using a photodiode array detector (HPLC-PDA). We also developed a rapid, simple, and low-cost thin layer chromatography (TLC) method for screening/detection of basic colorants and a liquid chromatography/mass spectrometry (LC/MS) approach for their identification.

The method is applicable to various processed foods such as fat-based food (curry paste and tandoori chicken), chili products (gochujang and chili sauce), and protein-based food (shrimp powder and powder soup).

Experimental

Reagents and chemicals

All solutions were prepared with ultrapure Milli-Q water (Milli-Q, Milford, MA), which was used for preparing the aqueous mobile phase. Ammonium acetate, sodium hydroxide, ammonium formate, sodium sulfate, hydrochloric acid, tetrahydrofuran (stabilizer free) (THF), ethanol (EtOH), and acetic acid were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan); 2-butanone, ethyl acetate, and hexane were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The high-performance liquid chromatography (HPLC) grade organic solvents methanol and acetonitrile were supplied by Merck (Darmstadt, Germany).

RB (purity 98.3%) was purchased from Wako Pure Chemical Industry, Ltd., whereas AO (purity 90.8%) and PA (purity 94.4%) were purchased from Chroma Technology Corp. and Acros Organics (Geel, Belgium), respectively. A saturated NaCl solution containing 0.1 mol/L NaOH was prepared by dissolving 4 g of NaOH in 1 L of the solution. A 1.6 mol/L ammonium formate solution (pH 2.5) was prepared by dissolving 10 g of ammonium formate in 50 mL of water and subsequently adjusting the pH to 2.5 with formic acid. Water was then added to the solution to obtain a 100 mL final solution volume.

Preparation of standard solutions

Stock standard solutions of RB, AO, and PA were prepared by dissolving each standard compound in methanol in a volumetric flask at a concentration of 1 mg/mL. The stock standard solutions were further diluted with methanol to give standard solutions for the recovery tests (with concentrations of 250 and 25 μg/mL). For the calibration curves, the stock solutions were diluted with a 1% acetic acid solution containing methanol to give five working standard solutions for analysis (with concentrations of 0.05, 0.1, 0.5, 1, and 2 μg/mL).

Preparation of sample solutions from food

All food samples, that is, curry paste, chili sauce, gochujang (hot pepper paste), tandoori chicken (roasted chicken prepared with yogurt and spices), shrimp powder, and powder soup were obtained from a market in Tokyo. Solid samples were finely cut or homogenized. A 5 g portion was accurately weighted and dissolved in 20 mL of a solution containing 0.1 mol/L HCl:EtOH (1:2). The sample solution was then shaken for 1 min, and ethyl acetate (20 mL) was subsequently added with further agitation for 1 min. The solution was finally centrifuged at 3000 rpm (1500–2000*g*) for 1 min, and the supernatant was collected into a separatory funnel. The same treatment was performed two times on the residual precipitates, and the supernatant liquid was collected into the separatory funnel for ethyl acetate extraction. After adding 1 mL of an NaOH solution (2.5 mol/L) to the ethyl acetate extraction layer (except for the shrimp powder), 50 mL of a saturated NaCl solution (containing 0.1 mol/L NaOH) was added to the ethyl acetate extraction layer in the separatory funnel. The mixture was then shaken, and the latter layer was removed. After adding 40 mL of hexane and 20 mL of 0.1 mol/L HCl to the residual ethyl acetate extraction layer in the separatory funnel and shaking the mixture again, the basic colorants were extracted to the latter layer and collected in a 100 mL measuring flask. A further amount of HCl (0.1 mol/L, 20 mL) was added to the remaining layer upon agitation, and this layer was also collected (together with the rest) into the 100 mL measuring flask. Water was then added to the flask to obtain a final solution volume of 100 mL.

A 20 mL aliquot of the prepared solution was carefully taken, and its pH value was adjusted to 10–12 using a 2.5 mol/L NaOH solution. This mixture was employed to precondition the Oasis HLB column using 10 mL of methanol and water. The column was washed with 10 mL of Mill-Q water and eluted using 4 mL of 1% acetic acid in methanol. A further amount of acetic acid containing methanol was added to the mixture to obtain a final volume of 5 mL as a sample solution for HPLC experiments.

Recovery tests and method validation

Recovery tests were performed to evaluate the accuracy of the developed method. A small amount of the standard solutions (0.1 mL, 25 μg/mL) was added to 5 g of shrimp powder, powder soup, curry paste, chili sauce, gochujang, and tandoori chicken (finely cut) in the absence of PA, AO, and RB. The samples were kept at room temperature for 30 min and then treated as described in the section "Preparation of sample solutions from food".

Calibration curves were prepared with the PA, AO, and RB standard solutions at concentrations of 0.05–50 or 100 μg/mL to examine the linearity of the calibration curves. Intraday precision (RSD$_r$) and interday precision (RSD$_R$) were assessed by analyzing duplicates of a curry paste spiked with PA, AO, and RB (0.5 μg/g) during a day and on five different days, respectively. The limits of detection (LOD) and quantification (LOQ) for PA, AO, and RB were estimated by the signal-to-noise $S/N > 3$ and $S/N > 10$ ratios of each peak in the standard solutions, respectively.

HPLC analysis

The LC system consisted of a Hewlett Packard 1100 series (Agilent Technologies, Palo Alto, CA) containing a G1315A PDA detector (monitored at 550 nm for PA and RB, and 450 nm for AO), the L-column (octadecylsilane (ODS); i.d.: 4.6 mm × 150 mm; particle size: 5 μm; pore size: 12 nm; Chemicals Evaluation and Research Institute, Tokyo, Japan), and a column heater set at 40°C. The mobile phase consisted of 20 mmol/L ammonium acetate, brought to pH 4.5 by dropwise addition of acetic acid (mobile phase A) and acetonitrile (mobile phase B). The gradient conditions were as follows: (1) a linear gradient from 20% to 60% mobile phase B (for 15 min) and (2) isocratic elution at 60% mobile phase B (for 5 min). The injection volume was 20 μL and the flow rate was 1.0 mL/min. The apparatus was controlled (and the data were collected and analyzed) by using the Agilent Chemstation software.

TLC analysis

TLC experiments were performed on 20 cm × 20 cm TLC RP-18 plates from Merck, which were cut into segments of 10 cm × 10 cm. Exactly 2 mL of the sample solutions was taken for HPLC, purged with nitrogen gas at room temperature, and concentrated to 0.2 mL. The sample solutions for TLC were spotted with 5–20 μL (~5–20 ng of each basic colorant), and standard solutions (5 μg/mL) of the basic colorants were spotted with 1–4 μL (~5–20 ng of each basic colorant) using a 5 μL capillary glass tube at 20 mm from the bottom of the plate.

The plates were developed up to 7 cm in a saturated developing chamber (Camag, Muttenz, Switzerland) for 10 cm × 10 cm plates. The developing solvents were 2-butanone–methanol–5 w/w% Na$_2$SO$_4$ (1:1:1, v/v/v) (solvent system A) and 2-butanone–methanol–1.6 mol/L ammonium formate (pH 2.5) (7:2:7, v/v/v) (solvent system B). After development, the plates were dried at room temperature and observed under white light for PA, AO, and RB, as well as at 254 nm for RB and 366 nm for AO and RB. The plates were documented by using a TLC visualizer (Camag).

LC/MS analysis

LC analysis was performed by using a HPLC–electrospray ionization-MS (HPLC–ESI-MS) instrument from Shimadzu (LCMS-2010; Shimadzu, Kyoto, Japan). Chromatographic separation was performed on a reversed-phase HPLC L-column ODS (i.d.: 2.1 mm × 150 mm; particle size: 5 μm; pore size: 12 nm). The mobile phase consisted of 20 mmol/L ammonium acetate (pH 4.5) (mobile phase A) and acetonitrile (mobile phase B). The gradient conditions were as follows: (1) a linear gradient from 20% to 60% mobile phase B (for 15 min) and (2) isocratic elution at 60% mobile phase B (for 5 min). The flow rate was 0.2 mL/min and the injection volume was 5 μL; the column oven was maintained at 40°C. The sample solution for HPLC was injected and analyzed in the ESI (+) mode with selected ion monitoring (SIM) using selected ion masses of *m/z* 288[PA–Cl], *m/z* 268[AO–Cl], and *m/z* 443[RB–Cl–H] for LC/MS detection.

Results and Discussion

Optimization of the sample solution preparation

To optimize extraction conditions, we examined the extraction time and the number of extractions using 0.1 mol/L HCl:ethanol (1:2) and ethyl acetate as the extraction solvent. As shown in Figure 2, the recovery rate of RB in chili powder was above 90% after three extractions.

To remove impurities from the extracted solution, we applied the salt-out method using a saturated NaCl solution containing 0.1 mol/L NaOH (basified saturated NaCl solution). This procedure helped in removing impurities in the solution extracted from chili powder.

We considered that impurities were effectively removed from the extracted chili-powder sample using a saturated NaCl solution containing 0.1 mol/L NaOH. When such a solution is added to the ethyl acetate extraction layer, the extracted solution is normally expected to have a pH of 9–11. However, in the case of the chili sauce, the pH appeared to be below 9. This observation indicates a loss in the amount of PA in the ethyl acetate layer because of the influence of the matrix. The presence of a thickener or vinegar in the chili sauce may cause an amount of PA to remain in the NaCl/NaOH layer. To prevent this loss, we added NaOH (1 mL, 2.5 mol/L) to the extracted solution in the ethyl acetate layer before adding saturated NaCl containing NaOH. Apparently, PA could be effectively extracted in the ethyl acetate layer using the proposed procedure. From this result, we can conclude that when the pH of the ethyl acetate extraction layer is not sufficiently alkaline from the effect of the food matrix alone, it is necessary to add a 2.5 mol/L NaOH solution adjust the pH to alkaline.

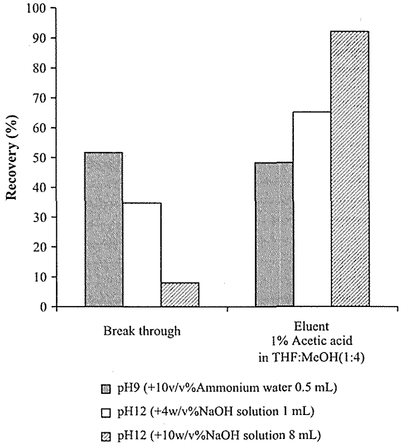

Figure 2. Recovery (%) of PA from an Oasis HLB under different pH conditions.

Optimization of the clean-up process on solid-phase extraction columns

Many solid-phase extraction columns such as octadecyl silica (ODS; Gagliardi et al. 1996), styrene–divinylbenzene polymeric surfaces (Strata-X, Strata-SCX, Oasis HLB, Sepabeads® SP70; Mitrowska et al. 2005; Lee et al. 2006; Chiang et al. 2011; Soylak et al. 2011), alumina and strong cation exchange columns (Halme et al. 2004), immune affinity columns (Xie et al. 2013), and polyamide (Dixit et al. 2011) have been used to clean-up basic synthetic dyes from foods, cosmetic products, and tissues.

Chiang et al. (2011) and Soylak et al. (2011) reported that stylene–divinylbenzene polymers are effective in cleaning basic dyes such as RB and malachite green from foods. Therefore, we attempted to clean-up the basic dyes from the prepared solutions using a cartridge containing a stylene–divinylbenzene polymer (Oasis HLB). We found that the basic dyes, except PA, could be retained in the cartridge, and we believe that the breakthrough of PA could be because of its positive charge. Therefore, we added an alkaline solution to the prepared solution after dilution with distilled water to neutralize the basic dye charge. As expected (see Fig. 2), we found that PA could then be retained in the cartridge and was recovered substantially from the sample.

The prepared solution was diluted three times with water to reduce the effects of the organic solvent. RB and AO were retained in the Oasis HLB, whereas PA was not. We therefore examined the effect of pH of the eluent on the retention of PA.

To achieve this, we adjusted the pH of a diluted solution prepared solution from chili sauce spiked with PA to 9 using 10% ammonium water, and to pH 12 using 0.5 mol/L NaOH and 8 mL of 2.5 mol/L NaOH solutions. The sample was then applied to the Oasis HLB, and a 1 mL fraction was collected. As shown in Figure 2, in the case of the diluted solutions prepared by using 10% ammonium water (pH 9) and 0.5 mol/L NaOH (pH 12), the pH was slightly acidic so that 30%–50% of the PA was not retained by the cartridge. On the other hand, the PA contained in the diluted solution using 2.5 mol/L NaOH (pH 12, 8 mL) solution was retained by the cartridge with less breakthrough. An improved PA recovery of 90% was achieved by elution with 1% acetic acid in methanol: THF (4:1).

This solution was tested as an eluent, and more than 90% of RB, AO, and PA was effectively eluted in the first 1 mL (Fig. 3A). However, as shown in Figure S1 of the chromatogram in the HPLC analysis of basic colorants after elution from the cartridge using 1% acetic acid in methanol:THF (4:1), the peaks corresponding to the basic colorants are broad, and the sensitivity to AO is low, even if the pH is changed from 3.5 to 6.5 to optimize the HPLC conditions. Since THF is generally unstable—it affects the peak shape in the HPLC analysis —we replaced the 1% acetic acid in methanol:THF (4:1) with 1% acetic acid in pure methanol eluent. As shown in Figure 3B, the shapes of the colorant peaks in the HPLC chromatogram improved, and the sensitivities were higher than those obtained by using THF as an eluent, although 1–2 mL of eluent was necessary to effectively elute all the color from the cartridge. Therefore, 1% acetic acid in methanol was used as an appropriate eluent for the purification of colorants from the cartridge.

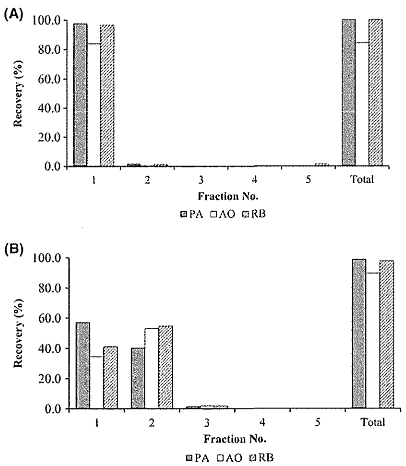

Figure 3. Recovery (%) of PA, AO, and RB from an Oasis HLB using different eluents: 1% acetic acid in THF:MeOH (1:4) (A) and 1% acetic acid in MeOH (B).

Optimization of HPLC conditions

According to previous reports on synthetic food colorant analyses by HPLC or LC/MS, an aqueous ammonium acetate solution (solvent A) and acetonitrile (solvent B) were used as the mobile phase for the gradient conditions in HPLC analysis (Suzuki et al. 2007). In this study, we tried to optimize the pH of solvent A.

As shown in Figure 4, the AO and PA peaks are broad, and the S/N ratios obtained by using solvent A at pH 6.5 (i.e., 5 for AO and 9.5 for PA) are lower than those obtained at pH 4.5 (8 for AO and 17 for PA) or pH 3.5 (5 for AO and 8 for PA), suggesting that the sensitivities for the determination of AO and PA using solvent A at pH 6.5 are lower than those at pH 4.5 or 3.5. In addition, in the case of RB, the retention time (RT) using solvent A at pH 3.5 is longer than that at pH 4.5 (Fig. 4), suggesting that the analysis of RB will take longer in the former case than in the latter one. The sensitivities of AO and PA at pH 4.5 are similar to those at pH 3.5, and therefore, we used 10 mmol/L ammonium acetate (at pH 4.5) as solvent A for the HPLC analysis of basic colorants.

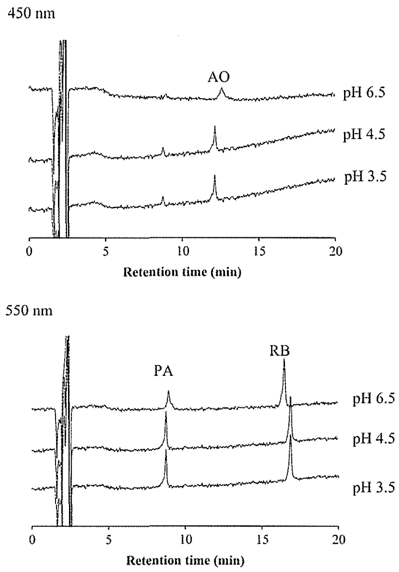

Figure 4. HPLC chromatograms (at 450 and 550 nm) of standard solutions of PA, AO, and RB (0.1 μg/mL) at different pH values (pH 6.5, 4.5, and 3.5).

Validation of the method

The analytical method developed herein was validated by determining its linearity, LOD, LOQ, trueness (by recovery tests), and precision. The calibration curve for PA exhibits linearity at the concentration of 0.05–50 μg/mL, whereas those for AO and RB are linear at 0.05–100 μg/mL. The regression coefficients were greater than 0.999 in all cases (PA, AO, and RB).

The LOD and LOQ were determined by using standard solutions. The LODs based on three times the S/N ratio were 0.0125 μg/g for PA, 0.05 μg/g for AO, and 0.0125 μg/g for RB. The LOQs based on ten times the S/N ratio were 0.05 μg/g for PA, 0.125 μg/g for AO, and 0.025 μg/g for RB. The confirmatory LOD for the three basic colorants based on visual evaluation of the PDA spectra was estimated to be 0.025 μg/g.

The accuracy and precision of the method were evaluated by recovery tests. Standard solutions of PA, AO, and RB were spiked with shrimp powder, powder soup, curry paste, chili sauce, gochujang, and tandoori chicken at a final colorant concentration of 0.5 μg/g. Table 1 shows the recoveries and relative standard deviations (RSDs) obtained by the developed analytical method. The recoveries and RSDs for PA, AO, and RB ranged from 70.2% to 102.8%, and from 0.8% to 8.0%, respectively, except for the shrimp powder, where the values ranged from 51.7% to 75.2% and 2.3% to 26.4%, respectively. The lower recoveries and precision values observed for the shrimp powder could be because of losses in PA, AO, and RB during purification using a cartridge, which involves precipitation induced by adding 1 mL of a 2.5 mol/L NaOH solution to the ethyl acetate extraction layer. Thus,

Table 1. Recoveries of pararosaniline (PA), auramine O (AO), and rhodamine B (RB) from spiked food matrices (shrimp powder, powder soup, curry paste, chili sauce, gochujang, and tandoori chicken).

	Recovery (%)[1]		
	PA	AO	RB
Tandoori chicken	102.4 ± 0.8	92.7 ± 5.7	91.3 ± 1.1
Gochujang	102.8 ± 3.3	92.6 ± 9.7	93.6 ± 0.9
Chili sauce	85.4 ± 8.0	92.7 ± 5.8	95.9 ± 1.9
Curry paste	86.2 ± 2.6	85.4 ± 6.3	92.8 ± 1.7
Powder soup	88.1 ± 3.2	70.2 ± 1.7	99.0 ± 3.6
Shrimp powder[2]	87.8 ± 3.9	76.8 ± 3.0	95.2 ± 2.9

[1] Mean ± SD.

[2] Recoveries from the shrimp-powder sample solution, which was prepared without adding NaOH.

Table 2. Intraday (RSD_r) and interday (RSD_R) precision data for curry paste spiked with PA, AO, and RB.

				Precision	
Analyte	Spiked level (µg/g)	Found (µg/g)[1]	Recovery (%)	Intraday RSD_r	Interday RSD_R
PA	0.50	0.40 ± 0.03	80.0	2.9	7.7
AO	0.50	0.43 ± 0.02	85.4	4.5	3.7
RB	0.50	0.49 ± 0.02	97.9	1.7	4.1

PA, pararosaniline; AO, auramine O; RB, rhodamine B.

[1] Mean ± SD.

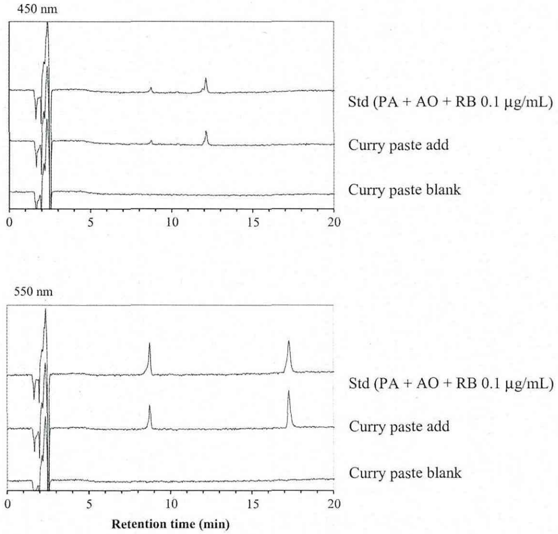

Figure 5. HPLC chromatograms (at 450 and 550 nm) of the standard solutions of PA, AO, and RB (0.1 μg/mL), a blank solution, and a sample solution from curry paste.

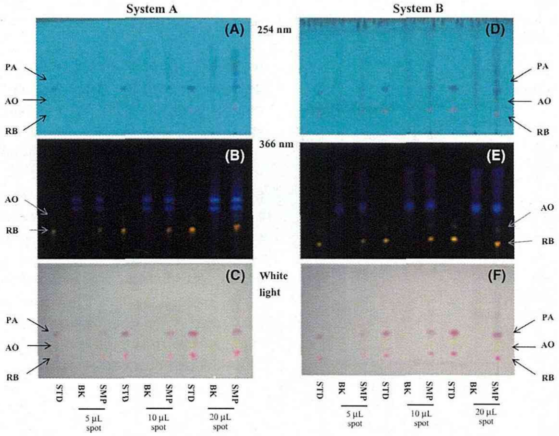

Figure 6. TLC chromatograms of a standard solution (STD), a blank solution (BK), and a sample solution (SMP) from curry paste at 254 and 366 nm, as well as under white light using the developing solvent systems A [2-butanone–methanol–5%Na_2SO_4 solution (1:1:1, v/v/v)] and B [2-butanone–methanol– 1.6 mol/L ammonium formate solution (pH 2.5) (7:2:7, v/v/v)].

to prevent precipitation, we did not add the NaOH to the solution prepared from shrimp powder; the prepared solution was purified using Oasis HLB. Consequently, the recoveries were improved to 87.8% for PA, 76.8% for AO, and 95.2% for RB.

The reproducibility of the results was assessed by determining both the RSD_r and the RSD_R of the recovery tests by spiking standard solutions of PA, AO, and RB with concentrations of 0.5 μg/g of each basic colorant in the curry paste. The RSD_r values ranged from 1.7% to 4.5%, and the RSD_R values ranged from 3.7% to 7.7% (Table 2).

Typical chromatograms of the analysis of PA, AO, and RB in recovery tests using the curry paste are shown in Figure 5. The peaks obtained for the basic colorants were well separated, with RT of 8.5 min for PA, 12 min for AO, and 17.5 min for RB. The PDA spectrum of the sample solution for the HPLC experiments agrees well with that of the standard solution. As shown in Figure S2A–E, we obtained well-separated HPLC chromatograms of the analysis of PA, AO, and RB in the recovery tests using tandoori chicken, gochujang, chili sauce, powder soup, and shrimp powder.

TLC

To apply the prepared sample solutions in conventional TLC experiments and assess the detection limit for basic colorants by TLC, we performed TLC analysis using the prepared sample solutions (5–20 μL, ~5–20 ng of basic colorant) in the recovery tests described in the section "Validation of the method." Typical TLC chromatograms obtained for PA, AO, and RB in recovery tests using a curry paste are shown in Figure 6A–F. As shown in Figure 6C and F, PA, AO, and RB were separately detected as red, yellow, and pink spots in systems A and B by spotting more than 5 μL of sample solution under a white light. However, it was difficult to visually detect the AO spot under the white light because of its yellow color. AO and RB were visually detected as fluorescent yellow and orange spots at 366 nm, separated from the fluorescent blue spots from impurities of the curry paste in systems A and B

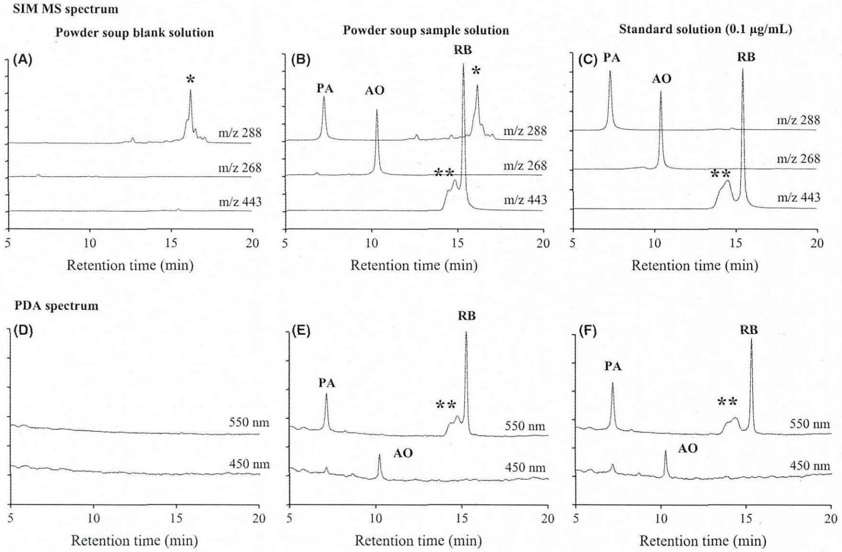

Figure 7. LC/MS SIM chromatograms of sample solutions from a powder soup spiked with PA, AO, and RB (each spiked level is 0.5 μg/g) and PDA chromatograms at 450 and 550 nm. The symbol "*" represents an artifact from the processed food and "**" represents an artifact from RB.

(Fig. 6B and E). A spot of AO was clearly detected at 366 nm in system B. Spots of PA, AO, and RB were also visually detected at 254 nm when more than 5 μL of sample solution was used in systems A and B. However, the fluorescence intensities of the AO and RB spots, as well as the visual intensity of the PA spot, were much lower than those observed under white light and at 366 nm. As shown in Figure S3A–E, we observed similar TLC of the sample solution from tandoori chicken, gochujang, chili sauce, powder soup, and shrimp powder.

LC/MS

To correctly identify PA, AO, and RB in sample solutions prepared from processed foods for the regulation of unauthorized basic colorants, a confirmation by LC/MS analysis is necessary. Therefore, we developed a qualitative LC/MS method to identify the basic colorants, although the quantitative performance of the method was not assessed. Figure 7A–F shows typical LC/MS SIM and HPLC-PDA chromatograms at 550 and 450 nm for the analysis of PA, AO, and RB in recovery tests using a powder soup. Peaks of the RT were detected at 8.5 min for PA, 11.7 min for AO, and 16.1 min for RB in the SIM (Fig. 7B and C) and HPLC-PDA (Fig. 7E and F) chromatograms of the standard solutions and sample solutions prepared from a powder soup spiked with basic colorants. As shown in Figure 7B, C, E, and F, interference peaks were detected in the case of RB before the RT peak, both in the SIM and HPLC-PDA chromatograms of the sample solution and the standard solution. These interference peaks appear to be derived from RB. Similarly, as shown in Figure S4, peaks for PA, AO, and RB were detected in the SIM and HPLC-PDA chromatograms of sample solutions prepared from other processed foods (i.e., chili sauce, curry paste, gochujang, tandoori chicken, and shrimp powder).

In the SIM chromatogram of a blank solution (Fig. 7A), multiple peaks were detected at approximately 16 min (*m/z* 288 for PA). However, the RT of these peaks was different from that of the PA standard solution, and since the peaks also appeared in the blank solution chromatograms of sample solutions prepared from other foods, we considered that they could probably be the impurities derived from the foods.

Conclusions

We developed an HPLC method for the determination of unauthorized basic colorants in processed foods. The recoveries achieved by this procedure ranged from 70.2% to 102.8%. The HPLC method offers a way to reduce interferences in fat-based food matrices (curry paste and tandoori chicken), water-soluble substances (chili color in gochujang and chili sauce), or protein-based products (shrimp powder and powder soup). This study shows that the proposed method is a simple and reliable way to determine unauthorized basic colorants such as PA, AO, and RB in processed foods. Furthermore, a TLC method for the screening/detection of the colorants, as well as an LC/MS approach to identify them, was also developed. The LC/MS approach was developed for qualitative purposes and has not been validated quantitatively. Further studies to determine basic colorants, both quantitatively and qualitatively, in processed foods by LC/MS would be necessary. We believe that these methods could be very useful for monitoring unauthorized basic colorants in inspection centers or quarantine laboratories in many countries.

Acknowledgments

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. HPLC chromatograms of standard solutions of PA, AO, and RB (0.1 μg/mL) at different pH values (6.5, 4.5, and 3.5) in 1% acetic acid in THF:MeOH (1:4).

Figure S2. HPLC chromatograms (at 450 and 550 nm) of the standard solutions of PA, AO, and RB (0.1 μg/mL), a blank solution, and a sample solution from curry paste.

Figure S3. TLC chromatograms of a standard solution (STD), a blank solution (BK), and a sample solution (SMP) from tandoori chicken, gochujang, chili sauce, powder soup, and shrimp powder at 254 and 366 nm, as well as under white light using the developing solvent systems A [2-butanone–methanol–5% Na_2SO_4 solution (1:1:1, v/v/v)] and B [2-butanone–methanol 1.6 mol/L ammonium formate solution (pH 2.5) (7:2:7, v/v/v)].

Figure S4. LC/MS SIM chromatograms of sample solutions from tandoori chicken, gochujang, chili sauce, curry paste, and shrimp powder spiked with PA, AO, and RB (each spiked level is 0.5 μg/g).

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ORIGINAL RESEARCH

Estimation of daily aluminum intake in Japan based on food consumption inspection results: impact of food additives

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Keywords
Aluminum, dietary intake, food additive, ICP-AES, provisional tolerable weekly intake

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Abstract

Dietary aluminum (Al) intake by young children, children, youths, and adults in Japan was estimated using the market basket method. The Al content of food category (I–VII) samples for each age group was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES). The Al content in processed foods and unprocessed foods ranged from 0.40 to 21.7 mg/kg and from 0.32 to 0.54 mg/kg, respectively. For processed foods in all age groups, the Al content in food category VI samples, sugar and confections/savories, was the highest, followed by those in category II, cereals. The daily dietary Al intake from processed foods was much larger than that from unprocessed foods. The mean weekly percentages of the provisional tolerable weekly intake (PTWI, established by the joint FAO/WHO Expert Committee on Food Additives in 2011) from processed foods for all age groups are 43.1, 22.4, 17.6 and 15.1%, respectively. Only the highest consumer Al exposure value (>P_{95}) of the young children group exceeded the PTWI.

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Kyoko Sato and Ippei Suzuki contributed equally to this study.

Introduction

Aluminum (Al) is the third most abundant element in the earth's crust and is widely applied to human life. However, the toxic effects of Al have been reported in several studies. Many researchers have reported that Al can be toxic to the central nervous, skeletal, and hematopoietic systems, and Al has been controversially implicated in Alzheimer's disease, osteomalacia, and dialysis encephalopathy (Nordberg et al. 2007; Aguilar et al. 2008; Peto 2010; She et al. 2012), although this remains to be clearly demonstrated.

The joint FAO/WHO Expert Committee on Food Additives (JECFA) established the provisional tolerable weekly intake (PTWI) for Al of 7.0 mg per kg body weight in 1989 (Joint FAO/WHO Expert Committee on Food Additives 1989). In 2007, the JECFA re-evaluated the safety of Al and lowered the PTWI to 1.0 mg per kg body weight because of the potential for Al to affect the reproductive and nervous system in experimental animals (Joint FAO/WHO Expert Committee on Food Additives 2007). However, the JECFA revised the PTWI to 2.0 mg per kg body weight in 2011 (Joint FAO/WHO Expert Committee on Food Additives 2011) as a result of new bioavailability and toxicological data (Poirier et al. 2011).

Al in the food supply comes from natural sources, the water used in food preparation, food ingredients, and utensils used during food preparation. The primary dietary sources of Al are foods and beverages, and these sources are either naturally existing or intentionally added (Pennington 1988; Greger 2007; Aguilar et al. 2008). Many researchers have reported estimates of Al dietary exposure in countries and regions, such as the United States (Pennington and Schoen 1995), Greece (Bratakos et al. 2012), Belgium (Fekete et al. 2013), South China (Jiang et al. 2013) and the European Union (Aguilar et al. 2008). However, there are large variations in Al exposure among the different countries and regions. These variations are due to differences in the survey methodologies employed, which take into account differences in population, dietary patterns, and consumption, as well as the reported Al concentrations in foods prepared using Al-containing food additives. Matsuda et al. (2001) reported that the average daily dietary Al intake in Japan, estimated from a total diet study between 1996 and 1998, was 3.5 mg, with a range of 1.8–8.4 mg. However, this estimated dietary Al intake is based on data collected more than 10 years ago and does not reflect current dietary intake. Therefore, it is necessary to assess the current dietary Al exposure in Japan in order to regulate Al intake from Al-containing food additives, given the JECFA revisions to the current PTWI exposure guidelines for Al.

In the present study, we determined the Al content of various food categories and its relation to Al intake by different age groups in Japan (young children, children, youths, and adults) using the market basket method. Samples were analyzed using inductively coupled plasma-atomic emission spectrometry (ICP-AES). The weekly dietary Al exposure from processed and unprocessed foods for all age groups was then estimated. Furthermore, the highest levels of consumer weekly Al exposure from processed foods for all age groups were estimated. Additionally, we discuss the estimated values and compare them with the JECFA revision to the PTWI in 2011.

Material and Methods

Reagents and chemicals

Hydrochloric acid (HCl) (35.0–37.0%) for measurement of toxic metals, nitric acid (HNO_3) (60–62%) for precision analysis, 0.1 mol/L HNO_3 for quantitative analysis, Al standard solution (100 μg/mL, certified by Japan Calibration Service System), and Ultrapure Water (H_2O) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Calibration standard solutions of Al (0.012, 0.06, 0.3, 1.5, and 7.5 μg/mL) were prepared by dilution of Al standard solution with 0.1 mol/L HNO_3.

Food consumption data and market basket method

The daily dietary Al intake was estimated using the market basket method (Ishiwata et al. 2002). For processed foods, a daily food consumption list of 189 types of processed foods in seven food categories for the four age groups was prepared using the data on the usual daily diet in Japan from the Special Survey Study of the Frequency and Level of Food Consumption (2005–2007) (government closed report), which was based on individual food intake surveys. The Special Survey Study calculated the average daily consumption of 189 types of food by the following age groups: 1–6 year olds (young children), 7–14 (children), 15–19 (youths), and older than 20 years (adults). The 189 food types are classified into seven food categories (I, seasonings and beverages; II, cereals; III, potatoes, legumes, nuts; IV, fish/shellfish, meat and hens' eggs; V, oils/fats and milk/milk products; VI, sugar and confections/savories; VII, fruits, vegetables, and seaweeds). Based on the prepared list, 286 processed food products (189 types of processed food) were purchased from a supermarket in Tokyo. For popularly consumed processed foods, several different brands were purchased, allowing the actual conditions of consumption (frequency and level of consumption) and processing methods to be taken into consideration.

We measured the required amounts of individual food products consumed by each age group, according to the Special Survey Study list. The collected food samples for each category were mixed, yielding 28 samples (4 [age groups] × 7[food categories]). Briefly, for each food category (I–VII), a predetermined amount of food was weighed for each food category for each age group. The amount for a specific age group for that particular food category was then homogenized with an equal weight of ultrapure water to yield a single sample using a blender (Knife Mill GRINDOMIX GM 300; Retsch, Haan, Germany). The one exception was food category I, which was homogenized without added water. In the Results and Discussion section, the determined Al concentration in each food category is shown as the amount of Al for the weight of the sample without added water. The 28 homogenized samples were separately bottled in 100 mL antioxidant-free polyethylene bottles and frozen below −20°C until analyzed.

For the unprocessed foods, we prepared a daily food consumption list of 33 types of unprocessed foods from four food categories (II, III, IV, and VII) based on the food consumption in the All Investigation Report of Daily

Intake of Food Additives in 2000 (http://www.ffcr.or.jp/Zaidan/mhwinfo.nsf/98a5d7b766af9bfb492565a10020c601/1100115ac3d23fd7492569d800171034?OpenDocument). Forty unprocessed food products (33 types of unprocessed foods) based on the All Investigation Report were purchased from a supermarket in Kanagawa Prefecture or through the internet.

We measured the required amounts of individual food products for each age group, according to the list for unprocessed foods. The measured food samples for each category were mixed, yielding 13 samples (4 [age groups] × 3 [food categories]) and a sample of food category III (since the sample for food category III is identical for all age groups). Briefly, for each food category (II, IV, and VII), a predetermined amount of food was weighed for each age group; then, all the food was homogenized to yield a single sample for each food category for each age group using blenders (Ultra Centrifugal Mill ZM 200, Knife Mill GRINDOMIX GM 300; Retsch) and BLIXER-5Plus (FMI Co., Osaka, Japan) and a food processor (MK-K58; Panasonic Co., Kadoma, Japan). The homogenized samples were separately bottled in 125.0 mL antioxidant-free polyethylene bottles and frozen below −20°C until further analyses.

Preparation of test solutions

Aliquots (2.0 g) of the homogenized samples were weighed and transferred into quartz beakers. The samples were dried and carbonized on a hotplate at 250°C. The carbide was then placed in a furnace and the temperature was raised to 485°C at 100°C/h and kept at 485°C overnight. After cooling, 5.0 mL of HNO_3/H_2O (1:1) mixture was added and the mixture was dried on the hotplate and incinerated in a furnace. The ash was wetted with H_2O, and 5.0 mL HCl was added and dried on the hotplate. The ash was dissolved with 0.1 mol/L HNO_3 and the volume was adjusted to 50.0 mL using Digi TUBEs (SCP SCIENCE Co., Baie-D'Urfé, Canada).

Al determination

Al concentrations in the food categories were analyzed using an Optima 5300DV ICP-AES (Perkin-Elmer, Waltham, MA). The ICP-AES operating condition parameters were: liquid uptake, 1.0 mL/min; plasma argon gas flow, 15.0 L/min; auxiliary argon gas flow, 0.2 mL/min; carrier gas flow, 0.7 mL/min; RF generator, 1300 W; optical viewing axially observed spectral lines, 395.153 nm.

Determination of dietary Al intake from processed and unprocessed foods

The daily Al intake, based on the Al concentration determined using ICP-AES for the food category samples and the daily consumption of each food category by each age group, was calculated using the following equations.

$$\text{Di (mg/person/day)} = \text{Ci (mg/kg)} \times \text{Xi (g/person/day) for each age group}/1000$$

Di (mg/person/day): the daily dietary intake of Al in each food category for each age group.

Ci (mg/kg): the determined level of Al in each sample of each food category for each age group.

Xi (g/person/day): the daily consumption of each food category for each age group

The daily intake of the processed food (categories I–VII) and unprocessed foods (categories II, III, IV, and VII) was separately determined and summed as the subtotal daily intake for that food category.

Total daily intake of Al for each age group was calculated using the following equation.

$$\text{Y (mg/person/day)} = \sum(\text{D}_{\text{N(I–VII)}}, \text{processed food category}) + \sum(\text{D}_{\text{N (II, III, IV and VII)}}, \text{unprocessed food category}) \text{ for each age group.}$$

The weekly Al intake for each age group was calculated using the following equation:

$$\text{W (mg/kg, bw (average body weight for each age group)/week)} = \text{Y (mg/person/day)} \times 7 \text{ (days)/the average body weight of each age group.}$$

The mean body weight of each age group is 16.0 kg for young children, 36.5 kg for children, 56.5 kg for youths, and 58.6 kg for adults.

Percentile analysis of Al exposure

Percentile analysis of Al exposure from processed foods for each age group was conducted to assess high-level dietary Al exposure in consumers. The P_{50}, P_{95}, $P_{97.5}$, and P_{99} dietary Al exposures were calculated as follows. The 287 types of processed foods in the individual daily food consumption data of the Special Survey Study of the Frequency and Level of Food Consumption (2005–2007) [government closed report], based on data from four age groups (young children [1–6 years old]: $n = 1619$; children [7–14 years old]: $n = 3419$; youths [15–19 years old]: $n = 2539$; adults [older than 20 years old]: $n = 32787$), were categorized into food categories I–VII. The individual consumption of each food category was calculated. The individual Al intake from the processed foods was then calculated by multiplying the individual consumption of each food category for I to VII food categories and the determined level of Al in that food category. Next, to calculate the individual daily Al intake (mg/kg bw/day), the individual Al intake from the processed food categories (mg/day) was divided by the individual body weight. The individual weekly Al intake (mg/kg bw/day) was calculated by multiplying by 7 (days). The percentile value of the individual weekly Al intake (P_{50}, P_{95}, $P_{97.5}$, and P_{99}) was extracted from the data of the individual weekly Al intake.

Results and Discussion

Validation method

The limit of detection (LOD) and limit of quantification (LOQ) of the developed method for Al determination was estimated based on the standard deviation (σ) of seven independent measurements of food category IV samples for processed foods (children) and food category II samples for unprocessed foods (young children), according to the quality assurance guideline announced by the Japanese Ministry of the Environment (2003). The LOD and LOQ are estimated to be 0.1 mg/kg (<3.143 σ) and 0.3 mg/kg (<9.429 σ), respectively.

The accuracy and precision of the developed method for Al determination were evaluated by recovery tests using three independent measurements of all food category samples for the adult age group for processed foods, and food category II, III, IV, and VII samples for all age groups for unprocessed foods.

For the mixed processed food samples of all food categories, the recovery of Al ranged from 83% to 113%, with a relative standard deviation (RSD) of <7.8%. The results of the recovery tests are shown in Table 1. For the mixed unprocessed food samples for categories II, III, IV, and VII, Al recovery with the level of quantification limit (0.3 mg/kg as Al) ranged from 81% to 106%, with an RSD of <18.5% (data not shown). The results show that the developed method is acceptable for determining the total Al concentration in all food samples in this study.

Table 1. Results of spike and recovery tests for each food group.

Food group	Spiked aluminum amount (mg/kg)	Recovered (%)	Relative standard deviation (%)
I	2	113.2	1.8
II	3	83.0	1.6
III	0.5	101.8	2.9
IV	0.5	106.9	6.6
V	0.5	84.5	7.8
VI	10	101.1	3.5
VII	3	92.8	3.7

Determination of Al concentration in processed and unprocessed foods using the market basket method

To estimate the Al content in processed foods, we determined the Al content of all food category samples for all age groups. As shown in Table 2, the Al content of processed food samples in all food categories ranged from 0.40 to 21.7 mg/kg. For samples of processed food categories for all age groups, Al content followed the rank order: category VI samples > II > VII > III. The main source of Al in category VI foods is thought to be food additives, such as leavening agents. In the category VI processed foods examined (55 products), 22 products were labeled as containing baking powder. In the category II processed foods examined (40 products), four products were labeled as containing a leavening agent. The Al detected in category VII foods is thought to be derived from natural sources such as soil and seawater. Researchers have reported increasing environmental contamination to be the cause of vegetable- and fruit-derived Al (Müller et al. 1998; Jiang et al. 2013), and that Al is derived from food additives such as aluminum ammonium sulfate and aluminum potassium sulfate used as color-fixing agents.

The Al detected in the category III foods is also thought to be derived from soil and other environmental factors because none of the labels on the category III foods indicated food additives containing Al. Furthermore, legumes such as lentils and red beans, raisins, and nuts such as almonds and hazelnuts, are reported to be rich sources of Al (Wang et al. 1991; Soliman and Zikovsky 1999; Cabrera et al. 2003; Bratakos et al. 2012).

The Al content in category IV foods containing processed fish products was lower than that of category III foods. In a previous Japanese study, Al levels (3.70–10.9 mg/kg) in fish samples were rather high (Matsuda et al. 2001). The high Al levels in processed fish products previously reported might be due to the presence of added stabilizers, such as food additives like aluminum ammonium sulfate and aluminum potassium sulfate, used in processed fish products in Japan, or due to differences in the varieties of fish used to manufacture the processed fish products.

To estimate the Al content from unprocessed foods, we further determined the Al content of unprocessed food samples of food category II, III, IV, and VII samples for all age groups. As shown in Table 3, the Al content of samples

Table 2. Al concentrations in processed foods (mg/kg).

	Food group						
	I	II	III	IV	V	VI	VII
Age group	Seasonings and beverages	Cereals	Potatoes, legumes, nuts	Fish/shellfish, meat and eggs	Oils/fats and milk/milk products	Sugar and confections/ savories	Fruits, vegetables and seaweeds
Young children	0.67	6.57	1.13	0.46	1.21	21.73	1.56
Children	0.76	5.49	1.07	0.41	1.09	17.03	1.68
Youths	0.86	7.68	1.05	0.66	1.24	20.57	2.35
Adults	0.99	4.93	1.13	0.40	0.49	19.12	4.45

Table 3. Al concentrations in unprocessed foods (mg/kg).

	Food group			
	II	III	IV	VII
Age group	Cereals	Potatoes, legumes, nuts	Fish/shellfish, meat and eggs	Fruits, vegetables and seaweeds
Young children	0.32	0.43	0.40	0.37
Children	0.43	0.43	0.33	0.50
Youths	0.36	0.43	0.44	0.50
Adults	0.39	0.43	0.33	0.54

of unprocessed foods in these categories ranged from 0.32 to 0.54 mg/kg. The results show that Al is widely contained in processed and unprocessed foods. Furthermore, the Al content in processed foods appears to be much higher than that in unprocessed foods. These results are consistent with previous reports (Jiang et al. 2013).

For unprocessed category II foods (cereals), the main cereal consumed by Japanese is rice. The expected Al levels in rice are consistent with the present results (0.32–0.43 mg/kg), but are considerably lower than the category III levels reported in Germany (8.5 mg/kg) (Müller et al. 1998), which were obtained for potatoes. However, the current result is similar to the levels (0.64 mg/kg) in potato reported in Greece (Bratakos et al. 2012). It should be noted that the differences between the values obtained in the present study and the reported values could be due to the environmental conditions in which the crops were grown.

In this study, the Al levels of food category IV samples, including unprocessed fish/shellfish, meat and hens' eggs, were less than 0.50 mg/kg. These results are similar to those previously reported (Bratakos et al. 2012; Fekete et al. 2013). It was reported that the Al levels in fish samples were below 1.0 mg/kg, whereas those in fresh meat were below 0.40 mg/kg. The Al levels in hens' eggs and egg products were reported to be less than 0.74 mg/kg (Pennington 1988; Müller et al. 1998; Soliman and Zikovsky 1999; Bratakos et al. 2012; Jiang et al. 2013).

For unprocessed category VII foods (fruits, vegetables, and seaweeds), the highest Al levels were present in fruits and vegetables. The present levels (0.37–0.54 mg/kg) were similar to the levels (typically less than 0.80 mg/kg) reported in Greece (Bratakos et al. 2012), and lower than the levels (2.41 mg/kg for fruits, 1.96 mg/kg for vegetables) reported in Belgium (Fekete et al. 2013). However, the Al levels in fruits and vegetables previously reported varied significantly (0.07–41.1 mg/kg) (Müller et al. 1998; Bratakos et al. 2012; Fekete et al. 2013; Jiang et al. 2013).

Estimation of dietary Al intake

Dietary Al exposure is related to both Al levels in foods and amount of food consumption. Therefore, the estimate of daily dietary Al intake was based on both the results of Al levels in food categories estimated by the market basket method, and the listed dietary intake amounts of various foods per age group. The daily dietary Al intake of processed and unprocessed foods is shown in Tables 4 and 5, respectively. As shown in Table 4, adults have the highest Al intake from food category I, youths and children showed the highest intake from food category II, while young children showed the highest intake from food category VI. For unprocessed foods, the dietary Al intake from food category VII was the highest for all age groups (Table 5).

Total daily Al intake from processed and unprocessed foods is shown in Figure 1. The daily dietary Al intake from processed foods is much larger than from unprocessed foods, and total daily Al intake in youths was the highest among all age groups. The study indicated that processed foods from food categories I, II, and VI significantly contribute to Al intake; however, the ratio of daily intake from unprocessed foods to the subtotal daily intake for food categories IV and VII is larger (77–79% for category IV foods and 67–88% for category VII foods). These findings suggest that the daily Al intake can be roughly estimated using only the processed food data for each age group.

Table 4. Daily Al intake from processed foods (mg/person/day).

	Food group						
	I	II	III	IV	V	VI	VII
Age group	Seasonings and beverages	Cereals	Potatoes, legumes, nuts	Fish/shellfish, meat, and eggs	Oils/fats and milk/ milk products	Sugar and confections/ savories	Fruits, vegetables, and seaweeds
Young children	0.23	0.56	0.08	0.01	0.09	0.83	0.01
Children	0.35	0.74	0.11	0.02	0.10	0.70	0.02
Youths	0.49	1.00	0.10	0.03	0.10	0.75	0.03
Adults	0.70	0.59	0.14	0.02	0.03	0.57	0.11

Table 5. Daily Al intake from unprocessed foods (mg/person/day).

	Food group			
	II	III	IV	VII
Age group	Cereals	Potatoes, legumes, nuts	Fish/shellfish, meat and eggs	Fruits, vegetables, and seaweeds
Young children	0.03	0.02	0.04	0.08
Children	0.05	0.03	0.06	0.17
Youths	0.07	0.02	0.09	0.17
Adults	0.07	0.02	0.06	0.22

The results of dietary Al intake estimates obtained in this study were similar to recent results from other national investigations, such as in Greece, China, and Belgium, although the survey measures of each investigation are unique and different. It was reported that nonalcoholic beverages are the most important contributor to Al intake in the Belgian adult population (Fekete et al. 2013). The present results show that the greatest contributor to Al intake in the adult Japanese group also appears to be beverages: the food group encompassing nonalcoholic beverages corresponds to food category I in this study (Fig. 1). In Greece, researchers reported that the major Al contributors are cereal products and vegetables, corresponding to 46.7% and 25.7% of total Al intake, respectively (Bratakos et al. 2012). The present results show that the major contributor to Al intake in the total group also appears to be cereal products, which correspond to food category II in this study. We also showed that category VI foods, such as sugar and confections/savories, in this study are major contributors to Al intake in the total group. However, confectionery products are small contributors (5.3%) to Al intake in Greece (Bratakos et al. 2012). We classified food products such as cake doughnut, butter cake and steamed bun as confectioneries, and placed them in food category VI. Food products similar to cake doughnut and butter cake, but not steamed bun, are consumed in Greece. These food products might be classified in cereal products in the study in Greece. Therefore, the observed differences for sugars and sweets between the study of Greece and the present study might be due to differences in food group categorization, and/or differences in consumption, and/or differences in food additive levels. The unique food products such as steamed bun are consumed mostly in Asian counties. Moreover, a similar study in South China showed that steamed flour products typically contain quantities of Al and are a major contributor to Al intake (Jiang et al. 2013). The Chinese study suggests that steamed flour products in South China contain food additives, including Al as leavening agents. The result of our study is thought to be consistent with a study in South China. While the daily Al intake in this study is significantly lower than that indicated in a previous Japanese report from 2001, the food categories that are the main contributors to daily Al intake in this study are quite similar to those of a previous report (Matsuda et al. 2001).

Determination of Al levels in individual food products of the high Al content food categories

To investigate in detail the kinds of processed food products contributing to high Al consumption, we determined the Al levels of individual processed food products in food categories I, II, and VI that are the main contributors to Al intake. The levels of Al and the daily dietary Al intake for all age groups are shown in Tables S1–S3. In food category I (Table S1), the processed food with the highest Al level was curry roux (10.7 mg/kg), followed by milk cocoa (9.69 mg/kg), and soluble coffee powder (6.12 mg/kg). In food category II (Table S2), the processed food with the highest Al level was tempura flour (222 mg/kg), followed by sweetened bun (156 mg/kg), Chinese steamed bun with a bean-jam filling (59.2 mg/kg), and Chinese steamed bun

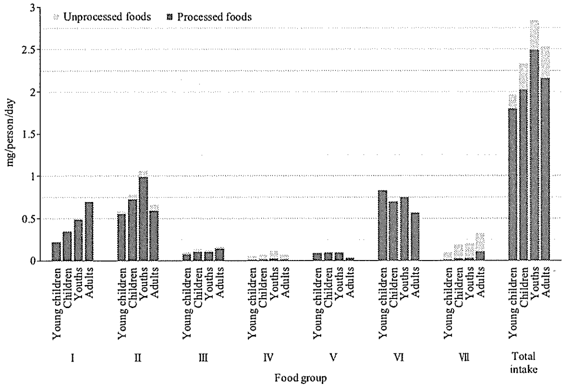

Figure 1. Total daily Al intake. The graph indicates the estimated daily intake of Al from each food category. Al intake from processed foods is indicated in black, and Al intake from unprocessed foods is indicated in gray.

with meat filling (57.5 mg/kg). In food category VI (Table S3), the processed food with the highest Al level was cake doughnut (258 mg/kg), followed by Japanese steamed bun (172 mg/kg) and butter cake (116 mg/kg). The range of Al concentrations in the various food products examined was 0.95–172 mg/kg for Japanese steamed bun products, 1.53–258 mg/kg for cake doughnut products, less than 0.3–59.2 mg/kg for shortcake products, and less than 0.3–116 mg/kg for butter cake products. In the present study, Chinese steamed bun is classified as a category II food because the product is processed using baker's yeast, and Japanese steamed bun is classified as a category IV food because the product is produced without using baker's yeast. Al levels in the present study are in line with previously reported results (Matsuda et al. 2001; Fekete et al. 2013).

In regions and countries such as the EU and the USA, sodium aluminum phosphate, used as a leavening agent in cereals and related products, and/or sodium aluminum silicate used as an anticaking agent in cake mixes and dried products, contribute highly to Al levels in wheat bread, cakes, cookies, and confectionaries. Food additives such as sodium aluminum phosphate and sodium aluminum silicate are approved for use as food additives (Jorhem and Haegglund 1992; Yang et al. 1994; Leblanc et al. 2005; Saiyed and Yokel 2005; Bratakos et al. 2012) in the USA and EU countries. However, in Japan, sodium aluminum phosphate and sodium aluminum silicate are not currently approved for use as food additives, whereas aluminum ammonium sulfate and aluminum potassium sulfate are currently used as food additives, without limits on the amounts used. Therefore, in Japan, the high Al levels in some samples of both categories II and VI might be attributed to the use of food additives such as aluminum ammonium sulfate and aluminum potassium sulfate as leavening agents.

In food category I, major contributors to daily Al intake include vegetable juice for young children (0.02 mg/person/day), curry roux for children (0.04 mg/person/day), and green tea for youths and adults (0.07 mg/person/day for youths and 0.16 mg/person/day for adults). For food categories II and VI, sweetened bun and cake doughnut are the main contributors to the intake of Al for all age groups. We suggest that the high Al levels in these processed foods contribute to high Al intake.

Percentile analyses of Al exposure in each age group

The weekly dietary Al intake due to processed foods was calculated by multiplying the daily Al intake value by 7 (7 days/week). The average weekly dietary Al exposure from processed foods for young children, children, youths, and adults is estimated to be 0.86, 0.45, 0.35 and 0.30 mg/kg bw/week, respectively. The percentages of those for all age groups to the PTWI established by the JECFA in 2011 are 43.1, 22.4, 17.6 and 15.1%, respectively.

The estimated high-level weekly exposures to Al (P_{50}, P_{95}, $P_{97.5}$, and P_{99}) are shown in Figure 2. The P_{95} is 2.0 mg/kg bw/week for young children, 1.0 mg/kg bw/week for children, 0.8 mg/kg bw/week for youths, and 0.6 mg/kg bw/week for adults. The percentages of P_{95} to PTWI (2.0 mg/kg bw/week) are 101% for young children, 49% for children, 40% for youths, and 31% for adults. As shown in Figure 2, high-level Al exposure (>P_{95}) that exceeded the PTWI was observed only in small children. To limit Al intake in young children, the population with the highest-level Al exposure, the government should regulate the use of food additives, such as leavening agents (including baking powder) in the manufacture of confectionaries.

While it is likely that cooking utensils and food packaging contribute to Al intake, intake through kitchenware materials is considered to be less important than intake from food (Fekete et al. 2013). However, further studies investigating Al intake from cooking utensils and food packaging are necessary.

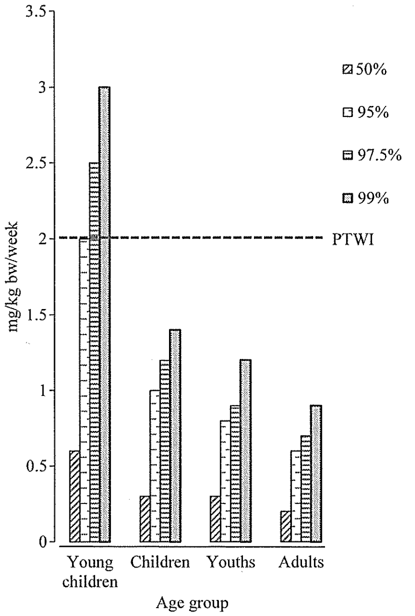

Figure 2. Percentile analysis of Al intake from foods. Each column indicates the estimated weekly Al intake for each percentile value. The dashed line indicates provisional tolerable weekly intake (PTWI; 2 mg/kg bw/week), established by JECFA in 2011.

Conclusion

The Al content of various food categories and the dietary estimated Al intake according to food category for young children, children, youths, and adults in Japan was determined using the market basket method, and Al analysis was conducted using ICP-AES. The Al content of samples of processed foods ranged from 0.40 to 21.7 mg/kg. For processed foods for all age groups, the Al content was highest in category VI samples, sugar and confections/savories, followed by category II samples, cereals. The daily dietary Al intake from processed foods is much larger than that from unprocessed foods. The average weekly dietary Al exposure from processed foods in young children, children, youths, and adults is estimated to be 0.86, 0.45, 0.35 and 0.30 mg/kg bw/week, respectively. High-level Al exposure (>P_{95}) that exceeded the PTWI was observed only in young children.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The levels of Al in the high Al content food category I and the daily dietary Al intake for all age groups.
Table S2. The levels of Al in the high Al content food category II and the daily dietary Al intake for all age groups.
Table S3. The levels of Al in the high Al content food category III and the daily dietary Al intake for all age groups.

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Scientific Research

A Comparative Study of the Hydroxyl and Saponification Values of Polysorbate 60 in International Food Additive Specifications

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ABSTRACT

We investigated the hydroxyl and saponification values of 27 samples of Polysorbate 60 products that were commercially available worldwide. We observed that the values of most of the studied samples were not within the range established at the Joint FAO/WHO Expert Committee on Food Additives (JECFA), while they did agree with the specifications described in the USA, the EU and Japan. We believe that purities of the new commercial Polysorbate 60 samples are higher than those of the older products which were available when the JECFA specifications were discussed (around 1973). The present study suggests that the hydroxyl and saponification values of the current JECFA specifications for Polysorbate 60 should be re-evaluated.

KEYWORDS

Polysorbate 60; Polyoxyethylene Sorbitan Monostearate; Hydroxyl Value; Saponification Value; Emulsifier; Specification

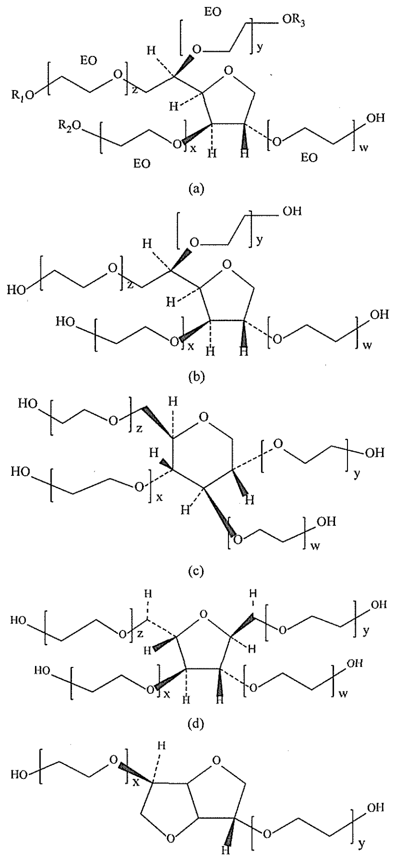
1. Introduction

Polysorbate 60 (polyoxyethylene sorbitan monostearate or Tween 60) is a sorbitol-based nonionic surfactant which is commonly employed as emulsifier, dispersant, and stabilizer in a wide variety of food, cosmetic and pharmaceutical products [1]. The compound primarily consists of partial fatty acid esters such as stearate and palmitate, sorbitol-derived cyclic ethers (sorbitans and sorbides) polymerized with approximately 20 molecules of ethylene oxide per molecule of Tween (Figure 1) [2]. Polysorbate 60 is widely used in various food products, including confectionery items, bread, dressings and shortenings [3-5].

Polysorbate 60 was approved as a food additive in the USA in 1960 and in the EU in 1995, followed by South Korea, China, Taiwan, Canada, Australia, New Zealand and Japan. Although the specifications and standards for Polysorbate 60 as a food additive have already been established in many countries, the ranges of hydroxyl and saponification values in the purity tests established at the 67th Joint FAO/WHO Expert Committee on Food Additives (JECFA) are somewhat different from those established in the USA, the EU, Japan and so on (Table 1).

Table 1. **JECFA specification deviation of Polysorbate 60 from EU, USA and Japanese in hydroxyl and saponification value.**

	Saponification value	Hydroxyl value
JECFA	41 - 52	90 - 107
JAPAN	45 - 55	81 - 96
USA	45 - 55	81 - 96
EU	45 - 55	81 - 96



EO = oxyethylene group (-CH$_2$CH$_2$-O-); w + x + y + z = approx. 20;
R$_1$ = Stearic acid (CH$_3$(CH$_2$)$_{16}$CO-) or Palmitic acid (CH$_3$(CH$_2$)$_{14}$CO-);
R$_2$ = R$_3$ = H.

Figure 1. **Typical structure of Polysorbate 60 (a) and sorbitol-derived cyclic ethers (b)-(e). (a) Polysorbate 60 (Polyoxyethylene sorbitan monostearate); (b) 1,4-sorbitan based polyethoxylate; (c) 1,5-sorbitan based polyethoxylate; (d) 1,4:3,6-isorbide based polyethoxylate; (e) 1,4:3,6-isorbide based polyethoxylate.**

Since Polysorbate 60 is commercially available worldwide as a food additive, multiple different specifications for this compound cause many troubles and affect the fair trade between countries. In addition, they might induce serious trade disputes. Therefore, it is important to investigate whether the hydroxyl and saponification values of the commercially available Polysorbate 60 food additives are within the range specified by the JECFA.

In this study, we determined the hydroxyl and saponification values of the commercially available Polysorbate 60 products. We observed that the measured values were not within the range specified by the JECFA, although they did agree with the specifications prescribed in the USA, the EU and Japan. We discuss some of the factors responsible for these differences.

2. Materials and Methods

2.1. Samples

Twenty seven commercially available Polysorbate 60 products were provided by the Japanese, American, and European manufacturers (Table 2). The data reported for samples 7 - 9 can be considered as reference values because these are industrial-grade samples.

2.2. Reagents and Chemicals

The following chemicals were all extra pure grade (unless stated otherwise) and purchased from Kanto Chemical Co. Inc., Japan: pyridine, acetic anhydride, ethanol, 0.5 mol/L ethanolic potassium hydroxide solution for the saponification analysis, phenolphthalein (10 g/L), diethyl ether, and 0.5 mol/L hydrochloric acid as well as a 0.1 mol/L potassium hydroxide solution for the volumetric analysis.

2.3. Analysis of the Hydroxyl Value

The hydroxyl value corresponds to the milligrams of potassium hydroxide (KOH) required to neutralize an equivalent amount of acetic acid combined with hydroxyl groups in 1 g of a sample. The analysis was conducted in accordance with Japan's specifications and standards for food additives. The procedure is as follows:

The sample was carefully weighed (approximately 1 g), placed in a 200 mL round-bottom flask, and mixed with 5 mL of the pyridine—acetic anhydride stock solution. A small funnel was placed at the neck of the flask and the temperature of the mixture was raised (95˚C - 100˚C) by using an oil bath (BK-33, HiTEC Corporation, Japan). After keeping the temperature constant for one hour, the flask was removed from the oil bath and the reaction mixture was cooled down to ambient temperature. Residual acetic anhydride was hydrolyzed to acetic acid by adding one milliliter of water. The reaction mixture was then reheated in the oil bath (for 10 min), and after cooling, the funnel and the neck were washed down into the flask using 5 mL of neutralized ethanol. The resulting mixture was titrated against 0.5 mol/L ethanolic KOH

Table 2. **Saponification and hydroxyl values in 27 commerical Polysorbate 60 products.**

No.	Manufacturers	Nationalities	Saponification value		Hydroxy value		Acid value	
				Ave. and S.D.		Ave. and S.D.		Ave. and S.D.
1	Company A	Japan	50.4	50.4 ± 0.25	96.8	97.3 ± 1.01	0.2	0.3 ± 0.15
2			50.7		98.5		0.5	
3			50.2		96.7		0.3	
4	Company B	Japan	53.2	52.8 ± 0.40	88.5	89.8 ± 1.22	0.4	0.3 ± 0.31
5			52.7		90.1		0.8	
6			52.4		90.9		0.2	
7*1	NOF	Japan	52.5	52.2 ± 1.53	79.3	83.8 ± 5.91	0.5	0.3 ± 0.20
8*1			52.7		78.2		0.4	
9*1			51.9		76.6		0.7	
10			50.9		81.5		0.2	
11			52.6		87.7		0.2	
12			54.0		91.0		0.2	
13			53.9		92.0		0.3	
14			52.0		85.0		0.1	
15			49.0		83.0		0.1	
16	Company C *2	USA	51.6	51.6 ± 0.40	84.4	85.8 ± 1.32	0.7	0.7 ± 0.25
17			52.0		86.1		1.0	
18			51.2		87.0		0.5	
19	Company D	Germany	52.2	52.1 ± 0.26	90.5	89.6 ± 0.86	0.5	0.6 ± 0.10
20			52.3		88.8		0.7	
21			51.8		89.4		0.6	
22	Company E	UK	49.7	49.7 ± 0.72	85.2	85.4 ± 2.02	0.3	0.6 ± 0.22
23			48.8		88.9		0.5	
24			49.9		83.9		0.4	
25			49.2		83.0		0.7	
26			49.9		85.8		0.9	
27			50.9		85.5		0.7	

*1Industrial grade; *2Company C now belongs to the other manufacturer.

using 1 mL of phenolphthalein as the indicator. The same procedure was followed without the sample (blank determination) and the hydroxyl value was calculated using Equation (1)

$$\text{Hydroxyl value} = \frac{(a-b)\times 28.05}{\text{Weight (g) of the sample}} + \text{Acid value}, \quad (1)$$

where a and b are the volumes (mL) of ethanolic KOH consumed in the blank determination and in the titration of the sample, respectively.

2.4. Analysis of the Saponification Value

The saponification value corresponds to the milligrams of potassium hydroxide (KOH) required to saponify the esters and neutralize the free acids in 1 g of a sample. The analysis was conducted in accordance with Japan's specifications and standards for food additives. The procedure is as follows:

The sample was carefully weighed (approximately 2 g), placed in a 200 mL flask, and mixed with 25 mL of 0.5 mol/L ethanolic KOH. A short reflux condenser (Vidrex Co. Ltd., Japan) was connected to the neck of the flask, and the mixture was gently heated for one hour (under frequent stirring) using a water bath (IWB-250, AS ONE Corporation, Japan). After cooling, 1 mL of phenolphthalein was added to the reaction mixture. The residual KOH in the mixture was immediately titrated against 0.5 mol/L hydrochloric acid. If the mixture was turbid, the titration was performed on warm samples (solution becomes clear when warmed). The same procedure was followed without the sample (blank determination), and the saponification value was calculated using Equation (2)

$$\text{Saponification value} = \frac{(a-b)\times 28.05}{\text{Weight (g) of the sample}}, \quad (2)$$

where a and b are the volumes (mL) of hydrochloric acid consumed in the blank determination and in the titration of the sample, respectively.

2.5. Analysis of the Acid Value

The acid value is the milligrams of potassium hydroxide (KOH) required to neutralize the free acids in 1 g of a sample. The analysis was conducted in accordance with Japan's specifications and standards for food additives. The procedure is as follows:

The sample (10 g) was weighted in a 250 mL glass-stoppered flask. 100 mL of a mixture of diethyl ether and ethanol (95) (1:1 or 2:1) was added as the solvent, and if necessary, the sample was dissolved by warming. Then, a few drops of phenolphthalein were added and the solution was and titrated against 0.1 mol/L KOH (until the solution developed a light-red color which persisted for 30 s). If the sample solutions were turbid at low temperatures, the titrations were performed on warm samples. The acid value was calculated using Equation (3)

$$\text{Acid value} = \frac{\text{Volume (mL) of 0.1 mol/L KOH consumed}\times 5.611}{\text{Weight (g) of the sample}} \quad (3)$$

3. Results and Discussion

The hydroxyl and saponification values of several Polysorbate 60 samples are shown in Table 2 and Figure 2. The results indicate that the average hydroxyl values of all the samples are lower than the median value of the JECFA specification. Only samples 1, 2, 3, 5, 6, 12, and 19 are within the range specified by the JECFA (41 - 52). Except for samples 1 - 3, the hydroxyl values of all the samples were close to the lower limit (41) of the JECFA

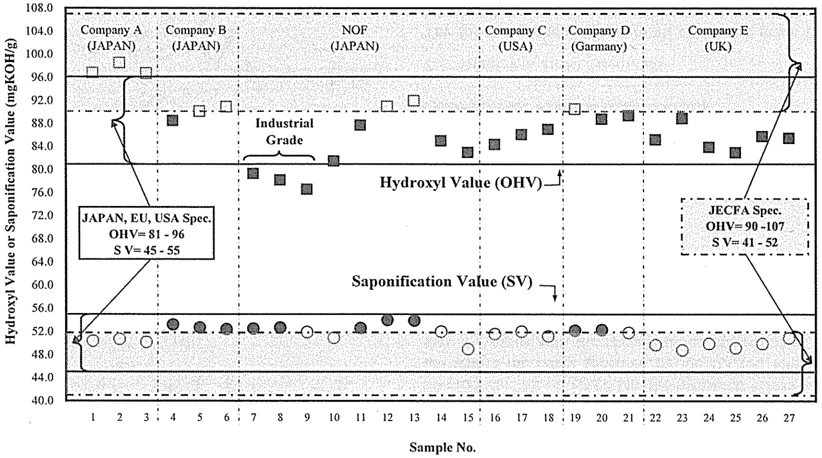


Figure 2. **Plot of the saponification and hydroxyl values of 27 commercial Polysorbate 60 products. The shaded areas represent the JECFA spec range of hydroxyl value and saponification value. Squares (■ or □) and circles (● or ○) represent saponification value and hydroxyl value, respectively. Closed circles (●) and squares (■) represent that they are out of the JECFA spec, and opened circles (○) and squares (□) represent that they are within the JECFA spec.**

specification. All the samples (except for samples 1 - 3 of company A and samples 7 - 9, *i.e.*, the industrial-grade probes) were within the range of the specification values accepted in the USA, the EU, and Japan (45 - 55). Since the hydroxyl value generally corresponds to the content of free hydroxyl groups in a compound, the hydroxyl values of Polysorbate 60 samples may give us an indication of the amount of free sorbitol-derived cyclic ethers present in those samples (Figure 1). This suggests that if the hydroxyl values in the samples are high, the total amount of free sorbitol-derived cyclic ethers as well as the acid values in the samples will also be comparably high. We showed that the average acid values determined for all the studied samples appear to be within the range (Not more than 2) specified by the JECFA (Table 2). The present results suggest that the commercial products available at the time when the JECFA specifications were established might contain higher amounts of free sorbitol-derived cyclic ethers compared with current commercial products.

Saponification values of samples 3 - 8, 11 - 13, 19, and 20 were within the range specified by the JECFA (90 - 107), whereas those of the remaining samples were not. However, the values of all the samples were within the range accepted in the USA, and the EU, and Japan (81 - 96). Since the saponification value generally indicates the content of ester groups in a compound, this value should decrease as the amount of free sorbitol-derived cyclic ethers increases in the Polysorbate 60 samples.

As shown in Table 1, the hydroxyl and saponification values of Polysorbate 60 specified by the JECFA are somewhat different from those accepted in several countries. Since impurities such as free sorbitol-derived cyclic ethers may raise the hydroxyl value and lower the saponification value of the samples, the two values measured for Polysorbate 60 will vary with the amount of impurities present in the samples. The hydroxyl value for Polysorbate 60 specified by the JECFA is higher than the values accepted in the USA, the EU, and Japan, whereas the JECFA-specified saponification value is lower than those accepted in the mentioned countries. The JECFA specifications for Polysorbate 60 were established at the 17th JECFA meeting, which was held in 1973, whereas the specifications in the USA (through the FDA), the EU, and Japan were established after 1995. We considered that the hydroxyl value for Polysorbate 60 in the JECFA specification is higher than that in the EU, whereas the saponification value is lower. This is because of the differences in amount of impurities (e.g., free sorbitol-derived cyclic ethers) present in different Polysorbate 60 samples. Accordingly, we believe that the purities of newer commercial Polysorbate 60 samples are higher than those of older products, which were available when the JECFA specifications were discussed (around 1973), although we could not investigate the purities of the compounds available at that time.

The results indicate that the quality of the Polysorbate 60 products that are currently available worldwide is not consistent with the JECFA specifications for this additive in terms of the hydroxyl and saponification values. However, these products do fulfill the quality requirements in the USA, the EU, and Japan. Because the JECFA specifications are very important for a fair trade, according to the Agreement on Technical Barriers to Trade (TBT) and the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS), these inconsistencies between the values determined for the studied Polysorbate 60 products and the JECFA specifications might induce serious trade disputes. In addition, according to the Agreements on TBT and SPS, if an international dispute happens, judgment will most likely be given based on an international standard.

Therefore, a revision of the current JECFA specifications for the hydroxyl and saponification values of Polysorbate 60 would be necessary for the international trade of current commercial products.

4. Conclusion

We investigated the hydroxyl and saponification values of Polysorbate 60 samples that are currently available worldwide as commercial food additives. Among the studied 27 samples, the hydroxyl values of 19 samples and the saponification values of 11 samples were observed to be outside the range specified by the JECFA. The results revealed that the JECFA specifications for Polysorbate 60 do not reflect the quality of the present samples. We would like to suggest a re-evaluation of the hydroxyl and saponification values of the current JECFA specifications for Polysorbate 60.

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