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Atmospheric pressure chemical ionization and atmospheric pressure photoionization for simultaneous mass spectrometric analysis of microbial respiratory ubiquinones and menaquinones

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An atmospheric pressure photoionization (APPI) source and an atmospheric pressure chemical ionization (APCI) source were compared for the selective detection of microbial respiratory ubiquinone and menaquinone isoprenologues using tandem mass spectrometry. Ionization source- and compound mass-dependent parameters were optimized individually for both sources, using the available quinone standards. Detection levels for the two ion sources were determined with ubiquinone-6 (UQ_6) and menaquinone-4 (MK_4, vitamin K_2) standards using flow injection analysis and selected reaction monitoring (SRM). With APPI the calculated lower limit of detection (LLOD) was 1.7 fmol μl^{-1} for UQ_6 and 2.2 fmol μl^{-1} for MK_4 at a signal-to-noise ratio of 3. These LLODs were at least three times lower than with APCI. The selectivity of detection afforded by SRM detection reduced complex mixture analysis to 3 min per sample by eliminating the need for chromatographic separations. The detection method was successfully applied to quinone quantification in a variety of environmental samples and cell cultures. Adequate amounts of respiratory quinones can be extracted and quantified from samples containing as low as 2×10^7 cells. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: atmospheric pressure photoionization; atmospheric pressure chemical ionization; ubiquinone; menaquinone; tandem mass spectrometry

INTRODUCTION

The ability of some microbes to adapt to the diverse conditions of their environment is a direct result of their capacity to form different respiratory quinones in response to the presence of different terminal electron acceptors. Aerobic growth with oxygen as the terminal electron acceptor induces Gram-negative bacteria and eukaryotes to form ubiquinones (UQ_n, 1-methyl-2-isoprenyl-3,4-dimethoxyparabenzoquinone) with the 2-isoprenyl chain containing 1–14 isoprene (n) units. Gram-negative bacteria when grown anaerobically, and also many other bacteria, form menaquinones (MK_n, 1-isoprenyl-2-methyl-naphthoquinone).[1] Some organisms form demethylated MK_n (DMK_n) if grown under anaerobic conditions with an electron acceptor such as nitrate. The UQ_n/MK_n ratio of microbial communities has proved to be an excellent measure of the terminal electron acceptor concentrations and fluxes that integrates over time.[2] In addition, the quinone isoprenologue profile provides insight into the composition of microbial communities.[3] Therefore, the respiratory quinone profiles of microorganisms are useful biomarkers to characterize both the community composition and, for Gram-negative bacteria, the *in situ* microniche redox environment. In studies of bioremediation, insights into the redox environments of the microbiota can be of vital importance in determining the success of the cleanup processes.

In previous studies, utilization of UQ_n/MK_n ratios as a measure of terminal electron acceptor flux has required serial

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extractions of large sample sizes and subsequent purification by thin-layer chromatography, because the concentrations per cell are low.[4] For example, UQ_n is about 0.1–0.6 μmol g^{-1} dry weight and MK_n about 0.02–0.1 μmol g^{-1} dry weight in an organism such as *E. coli*,[5] in contrast to the phospholipids, which are represent at about 100–150 μmol g^{-1} dry weight.[6] Quinone detection by single quadrupole mass spectrometry (MS) with a frit-fast atom bombardment (FAB) source has shown greater sensitivity than diode-array detection, but still requires 1–10 mg of dry cells.[7] The utility of electrospray tandem mass spectrometry (ES-MS/MS) in quantifying UQ_n from 10^7 *E. coli* cells has recently been demonstrated.[8] In that study, UQ_8 was found to be the dominant quinone if the organism was grown under aerobic conditions. The ES-MS/MS method takes advantage of the formation of the ammonium ion adduct of the ubiquinones, formed from ammonium acetate addition via a pre-column. This allowed direct UQ_n analysis in the neutral lipid extracts from several environmental samples dominated by a Gram-negative microbial community. Unfortunately, in our hands, MK_n and DMK_n were not detectable by ES-MS/MS. Tests for ionization of menaquinones using the electrospray source did not yield sufficient amounts of molecular ions, fragment ions, or ammonium adduct ions (data not shown), as also mentioned by Lytle *et al*.[8] Therefore, we tested other ionization sources for efficient ionization of menaquinones and ubiquinones. Here we show that the molar ratio MK/UQ, an extremely valuable biotechnological parameter, can be directly determined on neutral lipid samples of microbial communities, using atmospheric pressure chemical ionization (APCI)-MS/MS[9] or atmospheric pressure photoionization (APPI)-MS/MS in the selected reaction monitoring (SRM) mode.[10] Both types of quinones were readily ionized and detected with either ion source, and hitherto unrivalled small amounts of microbial cells (sample material) were needed to obtain UQ_n/MK_n ratios and profiles. However, APPI proved to be at least a factor of three times more sensitive than the APCI source, which is important for the detection of the small amounts of ubiquinones and menaquinones extractable from environmental samples.

EXPERIMENTAL

General MS

All experiments were carried out with an APCI or a prototype APPI source connected to an API 365 triple-quadrupole mass spectrometer (MDS SCIEX, Concord, Ontario, Canada) operated in the positive mode. The same heated nebulizer probe was used with each ion source and was maintained at 425 °C for both APCI and APPI, if not stated otherwise. Details of the prototype APPI source can be found elsewhere.[9] In brief, in APPI a vacuum ultraviolet lamp providing 10 eV photons is used rather than a corona discharge to initiate an ion–molecule reaction cascade producing reagent ions. Toluene (Baker, Phillipsburg, NJ, USA) or acetone (Baker), each of which is photoionizable, was added as a dopant (10% by volume of total the solvent flow) in-line with the auxiliary gas (nitrogen) to initiate the formation of reagent ions.[9,11]

High-performance liquid chromatography (HPLC)

An Agilent 1100 LC system was used for flow injection analysis (FIA) and for HPLC of quinones. Flow-rates were tested with the APCI and the APPI source ranging from 50 to 200 μl min^{-1} of methanol–chloroform (80 : 20, v/v) for FIA. Methanol or methanol–2-propanol (80 : 20, v/v) at 100 μl min^{-1} was used for chromatographic separation of quinones on HAISIL 300 30 × 1 mm i.d. (Higgins Analytical by Chromtech, USA)[8] and Hypersil RP-18 (ODS) 150 × 1 mm i.d. (3 μm) (Phenomenex, Torrance, CA, USA) microbore LC columns.

Quinones

The nomenclature for quinones designates the respective core structure, indicated by the number of units in the isoprenyl side-chain. Ubiquinone standards UQ_6, UQ_7, UQ_9, UQ_{10} and the menaquinone MK_4 (vitamin K_2), were obtained from Sigma (St. Louis MO, USA) and used as received. The ubiquinone UQ_8 was isolated in triplicate from 5 mg of *E. coli* lyophilized cells (Sigma) as described below. To obtain product ion scans, determine diagnostic ions and optimize source-dependent parameters the standards were dissolved in methanol–chloroform (80 : 20, v/v) and infused, through a tee into an additional solvent stream, at 10 μl min^{-1} using a syringe pump. The major solvent stream was provided by an Agilent 1100 quaternary pump to obtain a final flow-rate of 100–200 μl min^{-1} recommended for APCI and APPI, and suitable for the dimensions of the microbore LC columns used.

Flow cell biofilm

Acidovorax delafildii 670a, *Bacillus* sp 3h47e and *P. putida* sp were grown and then enriched in a biofilm accumulation chamber (BAC) using a constant stream of liquid delivering oxygen and nutrients as described previously.[12] To the biofilms grown for 48 h, *E. coli* cells were added at concentrations from 10^2 to 10^8 cells to simulate an environmental stress factor, and the supply of nutrients and oxygen was stopped to one out of four BACs. After 24 h the biofilms from the BACs were extracted and the neutral lipid fraction obtained by silicic acid column chromatography (see below).

Cell cultures

Enterococcus faecalis (ATCC 11 420) cells were grown in ATCC's 123 TYG medium under anaerobic conditions. After 24 h, 100 ml of culture medium (3.0×10^8 cells ml^{-1}) were centrifuged, the supernatant was removed and then 10 ml of one-phase extraction solvent were added to the cell pellet. *Wolinella succinogenes* and *Geobacter sulfurreducens* cell material was lyophilized in ammonium acetate buffer (pH 6.5, B. Schink, Konstanz, Germany) and 15 mg cell dry weight suspended in 10 ml of extraction solvent.

Sample preparation

Quinones were extracted from frozen samples or lyophilized cell material (*E. coli*) by a one-phase modified Bligh/Dyer extraction method. The organic phase extracts were dried under a gentle nitrogen stream. Residues were resolved in 100 μl of chloroform and fractionated on 500 mg of activated

silicic acid-packed glass SPE columns (SAC) using 5 ml of chloroform, 5 ml of acetone and 10 ml of methanol as described elsewhere.[13] The quinone-containing chloroform extracts were dried under nitrogen and stored at −20 °C. Extracts were resolved in 50–100 µl of methanol prior to measurement. The extracts, which also contain diglyceride, fatty acids and other neutral lipid species, were in general used without further purification. However, colored samples (i.e. extracts from environmental samples containing humic acids) were cleaned with 0.2 µm spin filters prior to injection on-column to keep the background signals low, enhance the separation and increase the column lifetime. Quinones are considered as photosensitive and sensitive to oxygen (MSDS Sigma-Aldrich), and therefore quinone-containing extracts or dry residues need to be protected from light and air. Quinone standards were dissolved in 100% methanol and kept in amber-colored vials at 4 °C. Aliquots of the standards were used to obtain a calibration curve for ubiquinones and menaquinones prior to sample measurements.

Table 1. Parameters for optimal detection of ubiquinones (UQ_n) and menaquinones (MK_n) with APCI and APPI[a]

Compound	Q1 (u)	Q3 (u)	DP[b] (V)	FP[c] (V)	CEP[d] (V)	CE[e] (V)
UQ_6	591.5	197.1	33	210	20.5	36
UQ_7	659.6	197.1	27	190	28.5	40
UQ_8	727.9	197.1	29	180	29.0	44
UQ_9	795.7	197.1	30	175	29.5	48
UQ_{10}	863.8	197.1	33	210	30.5	52
MK_4	445.6	187.0	25	165	13.8	31
MK_5	513.4	187.0	28	210	20.8	34
MK_6	581.3	187.0	28	210	20.8	39
MK_7	649.9	187.0	30	210	24.3	44
MK_8	718.0	187.0	30	210	27.9	48
MK_9	786.1	187.0	30	210	31.4	52

[a] The parameters were optimized using dilution of standards or using the purified neutral lipid fraction of lipid extracts from microorganisms with known quinone content. The scan time was 250 ms for each mass transition.
[b] Voltage between orifice and skimmer (ground).
[c] Voltage between skimmer (ground) and ring potential.
[d] Collision cell entrance potential.
[e] Collision energy for collision-induced dissociation.

RESULTS AND DISCUSSION

Optimization of APCI

For the compounds under study, the major ions observed with APCI were the protonated molecules, $[M + H]^+$. For ubiquinone-6 (UQ_6), the base peak in the spectrum of a Q1 scan was $[M + H]^+$ at m/z 591.7. The product ion spectrum of m/z 591.7 and of the other ubiquinone protonated molecules investigated showed a base peak at m/z 197 (Fig. 1). We assume that this fragment ion is the benzylium ion as it is a resonance-stabilized structure, and was identified previously by ES-MS/MS studies of these compounds.[8]

The mass spectrum of MK_4 (vitamin K_2) showed the protonated molecule $[M + H]^+$ at m/z 445. The major ion in the product ion spectrum of m/z 445 was detected at m/z 187, which represents the 2-methylnaphthoquinone core. All other menaquinones studied fragmented in a similar manner, producing this diagnostic ion (Fig. 1).

The SRM transitions used for the detection of 11 major UQs and MKs are listed in Table 1. Mass analyzer parameters were optimized individually for each of the available quinone standards to yield maximum signal intensity in the positive mode with the APCI source. For quinones without authentic standards available, optimum mass-dependent parameters such as voltages on orifice, collision energy and cell entrance potential were calculated assuming a linear change with increasing number of isoprene units in the side-chain. The collision energy (CE) needed to be increased with increasing mass of the parent quinones to maintain approximately the same center-of-mass collision energy, viz. 1.6 eV for UQ_n and 1.8 eV for MK_n. Optimum conditions were further verified and adjusted using quinone extracts of cell cultures from microorganisms with known quinone composition. For example, the parameters for UQ_8 were obtained using the neutral lipid fraction from *E. coli* (see sample preparation), as UQ_8 represented 90% of the total quinone content of cells grown under aerobic conditions.[1,5] MK_4 was used for initial optimization of

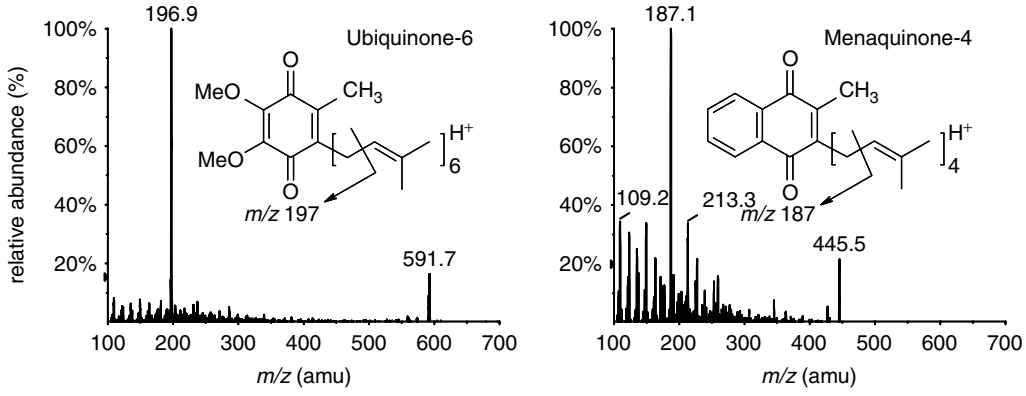

Figure 1. General structure of ubiquinones and menaquinones shown for UQ_6 and MK_4. The product ion spectra for m/z 591.7 (UQ_6) and m/z 445.5 (MK_4) revealed fragment ions, indicated with their m/z and structure, characteristic for the respective quinone class. The molecular ion $[M + H]^+$ and the respective diagnostic fragment ion, major ion in the mass spectra, were used for SRM.

detection parameters as it was the only menaquinone standard available. In a second step we obtained neutral lipid extracts of anaerobically grown *Wolinella succinogenes* and *Geobacter sulfurreducens* cultures (a gift from B. Schink, University of Konstanz, Germany), and determined the menaquinones in the extracts by monitoring the parent ions of m/z 187. For the *Wolinella* strain we found m/z 581 as the major parent ion, which matches the calculated mass for $[M + H]^+$ of MK_6. The *Geobacter* strain contained mainly m/z 718 and minor amounts of m/z 786 (10%), corresponding to $[M + H]^+$ of MK_8 and $[M + H]^+$ of MK_9, respectively. The settings for menaquinone detection were individually optimized for the following mass transitions in SRM: m/z 581/187, 718/187 and 786/187 and the values for m/z 513/187 (MK_5) and m/z 650/187 (MK_7) extrapolated afterwards (Table 1).

Optimization of APPI

The APPI source is based on the same heated nebulizer probe used for APCI and therefore most of the settings were used as optimized with the APCI source. However, the heated nebulizer at the APPI source has a spray voltage instead of the nebulizer current. The optimum APPI spray voltage was determined with UQ_6 and MK_4 using SRM and either acetone or toluene as the dopant. Signal levels were maximized at spray voltages of 1.3–1.7 kV with toluene as the dopant (Fig. 2). With APPI at 1.5 kV offset voltage the signal standard deviation was 15% lower ($n = 3$) than with the APCI source for all measured quinones, as indicated only for UQ6 in Fig. 2. With acetone as the dopant, the signal for UQ_6 showed a maximum at 1.5 kV spray voltage that was slightly lower than the signal obtained with toluene. Nonetheless, acetone was used in most experiments as recommended in the preliminary APPI source manual. A source gas temperature of APPI of ~325 °C yielded a slightly higher relative signal intensity for UQ_6 and for MK_4 (15 ± 5%) compared with the 425 °C determined as the optimum of APCI (not shown). An expected gain in detection sensitivity using APPI at lower temperatures can be compromised owing to sample carryover and peak tailing, therefore 425 °C was used in all further experiments for comparison of quinone quantification between the two ion sources.

With both APPI and APCI, the signal intensity at equal concentrations was always lower for MK_4 than for UQ_6, even after individual parameter optimization for the respective mass transitions. This is attributed to a lower stability of the naphthoquinone core, which is indicated by the following findings. First, in a Q1 scan (m/z 400–1000 u), with infusion of MK_4, an increase in the declustering potential increases the abundance of a fragment ion $[M - 14]^+$, which corresponds to an loss of the 2-methyl from the menaquinone core in the source ionization region. Second, the relative abundance of low-mass fragment ions other than m/z 187 (>30% for m/z 109, 149 and 213) was significantly higher than observed for low-mass fragment ions of UQ_6 (<10%; Fig. 1). Decreasing the collision energy for collision-induced dissociation (CID) did not change the signal intensity of low-mass ions relative to the fragment ion at m/z 187 (data not shown). Both findings cause a decrease in signal intensity in the SRM of $[M + H]^+/(m/z\ 187)$. The structure and formation pathways of the menaquinone low mass fragment ions are unknown.

Detection Limits

Calibration curves for the quinones MK_4 and UQ_6 showed a linear response in the range from <3 to 8999 and 6670 fmol µl^{-1}, respectively, for APPI. The range of linear response started at slightly higher quinone concentrations of 9.0 and 6.7 fmol µl^{-1}, respectively, for APCI. The correlations (r values) for all available quinone standard calibrations were $r > 0.9987$ and $r > 0.9957$ with APPI and APCI, respectively. The signal intensity standard deviations were <10% with APPI and <15% with APCI for the whole concentration range. Using FIA, the respective lower limit of quantification (LLOQ) with APPI and APCI at a signal-to-noise ratio (S/N) >9 was determined as 2.8 and 9.0 fmol µl^{-1} of MK_4, respectively. Compared with MK_4, the LLOQ for UQ_6 was slightly better with both ion sources (2.1 and 6.8 fmol µl^{-1}, respectively). The quantification of UQ_{10} was less sensitive than that of UQ_6, as nearly double the amount was needed with APPI (4.6 fmol µl^{-1}), and four times with APCI (23.2 fmol µl^{-1}) to obtain an S/N >9 (Fig. 3). Overall,

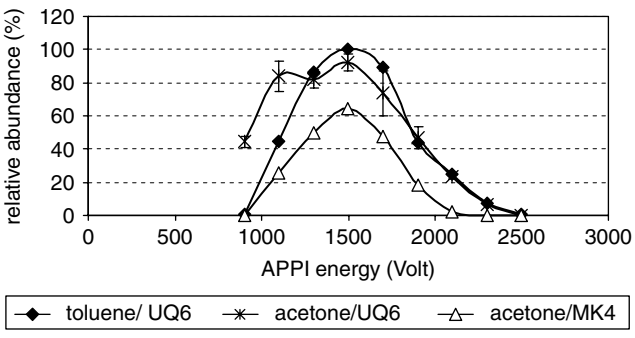

Figure 2. Relative signal intensity for UQ_6 (♦) and MK_4 (△) at increasing source energy determined with acetone as dopant for both quinone classes and with toluene for UQ_6 (∗). The highest value, obtained for UQ_6 with toluene as dopant at 1500 V, was set to 100%.

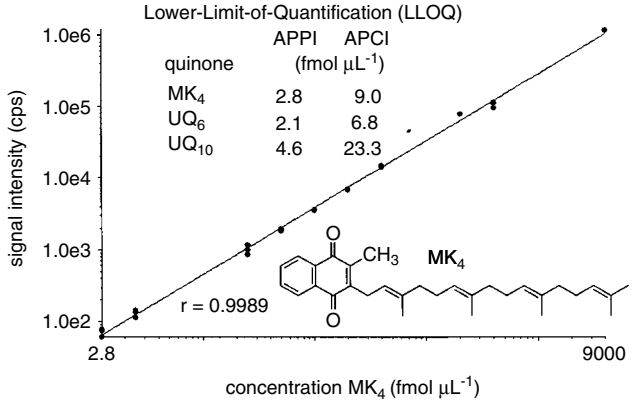

Figure 3. LLOQ for three respiratory quinones detected with LC/APPI-MS/MS and LC/APCI-MS/MS. Calibration curve of MK_4 measured with LC/APPI-MS/MS by MRM of m/z 445.6/187.0 (Q1/Q3). The structure of MK_4 is shown.

the APPI source proved to have better detection levels (at least a factor of three times better) than the APCI source, and also smaller standard deviations (in replicate analysis) for all quinones tested.

Separation of quinones

Partial separation of quinone isoprenologues can be achieved on a short microbore RP-18 column at low flow-rates, according to our existing method for the detection of ubiquinones by ES-MS/MS with the pre-column addition of 10 mM ammonium acetate as solvent modifier.[8] However, the ammoniated ion at m/z $[M + NH_4]^+$ known from ES of ubiquinones was not produced in the corona discharge gas phase according to an obtained Q1 mass spectrum of UQ_6 (APCI-MS data not shown). In the SRM mode (see Table 1), the signal intensities were comparable for a standard mix containing UQ_6, UQ_7, UQ_9, and UQ_{10}, whether 10 mM ammonium acetate was added or not, whereas addition of 20 mM ammonium caused a significant decrease in the signal intensities (Table 2). At the latter concentration of ammonium, the ionization of these molecules may be significantly favored over ionization of the ubiquinones. As ammonium acetate did not improve the ionization in either APCI or APPI, it can be omitted.

Whether ammonium acetate was added or not, the retention times of ubiquinones increased with their isoprenoid chain length (UQ_6 to UQ_{10}), and MK_4 eluted very shortly before UQ_6, indicating that menaquinones have a higher retention time than the respective ubiquinones (Fig. 4). The differences in retention time between the quinone peaks decreased significantly if the flow-rate was increased for APCI to 100 µl min^{-1}, e.g. 0.5 min elution time between the peak apex of UQ_6 and UQ_{10}, causing significant peak overlap (Fig. 4(A)). However, with SRM the individual quinones were easily resolved according to their unique mass transitions. Using 100% methanol as the solvent increased the retention time differences, but a complete baseline separation for all peaks could not be achieved on the short column (data not shown). With a longer column (Hypersil RP-18 (ODS), 150 × 1 mm i.d.) a baseline separation of ubiquinones and even of MK_4 and UQ_6 was possible with methanol–2-propanol (80 : 20) as solvent (Fig. 4(B)). However, significant peak broadening occurred, which compromises the detection sensitivity for ubiquinones with more than seven isoprenoid units and even later eluting respective menaquinones (not shown).

In any case, the relative abundances of quinones in mixtures separated by HPLC or simply determined by FIA were identical for quinone concentrations of 480 ppb. These data indicate that HPLC separation and the associated slower speed of analysis can be eliminated in favor of analysis by FIA with SRM detection. Apparently the ionization efficiencies among the various quinones at these levels are not compromised by simultaneous introduction into the ion source. Chromatographic separation of different quinones has been utilized in all currently established methods using an electrochemical electrode,[2] diode-array detector or single-quadrupole mass spectrometer as detection devices.[4,7] All these methods have the disadvantages of long analysis times, peak broadening for larger quinones and omitted detection of small menaquinones (MK_4 and MK_5).

To take advantage of the speed of analysis, FIA with SRM detection was employed in all subsequent analysis. Using isotopically labeled quinones as internal standards would provide the most exact method for quantification with MS/MS detection, but these were not commercially available. To obtain quantitative measurements of the

Table 2. Calculated concentration of quinone standard mix analyzed with APCI using 10 and 20 mM ammonium acetate as solvent modifier and without modifier

	Calculated concentration (ng ml^{-1})			
Modifier	UQ_6	UQ_7	UQ_9	UQ_{10}
10 mM NH_4 acetate	7.8	4.8	24.3	47.8
20 mM NH_4 acetate	6.2	3.7	17.9	29.9
None[a]	8.2	5.6	25.1	47.6

[a] Reference: values according to the respective standard concentration.

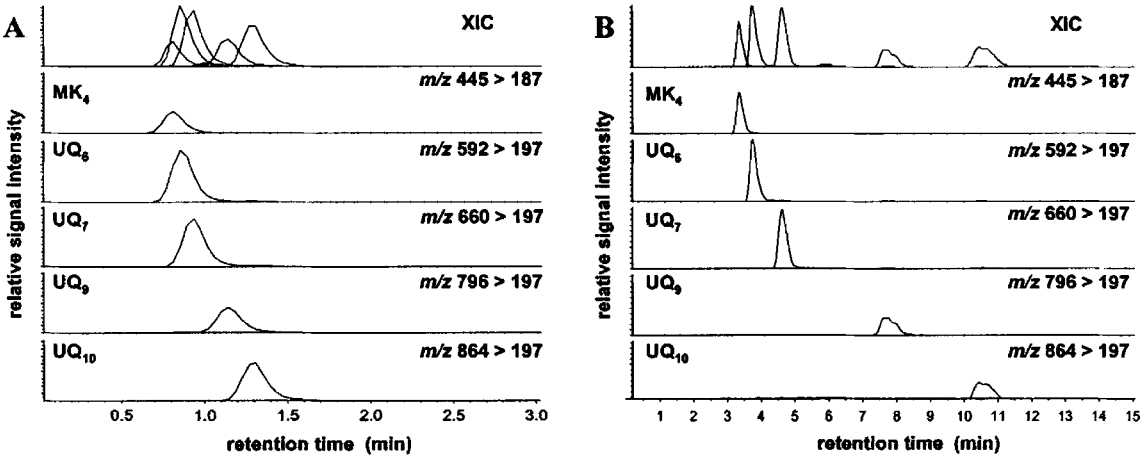

Figure 4. Separation of authentic quinone standards on an RP-18 HPLC column (A) in 3 min on HAISIL 300, 30 × 1 mm i.d. and (B) in 20 min on Hypersil RP-18 (ODS), 150 × 1 mm i.d. (100 µl min^{-1}, methanol–2-propanol (80 : 20)) detected with APCI-MS/MS. The signal intensities are relative to the highest peak (set to 100%) within the respective separation.

amount of quinones present in a mixture, an external standardization with a representative of each available quinone class was used. A stock solution of the quinones UQ_6, UQ_{10} and MK_4 were prepared at four concentrations (e.g. UQ_6 at 1.7, 6.8, 17.0, and 170.0 fmol μl^{-1}) and aliquots measured before each sample set. Although quinones are known to be photosensitive and to disintegrate if exposed to oxygen, with storage at 4 °C in MeOH and protection from light, measured concentration of standards showed no loss over a period of 6 months. This was verified by comparison of stored standards with freshly prepared solutions (data not shown).

Analysis of environmental samples

One of the most useful environmental parameters is the molar ratio of ubiquinones to menaquinones (UQ_n/MK_n). The absolute concentration in cells or other samples is less important. The simultaneous measurement of ubiquinones and menaquinones provides the best indication for relative quinone amounts within different samples. Peak area values for ubiquinones were normalized to UQ_6 standards and for menaquinones to MK_4 standards of known concentration (pg μl^{-1}) analyzed within the same sample sequence. Data were than converted to pmol values to obtain UQ_n/MK_n ratios based on the molar concentrations in the samples.

We quantified complex quinone compositions in environmental samples successfully using detection of 11 SRM transitions without a column separation. The chosen mass transitions embraced the ubiquinones and menaquinones directly accessible for quantification as available standards, or indirectly accessible owing to extrapolation based on the respective comparable standards. With FIA we could detect almost all of these monitored quinones in a single sample (Fig. 5), containing a calculated total quinone content of 81 pmol. This corresponds to 2.7×10^8 cells, or 1 nmol in 3.0×10^9 cells.[4] Based on profiles obtained from environmental samples and the determined detection limits, we propose suitability of the method for samples containing as low as 2.0×10^7 cells, or approximately two orders of magnitude less sample material than necessary for quantification based on diode-array detection.[7]

Menaquinones were induced in a Gram-negative bacterial community in microbial biofilm accumulation chambers[14,15] with interruption of the supply of oxygen and nutrients, driving the environment anaerobic after addition of 10^4 *E. coli* cells. The relative amount of MK_8 and MK_7 increased to 50 and 23%, respectively, within 24 h. If chambers were kept aerobic, addition of *E. coli* (10^2, 10^6, 10^8 cells) had no significant influence on the relative quinone distribution, clearly dominated by 80% UQ_9. The calculated molar ratio UQ_n/MK_n changed from 6.1 ($\pm$0.6) for aerobic conditions to 0.3 for anaerobic conditions (Table 3). These ratios were detected in biofilms containing $\sim 10^8$–10^9 cells cm^{-2} with the methods described here. We found comparable UQ_n/MK_n ratios published for 4 l batches of *E. coli* liquid cultures, where the ratio of MK_8 to UQ_8 (major quinones in *E. coli)* was 17.3 at the log phase with vigorous aeration, and 0.01 under consequent anaerobic growth conditions, but nearly 1.0 in the aerobic stationary phase.[5]

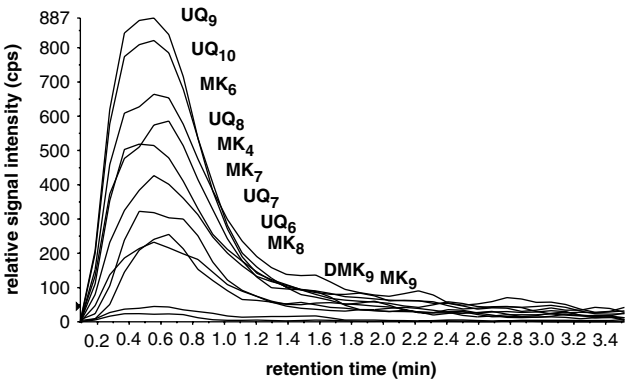

Figure 5. Quinone profile in an environmental sample extract measured with FIA coupled to APCI-MS/MS (at 50 µl min^{-1} solvent flow-rate). The calculated total quinone amount in the sample was 81 pmol.

If an aerobic culture (in artificial groundwater) is rendered anaerobic, menaquinones are rapidly produced, whereas the ubiquinones produced with aeration survive at least 2 weeks in an anaerobic environment.[14] Ubiquinones are not known to be produced by Gram-negative facultative aerobic microorganisms under anoxic conditions. This could possibly be reflecting the high redox potential of the ubiquinone–quinol system utilizing oxygen as a terminal electron acceptor. Therefore, we propose that quinone ratios indicate the redox state for a short-term history (weeks) of oxygen availability in the presence of Gram-negative aerobic, facultative aerobic or anaerobic bacteria. This hypothesis has been tested *in vitro* in reconstruction experiments.[2] The UQ/MK ratio near zero indicates long-term (months) exposure to strictly anaerobic conditions. Values below 1.0 indicate anaerobic conditions with a history of past oxygen

Table 3. Distribution of quinones detectable in a constructed tri-culture biofilm 24 h after addition of *E. coli* (10^2–10^8 cells) and aerobic growth followed by switch to anaerobic growth (10^4 *E. coli* cells added) (the respective ubiquinone to menaquinone ratio (UQ_n/MK_n) is also shown)

	Relative amount of quinones (%)								Ratio
Flow cell	UQ_6	UQ_7	UQ_8	UQ_9	UQ_{10}	MK_4	MK_7	MK_8	UQ_n/MK_n
Aerobic + 10^2 cells	0.8	0.8	1.0	81.0	2.4	2.1	8.4	5.2	5.5
Aerobic + 10^6 cells	0.8	0.8	1.8	83.0	2.2	1.5	8.5	3.0	6.8
Aerobic + 10^8 cells	0.8	0.9	3.8	79.0	2.3	1.5	8.5	4.5	6.0
Anaerobic + 10^4 cells	0.2	0.2	0.4	21.0	0.7	5.5	23.0	50.0	0.3

availability, whereas values around 1.0 and above indicate an aerobic or partial microaerophilic environment. A low UQ/MK ratio can also be an indicator for archaea as they produce menaquinones.[3] The presence of archaea would be reflected in a high MK/PLFA ratio.

Several respiratory quinones other than the major ubiquinones and menaquinones analyzed here have been reported in bacteria and archaea. Menaquinones showing single or multiple hydrogenations in the side-chain, expressed as $MK_n(H_x)$, are common in actinomycetes and known from analysis of sewage activated sludge.[4] Menaquinones with more than 10 isoprenoid units occur in *Bacteroides* spp.[16] and ubiquinones with 12–14 isoprenoid units in the side-chain (UQ_{12-14}) occur in *Legionella* spp.[1] Additionally, isoprenoid quinone structures containing sulfur, nitrogen, additional methyl groups in the core, heterocyclic cores and/or modifications in the side-chain (e.g. epoxy, ketone) or a loss of a methyl from the menaquinone core (demethylmenaquinones, DMKs) have also been described. Sensitive quantification of these specific quinones would be useful in taxonomic determinations and analysis of microbial systems characterized by elevated temperatures, sufficient light for photosynthesis, conditions suitable for methanotrophs or anaerobic growth with nitrate as electron acceptor.[16] The analysis of plastoquinones and phylloquinone will be important for aquatic environments.[17] APPI- or APCI-MS/MS can simplify the search for specific quinones in cell cultures or unknown samples.

Demethylmenaquinone-9 (DMK_9) was detected in neutral lipid extracts of *Enterococcus faecalis* by a precursor ion scan for m/z 173 (m/z $[187-CH_2]^+$), showing the expected peak for the protonated molecule $[M+H]^+$ at m/z 772. We could also detect thermoplasmaquinone[16] as a minor compound in a *Wolinella succinogenes* isolate with a similar approach using the fragment ion at m/z 201 as diagnostic ion (not shown).

CONCLUSIONS

The results presented in this paper show that both APCI- and APPI-MS/MS making use of SRM detection can be used for the selective, low-level and quantitative detection of both ubiquinones and menaquinones. These capabilities allow the determination of the environmentally diagnostic UQ_n/MK_n ratio. With APPI-MS/MS, the determined lower limit of detection (LLOD) was 1.7 fmol μl^{-1} for UQ_6 and of 2.2 fmol μl^{-1} for MK_4. The detection levels determined with APPI-MS/MS were at least three times better than with APCI-MS/MS. Therefore, APPI-MS/MS would be the most suitable method for the detection and quantification of the low levels of respiratory quinone isoprenologues in environmental samples. However, even the quantification of respiratory quinones with APCI-MS/MS is a promising tool. Owing to the high selectivity of quinone detection using the SRM mode, sensitive quantification was possible if either LC with baseline separation, LC with partial peak separation or simple FIA was used. The highest signal intensities were obtained with FIA and partial peak separation on a short column, whereas on a longer column peak broadening compromised the detection sensitivity. Using the here described APPI- or APCI-MS/MS approach for specific and sensitive detection, and quantification of the major isoprenoid ubiquinones and menaquinones, a wide variety of structurally modified respiratory quinones should be detectable with only small MS parameter alterations. The UQ_n/MK_n ratio can be used to access the redox state for a short-term history (weeks) of oxygen availability in the presence of Gram-negative aerobic, facultative aerobic or anaerobic microorganisms.

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