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Determination of Coenzyme Q10 and Q9 in Vegetable Oils

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A new sensitive and selective method has been developed for the quantification of the total coenzyme Q9 (CoQ9) and coenzyme Q10 (CoQ10) concentration in vegetable oil samples. The coenzyme Q fraction is isolated by solid-phase extraction (SPE) on amino phase eluting with a mixture of heptane:ethyl ether. The organic solvent is evaporated under nitrogen, and the residue is dissolved in a mixture of acetonitrile:tetrahydrofuran and finally is analyzed by reverse-phase high-performance liquid chromatography with a mass detector. The sensitivity of the method is based on the high efficient formation of the radical anions [$M^{-\bullet}$] of CoQ9 and CoQ10 by negative atmospheric pressure ionization. Interferences are minimized by using mass detection of the [$M^{-\bullet}$] ions ($m/z = 797.5$ for CoQ9 and $m/z = 862.5$ for CoQ10) in selective reaction monitoring mode ($m/z = 797.5 \rightarrow m/z = 779.5$ and $m/z = 862.5 \rightarrow m/z = 847.5$) using a triple-quadrupole mass spectrometer. The method was successfully applied to sunflower, soybean, and rapeseed oils, with a limit of quantification of 0.025 mg/kg for both compounds.

KEYWORDS: Coenzyme Q10; coenzyme Q9; vegetable oils analysis; HPLC-APCI-MS

INTRODUCTION

Ubiquinones (coenzymes Q or CoQ) are a group of lipid-soluble benzoquinones involved in electron transport in the mitochondrial respiratory chain. The ubiquinone structures are based on the 1-methyl-2-isoprenyl-3,4-dimethoxyparabenzoquinone nucleus with a variable number of isoprene units in the side chain. The ubiquinones are designated by numbers representing the number of isoprene units in the side chain (**Figure 1**). Naturally occurring members are the coenzyme Q6−coenzyme Q10 (CoQ10), and the differences in their properties are due to the length of the side chain (*1, 2*).

CoQ10 is the most common CoQ in animals and coexists with its reduced form ($CoQ10H_2$ or ubiquinol-10) that is the predominant form in the tissues of the living beings (**Figure 1**), but with age, its rate of production falls (*3, 4*). Epidemiological and biochemical evidence support the idea that $CoQ10H_2$ is, moreover, an important cellular antioxidant in vivo, inhibiting lipid peroxidation as well as regenerating other antioxidants such as α-tocopherol (vitamin E) (*2, 5, 6*). The antioxidant properties of CoQ10 allow reduction of free radical-induced oxidative damage of the low-density lipoproteins (LDL) and improvement of the endothelial function of the arteries and then a decrease in the susceptibility of cardiovascular disease (*7–10*). Furthermore, it has been shown that ubiquinones can be used as markers of several parameters: The $CoQ10/CoQ10H_2$ ratio is considered a marker of oxidative stress in coronary artery disease, and the LDL/CoQ10 ratio is considered a marker of the risk of coronary disease (*6*).

Taking into account its ability to reduce the oxidized LDL in vivo and in vitro, that its consumption is very safe (even at high doses), and that CoQ10 supplementation seems to protect mitochondrial membranes against free radicals, it is suggested that it has beneficial effects on the treatment of coronary disease (*4, 11–14*).

Moreover, there is also some evidence that CoQ10 supplementation could also help to prevent and/or treat other diseases including cancer, acquired immunodeficiency syndrome (AIDS), Parkinson's disease, and neurodegenerative diseases, and it also seems to be an energy booster and immune system enhancer (*15, 16*). Recently, CoQ10 has also been used as a nutraceutical supplement (e.g., in energy drinks) and as a cosmetic ingredient (antiwrinkle) (*17*).

Many procedures have been reported for the analysis of CoQ10 in different matrixes, mainly related to biological samples such as tissues, plasma, and serum. The authors determined not only CoQ10 but also $CoQ10H_2$, coenzyme Q9 (CoQ9), and coenzyme Q9 reduced form ($CoQ9H_2$), as well as some degradation products such as ubichromenol. They are usually extracted by liquid−liquid partition, mainly with hexane:ethanol, where the CoQ10 (a lipophilic compound) is extracted with the organic layer (*6, 10, 13, 17–22*). However, this procedure is not effective for fats and oils. In these cases, and in general for fatty samples, a saponification process (to remove

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the main interferences) as a previous step to the liquid–liquid partition (*2, 23, 24*), with a high solvent and time-consuming properties, is recommended. Subsequent analysis is carried out by reverse-phase (RP) high-performance liquid chromatography (HPLC) (usually C18), with or without previous purification of the fraction, coupled to electrochemical (ECD) or diode array (DAD) detectors. Although ECD is more sensitive than DAD (20-fold more), it used to be more affected by the interferences. The use of a mass detector (MS) can improve the results of this analysis since MS-MS increases the selectivity and increases the signal/noise ratio as compared to other detectors (*1, 20*). Quantification of CoQ10 can be obtained by internal or external standardization. Quantification by external standard is advisable only if the standard curve is obtained immediately before every series of analysis. The use of an internal standard prevents the determination of CoQ9 because this compound is often used as a reference (*20*).

Because CoQ10 is synthesized by living beings, it is present in most of foods, especially in meat. However, despite the evidence that CoQ10 consumption may have beneficial effects on human health, only few studies have assessed the contents of CoQ10 in foods (*2, 14, 23*). Moreover, only one survey about of the ubiquinone content in vegetable oils (*25*) was found, so the available information on CoQ analysis and contents in vegetable oils is scarce. Then, the objective of this survey is to develop and optimize a simple and fast analytical method for quantifying CoQ9 and CoQ10 in vegetable oils.

MATERIALS AND METHODS

Samples. Samples of crude (soybean and rapeseed) and refined oils (sunflower) were obtained from the pilot plant of the ITERG.

Materials and Reagents. For thin-layer chromatography (TLC), TLC silica gel 60 (0.25 mm) 20 cm × 20 cm glass plates (Merck, Darmstadt, Germany), HPLC quality *n*-heptane (J.T. Baker, Phillipsburg, NJ), and Pestinorm quality ethyl ether (VWR Prolabo, Barcelona, Spain) were used. For the cleanup procedure, 5 g of solid-phase extraction (SPE) cartridges with amino-propyl (NH_2) adsorbents (Varian, CA), HPLC quality *n*-heptane (J.T. Baker), and Pestinorm quality ethyl ether (VWR Prolabo) were used. For the chromatographic analysis, tetrahydrofuran, acetonitrile, acetone, methanol, and HPLC quality isopropanol (J.T. Baker) were used.

Individual standards of CoQ9 and CoQ10 were, respectively, obtained from Sigma-Aldrich and Fluka. The different solutions were prepared by appropriate dilutions in UV quality iso-octane (Merck, Darmstadt, Germany) of the pure standards. The stock solutions were protected from the light by aluminum foil and stored a 4 °C. Before their use, the accurate concentrations were determined by spectrophotometry at 275 nm, ε CoQ9 = 14440 and ε CoQ10 = 14020 L mol^{-1} cm^{-1}, respectively (*20*).

Apparatus. The analyses were performed by HPLC-MS. The HPLC equipment was a Dionex Ultimate 3000 series (Dionex, Sunnyvale, CA) composed of a vacuum degasser for the mobile phase solvents, a ternary pumping unit, and an autosampler. An Xterra MS RP C18 HPLC column (50 mm × 2.1 mm × 3.5μm) (Waters, MA) with an Xterra RP C18 HPLC guard column (10 mm × 2.1 mm) (Waters) was used. The detector was a triple-quadrupole mass spectrometer TSQ Quantum Ultra (ThermoFinnigan, Austin, TX) operating in selective reaction monitoring (SRM) mode. Xcalibur software (ThermoFinningan) was used for chromatographic and mass spectrometric analyses.

SPE Cleanup. In a 4 mL glass tube (provided with cap with a PTFE liner), 250 mg of oil was weighed (precision of 0.1 mg), and then, 0.5 mL of heptane was added. The mixture was homogenized by shaking.

The SPE column was conditioned by passing two volumes (2 × 10 mL) of heptane without leaving them to dry. The sample solution was charged onto the column, and the solution was pulled through. The tube was washed with two portions of 0.50 mL of heptane that were also poured onto the column. The column was eluted with 14 mL of a mixture of heptane:ethyl ether (80:20 v/v), and the eluted fraction was discarded. Then, the column was eluted with two fractions of 10 mL of heptane:ethyl ether (80:20 v/v) that had each been collected in a conical test tube of 12 mL. The fractions were evaporated using nitrogen (35 °C and 5 psi) up to 0.5 mL (approximately). The fractions were then combined, and the resulting extract was evaporated (in the same conditions) just until dryness.

Table 1. Solvent Gradient Used for HPLC Analysis of CoQ9 and CoQ10[a]

time (min)	% A	% B	% C
0.0	0	90	10
7.0	0	90	10
7.5	50	40	10
12.5	50	40	10
13.0	0	90	10
18.0	0	90	10

[a] A, acetonitrile:acetone (1:1 v/v); B, MeOH; and C, IsoProp.

HPLC Analysis. The residue was redissolved in 2 mL of acetonitrile: tetrahydrofuran (90:10 v/v) by shaking. An aliquot of 20 μL was injected into the HPLC using an autosampler. The HPLC system was set up maintaining the autosampler and column temperature at 20 °C and using a mixture of acetonitrile/acetone:methanol:isopropanol as the mobile phase at a flow rate of 200 μL/min. The solvent gradients are shown in **Table 1**.

The mass detector was equipped with an atmospheric pressure chemical ionization interface (APCI) running in negative mode. The operation conditions were as follows: vaporizer temperature of 314 °C, sheath gas (N_2) and auxiliary gas (N_2) of 20 and 5 arbitrary units, discharge current of 17 μA, and capillary temperature of 250 °C. The mass detector was running in SRM mode, in two scan events (m/z 797.5 → m/z 779.5 and m/z 862.5 → m/z 847.5) with a fragmentation energy of 17% and a collision argon pressure of 1.5 arbitrary units.

RESULTS AND DISCUSSION

Development of the Cleanup Procedure. Although the technique of direct injection in the HPLC after oil dilution in organic solvent has been proposed (*5, 25, 26*), it has not been selected in this work. Direct injection reduces the sensitivity of the method, since the amount of the injected oil in the HPLC should be low to obtain a proper work of the system. Moreover, most of the organic solvents used for oil dilution (acetone and 2-propanol) that are compatible with the HPLC, working in reverse mode, gave us peak shape distortion. Then, a cleanup step is required before HPLC analysis.

Silica gel column chromatography followed by recrystallization is the standard method for CoQ10 production in the industry (*17*), so we decided to use silica gel as stationary phase in our cleanup method. For a qualitative approach to the chromatographic condition, TLC assays were performed as follows: On the basis of the intense yellow color, we checked if a CoQ10 fraction free of the major constituents of the oils could be achieved. For this purpose, refined sunflower oil spiked with a high level of CoQ10 was used. The best resolutions were obtained using heptane:ethyl ether (80:20 v/v and 70:30 v/v).

We tried to extrapolate the TLC results to SPE using silica gel as a stationary phase to decrease the time consumption. Although we tested several mobile phases with various polarity levels and different amounts of stationary phase, it was not possible to get an effective separation between the triacylglycerides (TG) and the CoQ10 fraction. So, we tested SPE cartridges of silica gel modified with NH_2 groups, because of the affinity of the NH_2 groups with the aromatic rings and the double bounds. Finally, the best resolutions were obtained using SPE NH_2 cartridges of 5 g with heptane:ethyl ether (80:20 v/v) and heptane:ethyl ether (70:30 v/v).

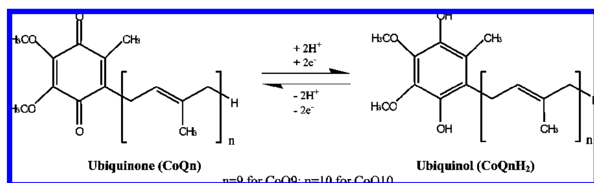

Figure 1. Depiction of the electrochemical transition between ubiquinone and ubiquinol.

After the solvent removal, a yellow oily residue remained in the extract (approximately 10% of the oil sample). To achieve a completely dissolution of the final extract, 2 mL of acetonitrile: tetrahydrofuran (90:10 v/v) was used.

Development of the HPLC Analytical Method. For the analytical determination, an HPLC coupled to a triple-quadrupole mass spectrometer was used. The triple quadrupole offers the capability of running in SRM detection mode, which provides high analyte selectivity by means of mass resolution of a precursor ion in the first quadrupole and subsequent filtration of the selected ion products in the third quadrupole. The advantage of SRM mode is its ability to remove interferences, not only of the parents but also of the product ions, increasing the signal-to-noise ratio, which improves limits of quantification and the confidence in the quantification results.

All of the available ionization sources for the mass detector were tested using heated electrospray ionization (H-ESI) and APCI in negative and positive modes; the best results were obtained using APCI in the negative mode. The signal was tuned in manual and automatic modes using the CoQ10 standard, according to the manufacturer's recommendations, to achieve the maximum sensitivity. A combination of both tuning methods gave us the best results.

After the optimization of the signal, the HPLC method proposed by Hansen et al. (*20*) for CoQ10 analysis [methanol: 2-propanol (80:20 v/v)] was tested, but the results were not so satisfying. The final elution gradient was a compromise between run time and a column cleaning step after the CoQ9 and CoQ10 elution since, although significant interferences were not observed in the chromatogram profiles, the remaining TG in the final extracts can increase the retention times. So, a cleaning step with acetonitrile:acetone (1:1 v/v) was included to avoid a change in the retention times.

Figure 2shows the chromatogram profiles (upper row) and the ion plots (lower row) of a refined sunflower oil purified following the proposed procedure. No interferences were observed in the chromatogram profiles, and the ions corresponding to CoQ9 (m/z = 779.5) and CoQ10 (m/z = 847.5) were the most abundant.

CoQ9 and CoQ10 Quantification in Vegetable Oils. The use of internal standard could not be used for CoQ9 and CoQ10 quantification because all of the target oils contained both compounds, and moreover, spiking experiments revealed significant signal suppression due to matrix effect of the final extract. Then, the matrix effect prevents the use of external standard quantification.

Therefore, for overcoming these problems, and after having checked the linear response of CoQ9 and CoQ10 in all of the final extracts, the standard addition approach was used for quantification. This approach is based on adding known amounts of the target compounds in the sample and calculating a subsequent linear regression to know the initial concentration. In this case, two different amounts of CoQ9 (11.1 and 22.5 mg/kg for rapeseed oil sample, 11.2 and 22.3 for soybean oil sample, and 109.5 and 222.1 for sunflower oil sample) and CoQ10 (26.2 and 51.5 mg/kg for rapeseed oil sample, 51.1 and 105.4 for soybean oil sample, and 10.5 and 15.9 for sunflower oil sample) were used to spike each sample, so three determinations were made to know the real CoQ concentration of each sample (the original sample as well as the two spiked levels). From the CoQ areas and the standard concentrations added, regression lines that give the initial CoQ concentrations were obtained. **Table 2** shows the regression lines, the correlation coefficients, and the CoQ9 and CoQ10 concentrations for each of the analyzed oils. The obtained correlation coefficient for each sample ranged between 0.90 and 0.99, and the concentrations ranged between 2.3 and 101.3 mg/kg for CoQ9 and 8.7 and 97.6 mg/kg for CoQ10, respectively.

In this case, the limit of detection (LOD) and the limit of quantitation (LOQ) were calculated from the signal-to-noise ratio of the peak height (*27*) of the oil extracts. The LOD was obtained by increasing dilutions of the final extracts until the CoQ peak height was three times higher than the average noise peak height, and LOQ was obtained by increasing dilutions of the final extracts until the CoQ peak height was 10 times higher than the average noise peak height. The LOD and LOQ obtained for the three oils were very similar and correspond to 18 and 60 pg, respectively, injected on the column. When expressed in analyte concentrations in the oil sample, LOQ (60 pg injected on column) is about 0.025 mg/kg (assuming 250 mg of oil sample, 2 mL of final extract, and 20 μL of injection volume).

Comparison with Other Available Methods. HPLC coupled to a MS detector has been widely introduced in analytical laboratories throughout the recent years, not only for molecular characterization but also for analytical purposes, due to its high sensibility and selectivity as well as the fall of its price. Therefore, this technique is widely used nowadays for the analysis of many compounds (triglycerides, polyphenols, etc.) that could be analyzed using other systems. Moreover, the use and maintenance of the modern HPLC-MS systems by a technician is easy, once the methods are ready. So, the use of this equipment for CoQ analysis is reasonable.

Mostly CoQ analysis methods need a cleanup phase to remove numerous interferences of the final extract. In food samples, the cleanup phase is based on organic solvent extraction (mainly with ethanol:*n*-hexane) that requires several steps and is highly time- and solvent-consuming (*1, 14*). However, direct ethanol−*n*-hexane solvent extraction is only effective for food samples other than fats and oils (*1*). Therefore, for fat and oil samples, a previous saponification step is needed to remove the interferences (*2, 23, 24*), which increase the time and solvent consumed. In the proposed procedure, the SPE cleanup allows a minimum sample manipulation (one step sample cleanup) as well as low solvent (lower than 45 mL/sample vs 115 mL/sample of the other methods) and time (plus than 10 samples/h) consumption. These facts increase the throughput of the CoQ analysis.

The combination of the high sensitivity and selectivity of the MS detectors running in SRM mode and the highly purified extracts brings to the proposed procedure lower LOD (18 pg/injection) values than those obtained by Mattila et al. in 2000 (0.2−6.0 ng/injection) and 2001 (3−5 ng/injection); LOQ values of Mattila et al. methods are not shown. Neither of the other authors that have analyzed CoQ in oil samples showed LOD and LOQ values. Besides, the low amount of sample needed (due to the high sensibility of the method), as well as the SPE cleanup that brings a highly purified extract, increases the HPLC column life. Moreover, benzene is the solvent most used in those

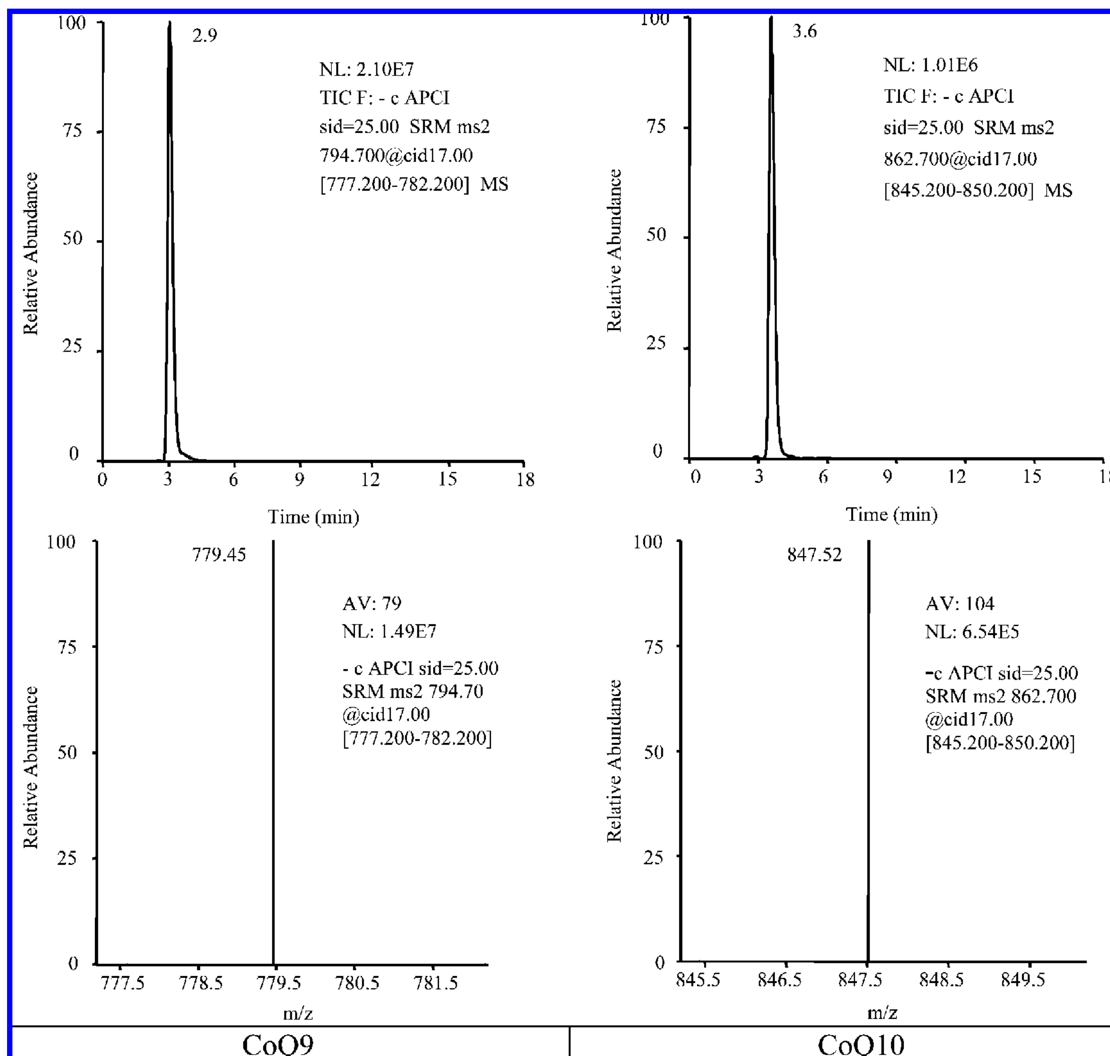

Figure 2. Chromatogram profile of a refined sunflower oil with CoQ9 and CoQ10 contents of 8.7 and 101.3 mg/kg, respectively.

Table 2. Standard Addition Method: Standards Added, Regression Lines, and CoQ Concentrations of Each Analyzed Oil[a]

oil		standard added (mg/kg)			regression line ($y = ax + b$) *a*	*b*	r^2	real content (mg/kg)
rapeseed (crude)	CoQ9	0.00	11.1	22.5	11368540	6424232	0.9993	2.3
	CoQ10	0.00	26.2	51.5	10045573	116261353	0.9510	46.4
soybean (crude)	CoQ9	0.00	11.2	22.3	4827908	29958131	0.9935	24.9
	CoQ10	0.00	51.1	105.4	6116005	148812445	0.8962	97.6
sunflower (refined)	CoQ9	0.00	109.5	222.1	12528416	316958388	0.9977	101.3
	CoQ10	0.00	10.5	15.9	5942408	12917627	0.9802	8.7

[a] *y*, μg added; *x*, peak area; and r^2, correlation coefficient.

methods that include a saponification step (*23, 24*). Unfortunately, benzene has been rated as "known to be carcinogenic to humans" by The International Agency for Research on Cancer (*28*), so its use in laboratories should be avoided.

In conclusion, we can say that a new method was developed for the analysis of the total CoQ9 and CoQ10 concentrations in vegetable oil samples. The SPE cleaning step allows removal of the main interferences of the oils using low solvent volume in a short time. The detection technique, based on the high efficient formation of its ions by APCI(−) and the minimization of the interferences by use of the selective reaction monitoring mode, increases the selectivity of the method. The impossibility of using internal standard and the presence of matrix effect were overcome by the standard addition method. Therefore, as compared to the previous methods, the proposed procedure is environmentally friendly and increases the throughput of the CoQ analysis.

ABBREVIATIONS USED

AIDS, acquired immunodeficiency syndrome; APCI(−), negative atmospheric pressure ionization; CoQ9, coenzyme Q9; CoQ9H_2, coenzyme Q9 reduced form; CoQ10, coenzyme Q10; CoQ10H_2, coenzyme Q10 reduced form; DAD, diode array detector; ECD, electrochemical detector; H-ESI, heated electrospray ionization; HPLC, high-performance liquid chromatography; LDL, low-density lipoproteins; LOD, limit of detection; LOQ, limit of quantitation; MS, mass detector; NH_2, amino-propyl; SPE, solid-phase extraction; SRM, selective reaction monitoring; TG, triacylglycerides; TLC, thin-layer chromatography.

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