Specific and rapid analysis of ubiquinones using Craven's reaction and HPLC with postcolumn derivatization

Haruo Shimada,[1,*] David Kodjabachian,[†] and Masami Ishida[*]

Frontier Science Laboratories,* H&BC Development Center, Shiseido Co., Ltd., Kanazawa, Yokohama 236-8643, Japan; and Ecole Nationale Supérieure de Chimie de Paris,† 75231 Paris Cedex 05, France

Abstract A new method for the analysis of ubiquinones in various samples was developed using an HPLC system with postcolumn derivatization. Craven's reaction, a specific color reaction for the analysis of ubiquinones, was used in the system. Because the reaction progressed in organic solvents that contained ubiquinones and ethylcyanoacetate under an alkaline condition, the selectivity for ubiquinone detection was higher than that for ubiquinone detection using the nonderivatized ultraviolet detection system at 275 nm, a system widely used for the analysis of ubiquinones. The new detection system can avoid the adverse effects of impurities. Furthermore, it can confirm specificity by stopping the color reaction under a neutral condition. The detection limit for ubiquinone-10 was 1 ng (1.2 pmol). A good linearity for the calibration curve was observed in the range of 11.7 pmol to 11.7 nmol. To investigate the possible application of this method, various samples, such as soybean capsules used as a dietary supplement and biological materials (rice as well as bovine plasma and liver samples), were applied to the system and their ubiquinone contents were quantified. This method is thought to be widely and conveniently applicable for determining the level of ubiquinones because of its high selectivity for ubiquinone detection.—Shimada, H., D. Kodjabachian, and M. Ishida. **Specific and rapid analysis of ubiquinones using Craven's reaction and HPLC with postcolumn derivatization.** ***J. Lipid Res.*** **2007.** 48: **2079–2085.**

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Ubiquinone, also known as coenzyme Q, belongs to the fat-soluble component in aerobic organisms and is widely distributed in nature with varying lengths of the side chain isoprene units (1, 2). Ubiquinone is important as an electron carrier in the mitochondrial electron transport system (1, 3, 4) and is useful for producing ATP. Ubiquinone-10, which has 10 isoprene units, is particularly important because it is found in the human mitochondrial respiratory chain. Ubiquinones are indispensable components for the maintenance of normal cellular functions, and their other biological functions, such as antioxidant functions, have been discussed (5–13). Rosenfeldt et al. (14) concluded that ubiquinone-10 administration improves the recovery of the mitochondrion and cardiac myocyte from stress. Thus, recently, various products containing ubiquinone-10 have been widely distributed, namely, foods for promoting body function, dietary supplements, cosmetics, and pharmaceutical products.

The general methods for the determination of the amount of ubiquinones in a substance are HPLC with ultraviolet detection (15–18) and HPLC with electrochemical detection (19–23). Electrochemical detection is used for high-sensitivity detection, whereas ultraviolet detection is most widely used because of its ease of application. The appropriate ubiquinone detection method should be selected depending on the purpose of the analysis. Other methods for ubiquinone analysis have also been studied (24–27). However, in some cases, much of the time involved in carrying out ubiquinone analysis is spent avoiding the adverse effects of impurities. Thus, the specificity of the detection method is important for the quantification of ubiquinones. Although there have been many opportunities for the analysis of various samples containing ubiquinones (e.g., ubiquinone-containing products and biological materials), the development of a widely usable analysis method for ubiquinones has been difficult. Various products and biological materials may contain many impurities that interfere with ubiquinone detection, and the contents of ubiquinones in various samples also differ. To overcome these constraints, we have applied a highly selective detection system for ubiquinones using an HPLC separation system and developed a new method of ubiquinone analysis for various products and biological materials.

Craven's reaction, a specific color reaction for quinones, was first reported by Craven in 1931 (28) and has been applied to the quantitative determination of ubiquinones (26, 27). This color reaction produces a blue product by

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Abbreviations: RSD, relative standard deviation.

[1] To whom correspondence should be addressed.
e-mail: haruo.shimada@to.shiseido.co.jp

displacing the methoxyl moieties of ubiquinones to active methylene compounds such as ethylcyanoacetate under an alkaline condition (29, 30). However, it is difficult to quantify the exact amount of ubiquinone accurately, because the blue product is not stable in the reaction mixture. Blue changes to green in several minutes (29). Moreover, Craven's reaction cannot distinguish specific ubiquinone species. To overcome these problems, Craven's reaction was applied to an HPLC postcolumn on-line system. The color reaction is fixed because it can be set to a suitable time in the reaction tube (the reaction time is dependent on the tube volume). Ubiquinone species, such as ubiquinone-9 and ubiquinone-10, can also be separated using an analytical column and distinguished by determining the retention time of each species. To assemble the HPLC ubiquinone detection system, expensive devices are not needed. To investigate the possible application of this method, various samples containing ubiquinones were tested using the system and their ubiquinone contents were quantified accurately.

Ubiquinone-10-containing capsules are widely distributed in the health food industry as a dietary supplement. However, detailed information on the contents of each product is not open to the public. Moreover, it is difficult to obtain a placebo sample, which is useful for validating the analytical method. Accordingly, we developed a ubiquinone-10-containing soybean capsule and a placebo sample using the methods of Andersson (16). Samples obtained from a soybean capsule were tested using the system, and the accuracy and precision of the new method were investigated.

To determine the ubiquinone content of biological materials, rice samples at two polishing stages were used as food samples, and bovine plasma and liver samples were used as mammalian samples. In this study, we aim to develop a specific method to accurately determine the ubiquinone content of various samples, such as dietary supplements and biological materials.

MATERIALS AND METHODS

Chemicals

HPLC-grade methanol, ethanol, chloroform, and hexane as well as ethylcyanoacetate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of analytical grade. Bovine plasma and liver samples were purchased from Funakoshi Co., Ltd. (Tokyo, Japan), and Tokyo Shibaura Zouki Co., Ltd. (Tokyo, Japan), respectively. The polished rice and brown rice (Koshihikari) were generous gifts from Mr. T. Ikeda.

HPLC apparatus and construction of the postcolumn derivatization system

The postcolumn derivatization system developed is shown schematically in **Fig. 1**. LC-10ADvp (Shimadzu Co., Ltd., Kyoto, Japan) and Nanospace SI-1 (Shiseido Co., Ltd., Tokyo, Japan) pumps were used for pumps A and B, respectively. An SIL-10ADvp autoinjector (Shimadzu) and an SPD-M20A diode array spectrophotometer (Shimadzu) were also used in the system. A postcolumn reaction was performed on a 50 μl mixer and a reaction coil (0.25 mm inner diameter × 7.5 m; Shimadzu). A regulator was used to stabilize the pressure of pump B (pressure was adjusted to 3.5 MPa). A CTO-10ADvp column oven (Shimadzu) was used to heat the analytical column and reaction coil at 45°C. A Capcellpak C18 UG 120A column (4.6 mm inner diameter × 750 mm, 3 μm particle size; Shiseido) was attached to the front of the mixer. The mobile phase, supplied by pump A, was composed of 100 mM ethylcyanoacetate in ethanol-methanol (1:1, v/v) at a flow rate of 0.4 ml/min. The reaction solution, supplied by pump B, was composed of 0.4 M potassium hydroxide in distilled water-ethanol (3:2, v/v) at a flow rate of 0.06 ml/min. Twenty microliters of a sample was applied to the system, and the chromatogram was plotted at an absorption frequency of 635 nm.

Characterization of the method

To confirm the effect of Craven's reaction, 234 pmol (202 ng) of ubiquinone-10 (**Table 1**) was applied to the system (reaction condition). Subsequently, the sample was reanalyzed using water-ethanol (3:2, v/v) instead of the reaction solution (nonreaction condition).

To investigate the detection limit and dynamic range for ubiquinone quantification, 1 mg/ml ubiquinone-10 was prepared in ethanol-*n*-hexane (95:5, v/v), and the solution was diluted to a suitable concentration from 0.03 to 504 μg/ml with the solution from the mobile phase, as shown in Table 1. The possibility for ubiquinone quantification was considered by determining the linearity of the calibration curve.

To investigate repeatability, 117 pmol (101 ng) of ubiquinone-10 was applied repeatedly six times to the system.

Extraction of ubiquinone-10 from the soybean capsule

A soybean capsule was prepared as follows. Solutions of 20, 100, and 200 mg/g ubiquinone-10 were prepared using soybean

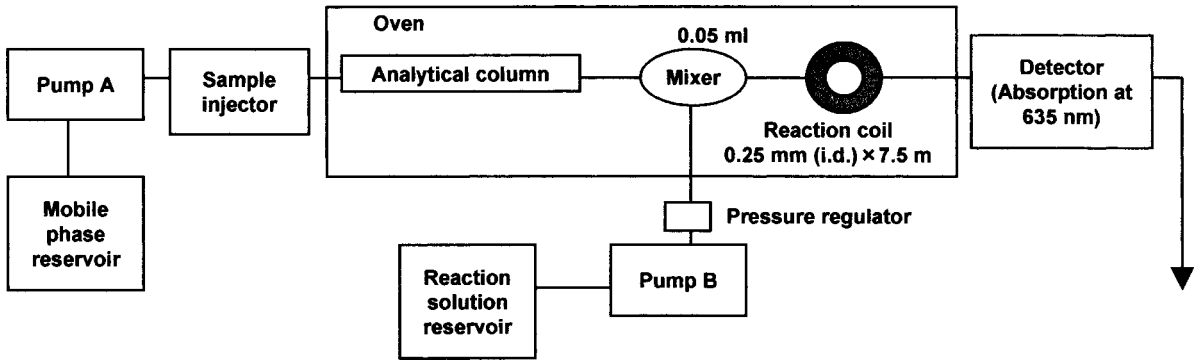

Fig. 1. HPLC postcolumn derivatization system for ubiquinone analysis using Craven's reaction.

TABLE 1. Standard preparation and amounts of sample injected

Ubiquinone-10	Amount Injected (20 μl)		Peak Area
μg/ml	*ng*	*pmol*	
0.0302	0.605	0.701	ND
0.0504	1.01	1.17	216
0.0756	1.51	1.75	410
0.101	2.02	2.34	486
0.202	4.03	4.67	895
0.504	10.1	11.7	2,215
1.01	20.2	23.4	4,710
2.02	40.3	46.7	9,389
5.04	101	117	23,920
10.1	202	234	47,390
20.2	403	467	95,912
50.4	1,008	1,168	240,433
101	2,016	2,335	482,532
202	4,032	4,670	969,282
504	10,080	11,675	2,447,005

oil warmed at 45°C. Aliquots of 0.3 g of solutions containing 6, 30, and 60 mg of ubiquinone-10 were placed into hard gelatin capsules, and the capsules were preserved in a container covered with aluminum foil. For a placebo sample, 0.3 g of soybean oil was placed into a hard gelatin capsule.

The capsule was opened and 50 ml of the *n*-hexane was poured into a volumetric flask using a funnel. Two and one-half milliliters of the solution was taken and diluted to a final volume of 50 ml with 1.2 mM $FeCl_3$ in ethanol-methanol (1:1, v/v). After allowing ubiquinol oxidation to occur (2 h), 20 μl of each sample was applied to the system. For ubiquinone quantification, standard solutions of 5, 15, 30, 50, and 75 μg/ml ubiquione-10 were also prepared.

Extraction of ubiquinones from rice samples

Approximately 1 g each of brown rice and polished rice samples was ground to fine flour on a mortar. Two-tenths of a gram of each sample was transferred to a test tube for centrifugation. The samples were suspended in 2 ml of methanol-1-propanol-*n*-hexane (3:2:1, v/v/v) and ultrasonicated for 3 min. The mixtures were then centrifuged at 2,500 rpm for 5 min, and the supernatant of each sample was collected. The residues were reextracted by adding 2 ml of methanol-1-propanol-*n*-hexane (3:2:1, v/v/v). The supernatants were evaporated to remove the organic solvent and dissolved with 0.1 ml of 1.2 mM $FeCl_3$ in ethanol-methanol (1:1, v/v). After allowing ubiquinol oxidation to occur (2 h), 20 μl of each sample was applied to the system. For ubiquinone quantification, standard solutions of 0.5, 2, 4, 6, and 10 μg/ml ubiquione-10 were also prepared.

Extraction of ubiquinones from bovine plasma

The procedure for extracting ubiquinones from bovine plasma was adapted from the method of Yamashita and Yamamoto (19). The recovery rates for ubiquinol-10 and ubiquinone-10 using the procedure were 102 ± 2% and 104 ± 4%, respectively (19). Bovine plasma (0.5 ml) was pipetted into a test tube, and then methanol (2.5 ml) and *n*-hexane (5 ml) were added to the test tube. The mixture was then shaken for 10 min and centrifuged at 2,500 rpm for 5 min. The upper layer (*n*-hexane layer) was collected. The lower layer was then subjected to reextraction two times by adding 2 ml of *n*-hexane. Subsequently, the *n*-hexane layer mixture was evaporated to remove *n*-hexane and dissolved with 0.1 ml of 1.2 mM $FeCl_3$ in ethanol-methanol (1:1, v/v). After allowing ubiquinol oxidation to occur (2 h), 20 μl of each sample was applied to the system. For the quantification, the calibration curve used for the quantification of rice samples was used because it was prepared within 1 day.

Extraction of ubiquinones from bovine liver

Bovine liver (0.04 g) was homogenized in 0.1 ml of 200 mM potassium phosphate buffer (pH 7.0). One milliliter of methanol and 2 ml of *n*-hexane were added to the homogenized liver, and the mixture was shaken for 10 min. The mixture was then centrifuged at 2,500 rpm for 5 min. The upper layer (*n*-hexane layer) of the mixture was collected. The lower layer of the mixture was then subjected to reextraction by adding 2 ml of *n*-hexane. Subsequently, the *n*-hexane layer mixture was evaporated to remove *n*-hexane and dissolved with 0.2 ml of 1.2 mM $FeCl_3$ in ethanol-methanol (1:1, v/v). After allowing ubiquinol oxidation to occur (2 h), 20 μl of each sample was applied to the system. For the quantification, the calibration curve used for the quantification of rice samples was used because it was prepared within 1 day.

RESULTS AND DISCUSSION

Characterization of the method

The theoretical reaction time (0.91 min) was calculated from the flow rate (those for pumps A and B were 0.4 and 0.06 ml/min, respectively) and volumes of the reaction coil (368 μl) and mixer (50 μl). The effect of Craven's reaction on the HPLC postcolumn derivatization system was confirmed by analyzing the content of ubiquinone-10 under the reaction condition, in which potassium hydroxide (0.4 M) in water-ethanol (3:2, v/v) was used as the pump B solution (**Fig. 2A**), and the nonreaction condition, wherein potassium hydroxide was removed from the solution (Fig. 2B). Because one peak (11.5 min) was observed in the chromatogram under the reaction condition and no peak was observed in the chromatogram under the nonreaction condition, derivatization was thought to be achieved using the system. The wavelength for the ubiquinone detection was determined to be 635 nm from the spectrum of the peak at 11.5 min (Fig. 2C). To investigate the dynamic range of ubiquinone concentration detection, solutions with a wide variety of ubiquinone-10 concentrations were prepared (Table 1). The detection limit, based on a signal-to-noise ratio of 3:1, was 1.2 pmol (1 ng). Because the lower limit of quantification, based on a signal-to-noise ratio of 10:1 [with an accuracy of 106.4% and a relative standard deviation (RSD) of 2.54%; n = 6], was 11.7 pmol (10 ng), the calibration curve was plotted using an injection amount from 11.7 pmol (10 ng) to 11.7 nmol (10 μg), as shown in Table 1 ($y = 209.47x - 2{,}319$; $r^2 = 1.0000$). The correlation coefficient ($r^2 = 1.0000$) indicates that the calibration curve is linear over the concentration range. The RSD of the standard solution at an injection amount of 117 pmol obtained from six experiments was 0.46% (the average of the peak area was 23,517).

Analysis of ubiquinone-10 from the soybean capsule

Soybean capsules containing ubiquinone-10 are widely distributed in the health food industry as a dietary supplement. **Figure 3A**, C show the chromatograms of the

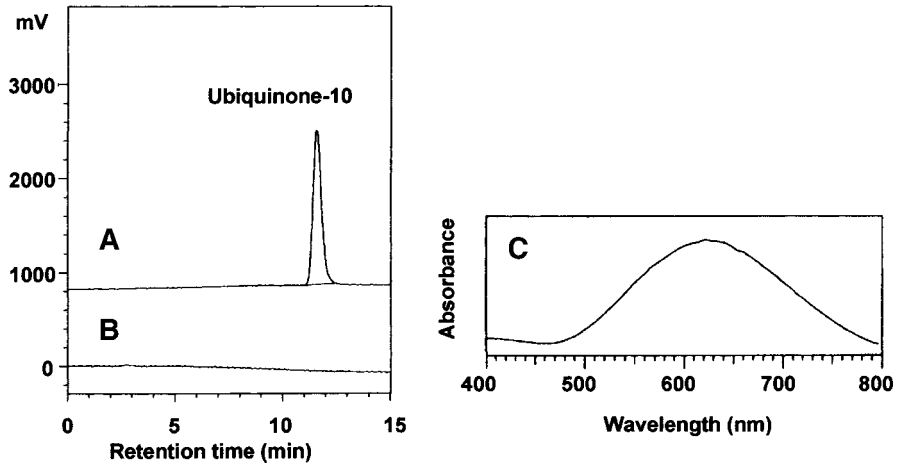

Fig. 2. Typical chromatograms of ubiquinone-10 by the HPLC postcolumn derivatization system. The HPLC conditions are described in Materials and Methods. A standard solution of ubiquinone-10 (234 pmol) was analyzed under two conditions. A: Reaction condition. KOH (0.4 M) in distilled water-ethanol (3:2, v/v) was used as the pump B solution. B: Nonreaction condition. Distilled water-ethanol (3:2, v/v) was used as the pump B solution. C: Absorption spectrum of the peak at 11.5 min (ubiquinone-10).

ubiquinone-10 contained in a soybean capsule under the reaction and nonreaction conditions, respectively. Figure 3B shows the chromatogram of a placebo sample under the reaction condition. Because no peaks were observed in the chromatogram of the placebo sample (Fig. 3B) under the reaction condition or in the chromatogram of the ubiquinone-10-containing capsule under the nonreaction condition (Fig. 3C), it was considered that no impurity affecting ubiquinone detection was present in the capsule. The chromatograms obtained from the removal of the analytical column are shown in Fig. 3D–F. No peak was observed when the nonreaction solution was used (Fig. 3F) or when the placebo sample was analyzed (Fig. 3E). The intraday recovery and RSD results obtained using the soybean capsule samples with the analytical column are shown in **Table 2**. The recovery and RSD results obtained at each known concentration of ubiquinone-10 were quite good (99.1–100.2% and 0.25–1.24%, respectively). Interday recovery and repeatability studies were carried out for 3 days using the 30 mg/capsule samples (five experiments for each day). The interday recovery and RSD results were also quite good (99.80% and 0.52%, respectively). These results indicate that the new method can be used to accurately quantify the ubiquinone.

The recovery test results obtained from the ubiquinone quantification at 30 mg/capsule with and without the analytical column were similar (99.9 ± 0.30% and 100.7 ± 0.59%, respectively). The calibration curves plotted using

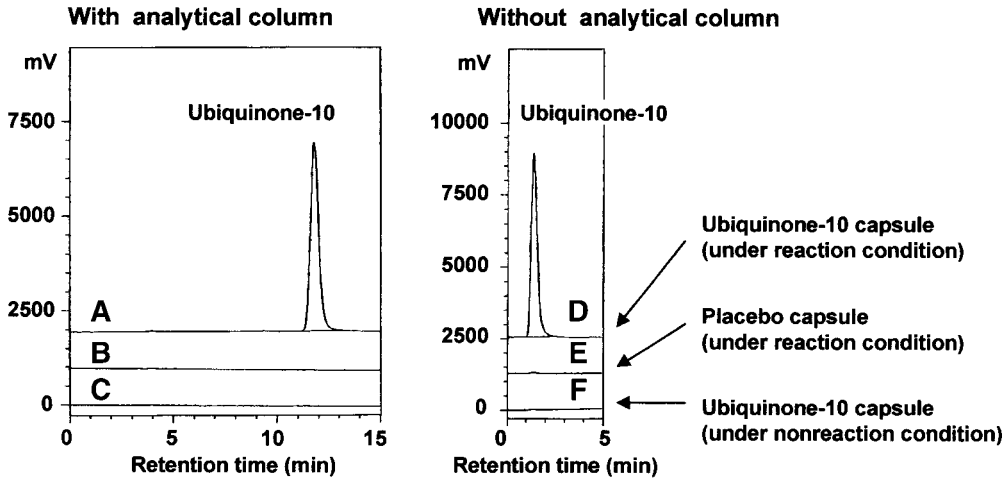

Fig. 3. Chromatograms of soybean capsules. Aliquots of the capsule samples (samples A, C, D, and F are all ubiquinone-10 capsules, and samples B and E are placebo capsules) were applied to the system under the reaction condition (samples A, B, D, and E) and the nonreaction condition (samples C and F). The left panel (samples A–C) shows the chromatograms obtained from the analysis using the analytical column. The right panel (samples D–F) shows the chromatograms obtained from the analysis carried out without using the analytical column.

TABLE 2. Intraday recovery and precision studies on soybean capsules (n = 5)

Ubiquinone-10 Amount in Capsule	Concentration Added	Concentration Measured	Amount Injected (20 μl)	Recovery	RSD
mg/capsule	*mg/g*		*ng*	*%*	
6	19.98	19.80	119	99.1	1.24
30	99.98	99.89	602	99.9	0.30
60	199.95	200.43	1,208	100.2	0.25

results obtained with and without the analytical column were both linear ($y = 205.0x + 27.9$, $r^2 = 1.0000$ and $y = 210.1x - 1221.7$, $r^2 = 1.0000$, respectively). In the case of the analysis of ubiquinone-10 in the capsule, there was no need for column separation. Thus, ubiquinone quantification can be performed rapidly without the analytical column (within 3 min). Because various ubiquinone-10-containing products have been distributed on the market recently, the use of the analytical column may depend on the composition of each product. Although specificity should be confirmed before ubiquinone quantification, specificity can be achieved easily using the nonreaction solution. This is a unique feature of the postcolumn derivatization method.

Application to rice samples

Rice is the staple energy source of many people. Ubiquinone-9 is the major quinone component of rice, and ubiquinone-10 is the minor component (31). Although only ubiquinone-10 is used for the human respiratory chain in mitochondria, ubiquinone-9 may also be important as an antioxidant (32). Polished rice is the final product of rice cleaning, and brown rice, which is obtained by the removal of the coarse and rather loose husk, is the intermediate product in the first step of rice cleaning. Whether ubiquinones are removed in the cleaning processes has been unclear. The extracted samples from polished rice and brown rice were applied to the system. The total amount of ubiquinone-10 (oxidized and reduced forms) can be determined by adding $FeCl_3$ to the sample solution. **Figure 4A–D** show the chromatograms of the two rice samples. The elution times for ubiquinone-9 and ubiquinone-10 were 9.3 and 11.5 min, respectively. Although ubiquinone-9 was the major quinone component of each sample, a small amount of ubiquinone-10 was also contained in each sample. It was confirmed that no interfering impurity was present in these rice samples, because no interfering peaks were observed in their chromatograms under the nonreaction condition (Fig. 4B, D).

To confirm the efficiency of our extraction method, the residues remaining after the polished rice and brown rice extractions were reextracted carefully using the same procedure and analyzed using the new system. No peak was observed in any of the chromatograms of the reextraction samples. Thus, the extraction is considered to be efficient. The calibration curve obtained from the ubiquinone quantification was also linear ($y = 201.7x + 26.5$, $r^2 = 0.9998$). The amounts of ubiquinone-9 and ubiquinone-10 in the two rice samples are shown in **Table 3**. The RSDs of ubiquinone-9, the dominant ubiquinone of rice, for polished rice and brown rice were 2.1% and 1.1%, respectively. The extraction efficiency was constant. The amounts of ubiquinones decrease as a result of rice cleaning. In the case of the conventional condition (ultraviolet detection at 275 nm), the amount of each ubiquinone was not determined owing to the many impurities present, and an analysis time of >20 min may be needed to avoid contamination in the next analysis (**Fig. 5**).

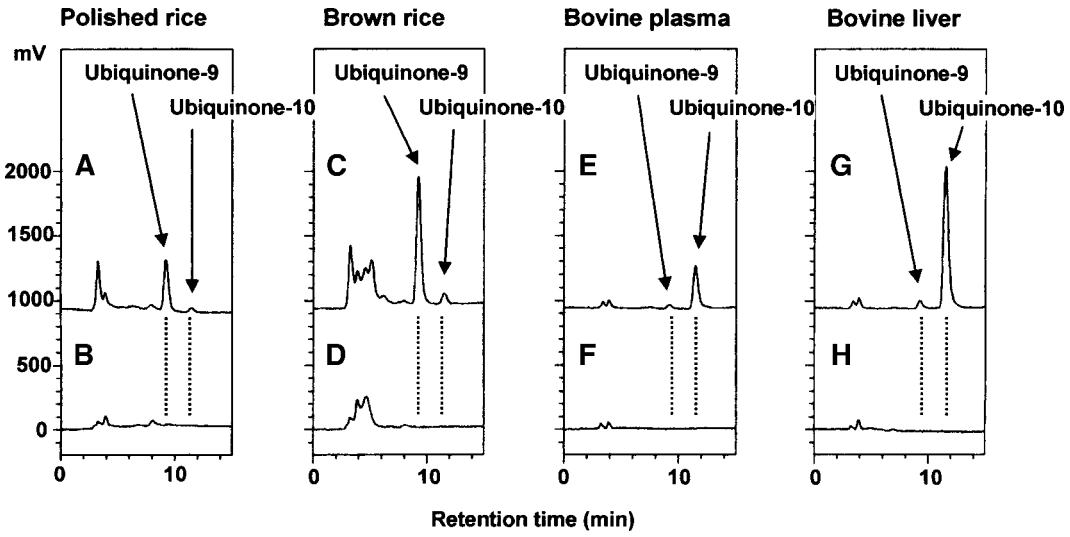

Fig. 4. Chromatograms of biological materials (samples A and B, polished rice; samples C and D, brown rice; samples E and F, bovine plasma; samples G and H, bovine liver) obtained using the HPLC postcolumn derivatization system. The upper sections (samples A, C, E, and G) show the chromatograms obtained from the analysis under the reaction condition. The lower sections (samples B, D, F, and H) show the chromatograms obtained from the analysis under the nonreaction condition.

TABLE 3. Ubiquinone amounts in biological materials

Quinone	Rice		Bovine	
	Polished	Brown	Plasma	Liver
Ubiquinone-9	2.84 ± 0.06 (2.1)	7.03 ± 0.08 (1.1)	0.040 ± 0.01 (13.5)	2.20 ± 0.19 (8.5)
Ubiquinone-10	0.22 ± 0.02 (7.7)	0.60 ± 0.03 (5.6)	0.470 ± 0.01 (1.9)	43.75 ± 1.94 (4.4)

Values indicate means (nmol/g for rice and bovine liver samples, nmol/ml for bovine plasma samples) ± SD and RSD (%) of three samples.

Application to bovine plasma and liver samples

Ubiquinone analysis of the bovine plasma and liver samples was performed using the system. The chromatograms of the bovine plasma and liver samples are shown in Fig. 4E–H. An impurity peak was not shown by these samples because no interference peak was observed in their chromatograms under the nonreaction condition (Fig. 4F, H). Because no ubiquinone peaks were observed in the chromatograms of the reextraction samples obtained from the residues remaining after the extraction of bovine plasma and liver samples, the extraction is considered to be efficient. The total amounts of ubiquinone-9 and ubiquinone-10 in the samples are shown in Table 3. The RSDs of ubiquinone-10, the dominant ubiquinone of bovine samples, for bovine plasma and liver samples were 1.9% and 4.4%, respectively. The extraction efficiency was constant. It was difficult to determine the amounts of ubiquinones by ultraviolet detection at 275 nm because of the impurities present (data not shown).

The postcolumn derivatization system is effective for determining the total amounts of ubiquinones without incurring much expense. Because of the high selectivity of the system, various products, namely, the soybean capsule and food and animal biomaterials, were analyzed under the same HPLC analysis condition, and the amount of each ubiquinone was quantified accurately. Many ubiquinone-10-containing products contain only ubiquinone-10 as their quinone component. If the impurities that interfere with the quantification are not contained in these products, there is no need to separate the quinone components. Thus, for these products, the use of an analytical column is not needed and the analysis time can be decreased to 3 min. This method may be advantageous for the simple determination of the total amount of ubiquinone in various products and foods.

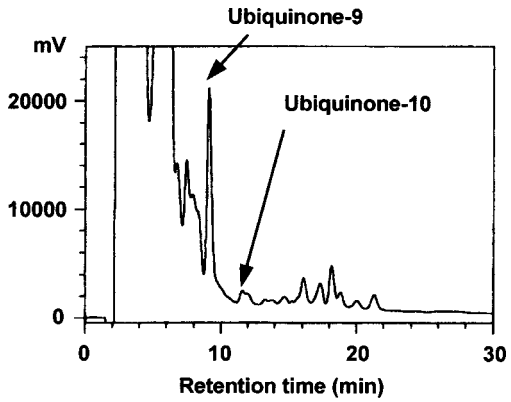

Fig. 5. Chromatograms of brown rice using the conventional ultraviolet detection method. The HPLC condition used was as follows: column, Capcellpak C18 UG 120A (4.6 mm inner diameter × 750 mm, 3 μm particle size, 45°C); mobile phase, 100 mM ethylcyanoacetate in methanol-ethanol (1:1, v/v); flow rate, 0.4 ml/min; detection absorbance, 275 nm.

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