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Short communication

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

Rodrigo A.C. Sussmann[a,1], Marcilio M. de Moraes[b,1], Gerardo Cebrián-Torrejón[b,d], Exequiel O. Porta[c], Antonio Doménech-Carbó[d], Lydia F. Yamaguchi[b], Alejandro M. Katzin[a], Massuo J. Kato[b,*]

[a] Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 1374, São Paulo, SP 05508-000, Brazil
[b] Department of Fundamental Chemistry, Institute of Chemistry, University of São Paulo, Av. Prof. Lineu Prestes, 748, São Paulo, SP, 05508-000, Brazil
[c] Instituto de Química Rosario (IQUIR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, S2002LRK Rosario, Argentina
[d] Departament de Química Analítica, Facultat de Química, Universitat de València, Dr. Moliner 50, 46100 Burjassot, Valencia, Spain

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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.

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

* Corresponding author.
E-mail addresses: majokato@iq.usp.br, massuojorge@gmail.com (M.J. Kato).
URL: http://mailto:majokato@iq.usp.br (M.J. Kato).
[1] These authors contributed equally to this work.

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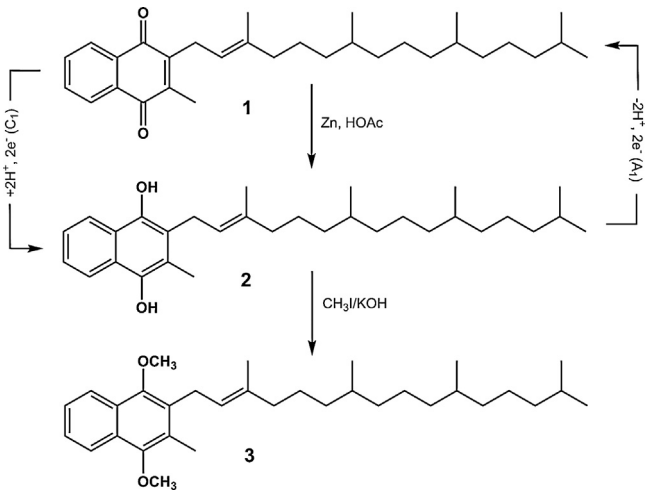

Fig. 1. Eletrochemical reduction/oxidation of hydrophylloquinone (**1**) to hydrophylloquinone (KH_2, **2**) and chemical reduction from **1** to **2** followed by methylation to di-*O*-methyl hydrophylloquinone (**3**).

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,

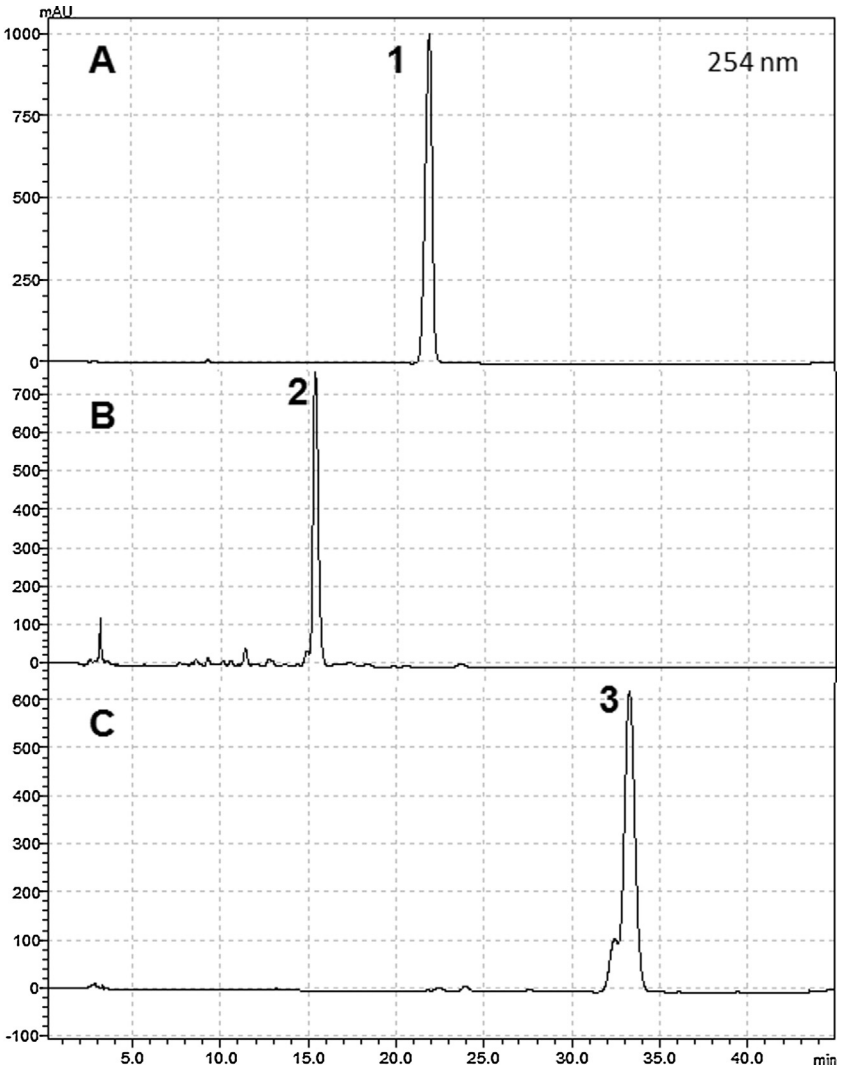

Fig. 2. HPLC profile of vitamin K1 and its derivatives: (A) vitamin K1 (**1**), (B) hydrophylloquinone (**2**) and (C) di-*O*-methyl hydrophylloquinone (**3**).

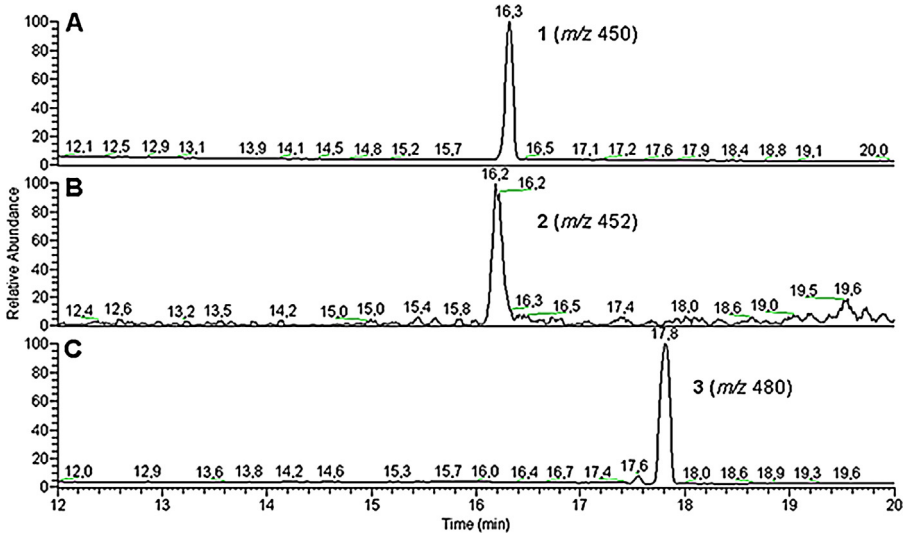

Fig. 3. GC profile of vitamin K1 and its derivatives: (**A**) phylloquinone (**1**) at 16.3min, (**B**) hydrophylloquinone (**2**) at 16.2 min and (**C**) di-*O*-methyl hydrophylloquinone (**3**) at 17.8 min.

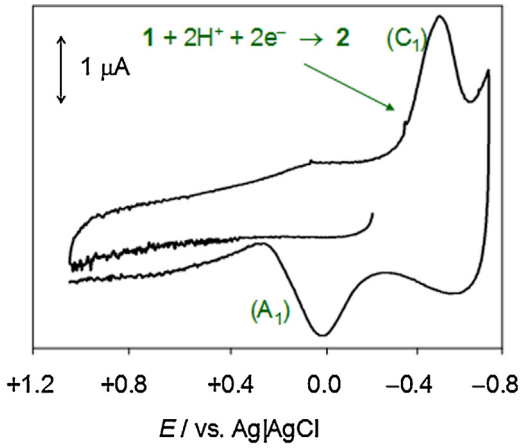

Fig. 4. Cyclic voltammogram, after semi-derivative convolution, of a vitamin K1 (**1**) film on glassy carbon electrode immersed into 0.10 M PBS, pH 7.4. Potential scan initiated at −0.25 V in the positive direction; potential scan rate 50 mV/s.

calc. 473.3390, EI *m*/*z* 450 $[M]^{+\cdot}$, 225 and 186 Da); hydrophylloquinone (KH_2, **2**) (HRESI$^+C_{31}H_{48}O_2$ $[M+Na]^+$, 475.3558, calc. 475.3546 Da; IE at *m*/*z* 452 $[M]^{+\cdot}$, 227 and 186 Da); and di-*O*-methyl hydrophylloquinone (**3**) (HRESI$^+C_{33}H_{52}O_2$, $[M+Na]^+$, 503.3858, calc. 503.3859 Da; EI *m*/*z* 480 $[M]^{+\cdot}$, 255 and 215 Da). Complete evidence for the structure of di-*O*-methyl hydrophylloquinone (**3**) was obtained from the analysis of ^1H and ^{13}C NMR data, which were assigned with the aid of DEPT 135, COSY, HSQC and HMBC data (Figs. S9–S14 in Supplementry data).

The cyclic voltammetry response of a phylloquinone (**1**) film on GCE in contact with PBS was obtained at physiological pH (Fig. 4). The potential scan was initiated at −0.25 V vs. Ag AgCl in the positive direction in order to test the presence of phylloquinone (**1**) as its reduced form KH_2 (**2**). As can be seen in the initial-going scan, no anodic peaks were detected indicating the purity of the quinone form. In the subsequent cathodic scan (Fig. 4), a reduction peak was recorded at −0.54 V (C_1) coupled, in the following anodic scan, by an oxidation peak at −0.04 V (A_1). The peak C_1 was unambiguously assigned to the proton-assisted reduction of the quinone form of phylloquinone (**1**) to the hydrophylloquinone (**2**) (Figs. 1 and 4). This is the first application of cyclic voltammetry to describe their interconversion.

The reduced KH_2 (**2**) was stabilized as its di-*O*-methylated derivative (**3**) using CH_3I, under N_2 atmosphere, yielding a quantitative conversion without detectable reoxidation to the quinone form as previously observed [3,14,15]. The compound **3** was fully characterized by spectroscopic methods and shows the same profile in HPLC and GC/MS analysis after six months at 0 °C in dark conditions (data not shown). Thus, this novel method is a reliable and alternative analytical tool for the indirect detection of **2** and for further research of this understudied metabolite.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2016.09.011.

References

[1] K. Sigfridsson, O. Hansson, P. Brzezinski, Proc. Natl. Acad. Sci. U. S. A 92 (1995) 3458–3462.
[2] W. Xu, P. Chitnis, A. Valieva, A. van der Est, Y.N. Pushkar, M. Krzystyniak, C. Teutloff, S.G. Zech, R. Bittl, D. Stehlik, B. Zybailov, G. Shen, J.H. Golbeck, J. Biol. Chem. 278 (2003) 27864–27875.
[3] C. Oostende, J.R. Widhalm, G.J. Basset, Phytochemistry 69 (2008) 2457–2462.
[4] J. Gross, W.K. Cho, L. Lezhneva, J. Falk, K. Krupinska, K. Shinozaki, M. Seki, R.G. Herrmann, J. Meurer, J. Biol. Chem. 281 (2006) 17189–17196.
[5] A. Lohmann, M.A. Schottler, C. Brehelin, F. Kessler, R. Bock, E.B. Cahoon, P. Dormann, J. Biol. Chem. 281 (2006) 40461–40472.
[6] M.M. Ulrich, B. Furie, M.R. Jacobs, C. Vermeer, B.C. Furie, J. Biol. Chem. 263 (1988) 9697–9702.
[7] C. Vermeer, M.J. Shearer, A. Zittermann, C. Bolton-Smith, P. Szulc, S. Hodges, P. Walter, W. Rambeck, E. Stocklin, P. Weber, Eur. J. Nutr. 43 (2004) 325–335.
[8] L.J. Schurgers, M.J. Shearer, K. Hamulyak, E. Stocklin, C. Vermeer, Blood 104 (2004) 2682–2689.
[9] P.H. Chu, T.Y. Huang, J. Williams, D.W. Stafford, Proc. Natl. Acad. Sci. U. S. A 103 (2006) 19308–19313.
[10] J. Li, J.C. Lin, H. Wang, J.W. Peterson, B.C. Furie, B. Furie, S.L. Booth, J.J. Volpe, P.A. Rosenberg, J. Neurosci. 23 (2003) 5816–5826.
[11] L.M. Vervoort, J.E. Ronden, H.H. Thijssen, Biochem. Pharmacol. 54 (1997) 871–876.
[12] K. Mukai, S. Itoh, H. Morimoto, J. Biol. Chem. 267 (1992) 22277–22281.
[13] K. Mukai, H. Morimoto, S. Kikuchi, S. Nagaoka, Biochim. Biophys. Acta 1157 (1993) 313–317.
[14] A.M. Zbierzak, P. Dörmann, G. Hölzl, Methods Mol. Biol. 775 (2011) 411–426.
[15] M. Shibata, H. Shimada, Methods Mol. Biol. 1153 (2014) 99–113.
[16] A.P. Marchand, G.M. Reddy, Synth. Stuttgart (1991) 198–200.
[17] A. Doménech-Carbó, L.M. de Carvalho, M. Martini, G. Cebrián-Torrejón, Rev. Anal. Chem. 33 (2014) 173–199.