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Short communication

Stabilization and detection of hydrophylloquinone as di-*O*-methyl derivative

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ABSTRACT

Phylloquinone is a redox active naphthoquinone involved in electron transport in plants. The function of this reduced form remains unclear due to its instability, which has precluded detection. Herein, a simple method that permits the stabilization of the reduced form of phylloquinone by di-*O*-methylation and HPLC detection is described.

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1. Introduction

Phylloquinone (**1**) (2-methyl-3-phytyl-1,4-naphthoquinone, vitamin K1) is a bifunctional molecule composed of a redox active naphthoquinone ring and a lipophilic phytyl side chain (Fig. 1). The phylloquinone (**1**) has a vital function in plants mediating electron transport in photosynthesis (photosystem I) [1]. The transfer of one electron, therefore, involves the quinone and semiquinone forms of phylloquinone, and usually occurs in the nanosecond time scale. The rate quinone/semiquinone (1:2) is classically studied by electron paramagnetic resonance [2]. Recently, other hypotheses for the functions of phylloquinone (**1**) in plants beyond electron transport were proposed. The reduced form of **1**, hydrophylloquinone (**2**, KH_2), was detected in smaller quantities in *Arabidopsis* leaves kept under light when compared with those kept in the dark suggesting that the KH_2 (**2**) is related to the photoactive pool of phylloquinone (**1**) and is involved in chlororespiration [3]. Other studies show that approximately 50% of the total phylloquinone (**1**) is not associated with photosystem I [4,5]. In vertebrates phylloquinone (**1**) is known as a cofactor for some carboxylases that convert specific glutamate residues on target proteins to γ-carboxyglutamates (Gla) [6] which are involved in blood coagulation, bone homeostasis and maintenance of vascular integrity [7,8]. In this process, **1** is converted to phylloquinone epoxide, which must be recycled to KH_2 (**2**) to complete the vitamin K1 cycle. This reduction is catalyzed by a vitamin K epoxide reductase (VKOR) [9]. The KH_2 (**2**) has been described as a potent biological antioxidant [10,11]. Furthermore, it has greater capacity than ubihydroquinone-10 ($Q_{10}H_2$) to regenerate α-tocopherol from the α-tocopheryl radical resulting from the major physiological free radical scavenging pathway [12,13].

Although the studies above have characterized different forms of phylloquinone (**1**), the characterization of KH_2 (**2**) remains unclear due to its instability [3,14,15]. Oostend and collaborators [3], described a protocol that employs sodium borohydride or sodium hydrosulfite as reducing agents of phylloquinone (**1**) in order to obtain the reduced form KH_2 (**2**) as a standard with

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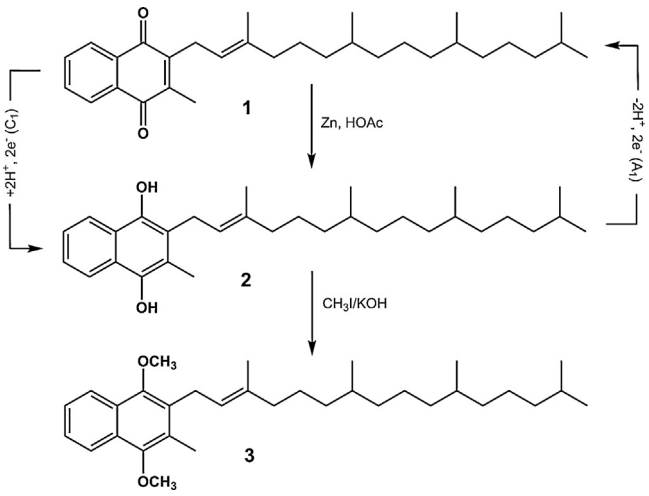

Fig. 1. Eletrochemical reduction/oxidation of hydrophylloquinone (**1**) to hydrophylloquinone (KH_2, **2**) and chemical reduction from **1** to **2** followed by methylation to di-*O*-methyl hydrophylloquinone (**3**).

a reoxidation rate of 2% per day. Furthermore, the authors suggest immediate analysis using fluorimetric detection of the samples because KH_2 (**2**) is only detectable for a few minutes after extractions.

The present article describes a straightforward and fast method to stabilize KH_2 (**2**) as di-*O*-methyl hydrophylloquinone derivative (**3**), which was characterized by HPLC-HRESI$^+$, GC/MS, ^{1}H and ^{13}C NMR (1D and 2D) and electrochemical method.

2. Materials and methods

2.1. Method for obtaining di-O-methyl hydrophylloquinone derivative 3

Phylloquinone (**1**) (1 eq., 0.044 mmol, 20 mg, 20 μL) was dissolved in 5 mL of anhydrous tetrahydrofuran (THF) under nitrogen atmosphere (N_2), resulting in a yellow solution. Zinc (4 eq., 0.177 mmol, 11.6 mg) was added to the reaction solution followed by an excess of glacial acetic acid (0.5 mL), resulting in a clear solution [16]. The formation of KH_2 (**2**) was monitored every 10 min by sampling 5–10 μL with a glass capillary, diluting in MeOH (100 μL), filtering in a cotton plug and injecting 1 and 5 μL into GC/MS and HPLC systems, respectively. After 30 min, when the reaction was completed, KOH (20 eq., 0.97 mmol, 54.7 mg) and methyl iodide (CH_3I) (20 eq., 0.97 mmol, 137.68 mg, 60.6 μL) were added. The reaction was stirred overnight, in the dark, at room temperature. The reaction mixture was partitioned between water (5 mL) and dichloromethane (3 × 5 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered and rotaevaporated yielding the compound **3** (20.9 mg, 98% yield).

2.2. GC–MS analysis

A gas chromatography model Trace GC coupled to mass spectrometer (electron impact Y2K ion trap (MS) PolarisQ System) (Finnigan, ThermoQUEST Inc., San Jose, CA) with a data analysis program (Xcalibur version 1.3) was used. The device was equipped with a TR-1MS column (30 m, 0.25 mm 0.25 μm, Thermo Scientific, USA). The injector temperature was 220 °C, equipped with a splitless liner, and kept at an initial oven temperature of 100 °C for 2 min and increased to 300 °C at a rate of 25 °C min. This temperature was maintained for 10 min and then cooled to initial conditions. The transfer line was maintained at 260 °C and helium flow was 1.5 mL/min. The mass spectrometer was operated in positive mode with ion source at 200 °C. The mass range monitored was *m*/*z* 40–500 (Full scan).

2.3. HPLC–MS analysis

Phylloquinone (**1**) and its derivatives were analyzed by high performance liquid chromatography–mass spectrometry (HPLC–MS) composed by a Shimadzu LC20AD pump, using a Phenomenex Luna C18(2) (150 mm × 2 mm × 3 μm), auto sampler SIL-20AHT, column oven CTO--20A, detector UV–vis SPD-20A (254 nm), CBM--20A controller. A linear gradient with acetonitrile (solvent A) and methanol (solvent B) was used at a flow rate of 0.2 mL min. The gradient starts at 0–5 min 60% (A) increasing up to 100% (A) from 5 to 30 min and held for 30–35 min. Finally, re-equilibrating from 35 to 40 min to 60% (A) and held until 45 min.

A MicrOTOF-QII (Bruker) mass spectrometer was used to determine the exact mass of phylloquinone and its derivatives. The capillary voltage was 4500 V, nebulization and dry gas 4 Bar and 9 L min, respectively, ion source temperature was 200 °C, quadrupole ion energy 6 eV, collision cell energy 12 eV, funnel 1RF 400 Vpp, funnel 2RF 350 Vpp funnel hexapole 350 Vpp and collision RF 350 Vpp.

2.4. Nuclear magnetic resonance

The ^{1}H and ^{13}C NMR spectra were recorded at a frequency of 500 MHz and 125 MHz, respectively, on a spectrometer Bruker DRX 500 using $CDCl_3$ as a solvent. Chemical shifts are given in parts per million (δ) using tetramethylsilane (TMS) as internal standard. ^{1}H NMR and ^{13}C NMR, DEPT 135 spectra, COSY, HSQC and HMBC correlations are presented in Supplementary data (Supplementary Figs. S3–S14, respectively).

2.5. Electrochemical study of phylloquinone (1)

Electrochemical measurements were performed at 298 ± 1 K in a thermostatic cell with a CH 660I equipment. A BAS MF2012 glassy carbon working electrode (GCE) (geometrical area 0.071 cm^2), a platinum wire auxiliary electrode and a Ag Cl (3 M NaCl)/Ag reference electrode were used in a conventional three-electrode arrangement. Cyclic (CV) and square wave voltammetries (SWV) were used as detection modes. Derivative convolution of data was performed for increase peak resolution.

Thin film of phylloquinone (**1**) on glassy carbon electrode was prepared according to reported procedure [17], by pipetting 10 μL of the solution of **1** in ethanol and allowing the solvent to evaporate in a fume hood (during approximately 2 min). As a result, a uniform, fine coating film was adhered to the basal electrode. Aqueous 0.10 M potassium phosphate buffer saline (PBS) at physiological pH, previously degasified by bubbling Argon for 10 min, was used as a supporting electrolyte.

3. Results and discussion

The hydrophylloquinone (KH_2, **2**) was obtained from reduction of phylloquinone (**1**) using Zn and acetic acid. Then, both phenolic groups were methylated with CH_3I under basic media to yield the di-*O*-methyl hydrophylloquinone (**3**) (Fig. 1). The formation of **2** and **3** was monitored by HPLC and GC/MS (Figs. 2 and 3). The retention times in the HPLC analysis were 15.3 min for KH_2 (**2**), 21.7 min for phylloquinone (**1**) and 33.2 min di-*O*-methyl hydrophylloquinone (**3**).

The compounds were characterized by HRESI$^+$ (Fig. S1 in Supplementry data) and GC/MS (Fig. 3, Fig. S2 in Supplementry data): phylloquinone (**1**) (HRESI$^+$ $C_{31}H_{46}O_2$ $[M + Na]^+$, 473.3379 Da,

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