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Miniaturized imprinted solid phase extraction to the selective analysis of Coenzyme Q10 in urine

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ABSTRACT

Coenzyme Q10 (CoQ10) is an important cofactor in the mitochondrial respiratory chain and a potent endogenous antioxidant. CoQ10 deficiency is currently associated with numerous diseases like mitochondrial and neurodegenerative pathologies, in which the earliest diagnosis and treatment with CoQ10 supplementation becomes paramount for patient's treatment. Consequently, the determination of CoQ10 levels in different biological matrices positions as a fundamental tool. Urine is an attractive and non-invasive alternative source to tissue, blood or other biofluids for CoQ10 analysis. However, it poses an analytical challenge, as it generally requires a complex sample preparation, with multiple steps. In this work we developed and validated a molecularly imprinted polymer solid phase extraction (MIP-SPE) followed by an HPLC-MS/MS method for the analysis of CoQ10 in urine. The MIP-SPE method developed is simple and fast compared to previously traditional reported methods, with reduced processing time, improved sample cleaning and excellent recovery values, along with its inherent high selectivity. The developed chromatographic method was validated according to FDA guidelines, and demonstrated to be suitable for the analysis of CoQ10 in urine samples with LOQ and LOD values of 0.6 ng/mL and 0.2 ng/mL of CoQ10 in urine respectively. Recovery values at three concentration levels were higher than 90.0 %.The proposed method is amenable to be applied in pediatric patients due to the low sample requirement and useful for diagnosis and post-treatment control

Keywords: coenzyme Q10, HPLC-MS/MS; imprinting; solid phase extraction; urine

Abbreviations

CoQ10: coenzyme Q10

CoQ9: coenzyme Q9

EGDMA: ethyleneglycoldimethacrylate

MF: matrix effect factor

MIP: molecularly imprinted polymer

MIP-SPE molecularly imprinted polymer solid phase extraction

MMA: methacrylic acid

MRM: multiple reaction monitoring

QC: quality control

1. INTRODUCTION

Coenzyme Q10 (Ubiquinone, CoQ10, Figure 1) is a lipophilic endogenous compound integrated in the mitochondrial respiratory chain where it acts as an electron carrier to produce cellular energy [1]. In addition, CoQ10 is recognized as a powerful antioxidant agent able to protect DNA, circulating proteins and cell membranes against oxidative damage [2–4]. The significance of CoQ10 deficiency has recently achieved clinical relevance and significance as a biomarker, particularly associated to metabolic and oxidative stress abnormalities [1,5] in a wide range of disorders, including cancer, muscular, neurodegenerative, cardiological, kidney and reproductive diseases [6–8], among others. Accordingly, the determination of CoQ10 in biological samples and the study between the correlation of its decreased levels with states of pathology are very important for early diagnosis and therapeutics [2,3,9,10].

Muscle biopsy is the gold standard matrix in the diagnosis of CoQ10 deficiency. Despite being more sensitive, this sample is not only highly invasive but also too traumatic for periodic clinical monitoring. Although blood CoQ10 measurements may provide a useful estimation for tissue CoQ10 levels, the procedure is still invasive, especially for pediatric patients undergoing continuous monitoring of their treatment. For that reason, urine is an attractive and non-invasive alternative for CoQ10 analysis.

In normal conditions, CoQ10 content in biological fluids and tissues is low, ranging in the ppm order, and is even lower in pathological conditions.

Given CoQ10 low concentration, the complexity of biological matrices and two molecular properties necessary for CoQ10 function like high hydrophobicity and easy oxidation, the analysis of CoQ10 has become technically challenging [1]. It is also necessary to consider the low sample availability in pediatric patients. Therefore, it is of the utmost importance to develop a simple and miniaturized sample preparation system of high selectivity, with minimum amount of sample required and fast enough to ensure molecule integrity throughout the entire analytical process. Moreover, it should be easily coupled to highly sensitive analytical techniques.

It is well known that the determination of trace level compounds in complex matrices requires a procedure of sample pretreatment to minimize matrix effects, removal of interferences, eliminate sample variability and increase sensitivity. Moreover, there are additional problems that must be solved before sample analysis when dealing with biological samples such as low sample volume availability which makes it indispensable to work with little quantity of sample and the need for rapid analysis to ensure stability of analyte

Biological matrices such as blood, plasma and urine are more complex than other matrices due to numerous compounds such as proteins, salts and even other chemically similar molecules to the analytes of interest that make analysis tedious [11].

Liquid-liquid extraction (LLE), protein precipitation and solid-phase extraction (SPE) are the traditional sample pretreatment methods used to isolate and concentrate analytes from biological matrices [12].

More recently, the analytical chemistry in the biological area has set new objectives, such as smaller initial sample volumes, improved extraction selectivity, automation facilitation and reduced glassware and organic solvents use in accordance with green chemistry principles. Keeping these observations in mind, it is evident that the traditional LLE does not fulfill most of the current requirements and it has been displaced by improved extraction techniques based on SPE in different miniaturized formats[13].

SPE surpassed traditional bioanalytical sample preparation methods given its efficient pre-concentration, reduced organic solvent usage, no foaming and emulsions formation, high recovery, ease of operation and automation. Sorbent choice is the main aspect in SPE as it controls parameters like selectivity, affinity and capacity. In this sense, selectivity has been significantly improved by the use of immune sorbents or molecularly imprinted polymers (MIPs) especially applied to modern preparation techniques [14].

In recent years, MIPs have become an attractive alternative to selective separation materials in SPE. MIPs have many advantages as polymeric sorbents for SPE because they are easy to synthesize, low-cost, robust, porous and highly selective for the target molecule. MIPs are also more stable over a wider pH range than traditional sorbents and can be used with a broader range of solvents which gives them the potential to be reusable, further lowering the costs.

On the other hand, mass spectrometry (MS) and tandem MS (MS/MS) are the most commonly used detection techniques for the identification and quantification of analytes in biological matrices due to their high sensitivity, selectivity and low time consuming. One of the problems in quantitative bioanalysis, particularly in electrospray ionization-mass spectrometry (ESI-MS), is the presence of ion suppression caused by matrix interferences. This phenomenon can be avoided by a previous sample preparation step with the appropriate sorbents to reach the required sensitivity, isolate the analyte and remove matrix components that interfere, even if they do not give a direct signal to the detector [15,16].

In this work, we developed and validated, for the first time, a miniaturized SPE procedure using a previously developed molecularly imprinted polymer (MIP) [17] as a packed sorbent in a column for urine sample concentration and selective recognition of CoQ10 with subsequent coupling to HPLC and an ESI-MS/MS detection system.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Coenzyme Q9 (CoQ9), CoQ10, dextran, methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA) and benzoyl peroxide were purchased from Sigma-Aldrich (Darmstadt, Germany). Acetonitrile, methanol, acetic acid, 1-propanol, Bakerbond SPE C18 and Silica gel SPE cartridges were supplied by J.T. Baker (New Jersey, USA). All solvents were of HPLC grade. Ultrapure water was obtained from an EASYpure^{TM} RF equipment (Barnstead, Dudubuque, IA, USA). Ammonium formiate were provided by Anedra (Argentina). Sodium Heparin 25000 units per milliliter was supplied by Northia, (Buenos Aires, Argentina), and saline sterile physiologic solution by Parafarm (Buenos Aires, Argentina). Polypropylene cartridges and frits for 1 mL SPE tubes 20 µm were supplied by Phenomenex® (California, USA).

2.2 Instrumentation and Chromatography

HPLC-MS/MS system comprised an UltiMate 3000 HPLC coupled to a TSQ Quantum Access MAX Triple Quadrupole Mass Spectrometer with electrospray ionization (Massachusetts, USA). CoQ9 and CoQ10 determinations were carried out using a micro HPLC-MS/MS method. The separation was performed using a C18 Hypersil Gold column (Thermo Fisher Scientific, 50x2.1 mm; particle size 3.0 µm) and a C18 Hypersil Gold precolumn (Thermo Fisher Scientific, 10x2.1 mm; particle size 3.0 µm) with column oven temperature set at 40°C. The mobile phase consisted of 10 mM ammonium formiate in methanol. The flow rate was set at 0.5 mL/min with an injection volume of 10 µL. Mass spectrometric detection was performed using a Thermo Scientific TSQ Quantum Access MAX triple quadrupole system (Thermo Scientific, California, USA) with ionization in positive ion mode. The spray voltage was set at 3500V. Nitrogen was used as the nebulizer and auxiliary gas, set at 60 and 20 arbitrary units, respectively. Vaporizer and capillary temperature were set at 350°C and 275°C, respectively. For collision-induced dissociation, high purity argon was used at 1.5mTorr. CoQ10 and CoQ9 were detected using multiple reaction monitoring (MRM) of the following specific transitions: $864 \rightarrow 197$ and $796 \rightarrow 197$, respectively (Figure 1).

2.3 MIP-SPE column preparation

Polypropylene cartridges of 1 mL capacity were packed with 10 mg of a MIP sorbent, previously developed by our laboratory [17]. The performance parameters of the developed MIP-column (minimum elution volume, recovery, load capacity per mg of sorbent, bed volume and reuse times) were determined in comparison with a traditional SPE C18 column.

2.4 Sample extraction

First morning urine sample (10 mL) was centrifuged at 14000 rpm during 10 min. Five milliliters of the supernatant was passed through the SPE column previously conditioned with 1 mL of a mixture of water:1-propanol (70:30) and 1 mL water. Then, the cartridge was washed with 2 mL of water and the CoQ10 was eluted with 1 mL 1-propanol. After elution, 40µL of CoQ9 (5µg/mL in 1-propanol) internal standard were added. The clean-up and preconcentration allowed 5-fold enrichment of CoQ10 in the samples. The procedure was used to analyze 22 samples of first morning urine in healthy subjects (11 female and 11 males, age range: 17-39 years).

This study complied with the Declaration of Helsinki and was approved by the Institutional Review Board and the Bioethical Committee of our Institution. Written consent from all volunteers was obtained.

2.5 Standards preparation, method quantification and validation

Validation was performed according to international guidelines [18,19]. The matrix effect and ion suppression were determined by calculating the percentage of matrix effect factor (%MF) as follows: %MF = b/a x100 being (a): standards injected directly in solvent and (b) the same amount of analyte added to post-extracted samples. If MF is 100%, there is no matrix effect (ideal situation).

Calibration standard solutions of CoQ10 were prepared in 1-propanol at the following concentrations: 3, 5, 10, 50, 100, 200, 500 ng/mL, corresponding to 0.6, 1, 2, 10, 20, 40, 100 ng/mL urine equivalent levels giving a 5-fold concentration factor. CoQ9 was used as internal standard (IS) at 200 ng/mL. Calibration curve was prepared in triplicate on three different days and were made by plotting the peak area ratios (analyte area/IS area) versus analyte concentrations.

Specificity of the current method was made by comparison of chromatographic runs between a CoQ10 standard solution and the urine sample.

Quantification was performed in multiple reaction monitoring (MRM) mode with the following mass transitions: for CoQ10 m/z 864 →197 and for CoQ9 m/z 796→197. Accuracy was evaluated by means of a recovery assay. The recovery assay was carried out by spiking urine samples with CoQ10 standard at three different levels (40, 10 and 1 ng/mL) by triplicate corresponding to the high (200 ng/mL), medium (50 ng/mL) and low (5ng/mL) point level of the calibration curve. The limit of detection (LOD) and limit of quantitation (LOQ) in urine were determined at signal-to-noise ratio of 3:1 and 10:1, respectively. Precision was evaluated at three levels for intraday (n=9) and interday assays (n=27) and it was expressed as RSD for the ratio CoQ10 area relative to internal standard.

To determine the effect of interferences on urine CoQ10 values, like red and white cells, different amounts of total blood and white cells were added to a healthy volunteer urine sample. White cells were isolated following traditional extraction [20].

The stability of CoQ10 in urine samples was evaluated in urine aliquots stored at -20 °C and -80 °C during 7, 15 and 30 days and during two frozen cycles, 8°C (2-24h) and room temperature (1-5 h). The percentage stability was calculated as mean values of three determinations as follows: % stability= (S_t/S_0)x100, where S_t is the concentration of analyte at time t and S_0 is the concentration at initial time.

2.6 Statistical analysis

Results were expressed as mean ± SEM. Shapiro-Wilks´W test of normality was used. Differences between groups were analyzed by Student’s t-test. Levels of significance were established at p= 0.05.

3. RESULTS

3.1 MIP-column optimization and performance parameters

In order to improve analyte interaction with the sorbent, the first stage was to optimize the loading step. Initially, solvent selection was based on the results obtained by *Contin et.al* [17] in which after evaluating different proportions of 1-propanol and water, 40:60 (v/v) was chosen as the optimal binding solvent ratio. Nevertheless, with this percentage of 1-propanol in the mixture, part of the CoQ10 was lost in the loading step. Hence, different proportions of 1-propanol were tested and finally we decided to eliminate the organic solvent to solve this problem. Following this reasoning, water was used as the chosen solvent for column washing.

The second stage was to assess the elution solvent. Ethanol, 1-propanol and 2-propanol (with and without 0.1% acetic acid), hexane and dichloromethane were tested. The highest recovery (over 90%) was obtained with 1-propanol, which is in accordance with the reported results of *Lucangioli et. al* [21].

The performance parameters of the developed MIP-column in comparison with traditional C18-column are shown in Table 1. Both column types were packed with the same amount of each sorbent (10 mg) and loaded with the same CoQ10 mass (2 µg). Loading, washing and elution solvents used were the optimized ones for MIP-SPE, for better comparison. These results demonstrate the superiority of MIP-SPE, especially in terms of recovery and minimum elution volume all of which enhances sensitivity. Moreover, the percentage recovery remains above 90% after four times of reusing the same column.

We also evaluated the percentage recovery with C18 based SPE previously reported for CoQ10 [22] in two different conditions: a) using the same BET area (corresponding to 20 mg C18 sorbent) respect to MIP sorbent, and b) with the amount of C18 previously reported (50 mg). In every case, the CoQ10 loaded mass was the same (2 µg). In both experiments, the percentage of recovery using C18 was between 50-68 %, (n=3 for each procedure).

3.2 Validation

3.2.1 Matrix effect assessment and common interferences

One of the main drawbacks in LC-MS/MS is the phenomenon known as matrix effect. This may enhance or suppress ionization of an analyte due to the competition with co-eluted components in MS source. Therefore, two samples with the same amount of analyte can produce signals with different intensities [23]. Even though the sample was previously passed through MIP-SPE and was eluted in 1-propanol, a minimum matrix effect (MF) could be still present. In order to quantify this, we used the procedure previously described by Matuskewzki et al [24] that includes the comparison of the peak area for CoQ10 standard in 1-propanol (a) and the peak area of the same CoQ10 concentration in a spiked sample after extraction (b). The ratio between (a) and (b) shows the extent of the absolute matrix effect.

The MF obtained with this procedure for CoQ10 at three different levels (5, 50 and 200 ng/mL in the final sample) in urine was 100.9 %, showing a negligible matrix effect.

To evaluate the relative matrix effect, we compared the MF values obtained from different individual urines from healthy volunteers with the ones contaminated with erythrocytes or white cells.

Contaminated urines were prepared with total blood to a final total concentration of 0.4 mg of protein/mL (equivalent to a covered field of red blood cells under microscope) and with white blood cells isolated from blood to reach 50-100 leukocytes per field under microscope. No interference in CoQ10 quantification was observed at those levels (Table 3).

The RSD for MF in all urine samples analyzed was < 3.0. This result shows that relative matrix effect on quantification is negligible and that the presence of red and white blood cells does not interfere in this procedure.

3.2.2 Sensitivity and linearity

According to guidelines, calibration curves should be prepared in the same biological matrix as the samples in the intended study by spiking the blank matrix with known concentration of the analyte.

Given that a negligible matrix effect was observed, and the sample was eluted in 1-propanol, this solvent was used for method validation. Linearity was tested from 3 to 500 ng/mL, corresponding to a range of

0.6–100 ng/mL in urine. The mean calibration curves were obtained on three different days in triplicate at seven different levels (y: 0.0255x+0.3873; $r^2 > 0.9950$). Mean values of standard error (SE) for the slope were lower than 1% and SE mean values for the intercept were lower than 11%. LOQ and LOD values were 3 ng/mL and 1 ng/mL respectively (0.6 ng/mL urine CoQ10 and 0.2 ng/mL urine CoQ10 equivalent levels).

3.2.3 Accuracy and precision

Accuracy and precision were assessed by the analysis of quality control (QC) samples prepared spiking urine with CoQ10 standard solution at three levels: 5, 50, 200 ng/ml in the final sample. Three samples per level were used and both intra-day and inter-day accuracy and precision were evaluated according to international guidelines. For accuracy and precision, the RSD at each concentration level should not exceed 15%, except for the LOQ where it should not exceed 20%, according to international bioanalytical guidelines. The results fulfilled all the established criteria (Table 2). For accuracy assessment, the concentration of QC samples prepared in urine was corrected considering the endogenous analyte concentration.

3.2.4 Stability

No significant CoQ10 decrease was observed during the first 4h at room temperature after collection (CoQ10>91%). After that, CoQ10 in the collected sample falls significantly (<80% at 5 hs). The sample could be stored at least 7 days at -20°C and 30 days at -80°C with CoQ10 > 90%. The sample should not be stored at 8°C as precipitation occurs with a consequent decrease in CoQ10 levels. Not more than one thaw cycle is recommended as CoQ10 levels could drop 30%, even if the sample is kept at -80 °C.

3.3 Application to real samples

Twenty-two urine samples were analyzed following the developed procedure. CoQ10 levels in urine shows no significant differences between males and females (20.9 ±3.7 vs 23.3±8.3 ng/mL urine, mean ± SEM, respectively).Figure 1 shows the chromatogram of CoQ10 in a healthy volunteer.

4. DISCUSSION

As it was previously mentioned, muscle biopsy is the gold standard matrix in the diagnosis of CoQ10 deficiency. Taking into account its invasiveness, it can be of value to analyze CoQ10 status in a wide range of sample types, as a deficiency may remain undetected if the appropriate specimen is not chosen. Yubero et al [25] summarized the advantages and limitations for the CoQ10 analysis in different biological specimens including urine. According to their discussion, it is important to emphasize that one of the major clinical phenotypes associated with CoQ10 deficiency is the nephrotic syndrome [8] and that clinical renal improvements following CoQ10 treatment were reported[26]. Although correlations with kidney CoQ10 status remains to be established, urinary tract CoQ10 analysis may help to fulfill the critical need for less invasive procedures and it could be especially useful for treatment monitoring purposes.

In this work we describe a miniaturized method to measure CoQ10 in urine, with the aim of detecting possible CoQ10 deficiency as well as to follow the treatment in urine which is a biological specimen obtained non-invasively. The micro-method involves solid phase extraction using a previously developed MIP [17], followed by HPLC-MS/MS.

Yubero et al [27] developed the first method to determine urinary CoQ10 status via HPLC with electrochemical detection with recovery values between 96.7-118.0%. However, it comprises a multiple step LLE sample preparation and requires at least 30 mL of sample to keep a constant cellular pellet. Moreover, samples with a high amount of blood contamination or with leukocyte counts higher than 30 per field are unfit to be analyzed for their CoQ10 content. On the other hand, Grossi et al [22] proposed a sample extraction procedure with n-hexane followed by a double clean up and concentration step on silica gel and C18 SPE cartridges applied to plasma samples. We compared this method against the proposed MIP-SPE. Different conditions were tested (section 3.1), and in all cases MIP-SPE showed the highest recovery values. This could be explained by the lower extraction selectivity of C18 versus MIP.

In the present work, only 10 mg of this polymer were needed to pack the polypropylene cartridges for the selective extraction of CoQ10 in small amounts of urine samples (5mL). The developed system was extensively optimized and validated to obtain adequate performance: low limits of detection and quantification, linearity in wide ranges of concentration, excellent reproducibility with high recovery percentages and without interferences of red and white cells. In addition, cartridges can be reused up to 4 times without loss of performance. To the best of our knowledge, this is the first report in which an effectively designed MIP in packed columns was evaluated for the selective isolation and determination of CoQ10 in urine sample.

5.CONCLUSION

The system developed in our work allows precise and accurate determination of CoQ10 in 5 mL of urine, which is very appropriate when collecting spontaneous samples from pediatric patients. The use of this solid phase extraction system does not present interference by proteins or elevated leukocytes, given the high selectivity of the MIP and its subsequent MS/MS analysis, which is extremely important in the case of a CoQ10 deficiency with renal impairment.

The advantage of the MIP-SPE procedure developed compared to a C18 traditional SPE sorbent was also shown: the traditional C18 SPE is surpassed in terms of reproducibility, sensitivity, selectivity, load capacity, reusability, speed and amount of sample required.

In this study we evaluated CoQ10 urine levels in healthy subjects divided by gender and age range. More studies are needed, however, to assess whether this determination could be used for diagnosis and if this matrix could replace the traditional ones.

In conclusion, the combination of miniaturized extraction procedures using a selective CoQ10 MIP followed by HPLC-MS/MS is appropriate for the reliable determination of CoQ10 in small amounts of urine that would be extremely useful for the diagnosis and post-treatment control of patients with CoQ10 deficiency.

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CONFLICT OF INTEREST

All authors certify that not actual or potential conflict of interest in relation to this article exists.

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Figure Legends

Fig.1. Chromatogram of (a) Coenzyme Q10 (CoQ10) and (b) Coenzyme Q9(CoQ9) internal standard in a in a healthy volunteer

Table 1

Performance parameters of the developed MIP-column in comparison to traditional C18-column

Parameter	Sorbent	
	MIP	C_{18}
Minimum elution volume (mL)[a]	1.0	2.5
Recovery (%)	109	69
Load capacity (µg per mg of sorbent)	>1	>1
Bed volume (µL)	60	140
Reuse (times)	4	1

[a] For a 2 µg CoQ10 load in 10 mg of sorbent

Table 2

Parameters of validation of the analytical method for determination of CoQ10 in urine

Parameter			
Precision (RSD) [a]			
Spiked levels	low (5 ng/mL)	middle (50 ng/mL)	upper (200 ng/mL)
Intra-day (n=9)	6.2	4.4	5.2
Inter-day (n=27)	11.1	4.8	7.4
Accuracy [b]			
Spiked levels	low (5 ng/mL)	middle (50 ng/mL)	upper (200 ng/mL)
Recovery %	89.0 (3.6)	95.3 (3.1)	94.0 (4.0)

[a] RSD values of normalized areas are the averages from three concentration levels of the calibration curves.

[b] Recovery mean values obtained from three individual samples on three different days at three concentration levels. RSD values in brackets.

Table 3

Potential interferences in urine matrix.

	CoQ10 concentration (ng/mL urine)
Sample A	6.1
Sample A + 80µL whole blood[a]	5.9
Sample A + Leukocytes[b]	6.6

[a] Total Protein 0.4 mg/mL

[b] 50 Leukocytes/field

Highlights

- First sample preparation method based on MIP-SPE for Coenzyme Q10.
- Low sample requirement of a non-invasive sample.
- MIP-SPE combined with HPLC-MS/MS is useful for diagnosis and post-treatment control.
- MIP-SPE yields higher quantitative performance parameters than traditional SPE sorbents.

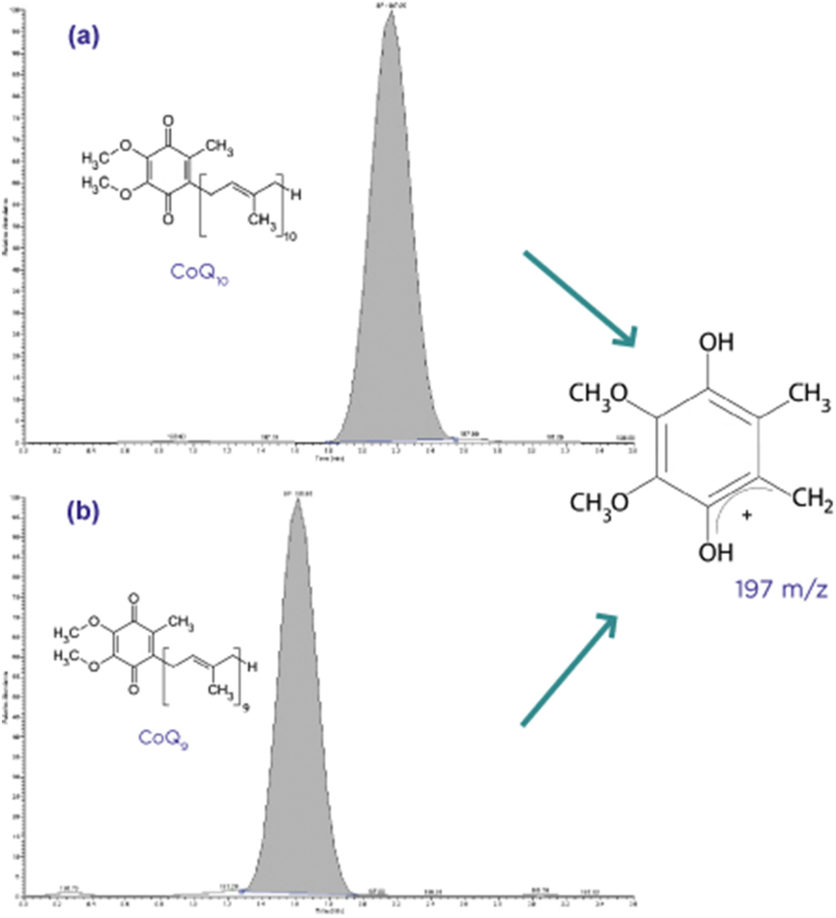
(a)
H3C O
H3C O
O
O
CH3
H
CH3
10
CoQ10
(b)
H3C O
H3C O
O
O
CH3
H
CH3
9
CoQ9
OH
CH3O
CH3O
CH3
CH2
+
OH
197 m/z

Figure 1