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FAILURE TO VERIFY HIGH LEVELS OF PYRROLOQUINOLINE QUINONE IN EGGS AND SKIM MILK

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SUMMARY: The levels of pyrroloquinoline quinone (PQQ) in eggs, skim milk and milk was re-examined by the use of gas chromatography/mass spectrometry. PQQ was extracted from the samples, after addition of [U-^{13}C]PQQ as internal standard, with n-butanol and Sep-Pak C_{18} cartridges. After derivatization of PQQ with phenyltrimethylammonium hydroxide, molecular peaks at m/z 448 and 462 were used for detection of PQQ and [U-^{13}C]PQQ, respectively, by selected ion monitoring. PQQ could be detected in eggs, skim milk and milk, but their levels were very low (e.g., egg yolk: 7.0 ng/ml; skim milk: 2.5 ng/g dry weight). Our data for eggs and skim milk are far below those measured by the redox cycling method of Gallop's group. © 1993 Academic Press, Inc.

Pyrroloquinoline quinone (PQQ) was discovered as a novel co-factor in various prokaryotic dehydrogenases in 1979 [1,2]. Recently, it has been reported that PQQ is nutritionally important as a vitamin or growth factor also in mammals [3]. The physiological significance of PQQ is now being a focus of interest for biochemists and nutritionists. Two groups have reported that very high levels of free PQQ are contained in eggs and skim milk, by the use of a redox cycling method [3-5]. In the present study, we have re-examined the levels of free PQQ in eggs and skim milk by gas chromatography/mass spectrometry (GC/MS) using stable-isotopic PQQ as internal standard, but have failed to confirm such high levels.

MATERIALS AND METHODS

Chemicals: [U-^{13}C]PQQ was synthesized microbiologically in *Hyphomicrobium methylovorum* described previously [6]. PQQ was obtained from

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Abbreviations: PQQ, pyrroloquinoline quinone; PTMA, phenyltrimethylammonium hydroxide; GC/MS, gas chromatography/mass spectrometry; SIM, selected ion monitoring.

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Mitsubishi Gas Chemical Company Inc. (Niigata, Japan); phenyltrimethylammonium (PTMA) hydroxide (20-25% methanol) from Tokyo Kasei Kogyo Co., Ltd. (Tokyo); other common chemicals were of the highest purity commercially available.

Biological samples: Eggs of domestic fowl and ducks were obtained from The Tokyo Metropolitan Livestock Experiment Station (Tokyo). Three brands of cow skim milk (lyophilisate) and milk commercially available in Japan were used.

Isolation of PQQ: To 1 g or 1 ml of samples to be analyzed, including 25 ng of [U-^{13}C]PQQ as internal standard, were added 4 ml of 1 M HCl solution, 50 µl of 2-mercaptoethanol, 0.1 ml of 10% potassium ferricyanide and 10 ml of n-butanol, and homogenized with a Polytron homogenizer for 5 min, being cooled with ice. After centrifugation at 800 x g for 5 min, the organic layer was transferred to another centrifuge tube containing 20 ml of n-heptane, 1 ml of pyridine, 0.1 g of NaCl, and 1 ml of distilled water and shaken for 5 min. The tubes were centrifuged at 800 x g for 5 min and the aqueous layer was evaporated to dryness in vacuo. The residue was dissolved in 10 ml of 0.1 M HCl and applied to a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA). The cartridge was washed with 20 ml of 1 mM HCl and finally 3 ml of 5 % pyridine solution was passed through it. The eluate was evaporated to dryness in vacuo.

Derivatization of PQQ: A 100-µl aliquot of PTMA hydroxide was added to the residue and heated at 100 ^{o}C for 15 min for methylation of PQQ; 1 µl of it was subjected to GC/MS analysis.

GC/MS conditions: The analyses were carried out on a HP-5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) coupled to a JMS-AX505H mass spectrometer (JEOL, Tokyo) with a computer-controlled data analysis system. GC separation was made with a DB-1 fused silica capillary column (15 m x 0.32 mm i.d., film thickness 0.25 µm; J & W Scientific, Folsom, CA). GC conditions were: column temperature 200-300 ^{o}C (20 ^{o}C/min); injection temperature 280 ^{o}C; and helium carrier gas 3 ml/min. The samples were injected in the splitless mode, and the splitter was opened after 1 min. The MS conditions were: electron energy 70 eV; accelerating voltage 3.0 kV; ionization current 300 µA; separator temperature 280 ^{o}C and ion source temperature 270 ^{o}C.

Protein assay: Protein concentrations were determined by the method of Lowry et al [7].

RESULTS AND DISCUSSION

As shown in Fig. 1, molecular peaks at m/z 448 and 462 for the authentic PQQ and [U-^{13}C]PQQ, respectively, constituted the base peaks in the spectra. There were fragment ions at m/z 417, 389, 357 and 329 for the authentic PQQ and at m/z 431, 402, 370, and 341 for [U-^{13}C]PQQ. The [U-^{13}C]PQQ gave no peak at m/z 448, showing no interference with endogenous PQQ by [U-^{13}C]PQQ, which had been added to the samples as internal standard; the spectrum of the authentic PQQ also had no peak at m/z 462. From these results, the molecular peaks at m/z 448 and 462 could be used for sensitive detection of PQQ and [U-^{13}C]PQQ, respectively, by selected ion monitoring

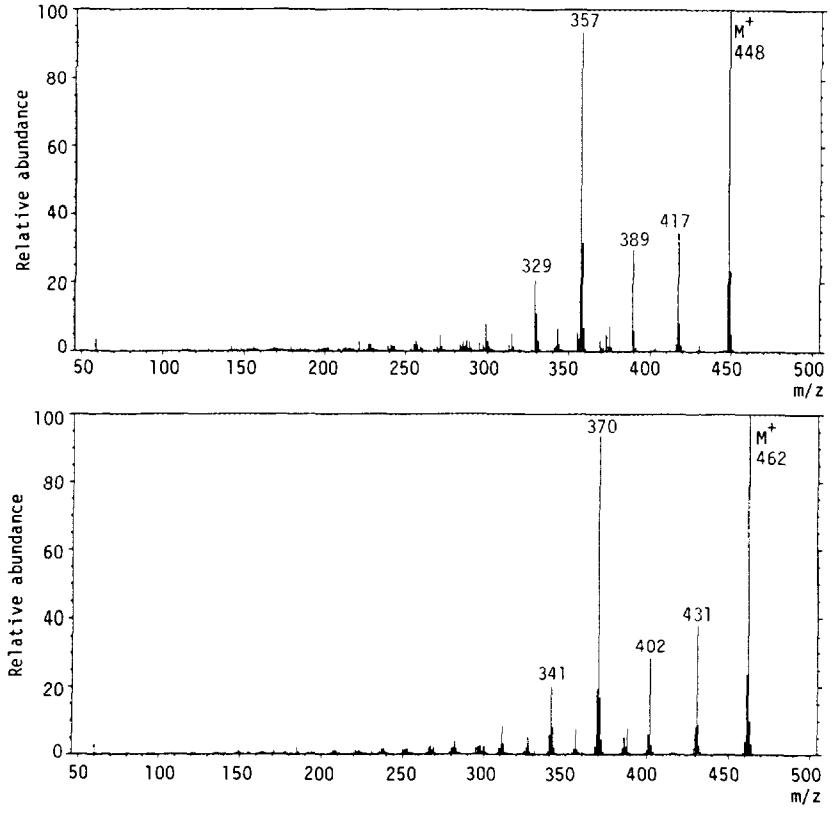

Fig. 1. Mass spectra of PTMA derivatives of the authentic PQQ (top) and [U-^{13}C]PQQ (bottom).

(SIM). The details of the specificity, quantitativeness and reliability of the present GC/MS method were described in our previous reports [8,9].

Typical SIM profiles for the authentic PQQ and for extracts from egg yolk and white of domestic fowl and ducks, and from skim milk and milk are shown in Fig. 2. Twenty five nanograms of internal standard [U-^{13}C]PQQ, which had been added to each 1 g or ml sample, appeared as a big peak in each SIM at m/z 462. For all samples, a small peak appeared on the channel at m/z 448 at exactly the same retention time as that of the internal standard, showing the presence of PQQ in the samples.

The concentrations of free PQQ were carefully quantitated in egg yolk and white of domestic fowl and ducks, and in skim milk and milk, as shown in Table 1. Trace amounts of free PQQ could be detected in all samples; it was highest in the egg white of ducks and lowest in the skim milk. Some data obtained by the redox cycling method of Gallop's group are also shown in Table 1 for comparison. By use of the redox cycling method, very high

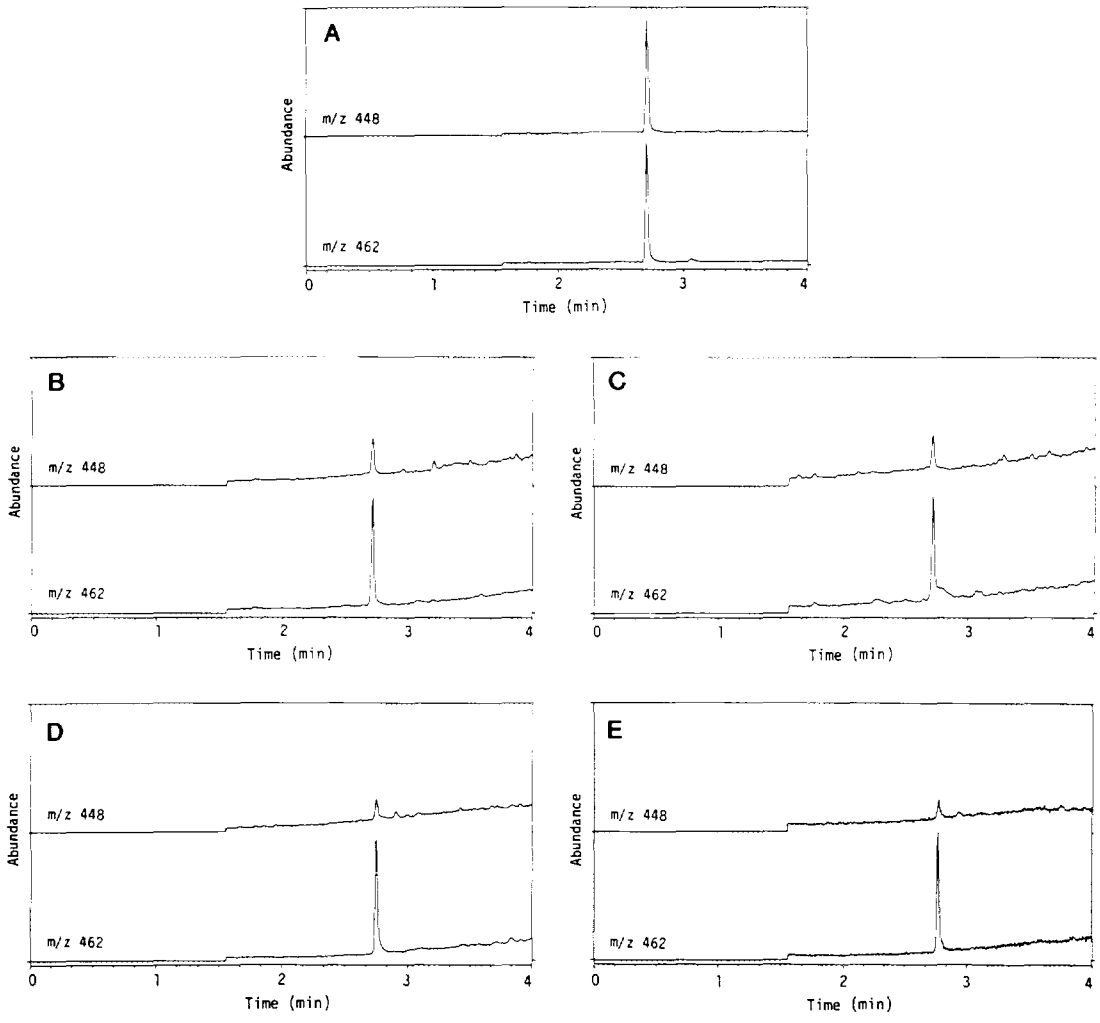

Fig. 2. SIM for PTMA derivatives of PQQ and the [U-^{13}C]PQQ as internal standard extracted from various biological samples. A: the authentic PQQ (250 pg/μl injection) and [U-^{13}C]PQQ (250 pg/μl injection) without extraction; B: egg yolk of domestic fowl; C: egg white of domestic fowl; D: skim milk; E: milk. The amount of [U-^{13}C]PQQ added to each sample was 25 ng. Typical data are presented in this figure.

levels of free PQQ had been detected in egg yolk and skim milk [3-5]. The PQQ levels of egg yolk and skim milk measured by their method are three to four orders of magnitude higher than those obtained in the present study. The method of Gallop's group is based on cyclic reaction of PQQ with glycinate-nitroblue tetrazolium to produce formazan for its colorimetry. It is obvious that such an oxidoreductive system is not specific for PQQ and is not suitable for detection of PQQ in crude biological samples; such reaction can easily be enhanced or suppressed by coexisting impurities.

Table 1. Concentrations of PQQ in eggs, skim milk and milk

Sample	Present study		Results by Gallop's method [3-5]
Domestic fowl (*Gallus gallus*)			(species not shown)
Egg yolk			
	7.0 ± 2.2 ng/ml	(4)	16500 ng/ml
	43.3 ± 11.0 pg/mg protein		85800 pg/mg protein
Egg white			
	4.1 ± 2.6 ng/ml	(4)	'PQQ not detectable'
	32.1 ± 21.1 pg/mg protein		
Duck (*Cairina moschata*)			
Egg yolk			
	19.3 ± 1.9 ng/ml	(3)	
	154 ± 17.8 pg/mg protein		
Egg white			
	28.3 ± 4.1 ng/ml	(3)	
	180 ± 24.2 pg/mg protein		
Skim milk (lyophilisate)			(liquid type)
	2.5 ± 1.4 ng/g dry weight	(4)	574 ng/ml
	4.7 ± 2.7 pg/mg protein		16500 pg/mg protein
Milk			
	3.4 ± 0.4 ng/ml	(4)	
	96.5 ± 16.7 pg/mg protein		

The amount of [U-^{13}C]PQQ as an internal standard added to each sample was 25 ng. Means ± SD are given. The number of samples are in parentheses.

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