Table 4
Inter-day recoveries and precisions of SA in two samples.

Sample	0.13 g kg^{-1} spiked		Maximum usage level spiked	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Noodle soup	83.6	5.1	91.7	3.6
Sausage	84.6	6.9	81.3	4.7

Each recovery value represents the mean of analysis in duplicate on five different days. RSD, relative standard deviation.

Table 5
Comparison of SA contents in commercial foods determined by two methods.

Sample	Proposed method (solvent extraction/qHNMR)		Conventional method (steam distillation/HPLC)	
	Content (g kg^{-1})	RSD (%)	Content (g kg^{-1})	RSD (%)
Cheese	0.25	5.5	0.27	4.3
Fish paste	1.46	2.9	1.42	3.1
Sausage	0.68	3.4	0.75	1.2
Dried cuttlefish	0.72	1.4	0.62	0.5
Syrup	0.66	1.6	0.66	0.9
Jam	0.62	2.8	0.59	2.8

Each value represents the mean of three independent experiments. RSD, relative standard deviation.

processed foods. In dried cuttlefish, the SA content determined by the proposed method (0.72 g kg^{-1}) was higher than that measured using the conventional method (0.62 g kg^{-1}). To understand this discrepancy, we examined the recovery experiment at a concentration level of 0.75 g kg^{-1} potassium sorbate. The recoveries were 94.5% for our method ($n = 3$) and 84.8% for the conventional method ($n = 2$), which could have been responsible for the observed difference in SA content. Moreover, the higher recovery for the proposed method indicates that this method is more accurate for the quantitative analysis of SA in dried cuttlefish than the conventional method.

4. Conclusion

Here an analytical method using a combination of solvent extraction and qHNMR analysis was applied and validated to determine SA levels in processed foods and we proved that the proposed method is useful for quantification of SA. This is the first report of the successful quantification of SA in processed foods using qHNMR. The proposed method has good accuracy, precision, selectiveness, and linearity in the assessed concentration range. In addition, it is an absolute quantification method with SI-traceability. The conventional method using steam distillation and HPLC require a long time for sample preparation (65 min), HPLC analysis including analysis of standard for creating calibration curve (50 min), and creation of calibration curve and determination of SA content (10 min). While, the proposed method takes 55 min (sample preparation: 35 min; qHNMR analysis: 15 min; data processing and determination of SA content: 5 min). In addition, the proposed method did not require multi-step purifications in pretreatment and a calibration curve to quantify SA in processed foods. Therefore, the proposed method is more rapid and simple than the conventional method. The LOQ was less than 12.5% of the maximum usage levels of all food regulated in Japan and by the Codex General Standard for Food Additives and also low enough for the purposes of monitoring SA. Therefore, the proposed method is applicable to the monitoring of SA in processed foods at the inspection center, regulatory laboratory, and quarantine stages.

Consequently, we consider that the proposed method is enabled to be an important and reliable alternative to the conventional method for the quantification of food additives in processed foods. In order to obtain the accurate data, different proton signals of SA in different processed foods should be selected to determine SA level, because the spectrum patterns are different depending on the processed food.

The proposed method could be applied to the identification and quantification of SA in samples with complex matrices, such as beverages, fruits, pharmaceutical formulations, and cosmetic products, as well as processed foods. Moreover, the results of the present study will aid in the development of future research, in fields such as food chemistry, pharmacognosy, natural product chemistry, and pharmacology, into the absolute quantification of main components or impurities in mixtures.

Acknowledgment

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Regular article

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Determination of cyanogenic glycoside linamarin in cassava flour using liquid chromatography-tandem mass spectrometry

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Abstract

A specific and reliable method was developed for determining the presence of linamarin, a cyanogenic glucoside in cassava flour and cassava starch, using liquid chromatography-electrospray ionization tandem mass spectrometry. Linamarin was extracted with acetonitrile and then purified by solid-phase clean-up using an NH_2 cartridge column. Isocratic HPLC was used to introduce samples for electrospray negative ionization tandem mass spectrometry. The multiple reaction monitoring (MRM) was performed using a characteristic fragmentation (*m/z* 246.1 → *m/z* 161.0) for linamarin. Calibration with a standard solution was linear over a working range of 0.001-0.1 ppm (r^2=0.995-0.999), which is equivalent to 0.18-18 μg/g in food samples. The mean recovery of linamarin from cassava flour was approximately 92-100%. The detection limits of the proposed method of linamarin in cassava flour and tapioca samples were 0.75 μg/g and 0.84 μg/g, respectively.

Keywords : cyanogenic glycoside, linamarin, cassava, tapiok, liquid chromatography-tandem mass spectrometry

I Introduction

Linamarin (phaseolunatin or acetone cyanohydrin-β-D-glucoside) (Fig 1.) is one of the major cyanogenic glucosides and toxic components found in many plants and is especially present in *Manihot utilissima*, also called cassava.[1, 2] The starch of cassava is consumed as a foodstuff worldwide. Owing to the toxic nature of linamarin, analytical methods have been developed for specifically monitoring linamarin as a means of regulation worldwide. Conventional analytical methods for linamarin detection based on its cyanide structure have used spectrophotometry, following endogenous enzymatic hydrolysis by β-glucosidase.[3-7] However, these methods involve time-consuming clean-up steps, making them inapplicable for processed foods such as cassava flour, as endogenous β-glucosidase could be removed or denatured during processing. Some studies have also analyzed linamarin as a cyanogenic glucoside using the post column high-performance liquid chromatography[8] and gas chromatography-flame ionization detector.[9, 10] However, as pretreatment for these methods is complicated, a simpler method is needed. In fact, quantitative and qualitative confirmation of linamarin contamination using reliable methods is necessary for regulation by governmental

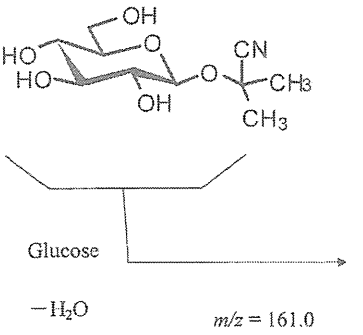

Fig. 1. Chemical structure and product ion of linamarin

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ordinances to ensure food safety.[11] In this study, we developed a method for the determination of linamarin as a cyanogenic glycoside based on liquid chromatography with tandem mass spectrometry (LC/MS/MS), and examined its applicability in determining the presence of linamarin in cassava flour and cassava starch. This study shows the development of a rapid and specific method for linamarin determination.

Ⅱ Materials and methods

1. Plant Samples

Cassava flour and cassava starch were obtained through the Ministry of Health, Labour and Welfare of Japan. All of the cassava flour samples were freeze-dried powder for 2 days using a freeze dryer (FD-81; Eyela, Tokyo, Japan).

2. Chemicals

Linamarin (Toronto Research Chemicals, Inc., Ontario, Canada) and all other chemicals and solvents were of high-performance liquid chromatography reagent grade (Kanto Chemicals, Tokyo, Japan).

3. Instrumentation

The LC system consisted of an Agilent Technologies HP 1100 series with a degasser, binary pump column oven, and autosampler (Agilent Technologies, Waldbroom, Germany). The MS detection system comprised an API 3000 triple quadrupole mass spectrometer (Applied Biosystems-MDS Sciex, Concord, Canada) equipped with a TurboIonSpray interface. The analytical data were processed by ANALYST software (version 1.4.1, Applied Biosystems-MDS Sciex).

4. LC/MS/MS conditions

Chromatographic separation of samples was performed in isocratic mode with a CAPCELL PAK AQ column (2.1 × 250 mm, Shiseido, Yokohama, Japan). The mobile phase consisted of 0.01% acetic acid : methanol (70:30 by vol.). The flow-rate was set at 0.2 ml/min. The injection volume was 10 μl and the run time was 40 min. Linamarin was eluted in approximately 5.5 min and then the column was washed completely using 0.01% acetic acid:methanol (10:90 by vol.) for approximately 15 min and equilibrated with the mobile phase for the rest of time. The turbo ion spray interface was operated in the negative ion mode at 5.4 kV and 400°C and supplied by an auxiliary gas flow of 4.0 l/min. The nebulizer gas flow was set at 1.49 l/min, the curtain gas flow at 1.25 l/min, and the collision gas at 5.2×10^{-5} hPa. Nitrogen gas was used in all cases. Samples were quantified by multiple reaction monitoring (MRM) of the deprotonated precursor ion and the related product ion.

5. Sample preparation

Cassava and tapioca samples (0.25 g) were shaken with 15 ml acetonitrile for 20 min at room temperature in a reciprocal shaker. The extracts were then centrifuged at 470 × g for 5 min and the supernatant was dried by evaporation at 40°C. The resulting residue was dissolved in 3 ml of acetonitrile-methanol (4:1, v:v) as a solvent and filtrated through a 0.45 μm filter (SYRINGE FILTER, 0.45 μm, GE Healthcare, Piscataway, NJ, USA) to make a sample solution. Bond Elut NH_2 cartridges (500 mg/3 ml; Varian, Palo Alto, CA, USA) were conditioned with the same solvent described above, loaded with the sample solution (1 ml), and then eluted with additional solvent. The first 3 ml elution was discarded, and the following 6 ml elution was collected. The collected elution was diluted tenfold with 0.01% acetic acid-methanol (9:1 by vol.). The final solutions (10 μl) were injected into the LC/MS/MS system. Linamarin was quantified using a linear calibration function that was established using the linamarin standard at concentrations of 0.001, 0.005, 0.01, 0.05, and 0.1 μg/ml (r^2=0.995-0.999) as standard solution, which is equivalent to 0.18-18 μg/g in food samples, because of 180-fold dilution during sample preparation.

Ⅲ Results

1. LC/MS/MS optimization

Mass spectrometry data were acquired in negative full-scan mode using the linamarin standard in continuous infusion. The deprotonated ion was *m/z* 246.1 $[M-H]^-$. The major product ions, *m/z* 161.0 $[glucose-H_2O]^-$ and *m/z* 187.6 [not determined] were monitored by successive MRM analysis. The product ion mass spectrum of the $[M-H]^-$ precursor ion is shown in Fig. 2. We considered that *m/z* 161.0, which was thought to correspond to glucose lacking H_2O, would be better than *m/z* 187.9 in terms of sensitivity. Acquisition in positive mode was also attempted, but a tenfold less sensitivity for the MS signal of the $[M-H]^-$ ion was observed compared to that obtained for the $[M-H]^-$ ion (data not shown). For optimization of chromatographic conditions, to achieve symmetrical peaks and a short analysis time, the mobile phase consisting of methanol and aqueous solution was used with the CAPCELL PAK AQ column. Although we also examined with normal phase columns, such as amide-type column and amino-type column, the reversed phase column (CAPCELL PAK AQ column) gave the best results in terms of the retention time and the separation.

2. Specificity

Generally, the LC/MS/MS method has high specificity since only ions derived from analytes are monitored. MRM

chromatograms of linamarin for a linamarin-free cassava flour sample and cassava flour samples spiked with 10 μg/g linamarin are shown in Fig. 3. No significant peaks interfering with the peak for linamarin were observed in the linamarin-free cassava flour and tapioca. The samples were diluted 180-fold during preparation as described previously, and it enabled to minimize the effect of contamination of foods, and to be the composition of the solvent almost same as the mobile phase.

3. Recovery and validation

The calibration curve of the linamarin standard solusion was shown in Fig. 4. The correlation coefficients for the standard curves in standard solution ranged from 0.995 to 0.999. Recovery of linamarin from cassava flour spiked at 10 and 100 μg/g linamarin, which was equivalent to approximately 0.056 and 0.56 μg/g in standard solusion, was examined. The mean recovery ranged from 92% to 100% for cassava flour and 100% to 105% for tapioca samples (Table 1).

Intra- and inter-day accuracy and precision for 10 and 100 μg/g linamarin determination in cassava flour (Table 2 A) and tapioca (Table 2 B) samples were also determined. Intra-day precision was obtained by measuring three replicate samples, which were fortified with 10 or 100 μg/g of the linamarin standard, on the same day. Inter-day precision was estimated by measuring three replicate samples on

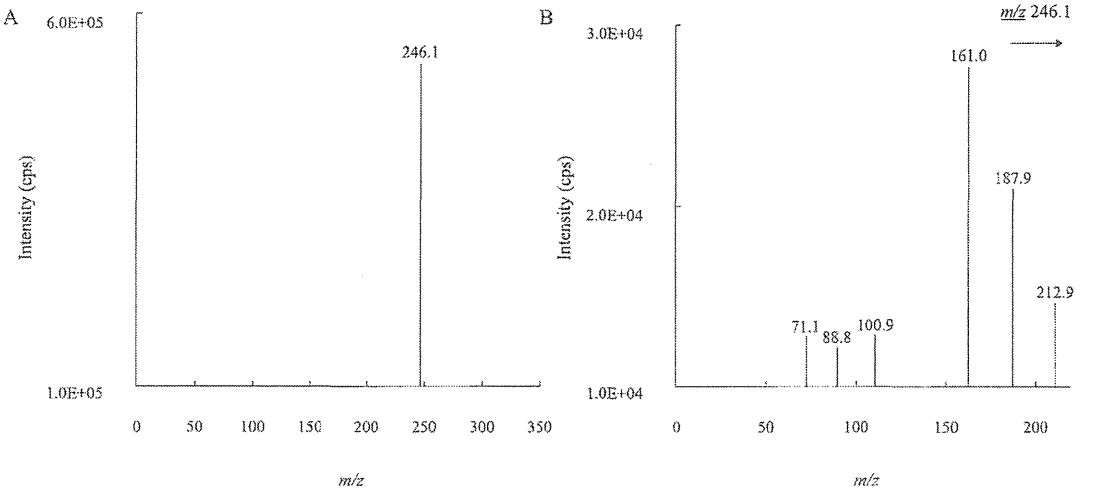

Fig. 2. Full-scan spectrum (A) and product ion mass spectra (B) of linamarin

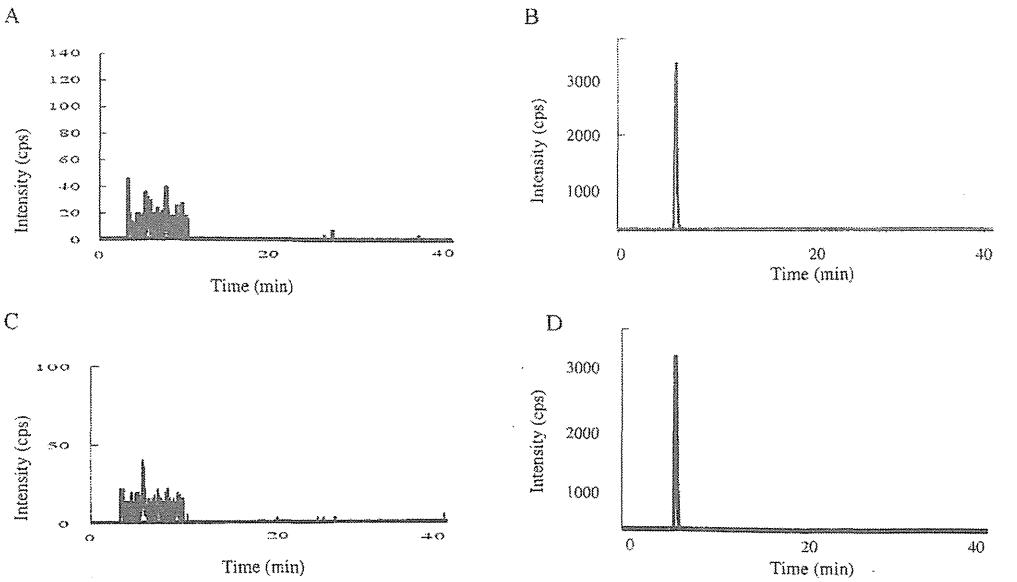

Fig. 3. Representative MRM chromatograms for (A) control cassava flour, (B) cassava flour spiked with 10 μg/g linamarin, (C) control tapioca, and (D) tapioca spiked with 10 μg/g linamarin

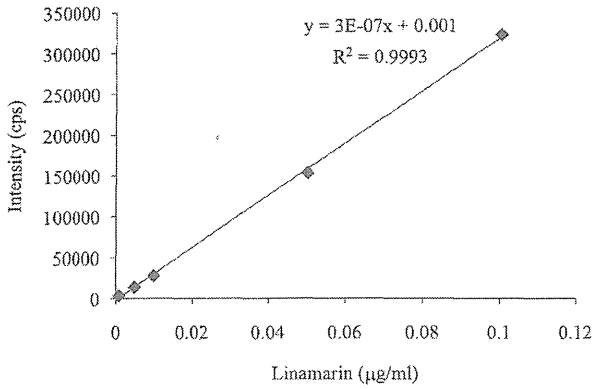

Fig. 4. Calibration curve of the linamarin standard solution

Table 1. Recovery of linamarin in cassava and in tapioca samples (n=6)

	Nominal concentration (μg/g)	Recovery (%)	R.S.D. (%)
Cassava flour	10	92.4	11.0
	100	100.0	5.1
Tapioca	10	105.3	11.9
	100	100.0	1.9

R.S.D., relative standard deviation

Table 2. Precision and accuracy results for linamarin in cassava flour samples (A) and tapioca samples (B)

(A) Cassava flour samples

Nominal concentration (μg/g)	Intra-day	Inter-day
10		
Mean (n=3) accuracy (%)	102.4	97.3
S.D. (%)	2.2	2.2
C.V. (%)	2.2	2.3
100		
Mean (n=3) accuracy (%)	99.9	100.4
S.D. (%)	1.1	1.1
C.V. (%)	1.1	1.0

(B) Tapioca samples

Nominal concentration (μg/g)	Intra-day	Inter-day
10		
Mean (n=3) accuracy (%)	93.9	111.8
S.D. (%)	1.5	0.7
C.V. (%)	1.5	0.6
100		
Mean (n=3) accuracy (%)	95.8	105.9
S.D. (%)	2.3	14
C.V. (%)	2.4	13

S.D., standard deviation; C.V., coefficient of variation

different days. For cassava flour and tapioca samples, intra-day precisions were 1.5% to 2.4% and 2.2% to 1.1%, respectively, while inter-day precisions were 0.6% to 13% and 2.3% to 1.0%, respectively. Only inter-day precision with 100 μg/g in cassava was between 10% and 15%, and we attributed this to the contamination of cassava. These values at all tested concentrations for both within- and between-run precisions were acceptable. The detection limit (LOD) and the quantification limit (LOQ) were examined using fortified samples based on S/N ratio of 3:1 and an S/N ratio of 10:1, respectively. The LOD of the proposed method in cassava flour and tapioca samples were 0.75 μg/g and 0.84 μg/g, respectively, while the LOQ were 2.49 μg/g and 2.79 μg/g, respectively. These results suggested that this proposed method is a sensitive and reliable way to determine the presence of linamarin in cassava flour and tapioca samples.

4. Application

Other cassava flour and tapioca samples were analyzed using LC/MS/MS to examine the applicability of the proposed method. As shown in Fig. 5A, linamarin can be detected at trace amounts (approximately 0.98 μg/g) in cassava flour, which is lower than LOQ in the proposed method. Although it can be detected in trace amounts in cassava flour, the LOQ[11] in these samples is approximately 50-fold lower than the regulated limit for cyanogens. For a tapioca sample, linamarin could be detected at less than the LOD (Fig. 5B).

Ⅳ Discussion

A specific and reliable method for the determination of linamarin in cassava flour and tapioca samples by LC/MS/MS was developed. In this study, we used the HPLC column, which is convenient for analyzing highly polar molecules[12], for retaining the linamarin, and we supposed that the proposed method is capable of determining linamarin rapidly and specifically in cassava flour and tapioca samples without matrix effects.

According to the safety regulation limit of the United Nations Food and Agriculture Organization (USDA) and World Health Organization (WHO), cyanogen levels in cassava flour should be less than 10 μg/g as cyanide equivalents.[11] This level of cyanogen is equivalent to approximately 91.5 μg/g as linamarin. Especially, the majority of cyanogen level is derived from linamarin in cassava flour.

Determination of the total cyanogen level in samples is very difficult, since the conversion from the determined cyanide level to cyanogen level is complicated because of cyanide production efficiency and the additional cyanide amount produced by endogenous enzymatic hydrolysis. Therefore, the determination of cyanogenic glycoside itself in the sample would be beneficial in confirming its presence in food samples, as well as in formulating food safety regulations. As linamarin is the major cyanide in cassava flour, we developed a specific and reliable method for the determination of linamarin as a cyanogenic glycoside. Accordingly, the rapid and specific method for quantifying linamarin could help to regulate its levels worldwide.

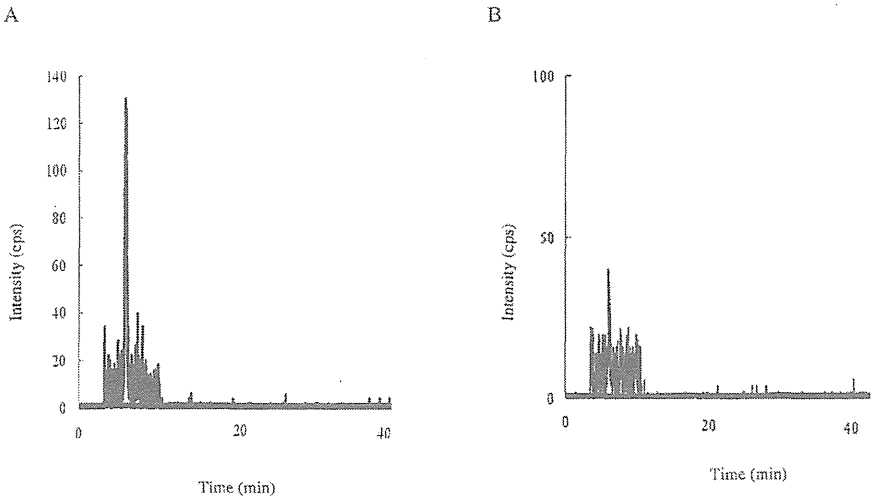

Fig. 5. Application of the determination method and detection method of linamarin in cassava flour and tapioca samples
(A) Cassava sample and (B) tapioca sample.

V Acknowledgments

Asako Watanabe-Ishitsuka and Hiroshi Akiyama contributed equally to this work. This study was supported by grants from the Ministry of Health, Labor and Welfare of Japan and from the Food Safety Commission of Japan.

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論　文

LS-MS/MS を用いたキャッサバ澱粉におけるシアン配糖体、リナマリンの定量

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キーワード：シアン配糖体、リナマリン、タピオカ、キャッサバ、LC-MS/MS

概　要

キャッサバ粉及びキャッサバ澱粉中のシアン配糖体であるリナマリンの定量について、LC-MS/MS を用いた特異的かつ信頼性のある方法を確立した。リナマリンをアセトニトリルで抽出し、その後アミノカートリッジカラムを用いて固相法により精製した。HPLC 分析はアイソクラティックで溶出し、イオン化はエレクトロスプレー法、ネガティブイオンモードで行なった。定量は MS/MS 測定で行い、プレカーサーイオン m/z 246.1、プロダクトイオン m/z 161.0 を用いた。標準溶液の検量線の直線性は 0.001-0.1 ppm (r^2=0.995-0.999) であり、サンプル中の濃度では 0.18-18 μg/g に相当する。キャッサバ粉における平均回収率は、約 92-100% であった。キャッサバ粉及びタピオカ製品中における検出限界はそれぞれ 0.75 μg/g および 0.84 μg/g であった。

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定量分析値の信頼性確保のためのqNMRを用いた市販試薬の純度決定

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Determination of the Purities of Commercial Reagent Products using qNMR for the Ensuring the Reliability of Quantitative Analysis

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Summary

In environmental analysis, the commercial reagent and reference material products of analyte compounds are indispensable for chromatography such as GC/MS and LC/MS. However, most of their purities are not certificated traceability to the International System of Units (SI). Hence the possibility that their obscure purities greatly ruin the reliability of the quantitative value is incontrovertible. In this study, the purities of forty one commercial pesticide reagent products (new or old) were determined by a quantitative analytical method which is traceable to SI using nuclear magnetic resonance (qNMR). qNMR is a rapid and simple quantitative analysis method and no reference compound of analyte is needed. The purities of ten commercial reagent products among our measured forty one products are different more than 5% to their labeled purities by the manufacturers, and the values were found in 47.9-94.8%. Therefore it consequently seems that the differences between SI traceable purities and labeled purities cause the error of 5.1-50.8% to the quantitative values of analytes. This result represents that qNMR analysis has potential to work as a bridge of SI traceability and the quality control of reagent product using qNMR is greatly important to secure the accuracy of analytical data.

Key words: quantitative NMR, standard, purity, reliability

1. はじめに

環境分析における水や大気等の時間軸上で流動的な試料は，同一の試料を再度採取することができないため，定量分析値の信頼性確保が特に重要である。現在，環境中の農薬等の有機物質の定量分析法には，分解能や微量分析能に優れた液体クロマトグラフィーやガスクロマトグラフィー等のクロマトグラフィーが広く採用されている。クロマトグラフィーは標準被検物質を用いて相対的に定量するため，測定対象化合物の標準物質が不可欠である[1,2)]。一方，物質量の絶対値は普遍的な国際計量標準である国際単位系（SI）にトレーサブルな測定によって得られると定義されているが[3,4)]，有機化合物については，この定義に従い，計量計測トレーサビリティの確保された純度が保証された標準物質として市販されているものは少ない。そのため，市販試薬が定量用標準物質の代用品として使用される。しかし，市販試薬の多くは，試薬メーカー独自の方法で調製および管理を行っており，これらの製品のラベルや成績書には，品質保証として，JIS規格に準拠したクロマトグラム上に観察される主成分のピーク面積百分率，あるいは吸光光度法による比吸光度等よ

り算出したものを「純度%」と示している場合が多い。ピーク面積百分率は，クロマトグラム上に観察されるすべての成分のピーク面積の総和に対する測定対象化合物のピーク面積を比率として表したものであり[1,2]，元素組成比の異なる不純物を含む混合物の純度は原理的に精確な測定が困難である。また，吸光光度法では，化合物の吸光係数が正確であることを大前提としているが，純度100 %の上位標準となる化合物が存在しなければ，精確な純度値を求めることが不可能と思われる。したがって，SIへの計量計測トレーサビリティが確保された標準物質が定量分析に用いられていない以上，得られた定量分析値は厳格なトレーサビリティの連鎖から外れ，試薬の純度値の誤差が定量分析値の信頼性を大きく損なっている可能性を否定できない。

実際に，我々は，純度が計量計測トレーサビリティの確保されていない市販試薬を定量分析に用いた場合，得られた定量分析値に非常に大きなばらつきがあり，結果として，メーカー間にある純度の差が定量分析値の不確かさを大きくし，室間精度に影響を与えていることを報告している[5]。また近年，各国の標準物質の同等性，化学計測の国際的整合性が議論されるようになり，得られた定量分析値について，計量計測トレーサビリティの欠如が問題視されるようになってきた。国際標準化機構(ISO)発行のISOガイドにおいても，測定値および分析結果の精確さの問題についての勧告があり，分析手順や校正手順，適切な認証標準物質の選択，トレーサビリティの確保等，トレーサビリティを実現し，精確な特性値を得ることを含んだ標準物質に関する指針が示されている[6-11]。このような背景から，環境分析の分野における分析精度の更なる向上のために，有機化合物の純度を計量学的に精確に測定する方法が早急に必要とされている。

我々は，これまでに定量分析値の高精度化を目指し，SIに基づく計量計測トレーサビリティが確保された新たな定量法として，一次標準測定法の資格を原理的に有する核磁気共鳴装置（NMR）を用いた定量分析法（quantitative NMR: qNMR）を開発してきた[12-19]。本研究では，qNMRの測定対象として，過去の全国水道事業体の農薬実態調査において，浄水での検出率や個別評価値が高い農薬等を30種選択し，標準物質として使用される可能性の高いこれら市販試薬の純度を測定し，同製品のラベルや成績書に記載されている純度値が分析精度に及ぼす影響について検討した。

2. 方法

2. 1 対象化合物

Table 1に測定に供した市販試薬30種41製品を示した。その化合物名，化学構造による分類，分子式，分子量，試薬メーカーを示す。

2. 2 試薬および試液

高純度hexadimethyldisilane（HMD）は和光純薬工業株式会社特注品，重溶媒はacetone-d_6（99.9 atom % D）およびmethanol-d_4（99.8 atom % D）（Isotec），diethyl phthalate（DEP）は，独立行政法人 産業技術総合研究所の認証標準物質（NMIJ CRM 4022-b：純度99.98 ± 0.01 w/w%（99.74 ± 0.09 mol/mol %））を用いた。

2. 3 装置

ウルトラミクロ天秤はXP2 U（メトラートレド株式会社）を使用した。試料の秤量値は，最小目盛0.0001 mgまで読み取った値を用いた。

核磁気共鳴装置（NMR）はオートサンプラー付きJNM-ECA（600 MHz，日本電子株式会社（現：株式会社JEOL RESONANCE））を使用した。qNMRのケミカルシフト値は，HMDを基準シグナル（0 ppm）とし，δ値をppm単位で表した。NMR測定条件はTable 2に示す。

なお，qNMR用標準液および試料溶液の調製には，化学用体積計（50，100 mLメスフラスコ）または電動オートピペッター（マルチピペットXstream（エッペンドルフ製），10 mL（不確かさ ± 0.4 %），3 ～ 5 mL（不確かさ ± 0.5 %））を用いた。

2. 4 qNMR用標準液の調製およびHMDの濃度校正

HMD約20 mgを精密に量り取り，acetone-d_6 100 mLに定容した。もしくは，HMD約100 mgを精密に量り取り，methanol-d_4 50 mLに定容し，この溶液をmethanol-d_4で25倍希釈した。これらHMDのaceione-d_6溶液またはmethanol-d_4溶液をqNMR用標準液とした。qNMR用標準液中のHMDの濃度を下記に従い，DEPを内標準物質として校正して求めた。すなわち，CRMであるDEP約5 -10 mgを精密に量り取り，qNMR用標準液1.0 mLに溶解した。この溶液0.6 mLをNMR試験管（5 mm ϕ × 200 mm，日本電子株式会社）に封入したものをHMD濃度校正用試料溶液とした。この溶液をqNMRに付し，DEPのCH_2 × 2およびHMDのCH_3 × 6に由来するシグナル面積，分子量，濃度等を式（1）に代入し，qNMR用標準液中のHMDの濃度を校正した。

$$W_{HMD} = \frac{M_{HMD} \times I_{HMD}}{H_{HMD}} / \frac{M_{DEP} \times I_{DEP}}{H_{DEP} \times W_{DEP}} \times \frac{P_{DEP}}{100} \quad (1)$$

ただし，W_{HMD}, W_{DEP} = HMDおよびDEPの濃度（mg/mL），M_{HMD}, M_{DEP} = HMDおよびDEPの分子量（146.3781および222.2337，分子量はIUPAC 2007年発表の原子量表[20]を用いて算出），H_{HMD}, H_{DEP} = HMDのCH_3 × 6およびDEPのCH_2 × 2のプロトン数，I_{HMD}, I_{DEP} = HMDのCH_3 × 6およびDEPのCH_2 × 2のシグナル面積，P_{DEP} = DEPの純度（99.98 w/w%）を示す。

2. 5 qNMRによる市販試薬の純度測定

市販試薬を約5 -10 mg精密に量り取り，予め調製したqNMR用標準液1.0 mLに溶解した。qNMR用標準液として，benzofenapおよびdalaponはmethanol-d_4溶液，その他はacetone-d_6溶液を使用した。これらの溶液0.6 mLをNMR試験管に封入したものを試料溶液とした。この試料溶液をqNMRに付し，HMDのシグナル強度面積，化合物に由来するそれぞれの特定シグナルの相対面積，分子量，濃度等を式（2）に代入し，農薬等の対象化合物の純度を算出した。

$$P_{target} = \frac{I_{target} / H_{target}}{I_{HMD} / H_{HMD}} \times \frac{M_{target} / W_{target}}{M_{HMD} / W_{HMD}} \times 100 \quad (2)$$

ただし，W_{HMD}, W_{target} = HMDおよび対象化合物の濃度（mg/mL），M_{HMD}, M_{target} = HMDおよび対象化合物の分子量[20]（146.3781およびTable 1），I_{HMD}, I_{target} = HMDおよび対象化合物の特定基のシグナル強度面積，H_{HMD}, H_{target} = HMDおよび対象化合物の特定基のプロトン数，P_{target} =対象化合物の純度（%）を示す。

2. 6 qNMR測定条件および解析処理

市販試薬41製品の純度は，既報のqNMR[16-19]により計量学的に精確に決定した。qNMRのケミカルシフト値は，HMDを基準シグナル（0 ppm）とし，δ値をppm単位で表した。qNMRデータ解析は，得られたFree Induction Decay（FID）信号データを定量解析ソフトウェア（Alice2 for qNMR，日本電子株式会社（現：株式会社JEOL RESONANCE））に導入して自動処理した。すなわち，

Table 1 Information of commercial reagent and reference material products

Compound	Class [a]	Formula	Molecular mass	Manufacturer [b]	Sample No.
Acetamiprid	NN	$C_{10}H_{11}ClN_4$	222.67414	Wako	1
Benzofenap	PZ	$C_{22}H_{20}Cl_2N_2O_3$	431.31180	Hayashi	2
Bromobutide	AA	$C_{15}H_{22}BrNO$	312.24528	Wako	3
Butamifos	OP	$C_{13}H_{21}N_2O_4PS$	332.35560	Wako, Kanto, Hayashi	4, 5, 6
Cafenstrole	AA	$C_{16}H_{22}N_4O_3S$	340.43588	Wako	7
Carbofuran	CM	$C_{12}H_{15}NO_3$	221.25240	Sigma	8
Dalapon	FA	$C_3H_4Cl_2O_2$	142.96866	Kanto	9
Dichlorvos (DDVP)	OP	$C_4H_7Cl_2O_4P$	220.97574	Wako	10, 11
Disulfoton	OP	$C_8H_{19}O_2PS_3$	274.40402	Wako, Kanto, Sigma	12, 13, 14
Diuron	U	$C_9H_{10}Cl_2N_2O$	233.09450	Wako	15
Fenobucarb	CM	$C_{12}H_{17}NO_2$	207.26888	Wako	16
Fipronil	PZ	$C_{12}H_4Cl_2F_6N_4OS$	437.14778	Wako	17
Fipronil sulfone	PZ	$C_{12}H_4Cl_2F_6N_4O_2S$	453.14718	Wako	18
Flutolanil	AA	$C_{17}H_{16}F_3NO_2$	323.30965	Wako	19
Iprobenfos (IBP)	OP	$C_{13}H_{21}O_3PS$	288.34280	Wako	20
Imidacloprid	NN	$C_9H_{10}ClN_5O_2$	255.66100	Wako	21
Isoxathion	OP	$C_{13}H_{16}NO_4PS$	313.30920	Wako, Kanto, Hayashi	22, 23, 24
MCPA	PA	$C_9H_9ClO_3$	200.61896	GL Sciences	25
Mecoprop	PA	$C_{10}H_{11}ClO_3$	214.64554	GL Sciences	26
Mefenaset	AA	$C_{16}H_{14}N_2O_2S$	298.35956	Wako	27
Fenthion (MPP)	OP	$C_{10}H_{15}O_3PS_2$	278.32806	Wako	28
MPP sulfoxide	OP	$C_{10}H_{15}O_4PS_2$	294.32746	Wako	29
MPP sulfone	OP	$C_{10}H_{15}O_5PS_2$	310.32686	Wako	30
MPP oxon	OP	$C_{10}H_{15}O_4PS$	262.26246	Wako	31
MPP oxon sulfoxide	OP	$C_{10}H_{15}O_5PS$	278.26186	Wako	32
MPP oxon sulfone	OP	$C_{10}H_{15}O_6PS$	294.26126	Wako	33
Pirimiphos-methyl	OP	$C_{11}H_{20}N_3O_3PS$	305.33356	Wako	34
Pretilachlor	AA	$C_{17}H_{26}ClNO$	311.84684	Wako, Kanto, Sigma	35, 36, 37
Pyroquilon	HF	$C_{11}H_{11}NO$	173.21114	Wako, Kanto, Sigma	38, 39, 40
Tricyclazole	HF	$C_9H_7N_3S$	189.23698	Wako	41

[a] The analyte compounds were classed by the chemical structure: neonicotinoid, NN; pyrazole, PZ; acid amido, AA; organophosphorus, OP; carbamate, CM; fatty acid, FA; urea, U; phenoxyalkanoic acid, PA; heterocyclic fungicide, HF.

[b] The compounds were purchased from five manufacturers: Wako Pure Chemical Industries, Ltd., Wako; Hayashi Pure Chemical Ind., Ltd., Hayashi; Kanto Chemical Co., Inc., Kanto; Sigma-Aldrich Inc., Sigma; GL Sciences Inc., GL Sciences.

Table 2 Instruments and acquisition parameters

Spectrometer	JNM-ECA600 (JEOL)
Probe	5 mm broadband autotune probe
^{13}C decoupling	Multi pulse decoupling with Phase and Frequency switching (MPF-8)
Spectral width	-5 ~15 ppm
Data points	64000
Auto filter	on (8 times)
Flip angle	90°
Pulse delay	60 s (>5*T_1)
Scan times	8
Sample spin	no spin
Probe temperature	22-25℃
Sample solvent	Acetone-d_6 or Methanol-d_4
qNMR reference material	HMD
Primary standard material	DEP
Window function	—

このソフトウェア上で，qNMR データをフーリエ変換および自動位相調整を行い，HMD および特定シグナルの積分範囲設定等を設定後，予め入力した HMD および対象化合物の濃度，分子量，特定基のプロトン数等の化合物情報から，純度を式 (2) に従い算出した。

3. 結果および考察

3. 1 qNMR の原理

^1H-NMR は，測定対象化合物の分子構造にかかわらず，すべての水素原子が個々に定量的な信号として観測され，スペクトル上に観察される異なる化合物のシグナル強度の比は化合物に寄与する水素のモル比に対応する特徴を有する。このことから，2 つのシグナルが異なる化合物 (A, B) に由来する場合には，個々のシグナル面積と化合物の濃度は関係式 (3) で表すことができる。言いかえれば，一方の化合物の純度が明らかで定量的に混合すれば，それぞれのシグナル面積と寄与する水素原子を勘案することで，両者のモル比の関係から測定対象の化合物の純度を決定する関係式 (4) が成り立つ。したがって，qNMR は，測定対象の化合物以外の標準物質を上位標準として定量分析値を得ることが可能であり，一次標

準測定法のうち，一次比率法すなわち「物質量の基準となる別の化学物質を用い，それとの比較において目的の化学物質の物質量を測定する方法」の資格を原理的に有する。故に本法は，国家標準物質や認証標準物質（CRM）を上位標準とすることで，SIにトレーサブルな定量分析値（純度）を得ることが可能である。

$$\frac{I_A}{I_B}=\frac{H_A m_A}{H_B m_B}=\frac{H_A W_A / M_A}{H_B W_B / M_B} \quad (3)$$

$$P_{sample}=\frac{I_{sample}/H_{sample}}{I_{std}/H_{std}}\times\frac{M_{sample}/W_{sample}}{M_{std}/W_{std}}\times P_{std} \quad (4)$$

ただし，I＝シグナル面積，H＝特定基のプロトン数，m＝モル濃度，W＝重量，M＝分子量，P＝純度（%），*sample*＝試料，*std*＝基準物質を示す。

本報告では，qNMR基準物質としてHMDを用いた。しかしながら，SIにトレーサブルな純度が証明されたHMDが流通していないことから，qNMRによる定量分析値のSIトレーサビリティの確保には，SIにトレーサブルなCRMであるDEPを一次標準として用い，qNMR標準液中のHMDの濃度を校正した後に，HMDを二次標準として測定対象化合物のqNMR測定を行う二段階方式を採用した。すなわち，HMDをqNMR基準物質として用いた際の測定対象化合物の定量分析値のSIトレーサビリティは，CRMのDEPを介して実現した（Fig. 1）。また，qNMRは1測定当たりの所要時間が約10-20分であり，得られた測定値は高い再現性を示し，測定結果の不確かさは概ね1%以内が達成されている。

3. 2　市販試薬製品の純度

市販試薬は，新品23製品，開封後冷蔵保存約2～5年の18製品の計41製品について，qNMRにより計量計測トレーサビリティの確保された純度を測定した。試薬メーカー3社から購入した農薬はそれぞれの試薬メーカーの頭文字で表した。また，同一製品の開封後冷蔵保存約5年と新品（未開封）の2製品を測定したDDVPはDDVP-old，DDVP-newと表記した。qNMRスペクトル上に観察された基準物質HMDおよび対象化合物に由来する各シグナル面積，水素数，濃度等を関係式（2）に代入し，それぞれの純度を算出した。なお，観察されたシグナルのうち，OHやNH基由来のプロトンは重水素置換が考えられるため，定量には用いなかった。また，低分子化合物のNMRスペクトルは，低磁場側より高磁場側のシグナルに予想される不純物のシグナルが重なる危険性が高い。qNMRの定量用シグナルは，不純物のシグナルと十分に分離していることが理想であるため，明らかに不純物のシグナルと重なっているシグナルを除外し，それぞれのシグナルより算出された平均値を対象化合物の純度とした。定量に用いたシグナルと算出された純度をTable 3に示す。qNMRを用いて得られた純度（± n = 3 RSD %）は47.9 ± 2.5～100.9 ± 0.2 %であった。純度の低い製品については，NMRスペクトル上の化合物と基準物質のHMDに由来するシグナル以外に不純物のシグナルが観察された。DDVP-oldに関しては，各シグナルから得られた純度のRSDが15.0 %と大きく，不純物を含んでいる可能性が高いと考えられた。従ってDDVP-oldは，すべてのシグナルによる定量値を用いず，最も低い定量値47.9 %を純度値とした。

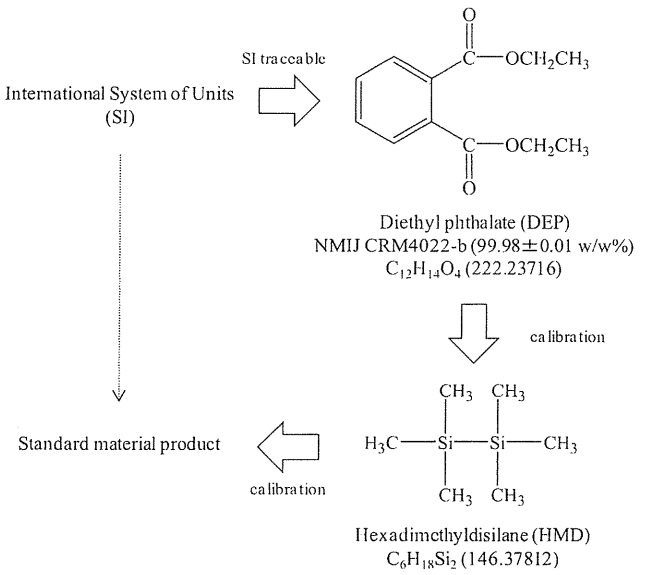

Fig. 1　Strategy of building up SI traceability of qNMR analysis
The concentration of HMD in qNMR solvent was corrected by DEP.

3. 3　純度が定量分析値の精度に及ぼす影響

対象化合物の計量計測トレーサビリティの確保された純度と成績書記載の純度値と比較した（Table 4）。その結果，市販試薬製品に記載の面積百分率による純度値は，95.3～100 %であり，41製品のうち73.2 %に相当する30製品（新品16製品，開封済み14製品）のqNMRによる純度は95 %以上で，それぞれの成績書記載の純度値と総じてほぼ等しい値を示した（Fig. 2）。一方，41製品のうち，新品のbutamifos-W, K, H，pretilachlor-W, K, Sの6製品，開封済み製品のacetamiprid，dalapon，DDVP-old，fipronilの4製品計10製品はqNMRによる純度と成績書記載の純度値との差が5 %以上あった。これらの10製品について，qNMRによる純度と成績書記載の純度値との比較をFig. 3に示す。開封済み製品では5.1～50.8 %，新品では5.2～22.8 %の差があった。

現状では，市販試薬製品の純度値は精確に値付けされていると前提して，定量用標準物質として使用することが多い。仮に市販試薬製品の成績書記載の純度値を信用してクロマトグラフィーにより定量分析を行ったとすると，qNMRによる純度と成績書記載の純度値に差があった10製品の得られる定量分析値は真値より5.1～50.8 %の誤差を生じることになると言える。

3. 4　純度評価の問題点

新品のpretilachlor 3製品はqNMRによる純度が76.9～77.1 %となり，添付の成績書に記載の面積百分率による純度値97.7～99.8 %と大きな開きがあった。得られたNMRスペクトルをFig. 4に示す。pretilachlorに由来するシグナルと基準物質のHMDに由来するシグナル以外に，分解物または製造原料に由来すると思われるシグナル（Fig. 4中*印のシグナル：0.89，1.12，1.55，3.38，3.78，4.55，7.12，7.20 ppm）が観察され，明らかに純度が低いことが予想された。この市販試薬製品をガスクロマトグラフ／質量分析計（GC/MS）により測定し，不純物のピークを観察した結果，クロマトグラム上にはpretilachlor以外のピークは検出されなかった（データ未収載）。つまり，qNMRスペクトル上には不純物のシグナルが明瞭に観察されていることを考慮すると，添付の成績書に記載されているクロマトグラフィーから得られた純度値を質量%純度として扱うことは不適切であると考えられる。

クロマトグラフィーによる面積百分率を用いて値付けられた試薬メーカーの成績書記載の純度値が，あらゆる不純物のレスポンス

Table 3 Calculated purities of samples from listed proton signals
The signals of OH, NH and overlapped protons with the impurities were not used for the quantification.

Sample No.	Compound	Signal	1	2	3	4	5	6	7	8	9	Average (%, RSD%)
		a)	3H,s	3H,s	2H,s	1H,d						
		b)	2.45	3.20	4.79	7.40	—	—	—	—	—	
1	Acetamiprid	c)	74.6	75.1	75.0	74.8						74.9 (0.3)
			3H,s	6H,2s	3H,s	2H,s	1H,d	2H,d	1H,d	2H,d		
			1.81	2.35	3.72	5.64	7.08	7.27	7.34	7.71	—	
2	Benzofenap		99.8	99.0	99.3	94.1	103.7	99.8	100.5	97.5		99.2 (2.7)
			9H,s	3H,s	3H,s	1H,s	1H,t	2H,t	2H,d			
			1.06	1.56	1.63	4.29	7.13	7.24	7.37	—	—	
3	Bromobutide		99.7	100.0	99.5	99.4	101.0	99.9	100.4			100.0 (0.6)
			1H,d	1H,s	1H,d							
			7.09	7.42	7.74	—	—	—	—	—	—	
4	Butamifos-W		94.4	95.0	94.7							94.7 (0.3)
5	Butamifos-K		90.1	90.4	90.5							90.3 (0.2)
6	Butamifos-H		94.5	94.9	94.9							94.8 (0.2)
			6H,s	3H,s	6H,s	4H,q	2H,s	1H,s				
			1.15	2.25	2.61	3.47	7.03	8.96	—	—	—	
7	Cafenstrole		98.5	97.7	97.9	98.2	97.9	97.4				97.9 (0.4)
			6H,s	2H,s	1H,t	1H,d	1H,d					
			1.37	2.99	6.68	6.80	6.92	—	—	—	—	
8	Carbofuran		100.2	100.3	100.8	100.4	100.1					100.3 (0.3)
			3H,s									
			2.16	—	—	—	—	—	—	—	—	
9	Dalapon		71.8									71.8 (—)
			3H,s	3H,s	1H,d							
			3.80	3.81	7.16	—	—	—	—	—	—	
10	DDVP-old		64.9	47.9	57.9							56.9 (15.0)
11	DDVP-new		96.6	93.4	93.5							94.5 (1.9)
			3H,t	6H,t	2H,q	2H,m	4H,m					
			1.18	1.28	2.55	3.04	4.11	—	—	—	—	
12	Disulfoton-W		99.6	99.9	99.9	99.4	99.7					99.7 (0.2)
13	Disulfoton-K		102.0	97.7	102.2	96.9	97.4					99.3 (2.6)
14	Disulfoton-S		100.6	99.3	100.1	98.4	99.2					99.5 (0.9)
			6H,s	1H,d	1H,d	1H,s						
			2.94	7.32	7.43	7.89	—	—	—	—	—	
15	Diuron		99.2	98.9	98.9	99.6						99.1 (0.3)
			2H,m	1H,m	2H,m	1H,m						
			1.51	7.11	7.21	6.97	—	—	—	—	—	
16	Fenobucarb		100.0	100.1	99.6	100.0						99.9 (0.2)
			2H,s									
			8.09	—	—	—	—	—	—	—	—	
17	Fipronil		92.9									92.9 (—)
			2H,s									
			8.13	—	—	—	—	—	—	—	—	
18	Fipronil sulfone		99.9									99.9 (—)
			6H,d	1H,m	1H,d	2H,m	1H,s	2H,2t	1H,t	1H,d		
			1.26	4.55	6.64	7.19	7.46	7.65	7.70	7.76	—	
19	Flutolanil		100.6	99.7	99.7	99.5	99.9	100.4	98.9	100.7		99.9 (0.6)
			12H,m	1H,s	1H,s	2H,m	1H,t	2H,t	2H,d			
			1.21	4.01	4.03	4.55	7.22	7.28	7.35	—	—	
20	IBP		99.8	100.3	98.0	100.2	99.8	99.7	98.8			99.5 (0.9)
			2H,t	2H,t	2H,s	1H,d	1H,d	1H,s				
			3.61	3.78	4.52	7.41	7.80	8.36	—	—	—	
21	Imidacloprid		99.3	99.2	98.4	98.9	99.0	99.6				99.1 (0.4)
			6H,t	4H,m	1H,s	3H,m	2H,d					
			1.33	4.28	6.82	7.50	7.83	—	—	—	—	
22	Isoxathion-W		98.7	98.5	98.0	99.2	98.0					98.5 (0.5)
23	Isoxathion-K		99.9	99.8	99.4	99.9	99.8					99.7 (0.2)
24	Isoxathion-H		98.8	98.7	98.3	99.2	98.3					98.6 (0.4)

Table 3 Continued

Sample No.	Compound	Signal 1	2	3	4	5	6	7	8	9	Average (%, RSD%)
		3H, s	2H, s	1H, d	1H, d	1H, s					
		2.18	4.71	6.83	7.08	7.12	—	—	—	—	
25	MCPA	100.1	100.2	99.2	100.4	99.1					99.8 (0.6)
		3H, d	3H, s	1H, q	1H, d	1H, d	1H, s				
		1.56	2.17	4.81	6.77	7.06	7.12	—	—	—	
26	Mecoprop	100.2	99.7	99.5	99.5	99.9	99.0				99.6 (0.4)
		3H, s	2H, s	1H, t	1H, t	5H, m	1H, d	1H, d			
		3.20	4.87	7.21	7.40	7.45	7.57	7.75	—	—	
27	Mefenaset	95.3	90.1	101.9	107.3	95.5	106.7	100.7			99.6 (6.4)
		3H, s	3H, s	3H, s	3H, s	2H, s+d	1H, d				
		2.24	2.42	3.79	3.80	6.98	7.17	—	—	—	
28	MPP	101.1	100.9	100.4	99.3	100.1	100.5				100.4 (0.6)
		3H, s	3H, s	3H, s	3H, s	1H, s	1H, d	1H, d			
		2.34	2.61	3.81	3.84	7.06	7.25	7.82	—	—	
29	MPP sulfoxide	100.8	100.7	100.1	98.8	100.2	99.8	99.8			100.0 (0.6)
		3H, s	3H, s	3H, s	3H, s	2H, s+d	1H, d				
		2.65	3.11	3.83	3.85	7.22	7.95	—	—	—	
30	MPP sulfone	99.3	99.4	99.8	98.9	99.3	99.8				99.4 (0.3)
		3H, s	3H, s	3H, s	3H, s	2H, s+d	1H, d				
		2.24	2.41	3.76	3.78	7.04	7.17	—	—	—	
31	MPP oxon	97.7	97.3	98.5	96.7	97.3	97.6				97.5 (0.6)
		3H, s	3H, s	3H, s	3H, s	1H, s	1H, d	1H, d			
		2.34	2.61	3.80	3.82	7.11	7.24	7.81	—	—	
32	MPP oxon sulfoxide	99.4	99.9	98.6	97.5	98.2	98.0	97.9			98.5 (0.9)
		3H, s	3H, s	3H, s	3H, s	2H, s+d	1H, d				
		2.65	3.10	3.82	3.84	7.26	7.95	—	—	—	
33	MPP oxon sulfone	99.6	99.8	97.7	96.9	99.0	99.6				98.8 (1.2)
		6H, t	3H, s	4H, s	3H, s	3H, s	1H, s				
		1.11	2.23	3.55	3.85	3.85	6.02	—	—	—	
34	Pirimiphos-methyl	99.2	99.6	97.2	99.8	99.7	98.7				99.0 (1.0)
		3H, t	6H, t	2H, m	2H, t	2H, t	4H, s+t	2H, d	1H, t		
		0.79	1.20	1.42	3.25	3.51	3.69	7.24	7.32	—	
35	Pretilachlor-W	79.1	79.5	73.4	79.2	79.1	79.0	78.5	78.3		77.1 (0.4)
36	Pretilachlor-K	78.6	80.2	71.6	79.3	79.9	78.7	78.2	79.8		77.0 (1.2)
37	Pretilachlor-S	78.9	79.6	72.8	78.9	78.9	78.4	78.1	78.3		76.9 (0.3)
		2H, t	2H, t	2H, t	2H, t	1H, t	1H, d	1H, d			
		2.49	2.89	3.11	3.92	6.82	6.93	7.01	—	—	
38	Pyroquilon-W	101.1	100.8	100.7	100.8	101.0	100.9	100.9			100.9 (0.1)
39	Pyroquilon-K	100.6	100.2	100.3	100.2	100.4	100.3	100.3			100.3 (0.1)
40	Pyroquilon-S	101.1	100.8	100.8	100.8	101.0	100.9	100.8			100.9 (0.1)
		2H, m	1H, d	1H, s							
		7.36	7.65	9.27	—	—	—	—	—	—	
41	Tricyclazole	99.6	99.8	98.8							99.4 (0.5)

a) Upper column shows a number of proton with the spin-spin coupling (s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet).
b) Middle column shows the signal region (ppm).
c) Lower column shows the purity of each signal (%).

ファクターが主成分と同じであると仮定して主成分の相対比を示すもので，絶対量を示しているわけではないことは明らかであるが，問題視されることはなかった。今回，qNMR を用いた純度測定により，市販試薬製品に記載の純度値が絶対量と異なることがあり得ることが証明された。なお，qNMR による純度試験に供した化合物は概ね 1 製品 1 ロットについてであり，市販試薬製品の純度について正確な情報を収集するためには，ロット間差についても今後検討する必要があると考えられる。

3. 5 開封済み製品の品質管理

市販試薬製品の純度の誤差が定量分析値の精度に及ぼす要因として，開封済の製品における品質管理について着目した。

DDVP は未開封の新品 DDVP-new の qNMR による純度が 94.5 % であるのに対し，開封後冷蔵保存約 5 年の製品 DDVP-old は 47.9 % であった。DDVP は熱には安定であるとされているが，長期保存により加水分解や酸化による純度値の低下が生じたと考えられた。化合物の安定性は化合物毎に異なり，保存期間や保存状態によっては

Table 4 Summary of commercial reagent products purities calculated by qNMR and labeled percentage of peak area on chromatogram by manufacturer

Sample No.	Compound		Purity (%) qNMR (n=3 average, RSD %)		Manufacturer[a]	Sample No.	Compound		Purity (%) qNMR (n=3 average, RSD %)		Manufacturer[a]
1	Acetamiprid	*	74.9	0.4	98.8 (GC/FID)	21	Imidacloprid	*	99.1	0.6	100 (LC/UV)
2	Benzofenap		99.2	1.9	99.6	22	Isoxathion-W		98.5	0.5	98.9 (GC/FID)
3	Bromobutide	*	100.0	0.2	—[b]	23	Isoxathion-K		99.7	0.3	99.1 (GC/FID)
4	Butamifos-W		94.7	0.8	99.8 (GC/FID)	24	Isoxathion-H		98.6	0.1	99.9 (GC/FID)
5	Butamifos-K		90.3	0.6	99.2 (GC/FID)	25	MCPA	*	99.8	0.2	—[b]
6	Butamifos-H		94.8	0.7	99.9 (GC/FID)	26	Mecoprop		99.6	0.7	—[b]
7	Cafenstrole	*	97.9	0.8	99.8 (HPLC/UV)	27	Mefenaset	*	99.6	0.2	99.9 (GC/FID)
8	Carbofuran		100.3	0.4	99.9 (HPLC)	28	MPP	*	100.4	0.4	99.9 (GC/FID)
9	Dalapon	*	71.8	0.8	99 (GC/FID)	29	MPP sulfoxide		100.0	0.3	99.8 (LC/UV)
10	DDVP-old	*	47.9	2.5	98.7 (GC/FID)	30	MPP sulfone	*	99.4	0.4	99.3 (GC/FID)
11	DDVP-new		94.5	0.4	99 (GC/FID)	31	MPP oxon	*	97.5	0.3	100 (GC/FID)
12	Disulfoton-W		99.7	0.4	98.6 (GC/FID)	32	MPP oxon sulfoxide		98.5	0.5	99.4 (GC/FID)
13	Disulfoton-K		99.3	0.2	95.3 (GC/MS, LC/DAD)	33	MPP oxon sulfone		98.8	0.5	99.8 (GC/FID)
						34	Pirimiphos-methyl		99.0	0.1	98.9 (GC/FID)
14	Disulfoton-S		99.5	0.3	98.6 (HPLC/UV)	35	Pretilachlor-W		77.1	0.5	99.2 (GC/FID)
15	Diuron	*	99.1	0.01	100 (LC/UV)	36	Pretilachlor-K		77.0	1.6	99.8 (GC/FID)
16	Fenobucarb	*	99.9	0.5	—[b]	37	Pretilachlor-S		76.9	0.4	97.7 (HPLC/UV)
17	Fipronil	*	92.9	0.2	98.1 (GC/FID)	38	Pyroquilon-W		100.9	0.2	100 (GC/FID)
18	Fipronil sulfone	*	99.9	0.4	—[b]	39	Pyroquilon-K		100.3	1.1	99 (HPLC/DAD)
19	Flutolanil	*	99.9	0.7	100 (GC/FID)	40	Pyroquilon-S		100.8	0.5	99.9 (GC)
20	IBP	*	99.5	0.3	100 (GC/FID)	41	Tricyclazole	*	99.4	0.1	100 (LC/UV)

[a] The purity means the area percentage of main peak on chromatogram.
[b] There was no mention of the purity in the certificate.
* Eighteen products are stored in 4℃ for about 2-5 years after first opening the pack.

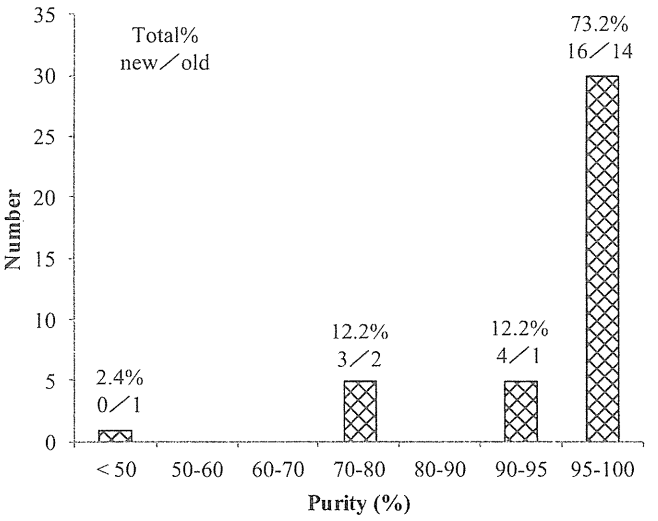

Fig. 2 Distribution of absolute purities of commercial reagent material products measured by qNMR

The 41 products were classified according to the absolute purities. The proportions and the number of new and old products were shown.

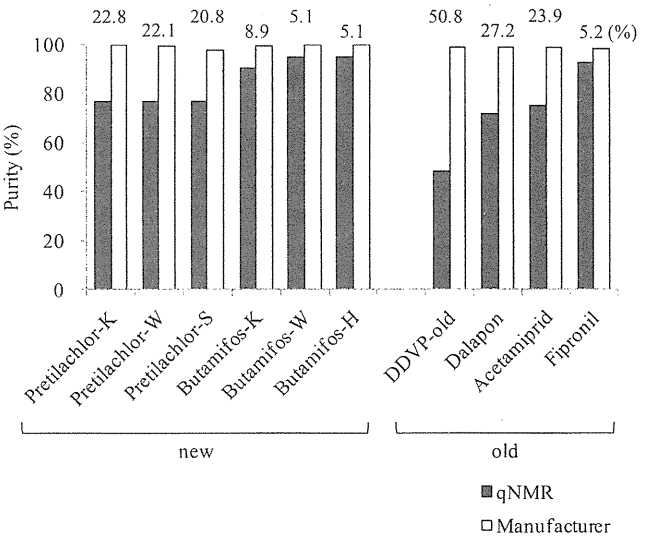

Fig. 3 Differences between manufacturer's labeled purities and the absolute purities measured by qNMR

Ten pesticide reagent products, which had difference more than 5 % between the labeled purities and absolute purities, were shown.

分解し，純度値が低下していく可能性を否定できない。この純度の低下が，結果として定量分析値の精度を悪くする恐れがあることが明らかとなった。

農薬等の試薬に記載されている有効期限は，開封前のメーカーの保証期間であり，開封後についてメーカーは保証していない。また，開封後の使用期限を設定しても，管理を徹底しなければ意味をなさない。つまり，現状では試薬の保管状態，開封試薬の廃棄期限等研究者自身が把握する必要がある。これらのことから，qNMRによる純度試験は，開封後の試薬の純度変化または有効期限の記載がない研究用試薬が，試験を行うことができる許容範囲にあるかを評価する手段としても有用である。さらに，市販製品の純度が低下していても，分析時に純度を測定することで，計量計測トレーサビリティの確保された純度値が得られるという利点がある。

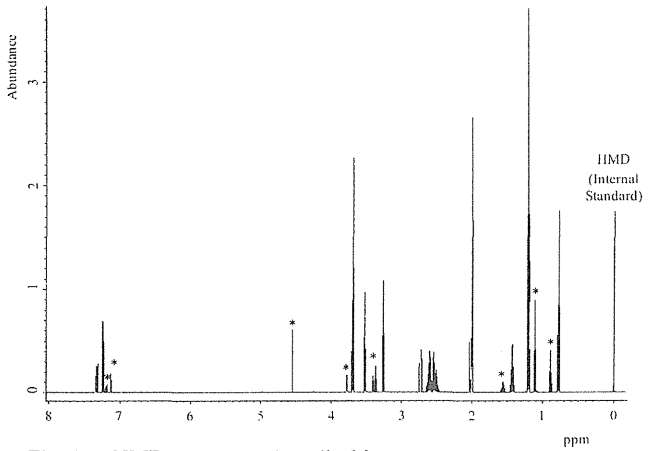

Fig. 4　qNMR spectrum of pretilachlor

HMD was used as a reference at δ 0 ppm and an internal standard for quantitative analysis. * Signals were impurities.

4. まとめ

環境分析に用いられるクロマトグラフィーは，測定対象の標準物質が必要であり，測定対象と同一の基準となる物質のピーク面積の比から求められる相対的な値のため，定量分析値の信頼性は標準物質の質に依存する。科学的な根拠に基づく定量分析値の信頼性確保には，市販試薬製品それぞれについて計量計測トレーサビリティの確保された純度の決定が最重要であると考えられる。そこで本研究では，qNMRの高い定量精度を応用し，市販試薬41製品について純度評価を行った。その結果，約75％が成績書記載の純度値と総じてほぼ等しい値を与えた。しかし，それ以外は成績書記載の純度値より5％以上低く，その純度の低下が定量分析値に誤差を与える要因となることが示唆された。本研究により，定量分析値の実験室間の相互比較には，純度が計量学的に証明された標準物質の精確な使用が重要であり，純度に対してSIトレーサビリティを確保しておけば，定量分析値の信頼性が飛躍的に向上すると考えられた。近年では，第三者の認定を受けた標準物質生産者が供給する認証標準物質や計量標準供給制度（JCSS）の体系内で扱われている認証標準物質の販売を行うようになってきている。しかし，環境分析において測定対象となる化合物は多種多様であるため，市販されている認証標準物質の数は未だ少ない。これらのことから，分析技術者が精確に値付けされた標準品を標準物質として使用する重要性を認識し，qNMRによる計量計測トレーサビリティの確保された定量法が標準品の純度や濃度の値付けや品質の保証を行う体制の整備に重要なツールとなることが期待される。

要　約

環境分野で広く用いられているクロマトグラフィーは測定対象化合物の標準物質が不可欠であるが，国際単位系（SI）への計量計測トレーサビリティが保証された市販の標準物質は少ない。SIトレーサビリティが確保された標準物質を用いない以上，標準物質の純度が定量分析値の信頼性を大きく損なっている可能性を否定できない。本研究では，核磁気共鳴装置を用いたSIトレーサブルな定量分析法を応用して，市販試薬41製品の純度を測定した。41製品のうち26.8％に相当する10製品の純度は，成績書記載の純度値との差が5％以上の47.9～94.8％であった。新品の純度の結果から，成績書記載の純度値を質量％純度として扱うことは不適切であると考えられた。また，市販試薬製品の純度および開封後の品質管理が定量分析値の室間精度に大きく影響を及ぼすことが示唆され，成績書記載の純度値を信用して使用すると，得られる定量分析値は真値より5.1～50.8％の誤差を生じることが明らかとなった。定量分析値の信頼性確保には，計量計測トレーサビリティの確保された純度が精確な標準物質を使用していくことで，飛躍的に向上すると考えられた。

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Specific Detection of Potentially Allergenic Peach and Apple in Foods Using Polymerase Chain Reaction

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ABSTRACT: Two PCR methods were developed for specific detection of the trnS-trnG intergenic spacer region of *Prunus persica* (peach) and the internal transcribed spacer region of *Malus domestica* (apple). The peach PCR amplified a target-size product from the DNA of 6 *P. persica* cultivars including 2 nectarine and 1 flat peach cultivar, but not from those of 36 nontarget species including 6 *Prunus* and 5 other Rosaceae species. The apple PCR amplified a target-size product from the DNA of 5 *M. domestica* cultivars, but not from those of 41 nontarget species including 7 Maloideae and 9 other Rosaceae species. Both methods detected the target DNA from strawberry jam and cookies spiked with peach and apple at a level equivalent to about 10 μg of total soluble proteins of peach or apple per gram of incurred food. The specificity and sensitivity were considered to be sufficient for the detection of trace amounts of peach or apple contamination in processed foods.

KEYWORDS: *food allergy, peach, apple, trnS-trnG intergenic spacer, internal transcribed spacer (ITS), polymerase chain reaction*

INTRODUCTION

Peach and apple are known to cause allergic reactions in certain populations.[1,2] The presence of undeclared apple or peach poses a risk to consumers allergic to these fruits. Thus, the methods for detecting trace amounts of peach or apple in food products should be of value to the food industries and regulatory agencies for reducing chances of unexpected exposure of the allergic consumers to the offending foods.

Peach and apple allergens belong to mainly four protein families: pathogenesis related (PR) 10 proteins, thaumatin-like proteins, lipid transfer proteins (LTP), and profilins.[3−5] Because of the occurrence of homologous proteins, wide cross-reactivity among fruits, especially those of the family Rosaceae, has been documented.[6−8] However, a patient who has developed an allergy to a particular fruit does not necessarily become clinically allergic to all other fruits having homologous and immunologically cross-reactive proteins. For example, Rodriguez et al. reported that 10 in 22 peach-allergic patients did not show clinical responses to any of the Rosaceae fruits tested, including apple, apricot, almond, plum, strawberry, and pear.[7] Therefore, a peach detection method that can differentiate peach from other Rosaceae fruits should benefit peach-allergic patients by reducing unnecessary avoidance of foods containing Rosaceae fruits other than peach.

Cultivars of peach belong to the species *Prunus persica*, which includes nectarine (*P. persica* var. *nucipersica*) and flat peach (*P. persica* var. *platycarpa*). Because accumulation of the major peach allergens, especially Pru p 3, has been confirmed not only in peach but also in nectarine and flat peach,[9−12] we chose *P. persica* including nectarine and flat peach as the target for our peach detection method.

For our apple detection method, we chose *Malus domestica* as the target, because it is the species of domesticated apple cultivated widely on all continents except Antarctica,[13,14] and its cultivars have been confirmed to possess allergenicity, although of various degrees.[15] Because no reports on their allergenicity could be found, and the chances that they would find their way into food products were thought to be slim, most of the "wild apples" or "crab apples" belonging to *Malus* spp. other than *M. domestica* and a few of its very close wild relatives were excluded from the target.

Among the currently available allergen detection methods, polymerase chain reaction (PCR)-based methods are highly specific and sensitive and are suitably used as confirmatory methods for positive ELISA tests to exclude false positives,[16,17] although they do not detect allergenic proteins per se. ELISA methods are sensitive, quantitative, and easy to perform and are especially suited as screening tests, but their possible cross-reactivity with homologous nontarget proteins may lead to false-positive results. Although ELISA methods for the quantitation of peach LTP (Pru p 3) and apple LTP (Mal d 3) have already been reported,[18,19] PCR-based methods should still be of value for confirmation of the positive results.

Under the Japanese labeling system for foods containing allergens, declarations of 7 food items are mandatory and those of 18 food items including peach and apple are recommended. The threshold for monitoring the mandatory labeling by ELISA is established at 10 μg protein/g food.[20] An ELISA result indicating the presence of total soluble proteins of an allergenic ingredient in the test sample at or >10 ppm (μg/g or μg/mL) is deemed positive, and, unless the presence of such allergenic

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ingredient is evident from the production records, a confirmation test is performed. Therefore, the PCR-based methods are expected to have a sensitivity equivalent to a contamination level of at least 10 ppm total soluble protein of the target allergenic ingredient.

For the PCR-based methods, target DNA sequences may be conveniently selected from the regions commonly employed in the molecular phylogeny studies (barcode gene regions), such as the trnS-trnG intergenic spacer, internal transcribed spacer (ITS), rbcL, trnL intron, matK, and psbA, to name a few. The abundance of interspecific sequence variations in these regions and the wealth of readily accessible sequence data for a wide variety of species make the target specific primer design easy to accomplish. Moreover, the multicopy nature of the sequences in these regions is helpful in making the detection methods achieve the required sensitivity.[21−24] Here, we report two PCR-based methods for the specific detection of peach (*P. persica*) and apple (*M. domestica*) using primer pairs designed on the trnS-trnG intergenic spacer (trnS-trnG IGS) region and the internal transcribed spacer-1 (ITS-1) region, respectively.

MATERIALS AND METHODS

Plant Materials. The fruits of three peach cultivars (*P. persica* cv. Hakuho, cv. Kawanakajima hakuto, and cv. Golden peach), two nectarine cultivars (*P. persica* var. *nucipersica* cv. Flavortop and cv. Shuho), one flat peach cultivar (*P. persica* var. *platycarpa* cv. Da Hong Pan Tao), five apple cultivars (*M. domestica* cv. Fuji, cv. Ohrin, cv. Jonagold, cv. Jonathan, and cv. Mutsu), cherry (*Prunus avium*), Japanese plum (*Prunus salicina*), apricot (*Prunus armeniaca*), Japanese apricot (*Prunus mume*), almond (*Prunus dulcis*), prune (*Prunus domestica*), pear (*Pyrus communis*), Japanese pear (*Pyrus pyrifolia*), strawberry (*Fragaria* × *ananassa*), raspberry (*Rubus idaeus*), loquat (*Eriobotrya japonica*), quince (*Cydonia oblonga*), juneberry (*Amelanchier* spp.), aloe vera (*Aloe vera*), pineapple (*Ananas comosus*), papaya (*Carica papaya*), orange (*Citrus sinensis*), satsuma orange (*Citrus unshu*), melon (*Cucumis melo*), Japanese persimmon (*Diospyros kaki*), fig (*Ficus carica*), mango (*Mangifera indica*), banana (*Musa acuminata*), avocado (*Persea americana*), blueberry (*Vaccinium corymbosum*), grape (*Vitis* spp.), and kiwifruit (*Actinidia deliciosa*) were purchased from local suppliers. Rice (*Oryza sativa*), soybean (*Glycine max*), maize (*Zea mays*), wheat (*Triticum aestivum*), potato (*Solanum tuberosum*), carrot (*Daucus carota*), onion (*Allium cepa*), Chinese cabbage (*Brassica rapa*), spinach (*Spinacia oleracea*), cucumber (*Cucumis sativus*), and tomato (*Solanum lycopersicum*) were also purchased from local supermarkets. Chinese quince (*Chaenomeles sinensis*) was obtained as a preserve and hawthorn (*Crataegus* spp.) as a dried fruit puree from local supermarkets.

Incurred Food Samples. For the sensitivity study, incurred food samples, strawberry jam and cookies, were prepared by spiking their raw ingredients with peach (*P. persica* cv. Hakuho) and apple (*M. domestica* cv. Fuji). The spiking materials were prepared by freeze-drying freshly peeled fruit and thoroughly mixing with an equal weight of calcium carbonate powder as a dispersant. Total soluble protein concentrations in the spiking materials were determined with the 2-D Quant Protein assay kit (GE Healthcare U.K., Ltd.) after extraction of the proteins with 0.1 M Tris-HCl buffer (pH 7.4, containing 0.5 M NaCl, 0.5% sodium dodecyl sulfate, and 2% β-mercaptoethanol) for 16 h at room temperature. They were 5.7 mg/g for peach and 2.7 mg/g for apple.

The strawberry jam was prepared as follows: Fifty grams of fresh strawberry was homogenized in a food processor to obtain a smooth puree, to which a calculated amount of each spiking material was added and mixed thoroughly. The mixture was cooked over low heat until the total weight was reduced to 65% of the initial weight. After 40 g of sugar was added to the mixture, the pH was adjusted to 3.2 with 0.3 g of citric acid. The mixture was then kept at 80 °C for 30 min.

The cookies were prepared as follows: One hundred grams of wheat flour, 75 g of sugar, 23 g of shortening, 15 g of butter, 1.5 g of salt, 0.12 g of baking soda, 0.37 g of cream of tartar, 25 g of water, and the calculated amount of the spiking materials were thoroughly mixed. The mixed dough was made into disks of approximately 3 cm in diameter and 0.5 cm in thickness and baked at 180 °C for 15 min. The baked cookies were crushed using a mixer mill IFM-650D (Iwatani Corp., Japan) to make a uniform powder. On the basis of the total soluble protein concentrations in the spiking materials as determined with the 2-D Quant Protein assay kit and the final weight of the incurred samples, the total soluble protein concentration of peach and that of apple in the incurred samples were calculated to be 12 μg/g each in the strawberry jam and 10 μg/g each in the cookies.

The final concentrations of slightly higher than 10 μg/g in the strawberry jam were due to evaporative loss of water during preparation. The incurred food samples were stored in a deep freezer at −80 °C until use.

DNA Samples. The DNA samples from soybean seed and maize leaf were extracted and purified with a DNeasy plant Mini Kit (Qiagen GmbH, Hilden, Germany). The DNA samples from the other plant materials (0.1−2.0 g of seeds, leaves, and fruit flesh) and the DNA sample from 2.0 g of incurred cookies were extracted with 20 mL of buffer G2 (Qiagen) supplemented with 20 μL of RNase A (100 mg/mL; Qiagen) and 200 μL of Proteinase K (Qiagen) and purified using Genomic-tip 20/G (Qiagen). The DNA sample from the incurred strawberry jam was prepared in the same way as the incurred cookies except that 0.8 g of autoclaved polyvinylpolypyrrolidone (Sigma-Aldrich Co., St. Louis, MO) was added to 20 mL of the supplemented buffer G2 to avoid possible PCR inhibition by phenolic substances in the sample.[25,26]

The DNA concentration was determined by measuring the absorbance at 260 nm. The DNA samples were diluted to 20 ng/μL with TE (pH 8.0) to make templates for PCR. For the sensitivity studies, DNA samples from a peach and an apple cultivar were serially diluted with a 20 ng/μL salmon testis DNA solution to obtain PCR templates containing 2 fg/μL−2 ng/μL of the target DNA.

The quality of all DNA samples was confirmed by amplifying a fragment of plant chloroplast DNA with the CP 03-5′ (5′-CGG ACG AGA ATA AAG ATA GAG T-3′) and CP 03-3′ (5′-TTT TGG GGA TAG AGG GAC TTG A-3′) primer pairs.[27]

Primers. We designed several candidate primers on various barcode gene regions and picked up the pairs that would achieve the desired sensitivity and specificity.

For peach PCR, the sense primer ppersica-F (5′-TGG TCG TAA TAA AAA GTC AAA A-3′) and the antisense primer ppersica-R (5′-CGT AAA CGC TCT AAT TTT AAT AG-3′) designed on the trnS-trnG IGS region of *P. persica* (GenBank accession no. AY500733) were selected. The second base from the 3′ end of the ppersica-R primer is a deliberate mismatch introduced to improve specificity.[28]

For apple PCR, the sense primer malus-F (5′-ATC ATT GTC GAA CCT GCA CG-3′) and the antisense primer malus-R (5′-ACA CGC GCC GGT GTA A-3′) designed on the ITS-1 region of *M. domestica* cv. Fuji newly sequenced for this work (GenBank accession no. AB636343) were selected.

PCR simulations were performed by using Amplify 1.0 (Bill Engels, Genetics, University of Wisconsin, Madison, WI)[29] to predict whether the designed primer pairs would give PCR products of the target size from the DNA sequences of the trnS-trnG IGS region and ITS-1 region deposited in GenBank.

PCR. PCRs were performed in 0.2 mL reaction tubes in a final volume of 25 μL, containing 1× buffer (PCR buffer II), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.625 unit of AmpliTaq Gold (Applied Biosystems, Foster City, CA), each primer pair (peach PCR, 0.5 μM each of ppersica-F and ppersica-R primer; apple PCR, 0.4 μM each of malus-F and malus-R primer), and 5 fg−50 ng of template DNA. Amplification was performed in a GeneAmp PCR System 9700 (9600 emulation mode, i.e., ramping speed of 1 °C/s) or 9600 (Applied Biosystems) by using the following conditions: for peach PCR, preincubation at 95 °C for 10 min, 45 cycles consisting of denaturation at 95 °C for 30 s and annealing/extension at 58 °C for 1 min, and a final extension at 72 °C for 7 min; for apple PCR, preincubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 1 min, and a final extension

at 72 °C for 7 min. The PCR products (7.5 μL) were electrophoresed on a 3% agarose gel containing ethidium bromide and analyzed with a ChemiDoc XRS illuminator (Bio-Rad Laboratories, Inc., Hercules, CA). The PCR with the CP 03-5′/CP 03-3′ primer pair[27] yielded the product of the expected size from all DNA samples. The DNA sequences of the PCR products from peach and apple were determined by direct sequencing with a BigDye Terminator v1.1 Cycle Sequencing Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

RESULTS

PCR Simulation. The specificity of the designed primer pairs was assessed by performing PCR simulations. For peach PCR, a total of 165 sequences of 125 species obtained from GenBank were subjected to the simulation. As shown in Table 1, with the ppersica-F and -R primer pair, the PCR product of the target size (74 bp) was predicted only from one sequence each of the trnS-trnG IGS region of *P. persica* and *P. mira*, but not from the other 163 sequences of 123 nontarget species, which include 50 *Prunus* spp., 59 Rosaceae species other than *Prunus*, and the other 14 plant foods.

The lower-than-maximum weight number of 4 predicted for the target *P. persica* was due to the deliberate mismatch introduced at the second base from the 3′ end of the reverse primer, ppersica-R, for improvement of the specificity.

For apple PCR, a total of 142 sequences of 125 species were subjected to the simulation. As shown in Table 2, with the malus-F and -R primer pair, the PCR product of the target size (134 bp) was predicted only from a total of 15 ITS-1 sequences of 6 *Malus* spp., that is, 10 sequences of *M. domestica* and one sequence each of 5 closely related *Malus* spp. (*M. asiatica*, *M. niedzwetzkyana*, *M. prunifolia*, *M. sieversii*, and *M. sylvestris*). The PCR product of the target size was not predicted from the 22 sequences of more distant 22 *Malus* spp. and the remaining 105 sequences of 104 nontarget species, which include 47 Maloideae species other than *Malus*, 9 Rosaceae species other than Maloideae, and the other 48 plant foods.

The lower-than-maximum weight number of 5 predicted for the target *M. domestica* sequences previously registered in GenBank was due to the fact that those sequences began only at the position corresponding to the eighth base from the 5′ end of the malus-F primer, which was designed on the new sequence (AB636343).

PCR Experiments. Specificity and sensitivity of the developed methods were confirmed by PCR experiments. As shown in Figure 1, the peach PCR method amplified a product of the target size (74 bp) from 50 fg of DNA extracted from three cultivars of peach (*P. persica* cv. Hakuho, cv. Kawanakajima hakuto, and cv. Golden peach), two cultivars of nectarine (*P. persica* var. *nucipersica* cv. Flavortop and cv. Shuho), and one cultivar of flat peach (*P. persica* var. *platycarpa* cv. Da Hong Pan Tao). However, as shown in Figure 2, it did not amplify such a product from 50 ng of DNA extracted from the other 36 fruits and vegetables tested, including 11 nontarget Rosaceae fruits. The nucleotide sequence analysis of the PCR product from *P. persica* cv. Hakuho confirmed that the target sequence of peach had been amplified (data not shown). A nonspecific product of >1500 bp in size was observed from spinach (Figure 2, lane 34), which was easily distinguishable from the target 74 bp product.

As shown in Figure 3, the apple PCR method amplified a product of the target size (134 bp) from 500 fg of apple DNA extracted from five cultivars (*M. domestica* cv. Fuji, cv. Ohrin, cv. Jonagold, cv. Jonathan, and cv. Mutsu). However, as shown in Figure 4, it did not amplify such a product from 50 ng of DNA extracted from the other 41 fruits and vegetables tested, including 16 nontarget Rosaceae fruits. The nucleotide sequence analysis of the PCR product obtained from *M. domestica* cv. Ohrin confirmed that the target sequence of apple had been amplified (data not shown). A nonspecific product of about 250 bp from pear (Figure 4, lane 10) and another of >1500 bp from spinach (Figure 4, lane 39) were observed, but were easily distinguishable from the target 134 bp product.

Table 1. Summary of Peach PCR Simulation Results

scientific name	common name	GenBank accession no.	weight no.[a]
125 species in total		165 sequences in total	
52 *Prunus* spp.		61 sequences in total	
Prunus persica	peach	AY500733	4
Prunus mira	smoothpit peach	AY500732	4
Prunus armeniaca	apricot[b]	AY500725	–
Prunus avium	cherry[b]	AY871252	–
Prunus domestica	prune[b]	AY500719	–
Prunus dulcis	almond[b]	AY500730	–
Prunus mume	Japanese apricot[b]	AY500726	–
Prunus salicina	Japanese plum[b]	AY500722	–
44 other *Prunus* spp.		53 sequences	–
59 Rosaceae species other than *Prunus*		90 sequences in total	
Malus domestica	apple[b]	AY461515 + 7 sequences	–
39 *Crataegus* spp.	hawthorn[b]	EF127091 + 53 sequences	–
Pyrus pyrifolia	Japanese pear[b]	AB545981	–
Amelanchier arborea	juneberry[b]	EF127115	–
Fragaria × *ananassa*	strawberry[b]	FJ422327	–
16 other Rosaceae species other than *Prunus*		25 sequences	–
14 plant foods other than the above		14 sequences in total	
Vaccinium myrtillus	blueberry[b]	DQ073200	–
Daucus carota	carrot[b]	NC_008325	–
Cucumis sativus	cucumber[b]	NC_007144	–
Solanum melongena	egg plant	AY555465	–
Vitis vinifera	grape[b]	NC_007957	–
Lactuca sativa	lettuce	NC_007578	–
Zea mays	maize[b]	NC_001666	–
Citrus sinensis	orange[b]	NC_008334	–
Solanum tuberosum	potato[b]	NC_008096	–
Oryza sativa (*japonica*)	rice[b]	X15901	–
Glycine max	soybean[b]	NC_007942	–
Spinacia oleracea	spinach[b]	NC_002202	–
Solanum lycopersicum	tomato[b]	NC_007898	–
Triticum aestivum	wheat[b]	AB042240	–

[a]An approximate indicator (ranging from 1 to 6) of the quality of matches and the strength of amplification predicted. The larger the weight number, the higher the probability of amplification. – indicates no PCR product of the target size was predicted. [b]Absence of amplification product of the target size was confirmed through PCR experiments as well.

Table 2. Summary of Apple PCR Simulation Results

scientific name	common name	GenBank accession no.	weight no.[a]
125 species in total		142 sequences in total	
28 *Malus* spp.		37 sequences in total	
Malus domestica	apple	AB636343	6
Malus domestica	apple	U16195 + 8 sequences	5
Malus asiatica		AF186494	5
Malus niedzwetzkyana	Niedzvetzky apple	AF186497	4
Malus prunifolia	plumleaf crab apple	AF186500	5
Malus sieversii	Asian wild apple	AF186493	5
Malus sylvestris	European wild apple	FJ899096	5
22 other *Malus* spp.		22 sequences	–
47 Maloideae species other than *Malus*		48 sequences in total	
Crataegus mollis	hawthorn[b]	U16190	–
Pyrus pyrifolia	Japanese pear[b]	AF287240	–
24 *Amelanchier* spp.	juneberry[b]	U83922 + 24 sequences	–
Eriobotrya japonica	loquat[b]	U16192	–
Cydonia oblonga	quince[b]	AF186531	–
19 other Maloideae species other than *Malus*		19 sequences	–
9 Rosaceae species other than Maloideae		9 sequences in total	
Prunus dulcis	almond[b]	AF318754	–
Prunus armeniaca	apricot[b]	AF318756	–
Prunus avium	cherry[b]	AF318737	–
Prunus mume	Japanese apricot[b]	AF318728	–
Prunus salicina	Japanese plum[b]	AF318725	–
Prunus persica	peach[b]	AF318741	–
Prunus domestica	prune[b]	AF318713	–
Rubus idaeus	raspberry[b]	AF055757	–
Fragaria × *ananassa*	strawberry[b]	AF163494	–
48 plant foods other than the above		48 sequences in total	
Aloe vera	aloe vera[b]	AF234345	–
Persea americana	avocado[b]	AF272322	–
Vaccinium myrtillus	blueberry[b]	AF382732	–
Daucus carota	carrot[b]	AY552527	–
Brassica rapa	Chinese cabbage[b]	AF128097	–
Cucumis sativus	cucumber[b]	AY833602	–
Ficus tonduzii	fig[b]	AY730140	–
Vitis vinifera	grape[b]	AF365988	–
Actinidia deliciosa	kiwifruit[b]	AF323830	–
Zea mays	maize[b]	DQ683016	–
Mangifera indica	mango[b]	AB071674	–
Cucumis melo	melon[b]	AJ488233	–
Allium cepa	onion[b]	AJ411944	–
Carica papaya	papaya[b]	AY461547	–
Solanum tuberosum	potato[b]	AY875827	–
Oryza sativa (japonica)	rice[b]	AP008225	–
Glycine max	soybean[b]	AF144654	–
Spinacia oleracea	spinach[b]	AF062088	–
Triticum aestivum	wheat[b]	AF521903	–
29 other plant foods		29 sequences	–

[a]See footnotes to Table 1. [b]See footnotes to Table 1.

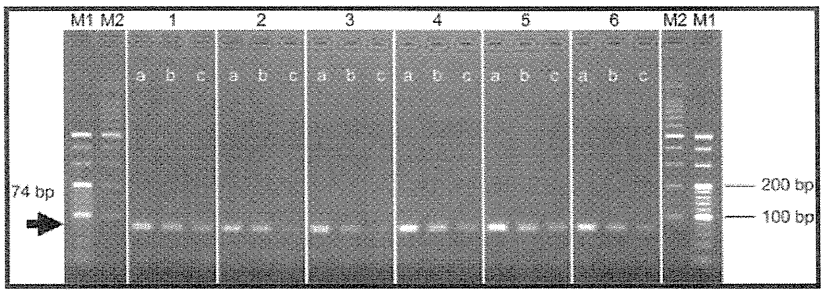

Figure 1. Amplification of the peach cultivar DNAs by the peach PCR method. The arrowheads indicate the expected size of PCR product. Samples 1–6 are amplification of genomic DNA extracted from *P. persica* cv. Hakuho (1), *P. persica* cv. Kawanakajima hakuto (2), *P. persica* cv. Golden peach (3), *P. persica* var. *nucipersica* cv. Flavortop (4), *P. persica* var. *nucipersica* cv. Shuho (5), and *P. persica* var. *platycarpa* cv. Da Hong Pan Tao (6). Lanes a–c show amplification of 500 fg (a), 50 fg (b), and 5 fg (c) of sample genomic DNA. Lane M1 is a 100 bp DNA ladder DNA marker (Takara Bio Inc.), and lane M2 is 20 bp DNA ladder DNA marker (Takara Bio Inc.).

Analysis of Incurred Food Samples. The sensitivity of the two PCR methods was tested by using the incurred foods containing freeze-dried peach and apple at a level corresponding to their respective total soluble protein concentration of about 10 ppm. As shown in Figure 5, PCR products of the target sizes were detected from 5–50 ng DNA samples extracted from the strawberry jam and the cookies.

DISCUSSION

Designing specificity is one of the most important issues in the development of allergen detection methods. A detection method for a particular allergenic food should be able to detect all species that are potentially allergenic to the patients known to be allergic to that particular food, but should not detect species that may not elicit clinical responses in a sizable proportion of the patients so that unnecessary avoidance of foods by those patients may be minimized.

Our primer pairs for peach PCR and apple PCR yielded the target size amplification product from cultivars of peach (including potentially allergenic nectarine and flat peach) and from those of apple, respectively. The absence of the target amplification product from nontarget fruits and vegetables was