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Simple and Sensitive Method for Pyrroloquinoline Quinone (PQQ) Analysis in Various Foods Using Liquid Chromatography/Electrospray-Ionization Tandem Mass Spectrometry

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Pyrroloquinoline quinone (PQQ) is believed to be an important factor for mammalian growth and development and has, therefore, been declared a vitamin by some researchers. However, this issue remains controversial, and from a nutritional viewpoint, accurate determination of PQQ levels in a variety of foods is very important. Here, we describe a simple, highly sensitive, and highly selective method for quantitative analysis of PQQ. Liquid foods or aqueous extracts of solid foods were analyzed using high-performance liquid chromatography (HPLC) combined with electrospray-ionization (ESI) tandem mass spectrometry (MS/MS). ^{15}N-labeled PQQ was added to the samples as an internal standard. Quantitative analyses of PQQ were performed by multiple reaction monitoring (MRM) with LC/MS/MS. Free PQQ was detected in almost all food samples in the range 0.19−7.02 ng per g fresh weight (for solid foods) or per mL (liquid foods). This method will enable the rapid and simple determination of PQQ levels in many samples.

KEYWORDS: Pyrroloquinoline quinone (PQQ); extraction; quantification; LC/MS/MS analysis

INTRODUCTION

Pyrroloquinoline quinone (PQQ) was initially identified as a novel cofactor of various bacterial dehydrogenases in 1967 (*1*), and its structure was fully elucidated in 1979 (*2*). Since the discovery of PQQ, functional studies have indicated that it is beneficial to mammalian growth and development (*3*, *4*). In 2003, Kasahara and Kato identified the first PQQ-dependent enzyme in mammals, 2-aminoadipic 6-semialdehyde dehydrogenase (AAS dehydrogenase), and asserted that PQQ acts as a vitamin in mammals (*5*). Whether PQQ is indeed a vitamin in mammals remains controversial (*6*−*8*).

To demonstrate the nutritional importance of PQQ, determination of PQQ levels in a variety of foods is indispensable. Various methods for instrumental analyses and bioassays for PQQ have been developed (*9*−*14*), but many of these methods are now recognized as unreliable for quantitative analysis of PQQ in crude samples. Of the reported methods, that of Kumazawa et al., who established a gas chromatography (GC)/mass spectrometry (MS) method for quantitation of free PQQ in crude biological samples and various foods (*15*−*17*), yields the most reliable data. Their method requires a lengthy PQQ purification and derivatization procedure and does not allow quantification of PQQ derivatives, some of which may exert PQQ-like activity. Therefore, development of an analytical method for the simultaneous quantification of both PQQ and PQQ derivatives is required to investigate the function of PQQ, but Kumazawa et al.'s (*17*) method is unlikely to be suitable for rapidly and accurately determining levels of PQQ and its derivatives from multiple samples. In this context, to achieve the determination of accurate levels of PQQ and PQQ derivatives in various foods, we developed a rapid, easy method. Some of the reports demonstrated that PQQ was converted into imidazolopyrroloquinoline quinone (IPQ) or its derivatives (IPQ/R) as a result of the reaction with amino acids in biological samples, and these derivatives are biologically active in some cases (*14*) (**Figure 1**). Furthermore, PQQ is very reactive with other nucleophiles, and this characteristic of PQQ could result in unknown adducts in foods. Therefore, a simple method for analyzing a wide variety of PQQ derivatives is also needed.

Kumazawa et al. reported that PQQ is ubiquitous in foods, and foods of plant origin contain much more PQQ than foods of animal origin (*16*, *17*). If PQQ is a vitamin in mammals, it must be present in mammalian diets. To analyze the relationship between the role of PQQ and its derivatives in mammals and

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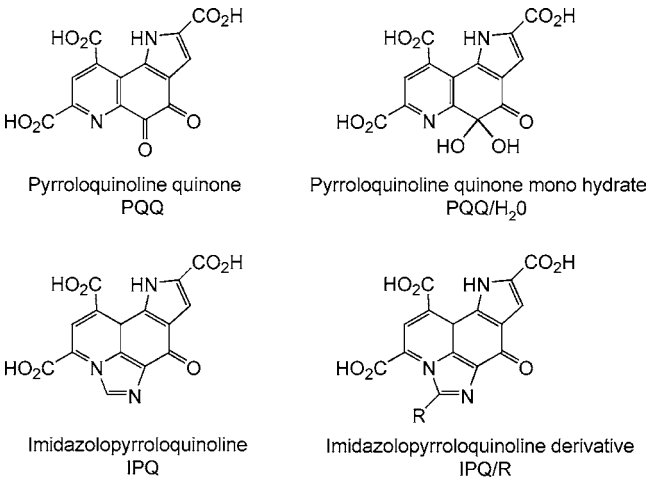

Figure 1. Structures of pyrroloquinoline quinone (PQQ) and its amino acid derivatives imidazolopyrroloquinoline quinone (IPQ) and IPQ/R.

the amount of PQQ and its derivatives in mammalian diets, a convenient analytical method for measuring PQQ levels in a variety of foods is needed. In this study, we report the development of a novel, simple, and reliable LC/MS/MS method for quantitative analysis of PQQ and its derivatives in foods of plant origin.

MATERIALS AND METHODS

Materials. PQQ was purchased from Wako Chemicals. Other common chemicals were of the highest purity commercially available. Food samples were purchased from local stores.

We used ^{15}N-labeled PQQ (MW 332) as an internal standard in which two nitrogens were replaced with ^{15}N nitrogen (**Figure 2a**). ^{15}N-labeled PQQ was synthesized microbiologically in *Hyphomicrobium* sp. DSM 1869 that was cultivated in mineral medium (pH 6.8) containing (per liter) 10 mL of methanol, 1.0 g of $(^{15}NH_4)_2SO_4$, 1.4 g of KH_2PO_4, 1.0 g of $MgSO_4 \cdot 7H_2O$, 5.0 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, 3.0 mg of $MnSO_4 \cdot 4H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 10 mg of NaCl, 0.2 mg of H_3BO_3, 0.1 mg of $CuSO_4 \cdot 5H_2O$, 0.1 mg of $CoCl_2 \cdot 2H_2O$, 0.1 mg of KI, 0.1 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 75 mg of $FeSO_4 \cdot 7H_2O$. The cultures were incubated for 10 days at 30 °C in a rotary shaker at 100 rpm and then centrifuged at 10000*g* for 5 min. The supernatant fractions were removed and acidified to pH 3.5 and then applied to a DEAE-Toyopearl chromatography column equilibrated with 2 mM KPO_4 buffer (pH 7.0). The column was eluted with a 0.100−1 M gradient of NaCl in 2 mM KPO_4 buffer (pH 7.0). Fractions that were red were collected and adjusted to pH 3.0, and the ^{15}N-labeled PQQ was salted out with NaCl at 4 °C. In total ion mode the ion peaks not from the ^{15}N-labeled PQQ were detected in the solution of this fraction, but the intensity of those peaks was less than 1/100 of the molecular ion peak of ^{15}N-labeled PQQ. In addition, in MRM mode for PQQ and the ^{15}N-labeled PQQ described in the latter part no detectable peaks were found. In this context, we decided to use ^{15}N-labeled PQQ prepared according to the above method in our analytical experiment.

Extraction of PQQ. We extracted PQQ from foods of plant origin using a modification of the method reported by Kumazawa et al. (*17*). For solid foods, ∼5 g of the food sample was transferred to a mortar, frozen in liquid nitrogen, and homogenized. Deionized water (20 mL) was added to the homogenate, which was then sonicated. ^{15}N-labeled PQQ (220 ng) was added to each 20-mL aqueous extract as an internal standard. The homogenate was clarified by centrifugation for 15 min at 10 000 rpm, and the supernatant was transferred to a new tube. Liquid foods were used in 20 mL aliquots. ^{15}N-labeled PQQ (220 ng) was also added to samples as an internal standard.

Then, the PQQ was purified from the solid and the liquid samples using the same procedure. One-half volume of 3 N HCl was added to the sample solution, and the solution was extracted with ethyl acetate. The extract was dried in an MG-1000 dry thermo bath (EYELA). The resulting solid was then dissolved in 500 μL of 100 mM acetic acid buffer (pH 4.8). Samples were filtered though 0.45 μm Ekicrodisc13 Versapor filters and transferred to 1 mL vials before analysis.

Mass Spectrometry. Standard mixtures of PQQ (10 μg/mL) and ^{15}N-labeled PQQ (3 μg/mL) were prepared in 9:1 100 mM acetic acid buffer (100 mM ammonium acetate/100 mM acetic acid 6:4 (v/v))/methanol (v/v). For selection of diagnostic precursor-to-product ion transitions, a syringe pump was used to directly inject standards at a flow rate of 10 μL/min into an electrospray-ionization (ESI) tandem mass spectrometer (Applied Biosystems 4000 Q-TRAP) equipped with an electrospray ion source (ESI-MS/MS). The ion spray voltage and temperature were optimized for production of the requisite precursor ions in negative- and positive-ion modes. Labeled and unlabeled PQQ were analyzed in negative-ion mode. The collision energy and gas pressure were then optimized for dissociation of deprotonated molecule ($[M - H]^-$) into diagnostic fragment ions. Once the characteristic precursor-to-product ion transitions had been determined, labeled and unlabeled PQQ were analyzed by reverse-phase HPLC and tandem mass spectrometry (LC/ESI-MS/MS) with multiple reaction monitoring (MRM) to determine retention times. To confirm the identifications, PQQ was analyzed in enhanced product-ion (EPI) mode.

High-Performance Liquid Chromatography. Crude samples were separated on an Agilent 1100 HPLC system equipped with an octadecylsilica (ODS) reverse-phase column (100 mm × 2 mm i.d.; Pegasil-ODS, Senshu Scientific). Elution was performed at a flow rate of 200 μL/min. The mobile phases used were 100 mM ammonium acetate/100 mM acetic acid 6:4 (v/v) and methanol. The total HPLC run time was 20 min. The gradient was increased linearly from 90% A, 10% B to 60% A, 40% B over 7 min, and then the initial conditions were restored and allowed to equilibrate for 13 min.

In Vitro Formation of PQQ Derivatives. PQQ derivatives were generated by incubating 2.5 μmol/mL PQQ in amino acid standard solution (Amino Acid standard H, PERBIO, France), which includes 1.25 μmol/mL of cysteine and 2.5 μmol/mL of other amino acids except tryptophan, asparagine, and glutamine, in 0.1 N HCl, at 25 °C for 24 h in the dark. The chemical reaction was stopped by freezing the sample to −80 °C.

RESULTS

In order to quantify PQQ and its derivatives, the LC/ESI-MS/MS system described here is preferable to the established GC/MS systems because liquid chromatography with a reverse-phase column allows quantification of PQQ and its hydrophilic PQQ derivatives without any chemical modification, and the LC/ESI-MSMS system has a wide dynamic range and MRM mode, which allows both high sensitivity and high selectivity. These merits rely on a distinct precursor-to-product ion transition that is diagnostic for the presence of the target compound(s) in a sample. MS/MS spectra of the products of unlabeled and ^{15}N-labeled PQQ are shown in **Figure 2b**. Analysis was performed in negative-ion mode. Molecular ion peaks were present at m/z = 329 and 331 for PQQ and ^{15}N-PQQ, respectively. The characteristic product ions for PQQ at m/z = 285 $[M - H - CO_2]^-$, 241 $[M - H - 2CO_2]^-$, and 197 $[M - H - 3CO_2]^-$ and for ^{15}N-PQQ at m/z = 287 $[M - H - CO_2]^-$, 243 $[M - H - 2CO_2]^-$, and 199 $[M - H - 3CO_2]^-$ are shown in **Figure 2**. PQQ hydrate and IPQ were also detected. The precursor and characteristic product ions used for qualitative and quantitative analysis of each compound are summarized in **Table 1**.

High-performance liquid chromatography was performed on an ODS column to allow simultaneous analysis of PQQ, PQQ hydrate, IPQ, and IPQ/R. Although several previous reports have described purification of PQQ using an ODS column, we found that PQQ was not sufficiently ionized by ESI in the reported

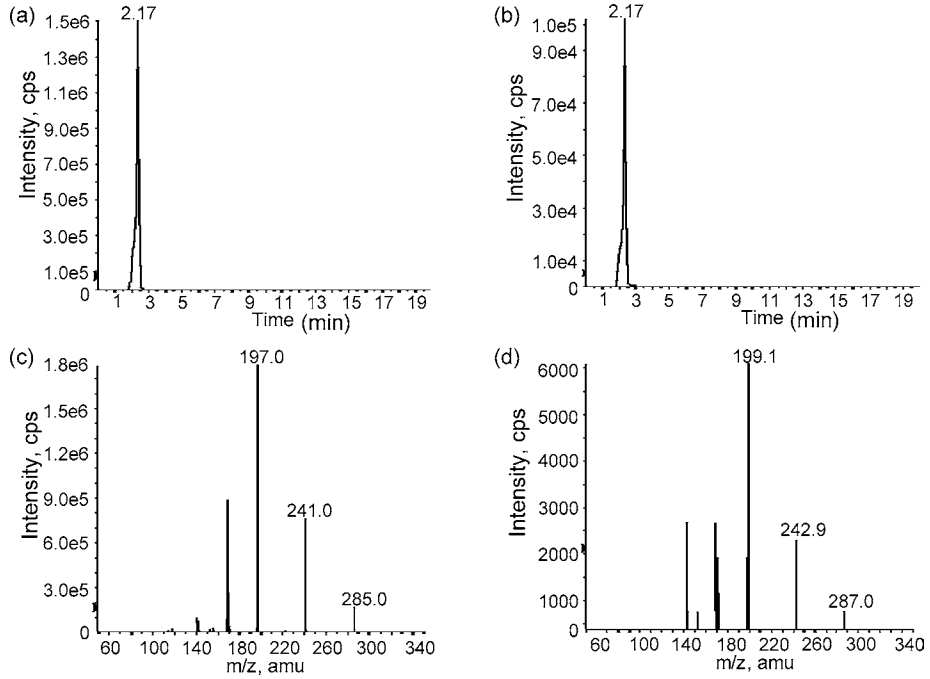

Figure 2. Negative-mode electrospray-ionization mass spectra of PQQ and ^{15}N-labeled PQQ, the internal standard. MRM chromatogram showing transitions for PQQ (**a**) and ^{15}N-PQQ (**c**) at m/z = 329/241 and 331/243, respectively. Enhanced product-ion spectra showing the (**b**) PQQ precursor (m/z = 329), PQQ product (m/z = 285, 241, 197), (**d**) ^{15}N-PQQ precursor (m/z = 331), and ^{15}N-PQQ product (m/z = 287, 243, 199) ions.

Table 1. Summary of Precursor-to-Product Ion Transitions Used in Quantitative Analysis of PQQ and PQQ Derivatives

analysis	retention time (min)	transition (m/z)		
PQQ	2.17	329/285	329/241	329/197
PQQ hydrate	2.17	347/329	347/259	
IPQ	5.01	340/296		

solvent systems. After screening several solvent systems, we selected acetic acid buffer as the eluent. The retention times for PQQ and its derivatives are summarized in **Table 1**.

Pyrroloquinoline quinone is in equilibrium with PQQ hydrate in an aqueous solution, and it also readily reacts with a variety of amino acids to form IPQ. Since PQQ and ^{15}N-PQQ behaved identically under all conditions tested, we used ^{15}N-PQQ as an internal standard in our analyses. PQQ levels in the test samples were determined by comparing the PQQ and ^{15}N-PQQ peaks. PQQ derivatives in the samples were treated as free PQQ in calculating total PQQ levels.

When solutions containing varying amounts of PQQ and ^{15}N-PQQ were used to create calibration curves, the curves exhibited excellent linearity in the range 50−100 000 pg (R^2 = 0.9965−0.9995), indicating a dynamic range far greater than that of GC/MS. Although the retention times for chromatography of PQQ and ^{15}N-PQQ were identical, the signals were resolvable in MRM mode. The detection limit was 0.5−2 pg. Absolute ion intensities in MRM mode were used to calculate recovery rates; more than 80% of the ^{15}N-PQQ was recovered from the standard solution throughout the entire separation procedure. However, the recovery rate of the internal standard in food samples was only 30−40%. Each experiment was carried out at least three times.

Using the method described here we determined PQQ levels in several foods of plant origin; these levels are shown in **Table 2**. The MRM chromatogram for beer is shown in **Figure 3**. Free PQQ was detected in almost all the food samples at concentrations ranging from 0.19 to 7.02 ng/g fw (or ng/mL).

Table 2. PQQ Concentrations of Various Foods (Mean ± SD)

sample	PQQ (ng/g fw or ng/mL)
field mustard	5.54 ± 1.50
spinach	7.02 ± 2.17
Chinese cabbage	nd[a]
green pepper	2.12 ± 0.40
broccli sprout	1.55 ± 0.37
Japanese radish	0.70 ± 0.42
tomato	nd
rape blossoms	5.44 ± 0.8
fermented soybeans	1.42 ± 0.32
green tea	0.16 ± 0.05
beer	1.66 ± 0.82

[a] Not detectable.

Although IPQ was not detected in the samples we tested, it was detected from a green tea sample when sufficient PQQ (0.3 μg/mL) was added to the tea sample exogenously (data not shown). This result clearly demonstrates that IPQ is derived from PQQ and the LC/ESI-MSMS system used in this report can detect IPQ included in samples.

DISCUSSION

To quantify PQQ levels, a variety of analytical methods have been developed. Among them, GC/MS analysis reported by Kumazawa et al. (*17*) has long been considered the most reliable method for quantitation of PQQ as well as practical. However, this method cannot allow quantification of hydrophilic derivatives of PQQ. To overcome this difficulty, we developed a new, highly sensitive, and highly selective LC/ESI-MS/MS method for quantitative analysis of both PQQ and its derivatives. This method allows rapid, straightforward analysis of the levels of PQQ and its derivatives in a variety of samples.

We used bacterially produced ^{15}N-labeled PQQ as an internal standard because we confirmed that its physicochemical properties were identical to those of unlabeled PQQ in our LC/ESI-MS/MS experimental system. For PQQ quantification both the

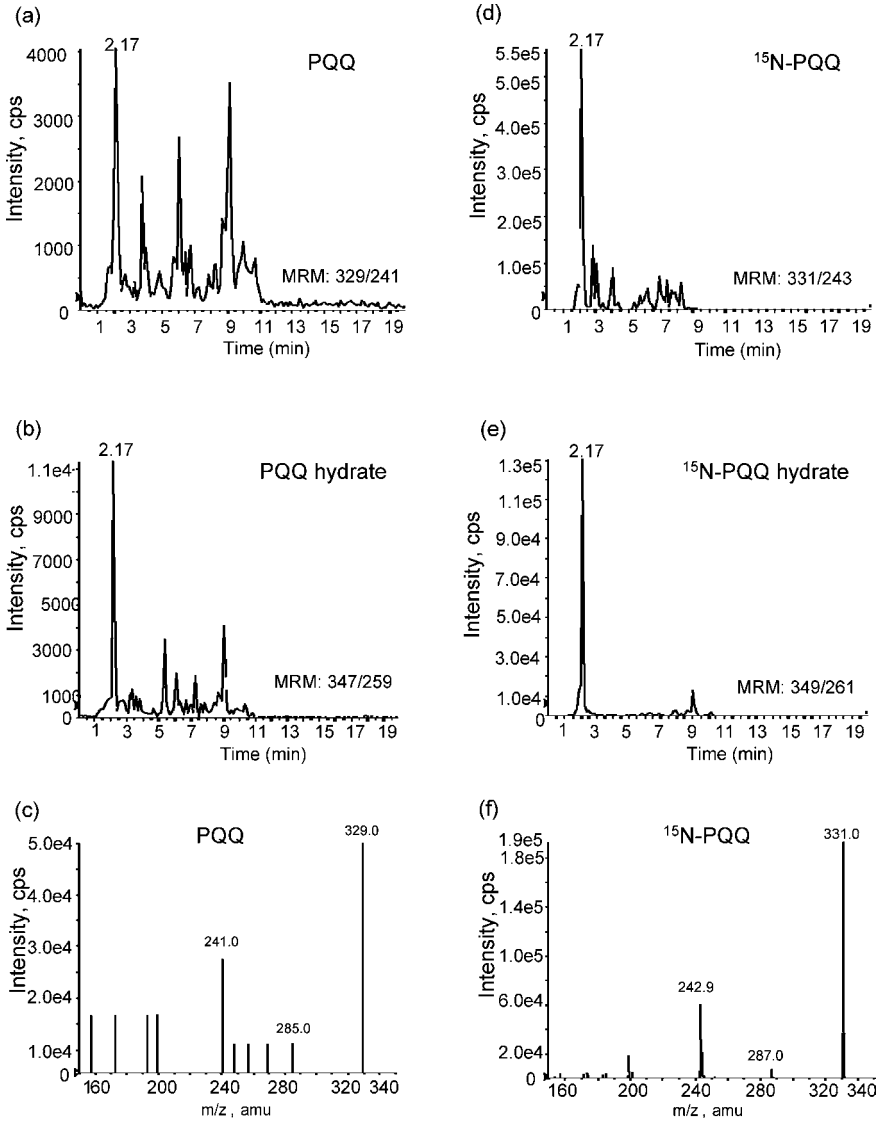

Figure 3. Mass spectra of PQQ, ^{15}N-PQQ (internal standard), and PQQ hydrate (from beer). Chromatogram peaks of (**a**) PQQ, (**b**) PQQ hydrate, (**d**) ^{15}N-PQQ, and (**e**) ^{15}N-PQQ hydrate. Fragmentation of the chromatogram peaks in **a** and **b** was confirmed by the EPI spectra of PQQ (**c**) and ^{15}N-PQQ (**f**), respectively.

high sensitivity and high selectivity of the MS/MS analysis in MRM mode did not require complete ODS-column separation of PQQ from other compounds; therefore, we used chromatography conditions that were insufficient to resolve highly polar PQQ from other polar compounds in the food samples. The LC run time was set to 20 min for quick analysis. Therefore, the run time per sample can be decreased, which is a great advantage over other analytical methods. Furthermore, our method does not require derivatization of PQQ and therefore decreases the overall length of time needed for analysis. Ultimately, the results are highly reproducible.

Kumazawa et al. (*17*) measured PQQ levels in a variety of foods using GC/MS and found that the range of free PQQ levels was 3.7−61 ng/g fw (or ng/mL). By contrast, the PQQ levels that we measured using LC/ESI-MS/MS were about 1 order of magnitude lower or 0.19−7.02 ng/g (or ng/mL). For example, using their GC/MS method Kumazawa et al. (*17*). found that fermented soybeans contain 60 ng PQQ/g fw, whereas we found that they contain 1.42 ng/g fw.

In environments containing organic nucleophilic compounds, such as amino acids, PQQ is immediately converted into IPQ. For example, Mitchell et al. showed that PQQ added to human milk predominantly produced IPQ (*14*). On the basis of this report the reason why our analytical result for the level of PQQ in fermented soybeans differs greatly from that of Kumazawa et al. (*17*) could be ascribed to the different degree of PQQ derivatization to IPQ in each sample. On the basis of this idea we tried to analyze both PQQ and IPQ simultaneously; however, no IPQ peak was detected. This result countered our expectations and differed from the result reported by Mitchell et al. (*14*). Therefore, we carried out a replication study based on the report by Mitchell et al. (*14*), i.e., a reaction between PQQ and the standard amino acid solution. The result is summarized in **Figure 4**.

The figure shows the ratio of each PQQ derivative to the total PQQ-related compounds derived by reacting PQQ with the mixture of amino acids. The ratio reported here is calculated based on the peak areas of LC/MSMS measured under the same conditions as used for PQQ quantification. IPQ was detected in the reaction mixture as one of the major products, but it constituted only 6.8% of the total compounds. IPQ/R, PQQ-derivatives reported by Mitchell et al. (*14*), constituted 7.9%

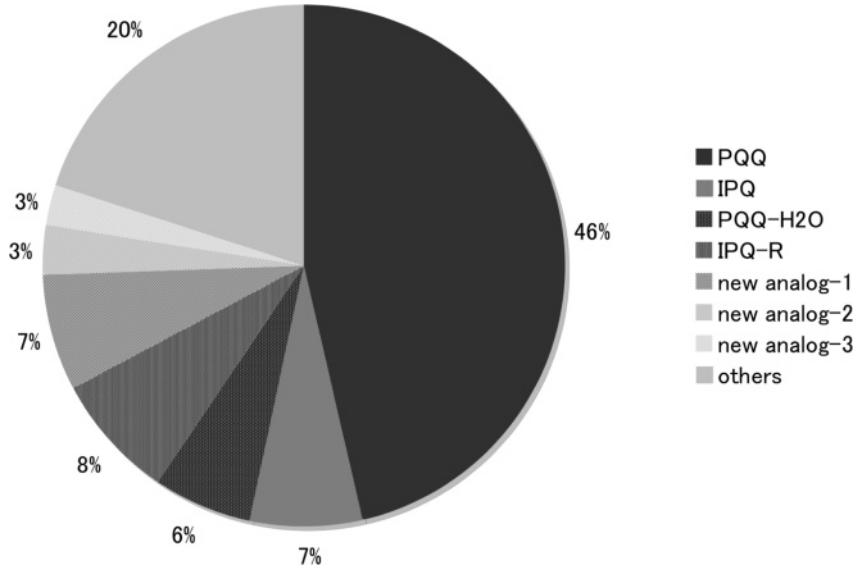

Figure 4. Ratio of PQQ and its derivatives derived by reacting PQQ and amino acids.

of the total compounds. In this experiment, three compounds thought to be PQQ derivatives were observed, but their structures were not elucidated. They constituted 7.1%, 3.2%, and 2.5% of the total compounds. In addition to these compounds a variety of unidentified and unresolved compounds were detected, which were derived from PQQ judging from their mass chromatogram patterns. This experiment was repeated several times and gave similar results under the same conditions, but the ratio depended on the pH and amino acid concentration. Mitchell et al. (*14*) reported that IPQ formation is pH and amino acid concentration dependent, and we confirmed that formation of other derivatives is also pH and amino acid concentration dependent. On the basis of these results we reconsidered the reason for the difference in the PQQ level in our data for fermented soybeans compared with that in Kumazawa et al. (*17*). Due to the abundant amino acids in fermented soybeans (*19*), PQQ must be converted not only into IPQ but also into various derivatives. Therefore, the PQQ levels should depend on the concentration of amino acids and the state of conservation of the samples.

Steinberg et al. (*4*) reported that mice fed a PQQ-deficient diet grow slowly, have fragile skin, and do not reproduce well. In 2003, Kasahara and Kato proposed that PQQ be classified as a new B vitamin based on their discovery of the mouse gene *U26*, which encodes an AAS dehydrogenase that requires PQQ as a cofactor (*5*). Kumazawa et al. (*17*) reported that PQQ is present in every human tissue at levels of 0.8−5.9 ng/g (or ng/mL) despite the fact that mammals cannot biosynthesize PQQ; therefore, they suggested that some of the PQQ in mammalian tissues is of dietary origin. Therefore, a comprehensive quantitative analysis of PQQ in food is important for elucidation of the role of PQQ in mammals. The method described in the present report is likely to significantly enhance progress toward this goal.

In conclusion, we developed a new LC/MS/MS method for quantitative analysis of levels of both PQQ and its derivatives and used this method to determine PQQ levels in a variety of foods. We suggest that the reaction of PQQ with amino acids and other nucleophilic compounds may yield a variety of PQQ derivatives under both experimental and physiological conditions. To reveal the nutritional importance of PQQ, an analytical method for identifying and quantifying unknown PQQ derivatives will be required.

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