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Determination of ubiquinone and related metabolites in zebrafish embryos by LC-HRMS

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Overview

• A fast and sensitive **LC-HRMS (MS^2) method to measure simultaneously cholesterol, tocopherol, vitamin K1, vitamin K2, coenzyme Q9 and coenzyme Q10** in zebrafish embryos samples was developed.

• The purpose of this study was to compare different ionization modes, mobile phases and stationary phases in order to **optimize lipid molecules separation**. After LC-MS parameters selection several **extraction conditions** were evaluated.

• The method was applied to the characterization of **wild-type zebrafish partial lipidome** with particular interest toward natural antioxidant molecules related to ubiquinone.

• We recently identified a zebrafish mutant, called *barolo*[1], showing a deficit of endogenous CoQ10. In this work a more extended lipidomic approach was performed.

Introduction

Characterization of lipidome is an emerging topic in metabolomics and the contribution of HPLC-HRMS is an important tool in the elucidation of biochemical pathways[1]. One goal of this work is to provide a rapid, selective and sensitive method to measure ubiquinone and related lipids concentration in zebrafish embryos and to apply it to metabolism studies. Ubiquinone (also known as coenzyme Q10) plays an essential role in the mitochondria electron-transport chain. It is an interesting molecule shown to play an important antioxidant role in the cardiovascular system and it is the only endogenously synthesized lipid-soluble antioxidant. Zebrafish (*Danio rerio*) is an established model for studying toxicology and understanding human diseases[2].

We selected for this study lipid molecules belonging to different classes that display different chemical properties and do variously ionize within LC-MS sources: a sterol, a phenolic derivative and some quinones.

The aims of the work are:

• selection of an **optimal liquid chromatographic method** to separate and measure zebrafish lipids involved in redox biochemical pathways (ubiquinones, cholesterol, vitamins E, K1 and K2),

• evaluation of the **best ionization mode** for the selected analytes (ESI in positive ion mode, MH^+ MNa^+ MLi^+ MNH_4^+ adducts; ESI in negative ion mode; APCI in positive and negative ion mode),

• assessment of **extraction conditions** in order to optimize analytical recovery,

• measure lipid profile in **wild-type and mutant zebrafish embryos and adults**.

Methods

Embryos and adult fishes were raised and maintained under standard laboratory conditions. Biological samples were extracted by LLE (liquid-liquid extraction) with various organic solvents. HPLC-HRMS analyses were accomplished on a Dionex Ultimate 3000 LC system coupled with a LTQ-Orbitrap instrument, with ESI and APCI interfaces. C4, C8 and C18 RP columns were tested for separation. Here we investigate the ionization modes, the ex vivo analytical sensitivity and the fragmentation mechanisms of ubiquinone and related compounds. The developed methodology will be applied to study samples of zebrafish embryos.

LLE tested conditions: ***procedure 1***: 35 embryos were washed, suspended and homogenized with 0.5 mL of water/2-propanol 40:60 v/v and extracted twice with 0.7 mL of hexane; ***procedure 2***: 35 embryos were washed, suspended and homogenized with 0.5 mL of Gibco® 7.4 PBS buffer and extracted twice with 0.7 mL of hexane. Then hexane phases were injected as they are (***1A, 2A***), after evaporation to dryness and reconstitution with methanol (***1B, 2B***) and after evaporation to dryness and reconstitution with hexane (***1C, 2C***).

LC: tested mobile phases (gradient conditions): Ammonium acetate 10 mM / Methanol; Formic acid 10 mM / Methanol; Formic acid 10 mM / Acetonitrile; Formic acid 10 mM / Methanol- Acetonitrile, from 70/30 to 0/100 in 15'. Tested columns: Phenomenex Luna C18(2) 100 A, 150 × 2.1 mm; Phenomenex Luna C8(2) 100 A, 150 × 2.1 mm; Phenomenex Jupiter C4 300 A, 150 × 2.1 mm.

ESI source conditions: source voltage 4.5 kV; capillary voltage 22 V; capillary temperature 265°C.

APCI source conditions: vaporizer temperature 450°C; source voltage 6 kV; capillary voltage 2 V; capillary temperature 250°C.

MS analyzer conditions: full scan FTMS positive/negative ion mode. 300-1000 *m/z* @ 30000 resolution. MS/MS precursor ions for quantitative method: cholesterol 369.4 *m/z* ($MH\text{-}H_2O^+$; collision energy, CE = 30) ; alpha-tocopherol 431.4 *m/z* (CE = 25); vitamin K1 451.4 *m/z* (CE = 25); vitamin K2 445.3 *m/z* (CE = 25); CoQ6 591.4 *m/z* (CE = 25); CoQ9 795.6 *m/z* (CE = 25); CoQ10 863.7 *m/z* (CE = 25)

Scheme 1: Lipid analytes with main fragmentation pathways observed for protonated molecular ions

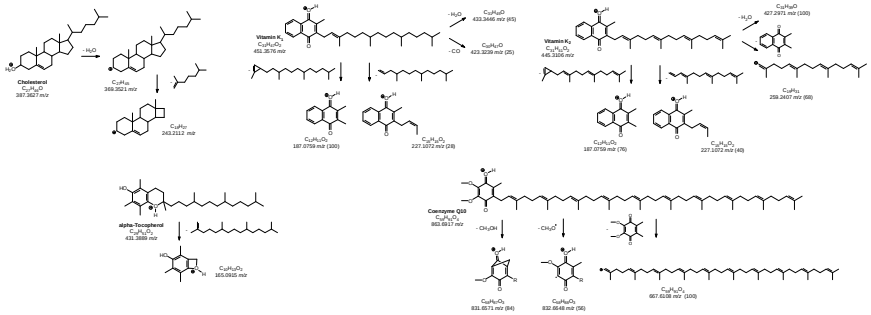


Figure 1: LC-HRMS chromatogram of selected analytes separation on RP-C4 stationary phase

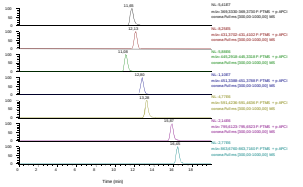


Figure 2: Correlation between logP values and retention times (C4 column / methanol as organic modifier)

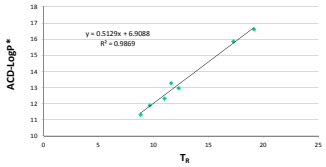


Figure 3: Extraction recovery of different LLE methods

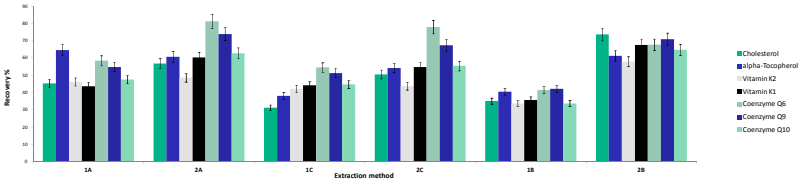


Table 1: Relative intensity of molecular ions obtained with different sources/polarity (peak height, direct infusion at 10 mg/mL)

	MH^+ (ESI)	$ML i^+$ (ESI)	MNa^+ (ESI)	MNH_4^+ (ESI)	$[M\text{-}H]^-$ (ESI)	MH^+ (APCI)	$[M\text{-}H]^-$ (APCI)
Cholesterol	n.d.	n.d.	n.d.	n.d.	n.d.	**1.34×10^8**	n.d.
Tocopherol	n.d.	n.d.	5.07×10^3	n.d.	2.69×10^4	**7.6×10^6**	2.21×10^2
Vit K1	1.793×10^5	1.48×10^7	2.27×10^7	n.d.	2.13×10^6	**2.37×10^8**	3.42×10^7
Vit K2	n.d.	3.28×10^7	1.45×10^7	3.48×10^7	n.d.	**1.49×10^8**	1.15×10^7
CoQ6	-	-	-	-	-	**2.8×10^8**	-
CoQ9	-	-	-	-	-	**1.2×10^8**	-
CoQ10	n.d.	2.58×10^7	1.75×10^8	n.d.	1.15×10^6	**2.38×10^8**	1.04×10^7

Table 2: Linearity and LLOQ of quantitative analysis method

	HRMS calibration curve	MS/MS calibration curve	HRMS LLOQ (ng/single embryo)
Cholesterol	Y = 0.2301 x - 0.5759 R^2 = 0.9979	Y = 0.0813 x - 0.1637 R^2 = 0.9988	0.57
Tocopherol	Y = 0.044 x - 0.0026 R^2 = 0.9998	Y = 0.111 x - 0.0064 R^2 = 0.9997	0.029
Vit K1	Y = 0.492 x – 0.0055 R^2 = 1	Y = 0.2434 x - 0.0182 R^2 = 0.9996	0.029
Vit K2	Y = 0.2671 x – 0.0205 R^2 = 0.9994	Y = 0.265 x - 0.0097 R^2 = 0.9996	0.029
CoQ6	Y = 0.2164 x – 0.0012 R^2 = 0.9999	Y = 0.2217 x - 0.0031 R^2 = 1	0.029
CoQ9	Y = 0.1084 x – 0.0027 R^2 = 1	Y = 0.039 x - 3 x 10^{-6} R^2 = 1	0.029
CoQ10	Y = 0.1433 x – 0.0099 R^2 = 0.9997	Y = 0.0248 x - 0.0025 R^2 = 0.9993	0.029

Results

In the first step of method development we optimized the chromatographic separation of highly lipophilic ubiquinone related compounds on RP-HPLC columns. We selected the RP-C4 stationary phase in order to shorten analysis time and the system acidic water/methanol as mobile phase to obtain the better sensitivity. The employ of acetonitrile as organic modifier reduced significantly ion intensity with the APCI source so it was not used. A model **chromatogram** is shown in **figure 1**. **Retention times** are well correlated to **logP** values found in ACD website[3] as shown in **figure 2**.

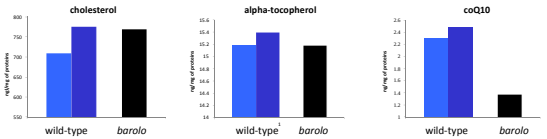
The investigation about the sensitivity of **different ionization modes** (positive vs. negative ion mode, APCI vs. ESI, proton vs. ammonium vs. sodium vs. lithium adducts formation) induced us to select positive ion mode APCI as the finest ionization mode. The relative intensity of molecular ion signals is reported in **Table 1**. It is noteworthy the difficulty to find a universal ionization mode in spite of the small number of studied analytes.

A **fragmentation** study of the analytes was also done and the main **pathways** are reported in **scheme 1**. A general tendency to eliminate the aliphatic chain could be observed. No common fragmentation pattern was observed for different molecular adducts. For example, CoQ10 MH^+ ion eliminates as well a molecule of methanol and a methoxy radical while a double neutral loss of formaldehyde is the major pathway of CoQ10 MNa^+ MS/MS breakdown.

A **validation study** was then completed to make possible quantitative determination on biological samples. Accuracy, precision, LLOQ, linearity, extraction recovery were evaluated and some values are reported in **Table 2** for full HRMS and MS/MS acquisition. Then to confirm the high similarity between zebrafish and higher vertebrates at the cellular and physiological levels we determined the variation of concentration of Coenzyme Q10 at various zebrafish growth steps. Extraction recovery of different tested procedures (1A-B-C, 2A-B-C) are reported in **Figure 3**, showing that 2B procedure provides the best results. **Figure 4** shows the mean values of ng of cholesterol, alpha-tocopherol, coQ10 / mg of total protein measure in wild-type zebrafish embryos at 72 hours post fertilization. Deficit of endogenous coQ10 synthesis in *barolo* mutant was confirmed.

Finally a study to characterize the **untargeted lipidome** of embryo samples is ongoing to resolve the complex mixture of compounds giving mono-charged ions in the range 700-1000 *m/z*. A tentative identification of some triacylglicerols was conducted bringing to identification of typical polyinsaturated fish triglycerides as 1,2,3-propanetriyl-1-hexadecanoate-2,3-di-docosahexaenoate (951.7457 *m/z*) or propanetriyl-1-hexadecanoate-2-docosahexaenoate-3-(9'-octadecenoate) (905.7618 *m/z*) .

Figure 4: minimum/maximum measured values of main lipid metabolites/weight of proteins in wild-type zebrafish embryos vs. *barolo*[1] mutant



Conclusions

A fast and selective high resolution method for zebrafish lipid profile has been developed and applied to wild-type and mutant biological samples. Lipids belonging to different classes (sterols, prenols, glycerides) has been analyzed in a single analytical run. Wild-type zebrafish embryos content of cholesterol, tocopherol and CoQ10 has been shown.

References

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