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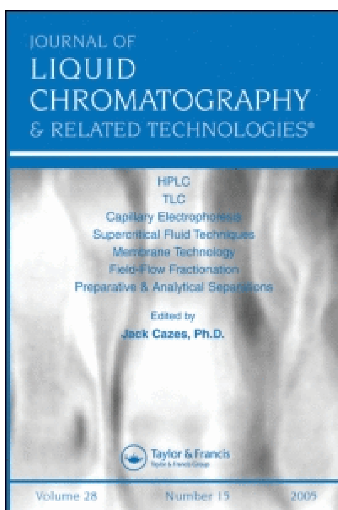

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Berger, G. , Kléo, J. , Breton, J. , Gilles, N. and Lirsac, P. N.(1994) '^{18}O and $U^{13}C$ Labeling of Photosynthetic and Related Quinones and Their Purification by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 17: 20, 4531 — 4539

To link to this Article: DOI: 10.1080/10826079408013635

URL: http://dx.doi.org/10.1080/10826079408013635

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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(20), 4531-4539 (1994)

^{18}O AND $U^{13}C$ LABELING OF PHOTOSYNTHETIC AND RELATED QUINONES AND THEIR PURIFICATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Different photosynthetic quinones have been labeled on the carbonyl groups by oxygen exchange with $H_2{}^{18}O$ and purified by high performance liquid chromatography on normal phase column with non aqueous solvent mixtures. $U^{13}C$ quinones have been extracted from Spirulina maxima, Synechocystis 6803 and E. coli, grown on ^{13}C labeled medium and purified on reversed phase columns. Vitamin K_1, plastoquinone PQA_{45}, ubiquinone Q_8 have been obtained in mg amounts, while different other quinones (menaquinone MK_8, ubiquinones Q_2, Q_7, Q_9, Q_{11}, plastoquinones A) were present only in trace amounts. Several of these quinones have been used in the reconstitution of the photosynthetic reaction centers, in order to assign the quinone bands in the light-induced Fourier transformed infrared difference spectra of the photoreduction of the primary quinone acceptor.

INTRODUCTION

The identification of the different bands observed in the light-induced FTIR difference spectra of the photoreduction of the primary quinone acceptor Q_A and their assignment to the protein or the quinone moiety are greatly facilitated by the use of bacterial reaction centers reconstituted with ^{18}O and ^{13}C quinones (1). In this work, the ^{18}O labeling has been obtained by isotopic exchange with $H_2{}^{18}O$ on commercially available quinones and the uniformly ^{13}C labeled quinones have been extracted from bacteria or algae grown on ^{13}C labeled culture medium. In the two cases the quinones have been purified in one or several steps by high performance liquid chromatography.

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MATERIAL AND METHODS

1) Labeling

a/ ^{18}O labeling

5 to 50 mg of quinone (vitamin K_1, Q_{10}, dimethylnaphtoquinone, 2-3 dimethoxy 5 methyl 1-4 benzoquinone, 2-3 dimethoxy 5-6 dimethyl 1-4 benzoquinone, tetramethyl 1-4 benzoquinone) (Sigma, Aldrich, Apin Chemicals) were incubated, in 1 ml vials stoppered with teflon faced liners, at 37°C during 1 to 15 days, under nitrogen, with 90 µl $H_2{}^{18}O$ (Service des Molécules Marquées CEA) and 60 µl trifluoroacetic acid (TFA, Merck), dissolved in 350 µl tetrahydrofuran (THF, Merck).

b/ $U^{13}C$ labeling

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E. coli strain BL 21 (2) was grown on mineral medium M9 added with 0.66 g/l of ammonium sulfate and 1.5 g/l of ^{13}C D glucose as sole carbon source. The medium was supplemented by 5 mg/l thiamine, minerals (0.5 mM $FeCl_3$, 0.1 mM $CaCl_2$) and contained 25 mg/l chloramphenicol and 150 mg/l ampicillin. The bioreactor was LSL Biolaffite 30 Al. The medium was oxygenated with air (12 l/min) at 37°C and the pH was continuously adjusted at 7.2 with 1 M NaOH. The final volume was 10.2 l.

Synechocystis PCC 6803 and Spirulina maxima were both cultivated in Zarrouk medium (3) containing 1.05 g/l of ^{13}C sodium carbonate as sole carbon source. Culture medium was maintained at 29°C pH 9.3 under 600 $\mu E/sm^2$ continuous incandescent illumination per absorbance unit at 366 nm. The final volume was 28.5 l.

For all samples, 50 µg of dried biomass were isotopically diluted with 1 mg casein and combusted in an oxygen/helium atmosphere. The gaseous product was analysed using a mass spectrometer. The isotopic enrichment of the preparation calculated from the $^{13}CO_2/^{12}CO_2$ ratio, was 98 % in the case of E. coli, 99 % in the case of Synechocystis and 96 % for Spirulina.

2) Purification

a) ^{18}O labeled quinones

THF, TFA and the mixture of $H_2{}^{16}O$ and $H_2{}^{18}O$ were evaporated under nitrogen stream and the residue of labeled quinone and impurities was dissolved in 500 µl chloroform (Lichrosolv, stabilised with 2 methyl 2 butene, Merck). High performance liquid chromatography was applied with a Waters Ass. liquid chromatograph composed of a M 510 pump, U_6K injector and 490 E detector. Preparative runs were performed on Partisil 10 M9 Whatman columns (9 mm x 50 cm),

with chloroform-ethanol mixtures (0.2 to 0.4 %) as eluent, or chloroform-hexane mixture (4/1 V/V), in the case of tetramethylbenzoquinone. Optical absorption was followed at the corresponding uv maximum.

b) $U^{13}C$ labeled quinones

- Quinones from E. coli

The culture medium was centrifuged for 20 min at 7,500 RPM. 25 g of wet bacteria were collected and extracted three times by agitation during 10 min with 100 ml of pure ethanol (eventually followed by acetone, then hexane). The suspension was filtered on glass filter, the different extractions were pooled and dried under vacuum. The residue was then extracted with hexane (2 ml), the solution was washed with water, separated and evaporated under vacuum. The new residue was dissolved in ethanol and injected on a Partisil 10 ODS_2 M20 Whatman column (20 mm x 50 cm). The different components were eluted with pure ethanol (14-18 ml/min). The quinone fractions were collected, evaporated and further purified on Ultrasphere ODS 5 µm (Beckman) (4,6 mm x 25 cm), with pure ethanol as eluent (1 ml/min), and finally on Partisil 10 M9 as above, with chloroform containing 0.2 % ethanol (6 ml/min).

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- Quinones from Spirulina maxima

175 g of wet centrifuged Spirulina were successively extracted with 250 ml of the following solvents : methanol (four times), ethanol (twice), acetone, heptane, chloroform-acetone-methanol mixture (1/2/1) (twice). The different filtrates (obtained by passage on glass sintered filters) were dried under vacuum, the residues were dissolved in minimal ethanol or isopropanol volume, filtered and injected on Partisil 10 ODS_2 M20, with pure or 2 % H_2O-containing ethanol as eluent (18 ml/m). Before chromatography, the methanol fractions, containing mainly chlorophyll a, were prepurified by absorption on a small column (diameter 2 cm, height 5 cm) of Bakerbond octadecyl (JJ Baker, BV), washing, and elution with the minimum of ethanol.

The optical density of the eluate was followed at two wavelengths, generally 260 nm and 450 or 530 nm. The fractions corresponding to a UV/visible absorption ratio greater than unity were repurified by one or two passages on µ Bondapak C18 Waters column (3,9 mm x 30 cm), with a mixture ethanol-water as eluent (gradient from 90 % to 100 % ethanol in 10 min, at the rate of 1 ml/min.

- Quinones from Synechocystis PCC 6803

175g of centrifuged Synechocystis (wet weight) were extracted four times successively with ethanol (total volume 2.4 l), then with a mixture chloroform-acetone-methanol (1/2/1) (total volume 800 ml). The different filtrates were pooled, dried under vacuum and the residue was

dissolved with ethanol, then isopropanol. These solutions were injected on a Partisil 10 ODS_2 M20 as above, with pure ethanol or 10 % isopropanol containing ethanol as eluent, at the rate of 18 ml/min.

In this case, the Millenium 2010 Chromatography Manager (Millipore) with the 996 Photodiode Array Detector were used to detect the minute quantities of vitamin K_1 and other quinones, which otherwise would not be visible among predominant pigments. The quinone containing fractions were pooled, dried under vacuum and rechromatographed on the same column as above, with 1 % H_2O-containing ethanol or pure ethanol as eluent, and finally on a μ Bondapak C18 column with a mixture ethanol-water (same gradient as for the quinones of Spirulina).

Analytical supercritical fluid chromatography was performed on a Jasco apparatus (880 PU pumps, 875 UV detector, 880-81 back pressure regulator), on a μ Bondapak C18 column, at 50°C, and 100 bars of pressure, with methanol as modifier (20 %).

UV-visible spectra were performed on a Shimadzu UV 160 A and infrared spectra were obtained on films using a Nicolet 60 SX FTIR spectrometer.

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RESULTS

1) ^{18}O labeled quinones

After unsuccessful efforts to label the CO groups of the quinones through the formation of acetals or oximes, we have tried the oxygen exchange route with $H_2{}^{18}O$. A previous report (4) related a 95 % exchange of oxygen by continuous stirring of Q_{10} with $H_2{}^{18}O$ in a dichloromethane - methanol mixture containing trifluoroacetic acid, during four months. In order to accelerate the exchange, we have studied the respective influences of the solvents (dichloromethane, methanol, dioxane, tetrahydrofuran) and of acids (trifluoroacetic, acetic or hydrochloric acids) or base (triethylamine). The incubation was performed at 37°C, to increase the velocities of exchange and to avoid separation of the mixture of solvents into two phases. In the conditions described under Material and Methods, the equilibrium was reached between 5 and 15 days. The maximum attainable percentage of labeling was theoretically around 82 %, mainly limited by the concentration of the TFA (bringing exchangeable ^{16}O) which was necessary for the exchange to occur. It was approximately that value (70 - 75 %) which was calculated from the infrared spectra (Fig. 1) and this labeling has been shown to be sufficient for the assignment of the bands in the light induced FTIR difference spectra (1). The purification was performed on a normal phase column, in non aqueous medium (chloroform containing a low concentration of ethanol). In these conditions, there is no oxygen exchange during the chromatography, contrary to the case where this operation would be done in aqueous medium, on reversed phase column.

The chemical yields decreased with time of incubation, due to slow degradation. With dimethylnaphtoquinone for instance, it was 85 % after 5 days and 65 % after 11 days.

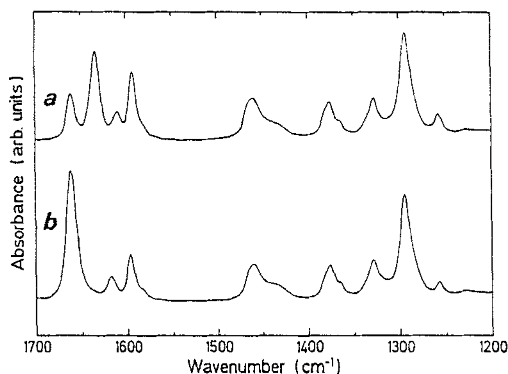

Fig. 1 Infrared spectra of Vitamin K_1 : a) ^{18}O, 70 % labeled, b) ^{16}O.

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2) U^{13} labeled quinones of E. coli

Several quinones have been shown to be produced by E. coli : menaquinone (MK8) and demethyl menaquinone (5), ubiquinones from Q_1 to Q_7 (6) which are synthetized in low amounts, and Q_8 which is the major quinone found (6-7). We have purified the main quinone by high performance liquid chromatography on reversed (Fig. 2) and normal phases columns, as described in Materials and Methods. It was identified as pure by uv-visible and infrared spectra and the length of the isoprenoid chain has been determined by comparison of the retention time on reversed phase column with those of different ubiquinones of known chain length. (Fig 3). The quantity of Q_8 purified from ^{12}C or ^{13}C E. coli was 0.063 ± 0,018 mg/g of wet weight of bacteria (7 preparations). This value is similar to that given by Bishop et al (7).

Q_7 and Q_9 were also separated and identified by their spectra and retention time on reversed phase column. Menaquinone was identified by its spectrum and retention time as MK8, in accord with (5). Their concentrations were respectively 2.66, 3.18 and 3.99 µg/g wet weight of bacteria. Contrary to Dave et al (6), we have found in E. coli an ubiquinone with a side chain with more than 8 isoprene units. The menaquinone concentration was much lower than that reported by Bishop et al (7).

3) $U^{13}C$ quinones from Spirulina

After chromatography on Partisil ODS_2 M20 preparative column of the different extracts of Spirulina, and one or two passages on µ Bondapak C_{18} column of the fractions showing a uv/visible absorption ratio greater than unity, several quinones were separated.

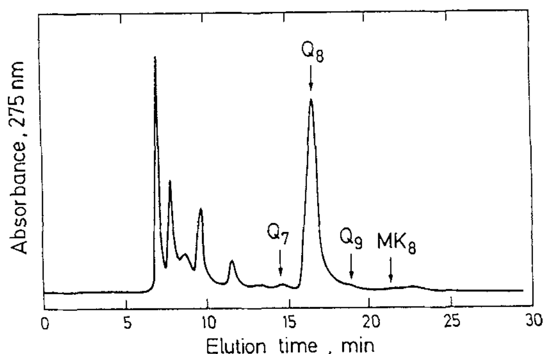

Fig. 2 Chromatogram of E. coli extract.

Conditions : Magnum 20 Partisil ODS_2 column (20 mm x 50 cm), elution with pure ethanol, 14 ml/min.

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A major quinone identified by its spectrum as plastoquinone, already proved to be PQA_{45} (8) the concentration of which was found to be 0.016 mg/g wet weight of algae.

Another plastoquinone, with a greater retention time on reversed phase column, in a ten times smaller amount.

Three ubiquinones, identified by their spectra and by their retention time : Q_2, Q_{11} and (probably) Q_{15},, by extrapolation of the curve of Fig 3. Their concentrations were respectively 0.048, 0.38 and 0.37 µg/g wet weight of algae.

Only a small quantity of vitamin K_1 could be separated and purified from Spirulina (0.03 µg/g wet weight). It is possible that some loss has occured during the chromatography, when vitamin K_1 absorbance was masked by other dominant pigments. The photodiode array detector was not yet available for these experiments. However, supercritical fluid chromatography of Spirulina extracts showed that the vitamin K_1 content was very low in this algae: 0.7 % of that of chlorophyll a, 4 % of that of PQA_{45}. Compared to Spirulina, Synechocystis 6803 was found to be much richer in vitamin K_1 (Fig. 4) : from 2.7 to 6 times, on the basis of the chlorophyll a or PQA contents. It is the reason why we have used this cyanobacteria for the purification of labeled vitamin K_1.

4) $U^{13}C$ quinones from Synechocystis 6803

The same protocol as above was used for the purification of the quinones of Synechocystis 6803, except that the detection system used was the Millenium 2010 Chromatography Manager (Millipore) equiped with the 996 Photodiode Array Detector (Waters). This system allows to visualize the spectrum of the eluate as it gets out of the column, and thus

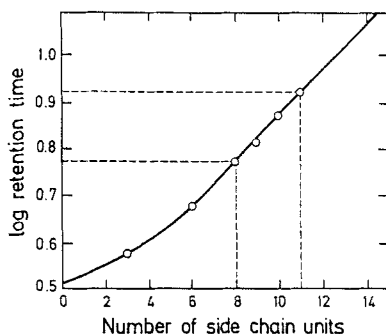

Fig. 3 Relation between retention time and length of the isoprenoid chain.

Conditions : μ Bondapak C18 column (3,9 mm x 30 cm), elution with ethanol containing 2 % H_2O, 1 ml/min.

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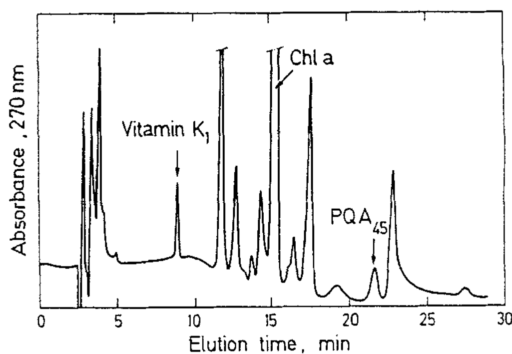

Fig. 4 Supercritical fluid chromatography of Synechocystis 6803 extract.

Conditions : μ Bondapak C18 column (3,9 mm x 30 cm), CO_2 pressure : 100 bars, temperature : 50 °C, Methanol concentration : 20 % during 5 minutes, then linear gradient to 25 % during 10 minutes, 1 ml/mn.

to collect more efficiently the fractions containing substances present only transiently, in low concentrations, and mixed with other strongly absorbing materials. Vitamin K_1 was purified from the ^{12}C and ^{13}C labeled algae (respectively 0.027 and 0.041 mg/g wet weight), as PQA45 (0.052 mg/g) for the normal culture, but surprisingly, 0.004 mg/g for the ^{13}C labeled sample). In addition, two other plastoquinones (255 nm absorbing materials) were eluted after PQA45, in very small amounts (respectively 0,072 and 0,12 μg/g). The reduced form of vitamin K_1, is also

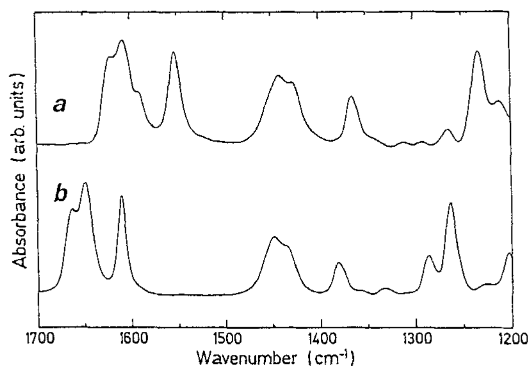

Fig. 5 Infrared spectra of Ubiquinone Q_8 : a) ^{13}C, b) ^{12}C.

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present in traces (0,27 µg/g)). It has been identified by its spectrum (maxima at 240 and 300 nm) and by formation of vitamin K_1as the major reaction product after oxidation with potassium ferricyanide (spectrum and retention time).

CONCLUSION

It is possible to label carbonyl groups of photosynthetic and related quinones by oxygen exchange with $H_2{}^{18}O$ and to purify them by HPLC on normal phase column, with non-aqueous solvents. $U^{13}C$ quinones as vitamin K_1, plastoquinone PQA45 and ubiquinone Q_8 have been extracted from Synechocystis Spirulina and E. coli respectively, grown on labeled medium and purified in mg amounts by HPLC on reversed phase column. These materials have been used then for the reconstitution of the photosynthetic reaction centers and for the assignment of the quinone bands in the light-induced FTIR difference spectra of the photoreduction of the primary quinone acceptor Q_A

ACKNOWLEDGMENTS

The authors are grateful to Mrs Bernadette Fournal for careful typing the manuscript.

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Received: May 12, 1994
Accepted: July 14, 1994