

Characterization of Pyrroloquinoline Quinone Amino Acid Derivatives by Electrospray Ionization Mass Spectrometry and Detection in Human Milk¹

A. E. Mitchell,* A. D. Jones,† R. S. Mercer,‡ and R. B. Rucker*²

*Department of Nutrition, University of California, Davis, California 95616-8669; †Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania 16802; and ‡Facility for Advanced Instrumentation, University of California, Davis, California 95616-8669

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We describe a HPLC method coupled to electrospray ionization mass spectrometry (ESI/MS) for quantification and identification of pyrroloquinoline quinone (PQQ) and condensation products formed upon incubation of PQQ with amino acids (IPQ; imidazolopyrroloquinoline and I/OPQ/R; imidazolopyrroloquinoline with attached R-group). More importantly, using these methods we demonstrate the presence of both PQQ and IPQ in human milk in nanomolar to micromolar concentrations. PQQ was incubated with amino acids and condensation products were separated by HPLC. Fractions corresponding to each product were collected and molecular masses were determined using ESI/MS. Ala, Asp, Arg, Cys, Gly, Glu, Ser, Thr, Trp, and Tyr form IPQ upon incubation with PQQ. Yields of IPQ were low (<5%) for Asp and Glu, yet high (>60%) for Thr. In addition to IPQ, Ala, Arg, Cys, Ser, Trp, and Tyr formed IPQ/R derivatives. His, Ile, Leu, Glu, Leu, Lys, Met, and Phe form only IPQ/R derivatives. Proline did not react with PQQ. Mass spectra indicate that PQQ forms stable hydrated carbonyls and decarboxylates easily. Although mass spectra were complicated by the oxidation state of the quinone and decarboxylation of PQQ, these methods are invaluable for the rapid detection of the full range of PQQ adducts in biological matrices. © 1999 Academic Press

Pyrroloquinoline quinone (PQQ,³ **1**) is a redox cofactor for several bacterial dehydrogenases, and is found in plants and animals at the nanograms/gram of tissue level (1–5). Although the biological function(s) of PQQ is not fully characterized in eukaryotes, PQQ can function as a cofactor for nitric oxide synthetase (6) and mitochondrial NADH-CoQ reductase (7, 8). Under physiological conditions PQQ reacts with amino acids to form imidazolopyrroloquinoline (IPQ) derivatives of PQQ (9). In micromolar amounts both PQQ and IPQ protect against CCl₄-induced liver injury, inhibit hydrocortisone-induced cataract formation, and display strong radical scavenging activities (10). In picomolar amounts PQQ and IPQ enhance DNA synthesis activity in human fibroblasts (11) and display nerve growth factor-inducing activity (12). Nutritional studies indicate that PQQ is a potent growth factor in mammals (13–15). Growth and indices of neonatal development are markedly improved when PQQ is added to chemically defined amino acid-based diets in nanomolar concentrations, i.e., in the same concentration range as biotin or folic acid.

PQQ is an aromatic heterocyclic anionic *ortho*-quinone that can be reversibly reduced through a semiquinone intermediate (PQQH) to the quinol (PQQH₂). In addition to amino acids, PQQ redox species readily react with nucleophiles such as thiols and alcohols to form stable condensation products (16–19). PQQ catalyzes, via Schiff base formation, the nonenzymatic ox-

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² To whom correspondence should be addressed at Department of Nutrition, 3135 Meyer Hall, University of California, One Shields Ave., Davis, CA 95616-8669. Fax: 530-752-8669. E-mail: rbrucker@ucdavis.edu.

³ Abbreviations used: PQQ, 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo-[2,3-*f*]quinoline-2,7,9-tricarboxylic acid; OPQ, oxazolopyrroloquinoline; IPQ, imidazolopyrroloquinoline; ESI/MS, electrospray ionization mass spectrometry; TFA, trifluoroacetic acid; MeOH, methanol; HCl, hydrochloric acid.

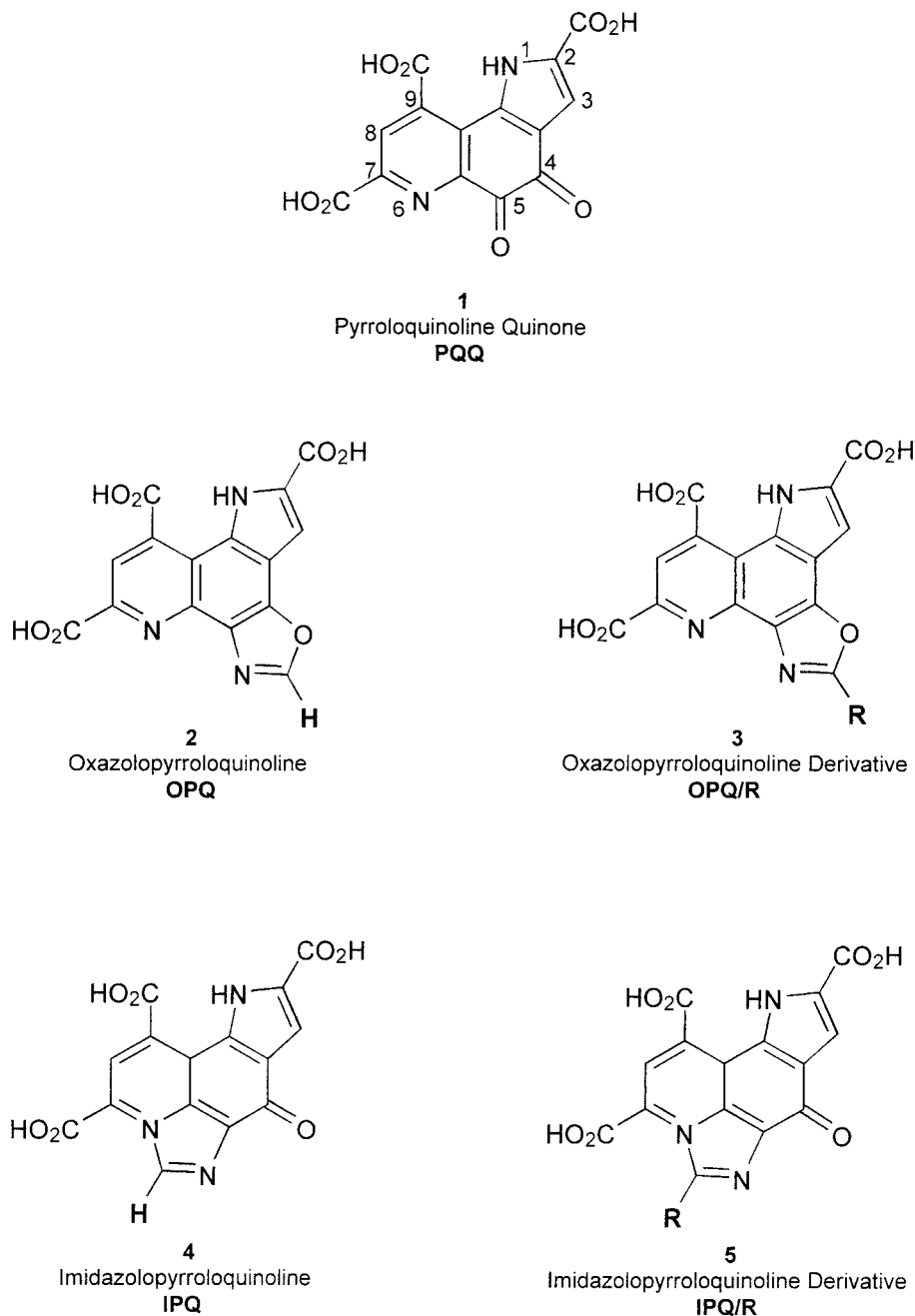


FIG. 1. (1) Structure of pyrroloquinoline quinone (4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid), (2) oxazolopyrroloquinoline (OPQ) and (3) its amino acid derivative OPQ/R, and (4) imidazolopyrroloquinoline (IPQ) and (5) its amino acid derivative IPQ/R.

idative decarboxylation or oxidative dealdolation of amino acids (20). As the cycle progresses, PQQ is converted into a compound with strong absorbance at 422 nm. This product was first described as oxazolopyrroloquinoline (OPQ, **2**), and its derivative (OPQ/R, **3**) (17, 20). However, recent X-ray crystal structures indicate

that this product is the IPQ (**4**) and its derivatives (IPQ/R, **5**; Fig. 1) (9, 24).

To date, quantification methods underestimate levels of PQQ in tissue because most measure only free PQQ and fail to determine contributions from IPQ adducts. For example, tissue levels of PQQ are fre-

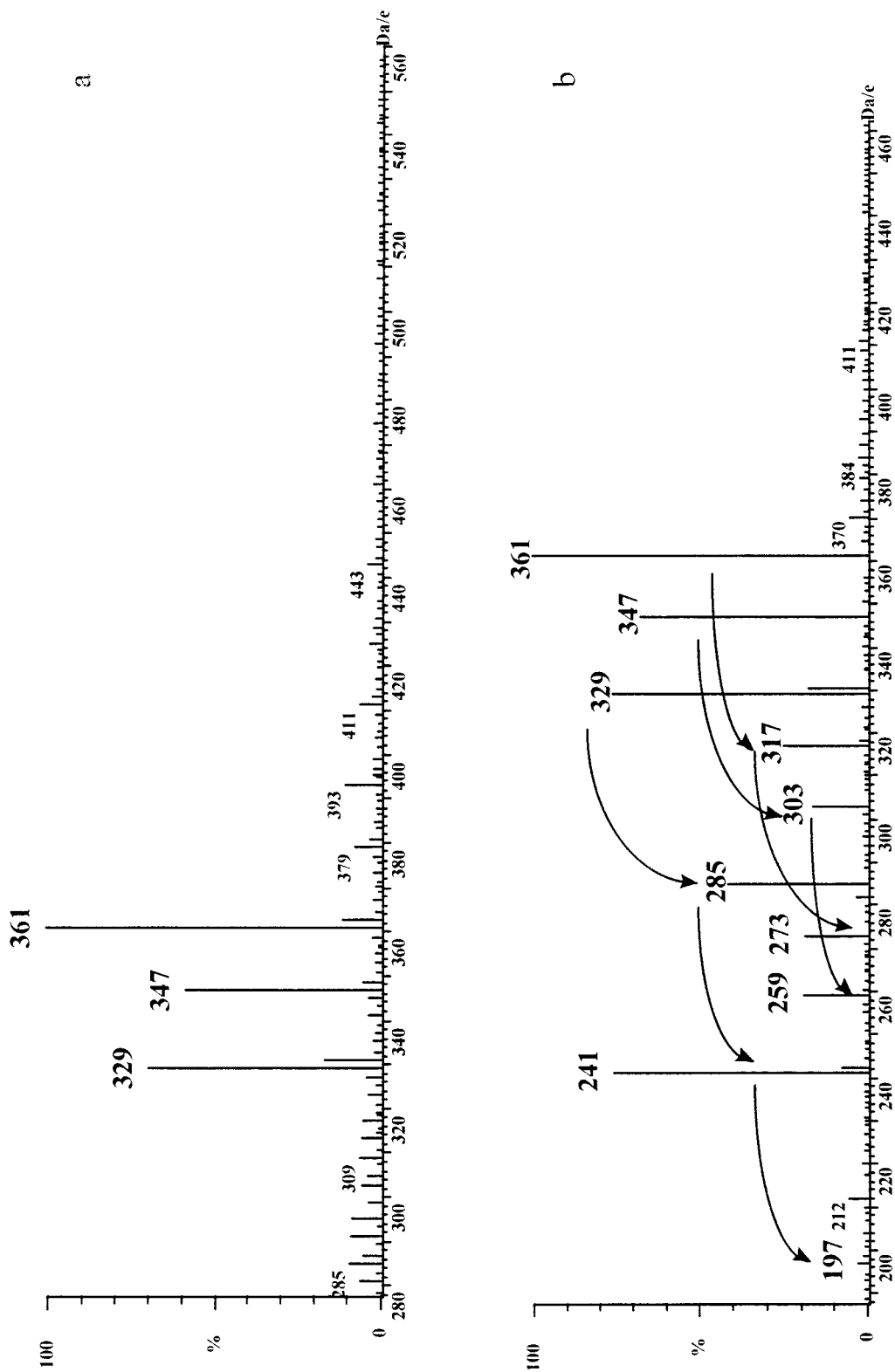


FIG. 2. (a) Negative-mode electrospray ionization mass spectrum of pyrroloquinoline quinone with a cone voltage setting of 30 eV and (b) negative-mode electrospray ionization mass spectrum of pyrroloquinoline quinone with a cone voltage setting of 40 eV.

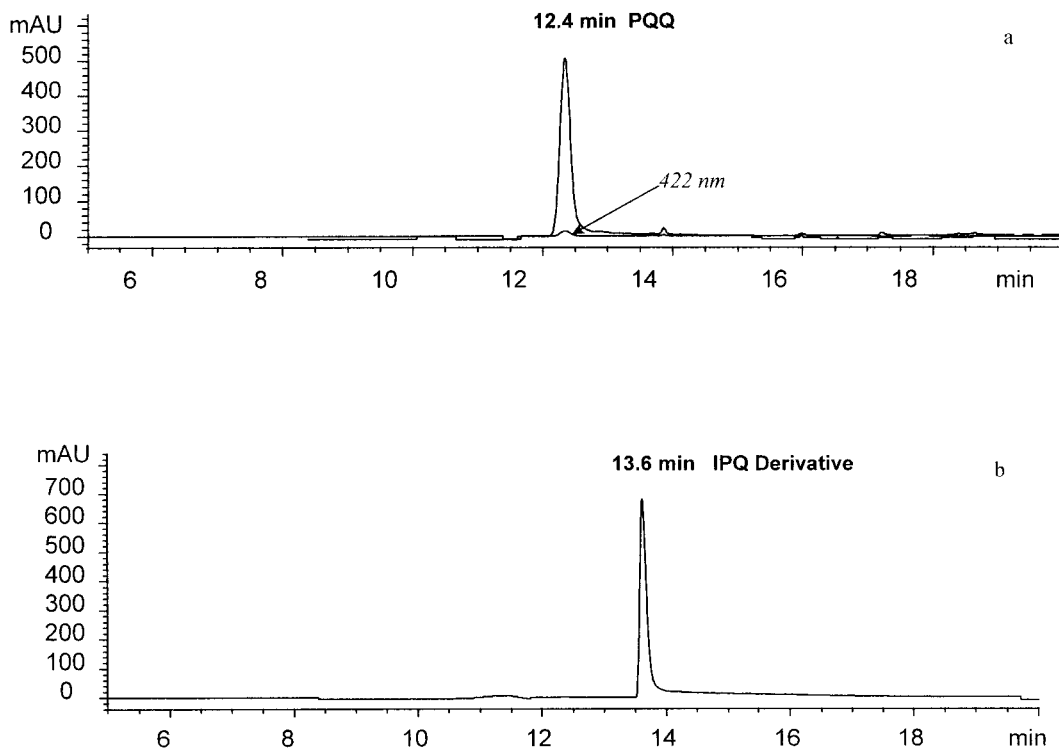


FIG. 3. Reverse-phase HPLC chromatogram of pyrroloquinoline quinone monitoring 422 and 249 nm and (b) reverse-phase HPLC chromatogram of pyrroloquinoline quinone incubated with the amino acid glycine monitoring 422 nm [IPQ derivative].

quently based on an enzyme assay that is unable to measure contributions from IPQ and IPQ/R derivatives (22), or on a sensitive redox cycling assay that lacks precision (16). Given the recent evidence that IPQ derivatives have redox and antioxidant activity as well as growth-promoting properties, a clear demonstration that PQQ and its associated derivatives exist in tissues has considerable importance. Earlier, GC/MS studies of PQQ added to biological samples, such as milk or serum, indicate that free PQQ disappears rapidly, and that the major product detected is an IPQ derivative (23, 24). These observations suggest that the preponderance of PQQ in tissues will be present as PQQ derivatives, and indicate a need for developing reliable methods for the detection of these derivatives in biological samples (9, 17). In the present study, we describe an improved diode-array reverse-phase HPLC method coupled to electrospray ionization mass spectrometry (ESI/MS), for rapid and reliable identification and quantification of PQQ, IPQ, and IPQ/R derivatives extracted from biological matrices. In addition, we show for the first time the presence of both PQQ and IPQ in human milk. The amount of PQQ and IPQ detected in milk samples is in excess of amounts shown previously to optimize growth in neonatal mice (>300 ng/g of milk solids) (15).

EXPERIMENTAL

Chemicals

PQQ, amino acids, and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile (ACN, Optima grade) was obtained from Fisher Scientific Corp. (Fair Lawn, NJ). All other chemicals used were of analytical grade.

In Vitro Formation of I/OPQ and IPQ/R Derivatives

I/OPQ derivatives were generated by incubating each amino acid with PQQ (1:5 mole ratio) under ambient atmosphere, in a 0.1 M phosphate buffer (pH 7.4). Incubations were allowed to proceed for 24 h at 25°C in the dark. Chemical reactions were stopped by freezing the samples to -80°C.

Reverse-Phase HPLC Separation of PQQ and I/OPQ and IPQ/R Derivatives

PQQ, IPQ, and IPQ/R derivatives were characterized using reverse-phase HPLC on a Phenomenex 5 μ Prodigy (250 cm \times 4.60 mm i.d., 10 nm pore size) C₁₈ column. Chromatographic analyses were carried out using a Hewlett-Packard series 1100 diode-array detector monitoring absorbance at 249 and 422 nm (the

TABLE 1

Condensation Products Formed upon Incubation of PQQ with the Amino Acids as Determined by Reverse-Phase HPLC and Negative-Mode ESI Mass Spectral Analysis

Amino acids ^a that form IPQ	% Yield (relative to PQQ total)	HPLC retention time (min)	Molecular mass ^b determined by ESI/MS	Amino acids ^a which form IPQ/R	% Yield (relative to PQQ total)	HPLC retention time (min)	Molecular mass ^b determined by ESI/MS	Amino acids ^a with no reaction
L-Alanine	7	13.6	341	L-Alanine	19	13.9	355	L-Proline
L-Aspartate	3	13.6	341	L-Arginine (-H ₂ O)	11	12.4	422	
L-Arginine	3	13.5	341	L-Asparagine	31	12.5	398	
L-Cysteine	5	13.6	341	L-Cystiene	6	12.8	387	
Glycine	98	13.6	341	L-Histidine	64	12.1	421	
L-Glutamate	4	13.6	341	L-Isoleucine	12	14.9	397	
L-Serine	67	13.6	341	L-Glutamine	13	13.8	412	
L-Threonine	64	13.6	341	L-Leucine	9	14.9	397	
L-Tryptophan	62	13.6	341	L-Lysine	5	14.4	409	
L-Tyrosine	54	13.6	341	L-Methionine	20	14.5	415	
				L-Phenylalanine	45	16.0	431	
				L-Serine	17	13.8	370	
				L-Tryptophan	37	15.9	470	
				L-Tyrosine	42	14.2	447	
				L-Valine	5	14.1	384	

^a Reactions of PQQ with amino acids (1:5 molar ratio) were carried out in a 0.1 M phosphate buffer (pH 7.4), for 24 h at 25°C in the dark. Chemical reactions were stopped by freezing the samples.

^b Molecular masses reported are the average of two separate determinations.

λ_{\max} for I/OPQ). PQQ and IPQ derivatives were separated using a flow rate of 1 ml/min with an analysis time of 25 minutes per sample. The initial mobile-phase composition was held at 5/95 ACN/water + 0.1% TFA (v/v) for 5 min followed by a linear gradient to 75:25 ACN:water + 0.1% TFA (v/v) at 25 min. Injections of 50 μ l were made using a Rheodyne injector. No memory effects were observed between samples in this concentration range. Fractions corresponding to each product were collected, concentrated under vacuum to dryness, and reconstituted in methanol:water (50:50, v/v) for ESI/MS analysis.

Mass Spectrometry

Molecular masses of reaction products were determined using a Quattro-BQ triple quadrupole mass spectrometer (VG Biotech, Altrincham, Greater Manchester, UK). The mobile phase was composed of methanol:water (50:50, v/v) delivered at 5 μ l/min by an Isco μ LC-500 syringe pump. Conjugates were analyzed by direct-flow injection of 10- μ l samples. Spectra were obtained in negative-ion mode with a capillary voltage -3.5 kV, with a source temperature of 80°C and a cone voltage of -50 V. Spectra were scanned over m/z 200-1000 at 20 s per scan and summed using the MCA acquisition mode in the Fisons Masslynx software. Mass calibrations were performed with oligomeric mixtures of polyethylene glycols with distributions centered at m/z 300, 600, and 1000. Extracts from milk

were characterized in positive-mode with a capillary voltage of 3.5 kV. ESI MS/MS experiments were conducted using argon as the collision gas at a measured pressure of 1×10^{-4} mbar. The collision cell was floated at -20 V.

Extraction of PQQ & IPQ from Human Milk

Prior to extractions all glassware was washed with 0.01 M HCl, MeOH and rinsed with nanopure water (3 \times). Human milk samples were obtained frozen from the San Jose Milk Bank (San Jose, CA). Samples were thawed on ice and combined. Two samples (200 ml) were extracted with equivalent amounts of hexane (3 \times) to remove lipids. Following hexane extractions, one sample was spiked with 1 ml of a 100 μ M PQQ solution (0.1 μ mol PQQ). Samples were dialyzed separately against 800 ml nanopure water at 4°C overnight. The dialyzable material was collected and run through pretreated solid-phase extraction columns (C₁₈, Varian) with the aid of vacuum. Extraction cartridges were pretreated by rinsing with 4 column volumes of 200 ml of 0.001 N HCl, followed by 100 ml of ACN + 0.1% acetic acid. This wash was followed by 100 ml nanopure water and another 100 ml of 0.001 N HCl. After the milk samples were loaded onto columns, the columns were washed with 200 ml of 0.001 N HCl in water. PQQ was eluted from columns with a 5% pyridine solution in nanopure water. Eluants were frozen and lyophilized for HPLC analysis. Relative percent-

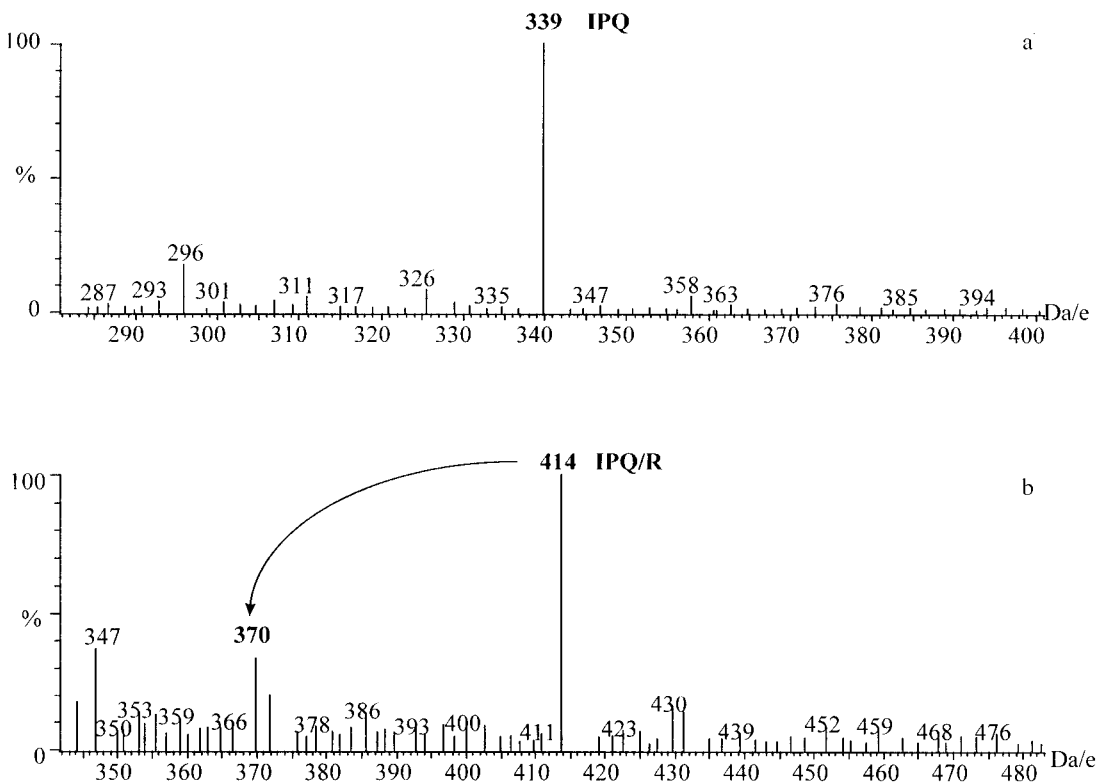


FIG. 4. Reverse-phase HPLC chromatogram of pyrroloquinoline quinone incubated with the amino acid tryptophan monitoring 422 nm and (b) reverse-phase HPLC chromatogram of pyrroloquinoline quinone incubated with the amino acid tyrosine monitoring 422 nm.

age yields of PQQ and IPQ were normalized to the peak area of riboflavin. Because riboflavin is naturally occurring in milk samples, and is extracted along with the PQQ it can be used as an internal standard.

RESULTS AND DISCUSSION

Conditions for ESI/MS analysis of PQQ were optimized using PQQ (1 $\mu\text{g}/\mu\text{l}$) dissolved in methanol:water (50:50, v/v). ESI/MS spectra indicate that PQQ ionizes as a mixture of PQQ with $[\text{M} - \text{H}]^-$ ions at m/z 329, the quinol PQQH₂ ($[\text{M} - \text{H}]^-$ ions at m/z 331), the PQQ hydrate ($[\text{M} - \text{H}]^-$ ions at m/z 347), and PQQ + 32 which gave $[\text{M} - \text{H}]^-$ ions at m/z 361 (Fig. 2a). Formation of the methyl hemiketal can account for the addition of 32 Da. Earlier studies indicate PQQ readily forms adducts with nucleophilic reagents such as methanol, aldehydes, ketones, urea, ammonia, and amines (19, 25, 26). These observations have encouraged the assumption that a covalent PQQ-substrate complex may be important in the reaction mechanism of bacterial methanol dehydrogenase. In the current studies, the hemiketal was not recovered when an HPLC separation step was carried out (to remove methanol) prior to ESI/MS analysis; indicating that

these additions are reversible. The C-5 carbonyl group that undergoes these reactions appears unusually reactive toward hard nucleophiles such as amino acids. Using semiempirical quantum mechanical algorithms (Chem3D Pro and MOPAC routines, CambridgeSoft) based upon the AM1 Hamiltonian, the theoretical structure (bond distances and angles) of PQQ was calculated. The resulting minimal energy configuration shows that the two quinone oxygens are bent out of the plane of the ring (dihedral angle of 39°), and that the carbons are bent out of the plane as well. The resulting bond angle strain facilitates addition of nucleophiles such as H₂O to the C-5 carbonyl group of PQQ.

Increasing the cone voltage of the mass spectrometer can produce fragmentation of labile bonds of compounds in the source region of the mass spectrometer. To help elucidate the site of hydration, we increased the cone voltage of the mass spectrometer over a range of 30 to 70 eV to induce fragmentation of PQQ during analysis. Mass spectra of the experiments show that PQQ, the hydrate, and the hemiketal decarboxylate at a cone voltages above 35 eV to give series of ions corresponding to consecutive losses of carboxyl groups (Fig. 2b). Decarboxylation results in a mass decrease of

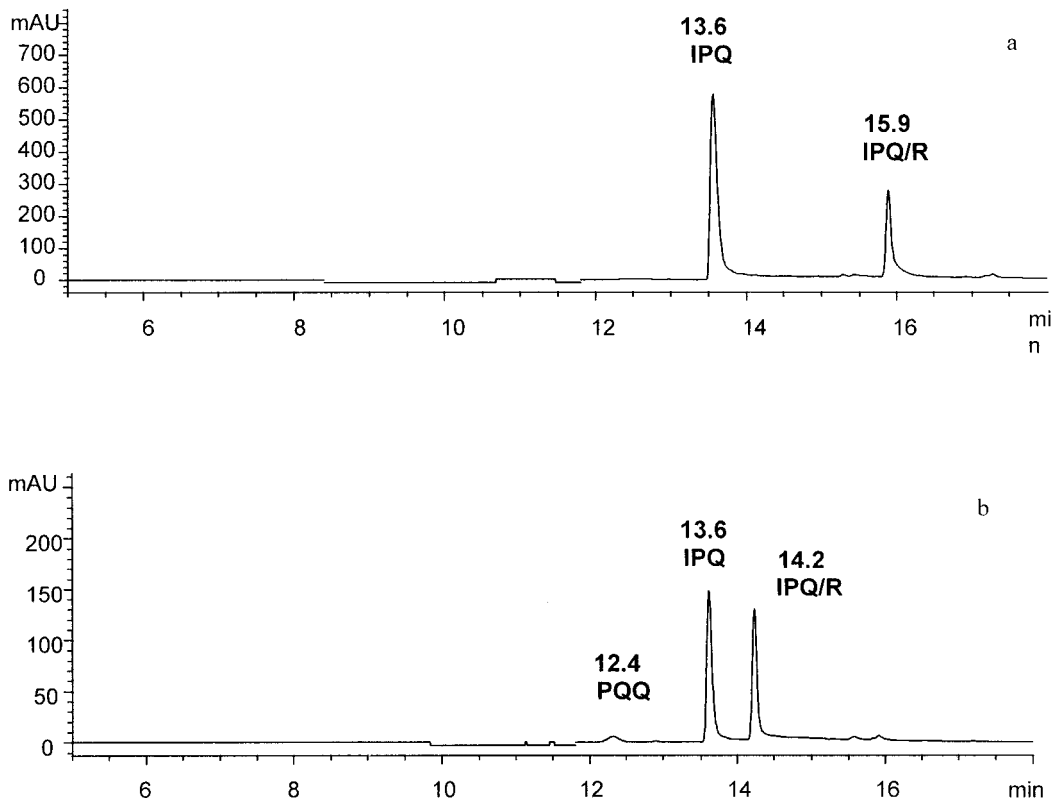


FIG. 5. Negative-mode electrospray ionization mass spectra of the IPQ conjugate formed upon incubation of PQQ with glycine m/z 340 Da and (b) negative-mode electrospray ionization mass spectra of the IPQ/R conjugate formed upon incubation of PQQ with methionine m/z 414 Da.

44 Da. Dissociation of PQQ at cone voltages above 40 eV results in the loss of all carboxyl groups and produces ions of m/z 285, 241, and 197. Complete decarboxylation was also observed with the hydrate and hemiketal at cone voltage setting greater than 40 eV. Dissociation of the hydrate results in ions of m/z 303, 259, and 215, whereas dissociation of the hemiketal produced ions of m/z 317, 303, and 259. Because higher cone voltages failed to induce the loss of water or methanol from the hydrate or methyl hemiketal, but induced decarboxylation of all carboxyl groups, we are able to conclude that the site of hydration is at the quinone, and that the hydrated forms of PQQ are stable species. The observations indicate that when performing mass spectral analyses of PQQ it is important to consider all ions in the mass spectrum which may arise from either the hydration of PQQ or decarboxylation of PQQ and PQQ hydrates.

Reverse-phase HPLC of PQQ standard incubated in phosphate (pH 7.4) buffer resolved one peak with a retention time of 12.4 min (Fig. 3a). Negative-mode ESI/MS analysis of fractions corresponding to this peak indicates that it is composed predominately of PQQ (m/z 329), the quinol PQQH₂ (m/z 331), and PQQ hydrate (m/z 347). In addition, several mass spectra

indicate that PQQ forms a minor product of dehydration with $[M - H]^-$ ions at m/z 311 (results not shown). This mass corresponds to formation of a stable lactam between the nitrogen at position 1 and the carboxyl group at position 9. Due to the complexity of the chemistry of PQQ and its ability to form hydrates, redox cycle, and dehydrate, we find that it is important to consider all ions in a mass spectrum when analyzing samples thought to contain PQQ.

Incubations of PQQ with glycine result in the formation of a yellow product with λ_{\max} at 422 nm and shift in the retention time to 13.6 min (Fig. 3b). Spectral changes in this peak correspond to formation of the IPQ derivative (17). ESI mass analysis of fractions corresponding to this peak indicates that it is IPQ with $[M - H]^-$ ions of m/z 340. Reverse-phase HPLC chromatograms of PQQ incubated with the amino acids indicated that at least 16 products are formed (Table 1). Ala, Asp, Cys, Gly, Glu, Ser, Thr, and Tyr form conjugates with λ_{\max} at 422 nm and HPLC retention times of 13.6 min. ESI mass spectra of HPLC peaks corresponding to these reaction products indicate that all are IPQ with $[M - H]^-$ ions of m/z 340 (Fig. 4a).

PQQ was most reactive toward Gly, yielding 98% of the IPQ product. All percentage yields reported herein

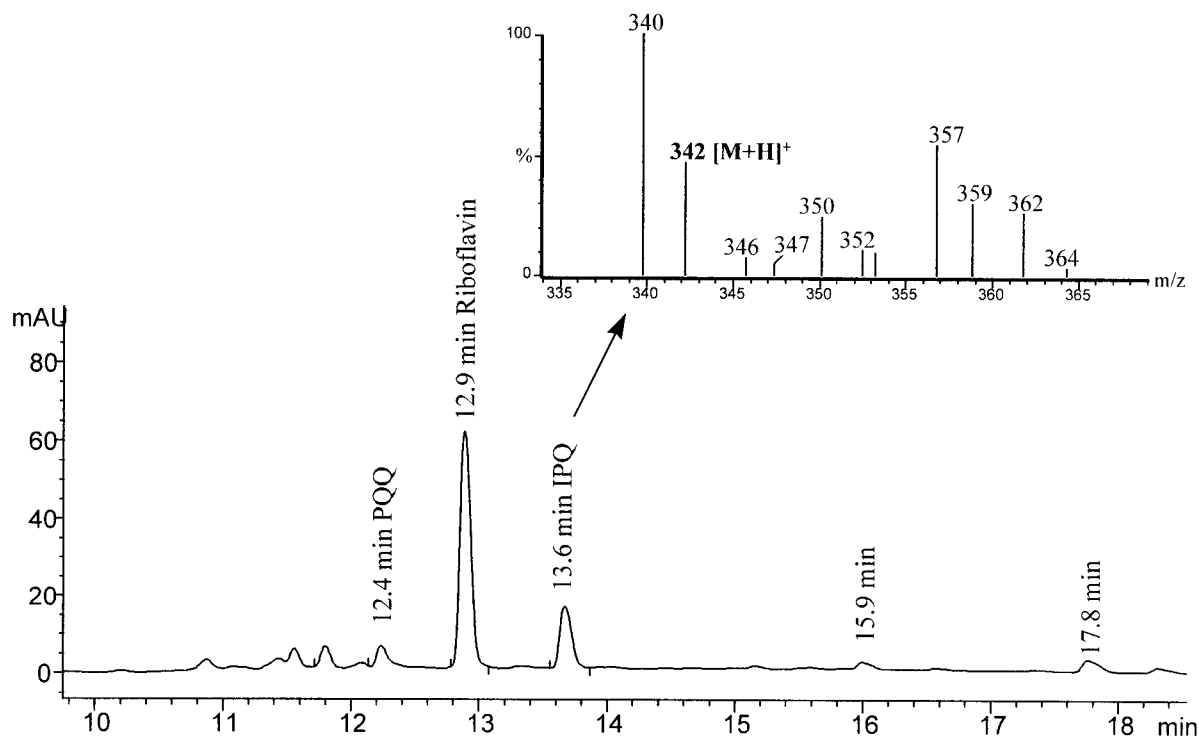


FIG. 6. Reverse-phase HPLC chromatogram of PQQ and IPQ extracted from human milk (200-ml sample).

are based on the HPLC peak area of the conjugate relative to the HPLC peak area of PQQ. Yields of IPQ were low (<5%) for Asp and Glu, yet relatively high (>60%) for Thr. PQQ formed both IPQ and IPQ/R derivatives upon incubation with Ala, Arg, Cys, Ser, Trp, and Tyr. IPQ/R derivatives were not observed in mass spectra of incubations of PQQ with either Asp or Glu; suggesting that the reactivity of PQQ toward these amino acids is low. The carboxyl groups of PQQ, Asp, and Glu are ionized at the pH of this reaction (pH 7.4). The low reactivity of PQQ with these amino acids may be due in part to charge repulsion between the carboxyl groups of the amino acids and PQQ or formation of intramolecular salt bridges between the carboxyl and amino groups.

PQQ forms both IPQ and IPQ/R derivatives upon incubation with Ala, Arg, Cys, Ser, Trp, and Tyr. The IPQ yields for Ala, Arg, Cys, Ser, Trp, and Tyr were 7, 3, 5, 67, 64, and 64%, respectively, whereas yields of the IPQ/R products were 19, 11, 6, 17, 37, and 42%, respectively. ESI mass spectra of His, Ile, Leu, Glu, Lys, Met, and Phe indicate that they form only the IPQ/R conjugates. The yields of IPQ/R products were 64, 12, 13, 9, 5, 20, and 45%, respectively. A representative ESI mass spectrum of PQQ incubated with a 5 M equivalent of Met can be found in Fig. 4b. Proline did not react with PQQ to give any products which is expected since Pro is a secondary amine which cannot

form the imine intermediate needed for condensation of an amino acid with PQQ.

Reverse-phase HPLC chromatograms for IPQ/R conjugates indicate that all are retained on the HPLC column and elute with retention times between 12.08 and 15.89 min. HPLC chromatograms of the IPQ/R derivatives of Trp and Tyr can be found in Figs 5a and 5b, respectively. As noted previously, Trp and Tyr form IPQ (13.6 min), as well as the IPQ/R derivative (15.89 min for Trp and 14.23 min for Tyr).

HPLC chromatograms of human milk samples spiked with 33 μg of PQQ indicate that spiked PQQ is recoverable as the IPQ derivative with a λ_{max} at 422 nm and HPLC retention time of 13.6 min. HPLC chromatograms of human milk (not spiked) demonstrate the presence of both PQQ and the IPQ derivative (Fig. 6). Fractions corresponding to these peaks were collected and analyzed by positive-mode ESI mass spectrometry. ESI mass spectra of the HPLC peak with a retention time of 12.4 min indicate that it is PQQ with $[M + H]^+$ ions at m/z 331 (data not shown). ESI mass spectra of the HPLC peak with a retention time of 13.6 min indicate that it is IPQ with $[M + H]^+$ ions at m/z 342 (inset to Fig. 6). Riboflavin is present at constant levels in milk; therefore, the sum of the peak areas for PQQ and IPQ was normalized to the peak area for riboflavin. Based on this response factor, we calculated the combined level of PQQ and IPQ in human milk to be 140–180 ng/ml ($\sim 0.5 \mu\text{M}$). This value for the

combined products of PQQ and IPQ is higher than earlier values reported for free PQQ in human milk (33 ng/ml) and demonstrates the need to consider both PQQ and IPQ products when quantifying levels of PQQ in biological samples (3).

That PQQ is present in milk in the range of 140–180 ng/ml may have physiological importance. On a milk solid basis, these values would correspond to 500 to 750 ng/g. In experimental animal models of mice fed chemically defined diets, approximately 300 ng/g of diet PQQ optimized neonatal growth and reproduction (15). PQQ in such diets is undoubtedly present as PQQ and IPQ derivatives. In general, mice fed chemically defined diets usually grow slower and have fewer pups than mice fed chemically defined, water-soluble diets (27). When PQQ is added at greater than 300 ng/g to an amino-based diet, reproductive outcome and growth are similar to those of mice fed stock diets. Moreover, the splenic cell response to mitogens, e.g., concanavalin A, or lipopolysaccharide is influenced by PQQ intake as is interleukin 1 and 2 production (15).

Over the past decade there has been considerable controversy as to whether PQQ has a definable role in eukaryotic organisms. Although PQQ and IPQ elicit important physiological responses, mechanistic features are not clearly defined, nor have the levels of PQQ or its derivatives been convincingly established in tissues or fluids. In this regard, establishing that micromolar amounts of PQQ and associated derivative are in milk is an important contribution. Concentrations of this magnitude, particularly in view of the growth promoting features of PQQ, suggest that additional work should be directed at the sources of PQQ in biological fluids and whether PQQ serves an unique biological role.

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