遺伝子組換え（GM）ダイズ系統A2704-12の系統特異的定量検知法の開発および性能指標の評価

誌名
食品衛生学雑誌
ISSN
00156426
巻/号
522
掲載ページ
p. 100-107
発行年月
2011年4月
Development and Evaluation of Event-Specific Quantitative PCR Method for Genetically Modified Soybean A2704-12

(Received July 23, 2010)

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A novel real-time PCR-based analytical method was developed for the event-specific quantification of a genetically modified (GM) soybean event; A2704-12. During the plant transformation, DNA fragments derived from pUC19 plasmid were integrated in A2704-12, and the region was found to be A2704-12 specific. The pUC19-derived DNA sequences were used as primers for the specific detection of A2704-12. We first tried to construct a standard plasmid for A2704-12 quantification using pUC19. However, non-specific signals appeared with both qualitative and quantitative PCR analyses using the specific primers with pUC19 as a template, and we then constructed a plasmid using pBR322. The conversion factor (Cf), which is required to calculate the amount of the genetically modified organism (GMO), was experimentally determined with two real-time PCR instruments, the Applied Biosystems 7900HT and the Applied Biosystems 7500. The determined Cf values were both 0.98. The quantitative method was evaluated by means of blind tests in multi-laboratory trials using the two real-time PCR instruments. The limit of quantitation for the method was estimated to be 0.1%. The trueness and precision were evaluated as the bias and reproducibility of relative standard deviation (RSDr), and the determined bias and RSDr values for the method were each less than 20%. These results suggest that the developed method would be suitable for practical analyses for the detection and quantification of A2704-12.

Key words: A2704-12; event-specific; genetically modified (GM); real-time PCR; soybean

Introduction

The use of genetically modified (GM) crops has been increasing since their commercialization in 1996. After more than a decade, the global area of GM crops has increased approximately 80-fold, from 1.7 million hectares in six countries in 1996, to 134 million hectares in 25 countries in 2009[1]. The utilization of GM crops has generated substantial economical benefits, but, nevertheless, has been subjected to rigid control. The presence of GM products in crops or foods is obliged to be labeled in the European Union (EU), Korea, Japan, Australia, and many other countries. In Japan, the genetically modified organism (GMO) labeling system has been defined by the “JAS law”[2] and the “Food Sanitation law”[3], and the thresholds for the unintentional commingling level for approved GM soy and maize were both set at 5%[4, 5].

The Japanese food self-sufficiency ratio on a calorie supply basis has been hovering around 40% in recent years, but the ratios among grains, except for rice, are extremely low[5]. Among them, soybeans are one of the most important crops in Japan. The domestic consump-
tion of soybeans as oil and food is over 4 million tons/year, but the self-sufficiency ratio for this crop is only approximately 5%*6, i.e., more than 90% of soybeans are imported into Japan. Most of them are imported from the United States, where GM soybeans account for more than 90% of the soybean cultivation areas. There are several approved GM soybean events in Japan*. The first commercial GM soybean was a glyphosate-tolerant soybean (GTS), event 89788, RRS, A2704-12 and A5547-127 were approved, and then the second generation of GTS, MON89788, was recently approved.

A2704-12 and A5547-127 contain the same transgene cassette consisting of cauliflower mosaic virus 3S promoter (P3S), the synthetic pat gene which codes phosphinothricin N-acetyltransferase derived from Streptomyces viridochromogenes, and cauliflower mosaic virus 3S terminator (T3S). Many methods for analyzing GTS and MON89788 have been published, but there is no report on quantification of A2704-12, although it is being commercially cultivated.

In this study, we developed a new quantitative method for A2704-12, using event-specific PCR, and then evaluated the method with two multi-laboratory trials. The development of the quantitative method was mainly carried out in the National Food Research Institute, and the interlaboratory collaborative study was independently conducted with the National Institute of Health Sciences (NIHS).

Materials and Methods

Plant materials

Regarding soybean and maize seeds, A2704-12 and a non-GM isoline of A2704-12 used as a non-GM soy sample were kindly provided by the developer, MON 89788, RRS, MON810, MON863, MON88017, and NK 603 by Monsanto Company (St. Louis, MO, USA), Bt11, Event176, GA21, and MIR604 by Syngenta Seeds AG (Basel, Switzerland), TC1507 and DAS59122 by Pioneer Hi-Bred International (Johnston, IA, USA), and T25 was directly imported from the USA. QC9651 maize from Quality Technology International, Inc. (Elgin, IL, USA) was used as a non-GM maize. Seeds of rice (Oryza sativa) variety Kinuhikari, wheat (Triticum aestivum) variety Haruyutaka, and barley (Hordeum vulgare) variety Harrington were harvested in Japan. A5547-127 was purchased from the American Oil Chemists' Society (AOCS) (Urbana, IL, USA) as DNA extract (AOCS 0707-C; above 999.9 ng/μL of GM DNA).

Oligonucleotide primers and probes

For the specific detection of A2704-12, a pair of primers (KVM175; 5'-GGATAGGCTGCGACACTGTT-3') and a fluorescent dye-labeled probe (TM031; 5'-CCCTGAGTGGCGCTTCC-3') were used for real-time PCR. The sequences of these primers and probe were taken from the report of the European Commission's Joint Research Centre (JRC, IRMM, Geel, Belgium)*8. Le1*13 was used as a soybean-specific endogenous reference DNA for quantitative analysis. For specific detection of Le1, the primers (Le1n02-5'; 5'- GCCCTTACTCCACCCCC-3' and Le1n02-3'; 5'- GCCCATCTGCAAGCTTCTT-TT'T-3') and fluorescent dye-labeled probe (Le1-Taq; 5'-AGCTTCGCCGCTCTTCAA CGTCCAC-3') were used. The oligonucleotide primers and TaqMan® probes were synthesized by FASMAC Co., Ltd. (Kanagawa, Japan) and Life Technologies (Carlsbad, CA, USA), respectively. The oligonucleotide probes were labeled with 6-carboxy-fluorescein (FAM) at the 5' ends and 6-carboxytetramethylrhodamine (TAMRA) at the 3' ends.

Preparation of calibrant plasmid

Specific sequence fragments from A2704-12 and the endogenous soybean Le1 gene were synthesized as a single oligonucleotide in tandem and inserted into a pBR322 vector. The constructed plasmid was purified by equilibrium centrifugation in a CsCl gradient to collect the covalently closed circular DNA, which was linearized by cutting at a restriction site located outside the integrated fragment. The concentration of the linearized DNA was calculated from the ultraviolet (UV) absorbance measured with a spectrophotometer, DU 800 (Beckman Coulter, Fullerton, CA, USA) as described previously, and converted to the molar concentration. The solution was then diluted to theoretical concentrations of 20, 125, 1,500, 20,000, and 250,000 copies per 2.5 μL, with 5 ng/μL of ColE1 DNA (NIPPON GENE, Tokyo, Japan) solution. Finally, the copy numbers of the diluted plasmids were adjusted based on the Le1 segment of pMUL3L2, which has been adopted in the Japanese standard analytical method*9, using quantitative real-time PCR analyses.

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*7 List of products that have undergone safety assessment and been announced in the Official Gazette (May 12, 2010) of the Department of Food Safety, Ministry of Health, Labour, and Welfare; http://www.mhlw.go.jp/english/topics/food/pdf/sec01-2.pdf

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Preparation of test samples and DNA extraction

To evaluate the quantitative method, we used six mixing levels of test materials containing 0%, 0.10%, 0.50%, 1.0%, 5.0%, and 10.0% A2704-12. To prepare the mixed samples, we ground A2704-12 seeds and non-GM seeds using MM200 and 2M100 grinders (Retsch, Haan, Germany), respectively, as described previously\(^6\), and then mixed the samples on a weight-to-weight basis. DNA was extracted from the ground materials using GM quicker (NIPPON GENE) according to the manufacturer’s manual. The concentration and quality of extracted DNA solutions were evaluated by measuring UV absorbance with a spectrophotometer, ND-100 (NanoDrop Technologies, Wilmington, DE, USA). Soybean genomic DNA solutions were adjusted to a concentration of 20 ng/μL.

Quantitative PCR

Qualitative PCR using a thermal cycler, GeneAmp PCR system 9700 (Life Technologies), and agarose gel electrophoresis were performed as described previously by Kuribara et al. (2002).

Quantitative PCR

TaqMan® real-time PCR assays were carried out using the Applied Biosystems 7900HT (AB 7900) or the Applied Biosystems 7500 (AB 7500) (Life Technologies), in 25 μL final volume, containing 50 ng of sample DNA, 12.5 μL Universal Master Mix (Life Technologies), 0.5 μM primer pairs, and 0.2 μM probe. The step-cycle program was as follows: 2 min at 50°C, 10 min at 95°C, 45 cycles, 30 s at 95°C, and 1 min at 59°C. In the reaction plate, each sample was measured in triplicate.

Multi-laboratory trial

Multi-laboratory trials were performed with the AB 7900 and the AB 7500 independently and consisted of 2 separate stages: measurement of the C\(_T\) value and a blind test. All measurements were conducted by 5 laboratories for the AB 7900 and 3 laboratories for the AB 7500. Experimental protocols were provided by the NIHS. Quantitative real-time PCR was performed with primers, probes, Universal Master Mix, and blind DNA solutions supplied by NIHS.

The first stage was the experimental determination of the C\(_T\) value as the ratio of the copy number of recombinant DNA (r-DNA) to the taxon-specific sequence in the GM plant genome. To calculate the C\(_T\) value for A2704-12, we extracted the genomic DNA from genuine GM seeds and determined the copy numbers of r-DNA and taxon-specific sequences. The measurement was conducted twice at each laboratory. The C\(_T\) value for each real-time PCR instrument was separately determined as the mean of the obtained values.

A blind test was carried out as the second stage. Blind samples designed as blind duplicates of the soybean genomic DNAs extracted from 6 different concentrations of A2704-12, 0%, 0.10%, 0.50%, 1.0%, 5.0%, and 10.0%, were sent to the participants. All participants were requested to submit the data from the real-time PCR analyses. All submitted data were analyzed by means of Cochran’s test\(^4\) and Grubbs’ test\(^15,16\).

Results and Discussion

Specificity of the PCR system for A2704-12

A2704-12 contains two copies of the pat gene cassette inserted in a head-to-tail configuration\(^*10\). The biotic transformation of soybeans was conducted by micro-particle bombardment with a pUC19-based plasmid containing the pat gene cassette. The pUC19 plasmid contains an antibiotic resistance gene; beta-lactamase (bla), and a lac operon, and several fragments derived from pUC19 DNA exist in the A2704-12 genome. Partial sequences derived from the bla gene and lac operon are integrated side-by-side between the two pat gene cassettes\(^*10\), and this site is unique to A2704-12. For specific detection of A2704-12, the junction site was used (Fig. 1). The specificity of the primer set was confirmed by qualitative PCR. The expected 64-bp product was detected using genomic DNA solutions from A2704-12, but not from non-GM soybeans; GM soybeans RRS, MON89788, and another glufosinate-tolerant soybean: A5547-127; non-GM maize; 11 lines of GM maize; rice, wheat, and barley; and the no template control (Fig. 2).

\(^*10\) Agbios database: http://www.cera-gmc.org/?action=gm_crop_database&
Fig. 2. Specificity test of the designed primer pair for genomic DNAs from several GM events or crops. Agarose gel (3.0%) electrophoretogram of the amplified PCR products corresponding to the 64 bp DNA. Arrowhead indicates the expected amplified product. Lanes 1–11, eleven GM maize events, namely NK603, Event176, T25, GA21, MON810, TC1507, Bt11, MIR604, MON88017, DAS59122, and MON863, respectively; 12 and 13, non-GM maize and non-GM soy, respectively; 14–17, our GM soybean events, namely, RRS, MON89788, A2704-12, and A5547-127, respectively; 18–21, rice, wheat, barley, and no template, respectively. Lane M shows 100 bp ladder size markers.

Fig. 3. Specificity test of the designed primer pair and probe for plasmid DNAs. A: Qualitative PCR analysis. Lanes 1–4, A2704-12 genomic DNA, CoIE1 DNA, pUC19, and pBR322, respectively. The arrow indicates a non-specific amplification product. Lane M shows 100 bp ladder size markers. B: Quantitative PCR analysis. pUC19, pBR322, or CoIE1-derived signals in the amplification profile using the AB 7900 are shown. Concentrations of DNA solutions of A2704-12 genomic DNA, CoIE1 DNA, pUC19, and pBR322 were 20, 5, 10, and 10 ng/μL, respectively.

Construction of calibrant plasmid for A2704-12

In Japan, one of the features of standard quantitative methods is the utilization of standard plasmid DNA as reference molecules and calibrators. We have developed several standard plasmids using pUC19 or pBR322. Although pUC19 is a useful plasmid containing a high-copy-number replicon, when the pUC19 plasmid was used as a template, a non-specific amplified product of around 1.0 kb was observed (Fig. 3A). Furthermore, a non-specific signal was detected by quantitative PCR analysis (Fig. 3B). As mentioned above, for the specific detection of A2704-12, the pUC19-derived sequences, KVM175 and SMO001, which are a part of the sequences of bla gene and lac operon, respectively, were used as primers. The unexpected band may have been caused by the sequences of these primers, indicating the possibility that inaccurate quantification could occur if pUC19-based plasmids were used as a calibrator for quantification. Thus, we used pBR322 plasmid for the construction of the standard plasmid, designated as pLLS. To prepare the pLLS plasmid, we removed a bla gene partial sequence that included KVM175 from pBR322 and then inserted the A2704-12 and Lei segments (Fig. 4). Figure 5 shows the amplification plots of the pLLS plasmids diluted to a concentration ranging from 20 to 250,000 copies per reaction (Fig. 5A and B). The

Quantitative PCR Method for A2704-12

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values from both A2704-12 and Le1 were above 0.999 (Figs. 5C and D), which is an acceptable level ($R^2$ should be above 0.990)\cite{footnote}. The slopes, representing the amplification efficiencies, were $-3.44$ and $-3.32$ for A2704-12 and Le1 standard curves, respectively. PCR efficiency was calculated by use of the following equation: PCR efficiency = $10^{(-1/slope)}$\cite{footnote}. The theoretical value with an efficiency of 100% in each cycle is 2.00, and, in this study, PCR efficiencies of 1.95 and 1.99 were obtained for A2704-12 and Le1, respectively.

**Determination of the $C_l$ value for A2704-12**

To determine the experimental $C_l$ value for A2704-12, we measured the copy numbers of Le1 and A2704-12 in the extracted DNA from A2704-12 seeds. The $C_l$ value was determined with two real-time PCR instruments (the AB 7900 and the AB 7500) independently, from the results of 5 laboratories for the AB 7900 and 3 laboratories for the AB 7500. The measurement was repeated twice, and the $C_l$ value was determined as the mean of values measured by these laboratories. The determined $C_l$ values with the AB 7900 and the AB 7500 were similar; in fact, they took the same value of

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SD: Standard deviation
RSD: Relative standard deviation

**Fig. 4.** Schematic diagram of the construction of pLLS plasmid

The region corresponding to positions 3613–3843 in pBR322 was removed, and the specific sequences of A2704-12 and the Le1 were inserted in tandem. The nucleotide sequence of pBR322 is available under accession number J01749.

**Table 1.** Experimental conversion factor for A2704-12

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SD: Standard deviation
RSD: Relative standard deviation

**Fig. 5.** Amplification plots for dilution series of pLLS plasmid and corresponding curves

Typical amplifications of A2704-12 and Le1 segment are shown in A and B, respectively. The respective standard curves are shown in the panel below, and the $R^2$ and slope values of each standard curve are indicated at the upper-right corners. The dilutions contained 250,000, 20,000, 1,500, 125, and 20 initial template copies per reaction, respectively. These analyses were performed with the AB 7900.
0.98 rounded to the nearest hundredth of a unit (Table 1). From these results, we used 0.98 in the following quantifications as the common Ct value for the two instruments.

**Evaluation of the PCR quantification by multi-laboratory trials**

We performed multi-laboratory trials to evaluate the developed quantitative method for A2704-12 as a blind test using the AB 7900 and the AB 7500 instruments in 5 and 3 laboratories, respectively. We used DNA solution-based blind samples as described previously. For PCR quantification, analytical procedures would be divided into two main steps, that is, DNA extraction from samples and real-time PCR measurements. In our previous study, the Japanese standard analytical methods which were developed to quantify RRS and several GM maize events, were validated with an interlaboratory study which consisted of 2 steps, namely, DNA extraction and PCR quantification. After that, the established methods were evaluated with another interlaboratory study which was performed using the same materials without the DNA extraction step. However, the obtained precisions from the two studies were almost the same, suggesting that, at least in our system, the repeatability of relative standard deviation (RSDr) and reproducibility of relative standard deviation (RSDR) of the DNA extraction step were significantly smaller than those of the PCR quantification step. It was also suggested that the relatively large RSDr and RSDR values which were obtained from GMO quantification, may be attributed to the principle of real-time PCR, which is based on relative quantification between target and taxon-specific sequences, rather than absolute quantification.

All the participants received primers, probes, and test samples consisting of six different concentrations of A2704-12, and the measurement was performed twice. All the submitted data were examined for outlier laboratories with extreme variation using Cochran's test (p < 0.025) and with an extreme average level using Grubbs' test (p < 0.025) for the AB 7900 and the 7500 independently, as previously described and no outlier was found. The blank sample, 0% A2704-12, was used to estimate invalid laboratories, and no laboratory was eliminated. We then used all of the submitted data obtained from mixed samples with five different GM contents (0.1%, 0.5%, 1.0%, 5.0%, and 10.0% concentrations) for further statistical analyses. The trueness and precision were determined for the AB 7900 and the 7500 as previously described. The mean and relative standard deviation (RSDr) of blind samples were measured (Table 2). The determined bias, RSDr, and RSDR for the AB 7900 ranged from −14.6% to 1.0%, from 6.4% to 9.9%, and from 7.5% to 10.7%, respectively. The determined bias, RSDr, and RSDR for the AB 7500 ranged from −7.1% to 11.7%, from 3.0% to 13.2%, and from 10.7% to 10.7%, respectively. The obtained bias, RSDr, and RSDR here were similar to or within a narrower range than those in previously reported GMO events.

The data below 20 copies were extrapolated from the standard curve in our method because there was no calibrant below 20 copies. In Table 2, all the measured copy numbers of 0.10% samples were over 20 copies. Therefore, we estimate that the limit of quantitation (LOQ) for A2704-12 is 0.10% in this method.

We developed a specific quantification method for
GM soybean A2704-12. The experimentally determined Crr value was 0.98. The LOQ, trueness, and precision of this method were similar to or better than those of previous methods (20-23). ISO 24276 specifies the LOQ in GMO analysis, and the values are generally observed to have a RSD of 25% or less at the lowest level*11; all the RSD obtained in this study met this criterion. Therefore, we concluded that the developed method would be applicable for the detection and quantification of A2704-12 to monitor the validity of the food labeling system in Japan.

Acknowledgements

We would like to thank the following collaborators for their participation in these studies:
FASMAC Co., Ltd., Kanagawa, Japan
Food and Agricultural Materials Inspection Center, Saitama, Japan
Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan
Hiroshima Prefectural Technology Research Institute, Public Health and Environment Center, Hiroshima, Japan
Japan Food Research Laboratories, Tokyo
Kanagawa Prefectural Institute of Public Health
National Food Research Institute, Tsukuba, Japan
Tokyo Metropolitan Institute of Public Health

This work was supported by the Ministry of Agriculture, Forestry, and Fisheries of Japan Research Project, “Assurance of Safe Use of Genetically Modified Organisms”, and by a grant from the Ministry of Health, Labour and Welfare of Japan and by a grant from Consumer Affairs Agency, Government of Japan.

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6) De Beuckeleer, M. Elite event A5547-127 and methods and kits for identifying such event in biological samples.

*11 International Standard 24276, Foodstuffs—Nucleic acid based of analysis for the detection of genetically modified organisms and derived products—General requirements and definitions.


PETボトルおよびガラス瓶のリユースに関する検証（ノート）

林英一* 井井利男 新美宏一 食誌誌52(2), 112~121 (2011)

PETボトルおよびガラス瓶のリユースに際し、汚染物質に対するリユースシステムの安全性を検証するため、汚染物質5種類を用いて検証試験を行った。処理汚染物質として1,1,1,2トリクロロエタン、クロプロビル、トルエン、ペンゼンおよびフェニルプロピオンを用い、これらをPETボトルに充てんし50℃で7日間保存後、その溶液を取り出し、そのときの各物質濃度を測定した。試験結果より、各物質はリユースすることが確認された。さらに、PETボトルの耐熱性を確認するために、60℃で100時間保持したところ、PETボトルは破損することなく、内容物の品質保持も確認された。

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LC-MS/MSによる味付け海苔中のチアベングザルの分析法の検討（ノート）

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味付け海苔のチアベングザルの検出事例を経験した。原因調査の結果、味付け工程で津軽油を添加する際に使用されるスプングザルに、チアベングザルが混入していたことが判明した。今回、LC-MS/MS法を用いて味付け海苔中のTBZの定量法を検討した。味付け海苔の製造工場でのスプングザルが広く普及していたことから、改良した定量方法を用いて同時期と翌年の市販品についてTBZの調査を実施した。実験事例のあった平成20年3月までに6検体中5検体で0.014~1.376μg/g、翌年の平成21年7月に6検体中1検体で微量のTBZが検出された。以上のことから味付け海苔の製造工場が見直され、現在では改良していると考えられた。

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新規魚体食品用容器PID（Pouch in Dispenser）の微生物に対する保鮮特性の検討（報告）

長谷川 弘田中和子 篠田雄馬 鈴木均良 食誌誌52(2), 108~111 (2011)

魚体食品用容器PID（Pouch in Dispenser）の通常使用時、および人為的汚染時における微生物保鮮特性の検討を行った。開封後、静置あるいは風を当たせる場合、または中に内容物を注ぎ出した場合、PDEへの侵入が確認された。また、人为的に風を注ぎ口に吹きつけた場合、供試体の外側に侵入した。乾燥させた布を用いて供試体を操作した場合、内部への侵入が確認されなかった。また、清潔な乾燥した布で注ぎ口をふれた場合は微生物が侵入する可能性は低いと考えられた。注ぎ口に触れず注意喚起表示や、内容物によっては水分活性、pH等の調整、保存条件の選定が必要であると考えられた。

*北海道大学水産科学研究所

輸入冷凍野菜・果実中の残留農薬実態（1984年4月~2008年3月）（調査・資料）

小林敏雄・大村健治 田村康康 福澤博幸 上条健司 岩本紀子 彩山百合子 永山敏広 高野知郎 食誌誌52(2), 121~129 (2011)

1989年4月から2008年3月にかけて東京都内市販されていた輸入冷凍野菜・果実559検体について農薬の残留調査を行った。186検体から43種類の有機リン系農薬、有機ホルマリン農薬、ピレスロイド系農薬、カルボメート系農薬、その他の農薬が検出された。検出された農薬の検出濃度は、1.0ppm未満〜46ppmの範囲で検出された。農薬類（こまつなおよびほうれんそう）では、クロルピロフィル、ペルメトリンおよびオメトエート、豆科野菜（えだまめおよび蒸パンげん菜）では、ジルペンタリンおよびヘテロカプサロフ、野菜類（ブロッコリー）では、ピメジン酸およびパルカルフィル（NAC）の検出濃度が高かった。また、ライン豆での、果肉内の水溶性の高いセンハンドソース検出及びミシモ検出が検出された。検出された農薬は処理冷凍野菜および果実を嗅味された場合の農薬の推定検量線を算出し、一日摂取許容量（ADI）と比較したところ、各農薬のADI値の0.5%未満から30%の範囲であり、通常の嗅味による健康影響はないと考えられた。

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鰤魚中の一酸化炭素分析法の改良（調査・資料）

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通知で規定されている鰤魚中の一酸化炭素（CO）分析法のうちA法（通知A法）は、試料を多量に必要とし、また酸化炭酸製造時に鰤魚中のCOの一部が散逸するなどの問題が指摘されている。そこで本研究では、これらの問題点の解決を目指して、試料の改良を行い、分析方法を一部変更した分析法（改良A法）の適用性を検討した。また、改良法を用いて通知管理されているマグロ、ブリ、ハマチおよびチマヨソブリ中のCO濃度のパックグラウンド値を調査した。その結果、改良法は、試料製造段階時のCOの散逸抑制、試料の減圧操作の簡易化により適用可能であることが確認された。さらに、4種類の魚を共同で実施した各魚種中のCO濃度のパックグラウンド値については、改良法が通知A法と比較してCOの回収率が向上することから、特にCO未処理のチマヨソブリ中のCO濃度が現在の規制値を超えることが判明した。したがって、改良法を今後新たに鰤魚中のCO分析法として適用する場合には、チマヨソブリの規制値の変更が必要であると考えられた。

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