Waters™

Nota applicativa

Solid Phase Extraction and Analysis of Coenzyme Q10 from Plasma Using Oasis PRiME HLB for Clinical Research

Jonathan P. Danaceau, Pamela C. Iraneta, Erin E. Chambers

Waters Corporation



For research use only. Not for use in diagnostic procedures.

Abstract

This application note describes the extraction of CoQ10 from plasma samples and its subsequent analysis by UPLC-PDA detection.

Benefits

- Simplified SPE extraction without conditioning and equilibration of the SPE sorbent
- Minimization of residual phospholipids from plasma samples
- On column concentration of CoQ10, without evaporation and reconstitution
- Chromatographic separation of CoQ10 from all endogenous interfering substances

Introduction

Coenzyme Q10 (CoQ10) is a ubiquitous lipid soluble molecule that is a key component of the mitochondrial electron transport chain and is necessary for ATP synthesis. It also can act as an important antioxidant.[1-3] CoQ10 deficiency has been associated with many diseases including neurologic disorders,[4] but is of particular interest as a cardioprotective molecule. Deficiencies in CoQ10 have been associated with heart failure and supplementation with CoQ10 has been found to improve cardiac function.[5] Because of this, there is an ongoing research interest in the quantification of CoQ10 in human plasma.

From an analytical standpoint, CoQ10 tends to be found at relatively high concentrations in plasma and can often be readily detected by non-specific HPLC detection methods such as electrochemical or UV detection. While this can be an advantage for research laboratories that may have limited mass spectrometry capabilities, it does mean that chromatography and sample preparation become critical, as CoQ10 must be adequately separated from endogenous interferences in order to achieve accurate and reproducible quantification.

CoQ10 has some properties that make it challenging to extract from plasma samples. It is highly protein bound, requiring strong protein disruption in order to make it accessible for solid phase extraction. It is also extremely hydrophobic. Many common solvents used for solid phase extraction (SPE), such as methanol, are not strong enough to elute CoQ10 from SPE sorbents. Due to this and other factors, traditional SPE methods

need significant optimization to ensure efficient and consistent extraction of CoQ10 from plasma.

This application note describes the extraction of CoQ10 from plasma samples and its subsequent analysis by UPLC-PDA detection. CoQ10 is isolated from plasma by protein precipitation followed by SPE using Oasis PRiME HLB µElution plates. Sample pretreatment, including protein precipitation to disrupt protein binding, has been optimized for CoQ10. The SPE procedure has also been carefully optimized for fast and efficient extraction, resulting in high recoveries and clean extracts. Residual phospholipids are minimized through the use of an Oasis PRiME HLB µElution Plate. Finally, the extracts are analyzed using a CORTECS UPLC T3 Column, which provide baseline resolution of CoQ10 from all endogenous interfering peaks. The result is a clinical research method that is rapid, efficient, and clean, with excellent accuracy and precision that is ideally suited for the analysis of endogenous CoQ10 from plasma samples.

Experimental

Sample pretreatment

150 µL plasma samples were spiked with 1 µg/mL CoQ9 as a retention time marker and pretreated with 20 µL of 4 µg/mL benzoquinone in methanol. Samples were vortexed and allowed to sit for 10 minutes at room temperature to fully oxidize all CoQ10 in the samples. All samples were then precipitated with 900 µL of 50:50 ACN:IPA. Precipitated samples were vortexed and centrifuged at 12,500 rcf for five minutes at 10 °C. The supernatant was then transferred to a 96 well collection plate and diluted with 500 µL of 12% aqueous phosphoric acid prior to solid phase extraction.

Solid phase extraction

Diluted samples prepared as described above were directly loaded on to an Oasis PRiME HLB µElution Plate in two aliquots without conditioning or equilibration. After the samples had been loaded onto the sorbent, all samples were washed with 200 µL of 5% strong ammonia in water followed by 200 µL of MeOH. All wells were then dried under high vacuum (15 in. Hg) for one minute prior to elution. Samples were then eluted with 2 x 25 µL aliquots of 50:50 ACN:IPA and diluted with 25 µL of 50:50 MeOH:H_2O. Of the final extract, 7.5 µL was injected onto the UPLC system.

LC conditions

LC system:	ACQUITY UPLC I-Class (FL)
Detection:	PDA (275 nm)
Collection:	ACQUITY 96-well 1 mL collection plates 800 µL round well 50/pkg [P/N 186002481]
Column:	CORTECS UPLC T3 1.6 µm, 2.1 x 50 mm
Column temp.:	40 °C
Sample temp.:	10 °C
Injection volume:	7.5 µL
Flow rate:	600 µL/min
Mobile phase:	80:20 ACN:IPA with 0.08% formic acid
Gradient:	Isocratic

Data management

MassLynx Software v4.1 with TargetLynx Application Manager

Results and Discussion

Extraction method development

Coenzyme Q10 (CoQ10) exists as two major forms in the body. In its reduced form, known as ubiquinol, the carbonyl oxygens on the quinone ring exist as hydroxyl groups. In its oxidized form, as shown in Figure 1, they form double bonds with the quinone ring. To simplify quantification, plasma samples were first treated with benzoquinone to fully convert all ubiquinol and semi-quinone forms to the fully oxidized ubiquinone shown in Figure 1.

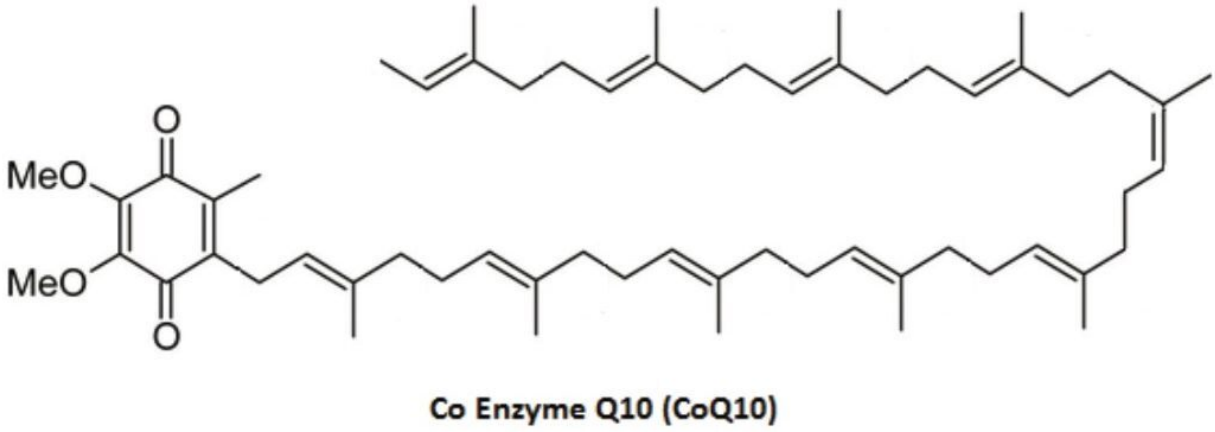


Figure 1. Structure of Coenzyme Q10. CoQ10 consists of a dimethoxylated methylquinone ring with a tail consisting of 10 repeating isoprene units.

CoQ10 is a lipophilic molecule which is highly protein bound. As such, the first step in extracting this molecule from plasma is precipitation with organic solvent to disrupt protein binding. We investigated both the nature of the precipitation solvent as well as the ratio of solvent to plasma for optimal extraction. Studies evaluating pure acetonitrile and a combination of acetonitrile (ACN) and isopropanol (IPA) reveled that a 50:50 ACN:IPA mixture resulted in significantly higher recoveries than ACN alone. Moving forward, the optimal ratio of precipitation solvent to plasma was investigated. Plasma samples fortified with 1 µg/mL CoQ9 and CoQ10 were precipitated with increasing volumes of 50:50 ACN:IPA. Figure 2 shows that 100% recovery could be achieved with a ratio of 6:1 solvent:plasma. This combination was used for the remaining experiments.

Following protein precipitation, samples were loaded onto an Oasis PRiME HLB µElution Plate. However, the precipitated supernatants needed to be diluted with water to avoid analyte breakthrough due to their high organic content. Dilution with pure water resulted in poor recoveries from the SPE plate. This is likely due to the hydrophobic nature of CoQ10. In a low ionic strength solution of pure water and supernatant containing a high organic solvent content, the water molecules would tend to form a highly ordered structure around the hydrophobic CoQ10 molecule, or the molecules could self associate, making their interaction with the PRiME HLB sorbent energetically unfavorable. Increasing the ionic strength of the aqueous diluents appeared to overcome this problem.

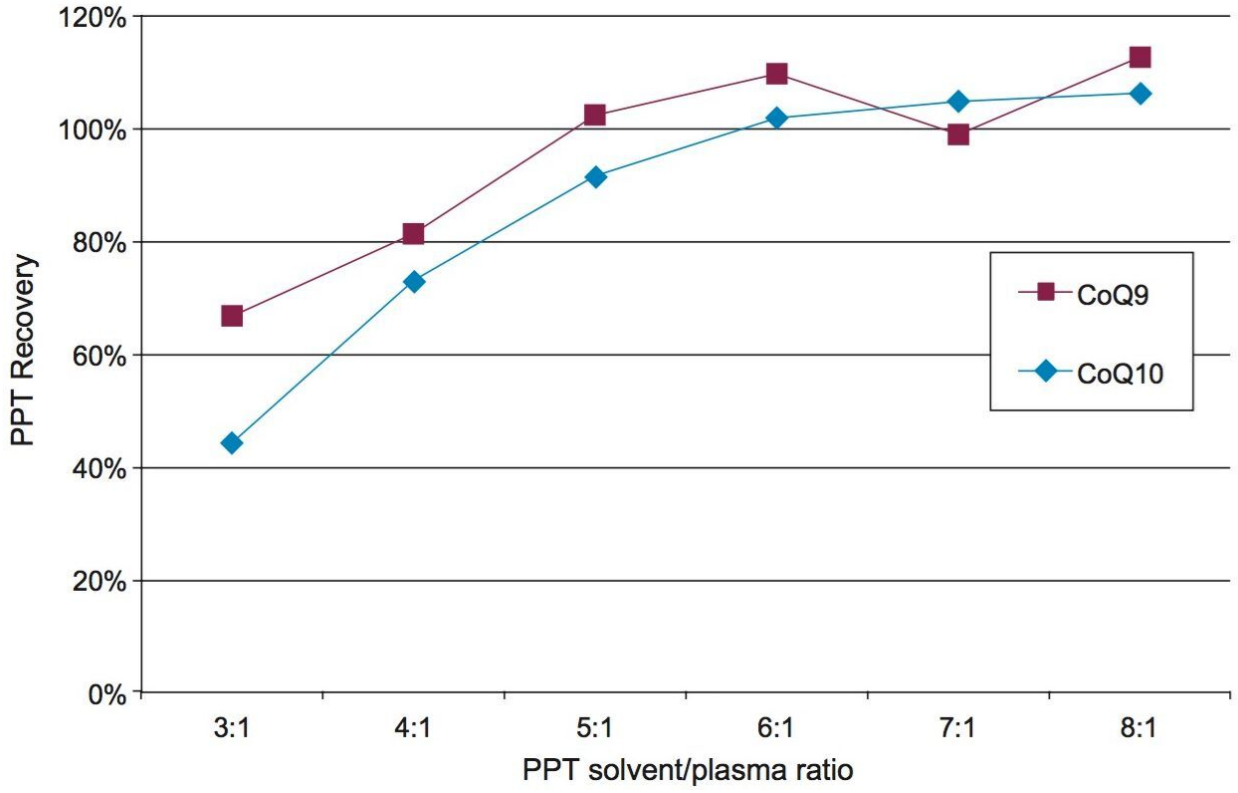


Figure 2. Optimization of precipitation solvent. Recovery of CoQ9 and CoQ10 from plasma is shown related to the volume of precipitation solvent used. Both compounds showed increased efficiency which reached a maximum at approximately a 6:1 ratio of solvent:plasma. The solvent used was 50:50 ACN:IPA.

Figure 3 demonstrates that compared to dilution with pure water, dilution with either 2x PBS or 4% H_3PO_4 substantially improved recovery on the Oasis PRiME HLB sorbent. As shown in Figure 3, phosphoric acid dilution resulted in higher recoveries than PBS dilution and was used for the remainder of the experiments. We subsequently investigated the optimal ratio and concentration of phosphoric acid for consistent recoveries. Dilution with 12% H_3PO_4 gave the most consistent recoveries compared to other concentrations of acid ranging from 2–16%. In addition, a volume of 500 µL was used to dilute the final supernatant. This final solution had enough aqueous content to avoid analyte breakthrough during the load step and was a small enough volume to enable sample loading in only two 750 µL aliquots.

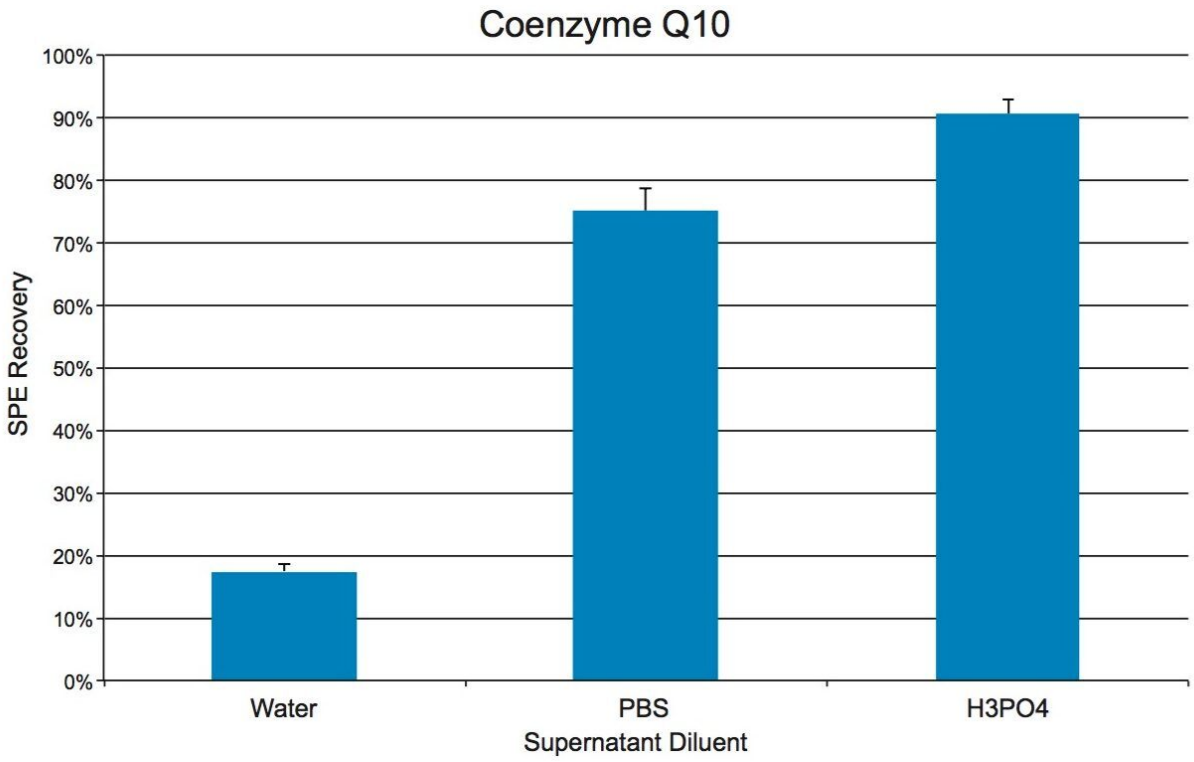


Figure 3. Optimization of post-precipitation sample diluents. SPE recovery of CoQ10 is shown as a function of the sample diluents used. Diluting the sample with water gave poor recovery. 2X PBS dramatically increased recovery and the best recovery was seen when the samples were diluted with 4% phosphoric acid. The concentration of phosphoric acid was later changed to 12% to improve the consistency of the extraction.

The wash steps were also optimized. Typically, final washes in reversed-phase SPE procedures should have enough organic solvent to clean up the sample without eluting the analyte of interest. A series of experiments revealed that a final wash with 100% methanol accomplished this goal. As the final results will show, recoveries were efficient and consistent. In addition, washing with methanol helped to minimize the amount of residual phospholipids in the final extract, which can contribute to reduced column lifetimes. Figure 4 shows combined phospholipid traces from precipitated plasma and from a sample that had been extracted using Oasis PRiME HLB µElution plates. The intensity and area of the phospholipid traces in the extracted sample were approximately 5% of those seen in the sample that had only been subject to protein precipitation. The actual phospholipid removal was even greater considering that the extracted sample had been concentrated 2X from 150 µL to 75 µL (the final eluted sample volume). By contrast, the precipitated sample was actually diluted 7X due to the addition of six parts ACN:IPA to one part plasma. When corrected for final sample volume, the Oasis PRiME HLB extracted samples had less than 1% of the residual phospholipids when compared to precipitated plasma.

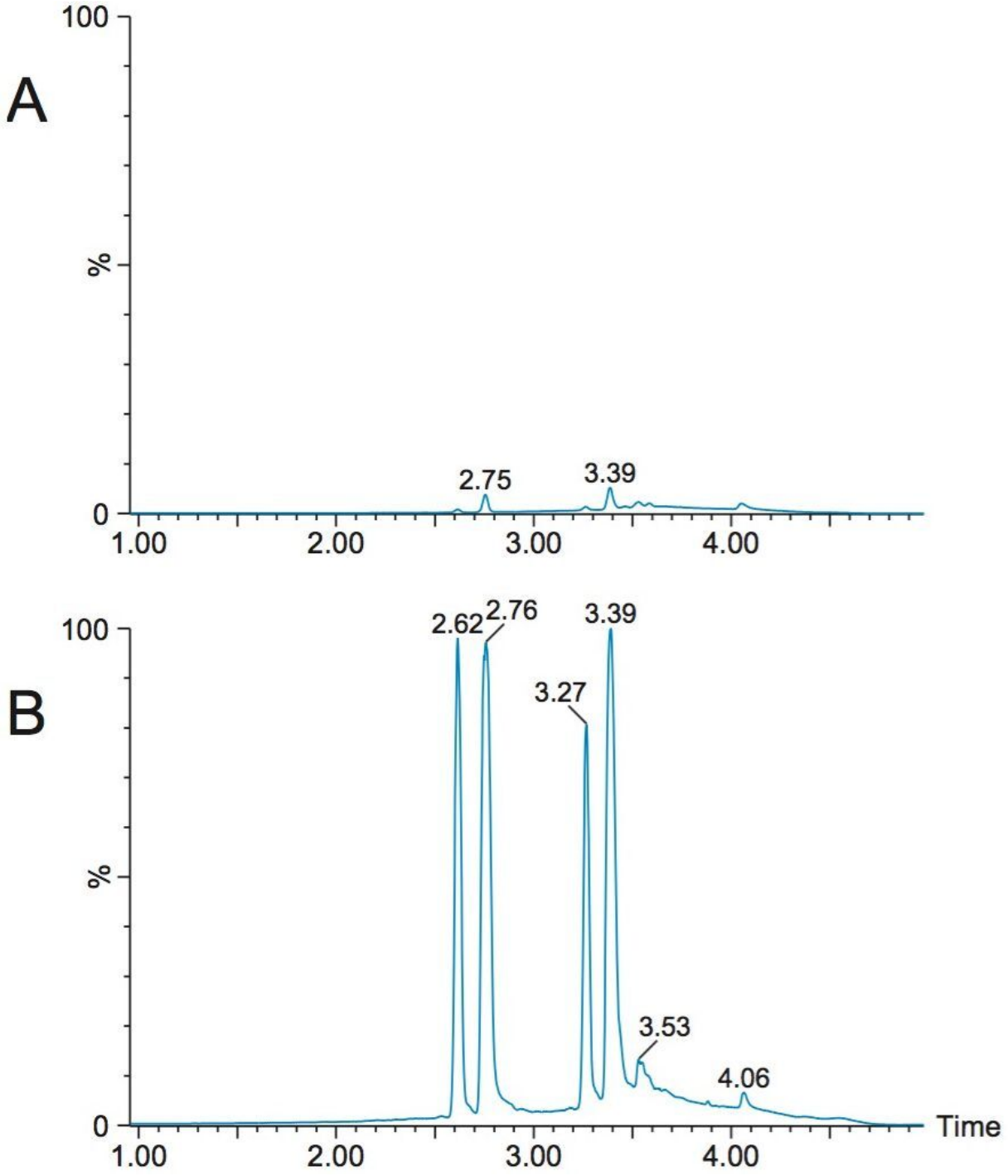


Figure 4. Pospholipid removal using Oasis PRiME HLB. A. Residual phospholipids remaining in plasma samples prepared using an Oasis PRiME HLB µElution Plate. B. Residual phospholipids in plasma samples prepared using protein precipitation only. Traces are a combination of five common phospholipids. Scales are linked to compare the magnitude of phospholipids present in each sample.

Finally, the elution solvents and volumes were optimized. Chromatographic method development had shown that a protic solvent such as IPA was required for elution of CoQ10. Methanol was tested, but was not a strong enough organic solvent. We tested elution solvents consisting of 25:75, 50:50, and 75:25 ACN:IPA. The combination of 50:50 ACN:IPA gave the best recovery of CoQ9 and CoQ10 compared to the other solvent combinations. This was a preferable combination versus a larger proportion of IPA, as IPA can cause undesirable solvent effects such as peak broadening during the chromatographic analysis. We also evaluated the optimal volume and number of elution aliquots. Adding a third 25 µL elution slightly increased the recovery of CoQ10, but at the expense of diluting the sample and reducing overall analytical sensitivity. Using the final extraction method, the recovery of CoQ10 was 64%, which included the entire extraction procedure, including protein precipitation and SPE.

Chromatography

Figure 5 shows the separation of CoQ10 on the CORTECS UPLC T3 Column. CoQ9, which is used as a retention time marker in this method, elutes at 1.3 minutes. CoQ10 elutes at 1.98 minutes and is well separated from the large peak at 1.7 minutes. Figure 5B shows the separation in greater detail and highlights the baseline separation of CoQ10 from the other peaks in the chromatogram. The top trace is from an unfortified plasma sample and demonstrates that CoQ10 can easily be quantified as baseline levels in plasma samples. Previous work, in which other columns were evaluated, revealed that the CORTECS UPLC T3 Column was superior to other chemistries and base particles for this separation. For example, the ACQUITY UPLC HSS T3 Column was unable to separate CoQ10 from the large interfering peak at 1.7 minutes. When a CORTECS UPLC C_{18} was used, other minor peaks were present that interfered with CoQ10. The ACQUITY UPLC BEH C_{18} Column was able to separate CoQ10 from the interfering peaks, but it still eluted before the baseline was stable, compromising integration and quantification. Only the CORTECS UPLC T3 Column was able to separate CoQ10 from all interferences while maintaining a rapid and efficient separation.

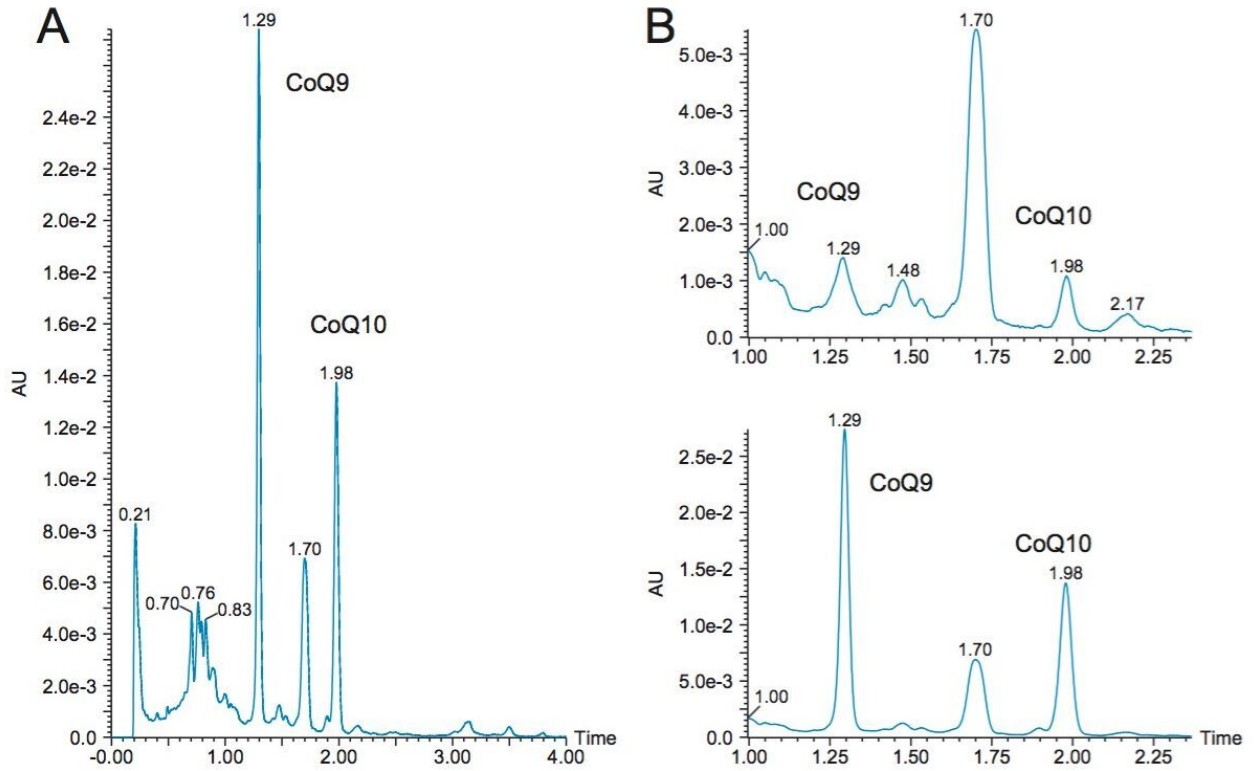


Figure 5. Chromatography of Coenyme Q10 on CORTECS UPC T3 Column. A. Chromatography of the PDA trace (275 nm) from a plasma sample spiked with CoQ9 and CoQ10. B. Chromatography of a blank, unspiked sample (top trace) and a spiked sample (bottom trace) showing the separation of CoQ10 from other peaks. The blank trace (top) shows that endogenous levels of CoQ10 can be easily detected and quantified using this method.

Quantitative results

In order to determine quantitative performance, plasma samples were fortified with concentrations ranging from 50–5000 ng/mL CoQ10. Four levels of quality control samples were also prepared at 150, 600, 1500, and 3000 ng/mL. These concentrations cover the endogenous range expected in plasma samples. The calibration curve and the quantitative values were all corrected for the endogenous CoQ10 concentration of 203 ng/mL in the plasma sample used to prepare them. Figure 6 shows the calibration curve and Table 1 lists the accuracy of the individual calibrators. The calibration curve was linear with an R^2 value of 0.995 for a 1/x weighted linear fit. All of the calibrators were within 15% of their target values and most were within 10%, with an average deviation of only 5.56% demonstrating excellent linearity of the method.

Quality control results were both accurate and precise. Table 2 shows that the mean of each calibration level was within 6% of the target value with excellent precision. The largest %RSD was 12.1% for QC level 3 and

the remaining levels had %RSDs less than 8%.

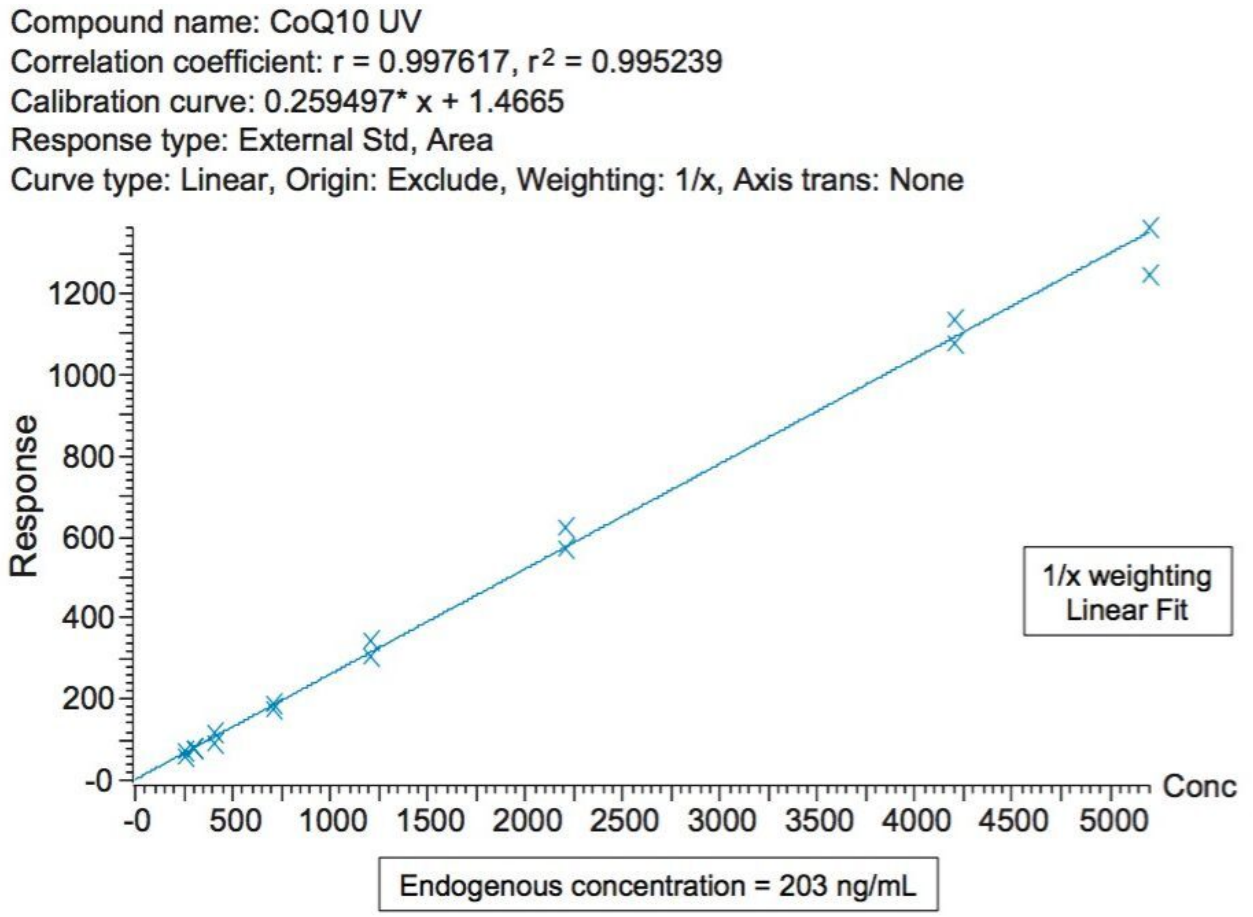


Figure 6. Calibration curve of CoQ10. Calibrators were prepared from 50–5000 ng/mL. The standard addition algorithm in TargetLynx XS was used to correct for the endogenous concentration of CoQ10 in the plasma sample (203 ng/mL). The curve was linear with an R^2 value of 0.995. All individual calibrators were within 13% of target values with an average deviation of 5.6%.

Table 1. Calibration results for CoQ10

Calibration addition	Replicate	Corrected concentration	Result	%Dev.	%Acc
50	1	253	265.2	4.8	104.8
	2		227.2	-10.2	89.8
100	1	303	284.5	-6.1	93.9
	2		298.3	-1.5	98.5
200	1	403	355.1	-11.9	88.1
	2		455.0	12.9	112.9
500	1	703	681.8	-3.0	97.0
	2		722.1	2.7	102.7
1000	1	1203	1174.2	-2.4	97.6
	2		1323.3	10.0	110.0
2000	1	2203	2202.9	0.0	100.0
	2		2405.3	9.2	109.2
4000	1	4203	4148.3	-1.3	98.7
	2		4375.6	4.1	104.1
5000	1	5203	4785.6	-8.0	92.0
	2		5243.8	0.8	100.8
		Mean % Dev. (Abs)		5.56%	

Calibration summary of CoQ10. The corrected concentration has had the endogenous level of CoQ10 (203 ng/mL) added to the spiked concentration listed in the first column. All % deviations were less than 13% and the mean % deviation for all calibrators was 5.6%.

Table 2. QC results for CoQ10

	Quantification (N=6)			% Accuracy			%Dev
QC Level (spiked conc.)	Mean	S.D.	%RSD	Mean	S.D.	%RSD	Mean
353 ng/mL (150)	347.2	26.2	7.5%	98.4	7.4	7.5%	-1.7%
803 ng/mL (600)	846.8	40.7	4.8%	105.5	5.1	4.8%	5.5%
1703 ng/mL (1500)	1708.0	206.9	12.1%	100.3	12.2	12.1%	0.3%
3203 ng/mL (3000)	3096.3	190.4	6.1%	96.7	6.0	6.2%	-3.4%

Quality control summary for CoQ10. QC values were corrected for the endogenous CoQ10 concentration of 203 ng/mL in the plasma sample used. All calibrators were accurate, with % deviations of less than 6% (N=6). Results were precise across all levels. The maximum %RSD was 12% with most %RSDs under 8%.

Conclusion

This application note details the efficient extraction and analysis of CoQ10 from plasma samples. CoQ10 is a uniquely hydrophobic molecule that is highly protein bound, so the processes of optimizing sample pretreatment, solid phase extraction, and chromatography are highlighted. The use of an Oasis PRiME HLB µElution Plate resulted in efficient extraction from plasma, while removing at least 95% of residual phospholipids. The µElution format enabled concentration of the sample without evaporation and reconstitution. This saves time over traditional liquid-liquid extraction methods and minimizes the potential for adsorptive loss during this step. Chromatography was optimized on a CORTECS UPLC T3 Column that enabled rapid analysis while maintaining baseline separation from interfering peaks present in the final extracts, a key consideration for any separation employing relatively non-selective detection such as UV. This optimization resulted in quantitative results that were analytically sensitive, linear, accurate, and precise. Endogenous levels were easily quantified, and calibration and quality control samples were accurate over a wide range of concentrations.

References

1. Stocker, R., Bowry, V.W., Frei, B. Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does alpha-tocopherol. *Proceedings of the National Academy of Sciences*. 1991;88(5):1646–1650.
2. Sohal, R.S. Coenzyme Q and Vitamin E Interactions. Methods in Enzymology, *Academic Press*. 2004;146–151.
3. Mohr, D., Bowry, V.W., Stocker, R. Dietary supplementation with coenzyme Q10 results in increased levels of ubiquinol-10 within circulating lipoproteins and increased resistance of human low-density lipoprotein to the initiation of lipid peroxidation. Biochimica et Biophysica Acta (BBA) – *Lipids and Lipid Metabolism*. 1992;1126(3): 247–254.
4. Duberley, K.E.C., I.P. Hargreaves, K.-A. Chaiwatanasirikul et al.: Coenzyme Q10 quantification in muscle, fibroblasts and cerebrospinal fluid by liquid chromatography/tandem mass spectrometry using a novel deuterated internal standard. *Rapid Communications in Mass Spectrometry.* 2013;27(9): 924–930.
5. DiNicolantonio, J.J., Bhutani J., McCarty M.F., O'Keefe J.H. Coenzyme Q10 for the treatment of heart failure: a review of the literature. *Open Heart*. 2015; 2(1), e000326.

Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

MassLynx MS Software <https://www.waters.com/513662>

TargetLynx <https://www.waters.com/513791>

ACQUITY UPLC PDA Detector <https://www.waters.com/514225>

720005966, April 2017

©2019 Waters Corporation. All Rights Reserved.