

Rapid and simple analysis of paraquat in tissue homogenate by ultra-high performance liquid chromatography

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We present a method for the rapid and accurate quantitative analysis of paraquat (PQ) in biological samples. Paraquat, also known as methyl viologen, is commonly used to induce oxidative stress in laboratory studies. Our method uses classical standard addition methodology and ultra-high performance liquid chromatography (UPLC) with photodiode array detection to estimate recovered PQ in whole-organism homogenates. The method facilitates simple and accurate quantification of PQ present in a biological sample, a much more biologically meaningful parameter than the dose of PQ administered in an experimental treatment. In an example using 4 different genotypes of *Drosophila melanogaster* exposed to 10 mM PQ, we measured concentrations from 2.8 to 6.3 μM , corresponding to 1.2 to 2.6×10^{-12} mol PQ per mg of fly. Analysis is simple, uses minimal sample preparation, and exploits the resolution and speed of UPLC to provide rapid (3 min) assays of homogenate samples.

Introduction

Paraquat (PQ), or methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride), was developed as an agricultural herbicide, but it is now commonly used in laboratory settings to induce reactive oxygen species (ROS). PQ's agricultural use is in decline, but its use in the laboratory has become commonplace and PQ has become well established as an inductive agent in laboratory studies of *in vivo* oxidative stress.^{1–3} Upon ingestion, PQ undergoes a NADPH-dependent reduction, yielding the PQ radical.⁴ This radical then rapidly reacts with oxygen to re-form the parent molecule, concurrently generating superoxide, a potentially damaging ROS. PQ is continually reduced and oxidized and this redox-cycling results in further ROS generation and NADPH depletion.⁴

Reactive oxygen species are produced naturally in the cell and have essential biological roles, but ROS levels may exceed a biological system's ability to neutralize and eliminate them resulting in oxidative stress.^{5,6} Excess ROS has been shown to induce damage to essential macromolecules: lipid peroxidation can disrupt cell membranes and lead to cell damage and death; formation of protein carbonyl groups on amino acid side chains can lead to protein misfolding and inactivation; DNA strand breaks and base damages can lead to a number of diseases and the induction of cancer.^{4,6–9} Oxidative stress can also disable a system's ability to easily repair the damage done previously by ROS, resulting in even further increases in cellular ROS.

Oxidative stress is thought to play a major role in aging and age-related disease and decline and studies of ROS and oxidative stress are central in modern biology.

ROS are produced as a by-product of natural cell processes, but ROS production can also be induced by extracellular agents—such as PQ.^{6,10,11} In the laboratory, PQ is commonly administered to induce ROS production and oxidative stress.^{4,6,12} *Drosophila melanogaster*, the common fruit fly, has emerged as a dominant model system in these studies because of its ease of culture and our knowledge of this species' genetics and genetic diversity. In laboratory studies, the administered PQ dosages are generally known, but the effective biological concentrations in tissue homogenates have seldom, if ever, been quantified. Here we demonstrate a simple and rapid method for the quantification of PQ in biological samples using ultra-high performance liquid chromatography with photodiode array detection. We have developed the method using *D. melanogaster*, but it is equally applicable to many biological systems. The method is based on a classical standard addition approach and involves minimal sample preparation, while providing a linear analytical response over the range of 1–50 μM . The analytical speed (total isocratic UPLC run times of roughly 3 min per sample) provides for fast and accurate quantification of PQ in biological samples for use in studies of oxidative stress.

Materials and methods

Chemicals

PQ (1,1'-dimethyl-4,4'-bipyridinium dichloride) was reagent grade and obtained from Sigma-Aldrich (Cat. No. 856177, St Louis, MO). Sodium 1-octanesulfonate monohydrate (NaOS)

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was analytical grade and used as an ion-pairing reagent for UPLC (Sigma-Aldrich, St Louis, MO, Catalog no. 74884). Water used in UPLC mobile phase was ultra-pure (18.2 M Ω) grade for liquid chromatography applications.

Fly stocks

Iso-third chromosome lines were used as by Merritt.^{13,14} These fly stocks have different 3rd chromosomes in identical genetic backgrounds (1st and 2nd chromosomes) and represent a snapshot of the genetic diversity present in North American *D. melanogaster*. Lines were constructed from 3rd chromosomes extracted from the isofemale lines and placed in the w; 6326; TM3,SB genetic background. Genotypes were w; 6326; *JFL12/TM3*, w; 6326; *MFL42/TM3*, w; 6326; *NC25/TM3*, w; 6326; *VA29/TM3*, and w; 6326; *VT26/TM3*; herein referred to as JFL12, MFL42, NC25, VA29, and VT26. All stocks were maintained on standard cornmeal media with 12 hour light and 12 hour dark cycles at 25 °C.

Paraquat delivery method

Adult males were fed 10 mM PQ in a simple sugar diet (1% sucrose solution), following established protocols.¹⁵ In brief, two $\frac{3}{4}$ inch diameter discs of Whatman Grade 2 filter paper were saturated with PQ/sucrose solution and placed in the bottom of a standard *Drosophila* rearing vial. Flies ($n = 15$) were then placed in each vial, with the saturated discs, for 24 hours. Control flies were fed sucrose only by placing them in vials similar to those containing exposed flies, but with filter paper discs saturated solely with 1% sucrose.

Quantification of paraquat by UPLC and the standard addition method

Ingested PQ was measured through determination of PQ concentrations in whole fruit fly homogenates using an Acquity UPLC system with photodiode array (PDA) detection (Waters, Milford, MA), equipped with a long pathlength (2.5 cm) detector flow cell. Newly emerged adult flies were collected from fly stock vials, aged 3–5 days, and ~15 flies placed in vials with either PQ or control treatments. After treatment, flies were gently anesthetized using CO₂ and samples of 5 flies collected and frozen in individual microcentrifuge tubes at –80 °C for storage until analysis. Frozen samples were placed on ice and homogenized using a hand homogenizer at ~1000 rpm for 10 s in 500 μ L of freshly prepared solution of 2 mM sodium 1-octanesulfonate monohydrate, 0.1% (v/v) formic acid, and 10% (v/v) acetonitrile in ultra pure (18.2 M Ω) grade water. Homogenates were centrifuged twice at 16 000g for 5 minutes at 4 °C to pellet any solids, with the supernatant being collected and transferred to new microcentrifuge tubes between centrifugations. The final supernatant was collected from each sample and split into three aliquots. Two of the three aliquots were spiked with PQ (5 μ L or 10 μ L of a 100 μ M solution of PQ in mobile phase A; A—2 mM NaOS/0.1% Formic acid in 9 : 1 acetonitrile : water) in order to create a standard addition calibration curve.

Prepared aliquots were injected (15 μ L) and analyzed using an isocratic UPLC program, with mobile phase composition set to 80 : 20 A : B; (B—acetonitrile). The flow rate was constant and

set to 200 μ L min^{–1} at a temperature of 50 °C. The column used was an ACQUITY UPLC BEH C18 column (1.7 μ m \times 2.1 mm \times 50 mm). Chromatograms were collected using both 257 nm and 395 nm, and the UV spectra of putative PQ peaks were used in analyte verification. Finally, the standard addition method served as a means to confirm PQ retention time in each analytical run and to quantify PQ in a given sample. Total run times were 3 min each.

Biological replication

In experiments assaying ingested PQ concentration, each genotype was raised in 2 independent vials and 2 samples of 5 flies collected from each vial, for a total of 4 samples per treatment per genotype.

Results

Verification of linearity and precision

The linearity and precision of replicate assays were characterized by generation of standard curves using triplicate sample preparations, each sample containing a homogenate of 5 flies in 500 μ L of UPLC mobile phase A, and PQ, ranging in concentration from 0–50 μ M. Using a wavelength of 257 nm, integrated peak areas were linear over the entire range ($R^2 = 0.9988$). Replicate analyses ($n = 3$) over this concentration range displayed precision (relative standard deviation) ranging from 1.2 to 3.1%. Replication of standard curves showed that the linearity of response was consistent over 3 different curves ($R^2 = 0.9981$ – 0.9997). The standard curve illustrating linearity of analytical response is shown in Fig. 1. Exemplar chromatograms showing analysis of PQ-free and PQ-spiked (10 μ M) fly homogenate are shown in Fig. 2.

Analysis of homogenates of paraquat-free and paraquat-fed flies

In addition to the verification of assay linearity over the range of 0–50 μ M, random screening of PQ-free (PQ–) and PQ-fed (PQ+)

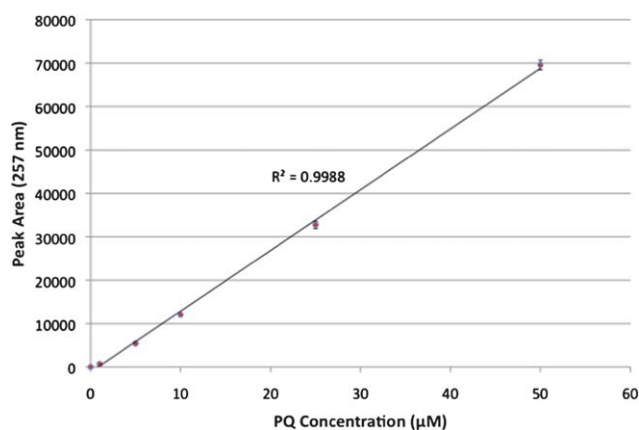


Fig. 1 Concentration dependence of paraquat (PQ) assay across a concentration range of 1–50 μ M. Each concentration level assayed in triplicate, points are the mean of the replicates with error bars indicating the standard error.

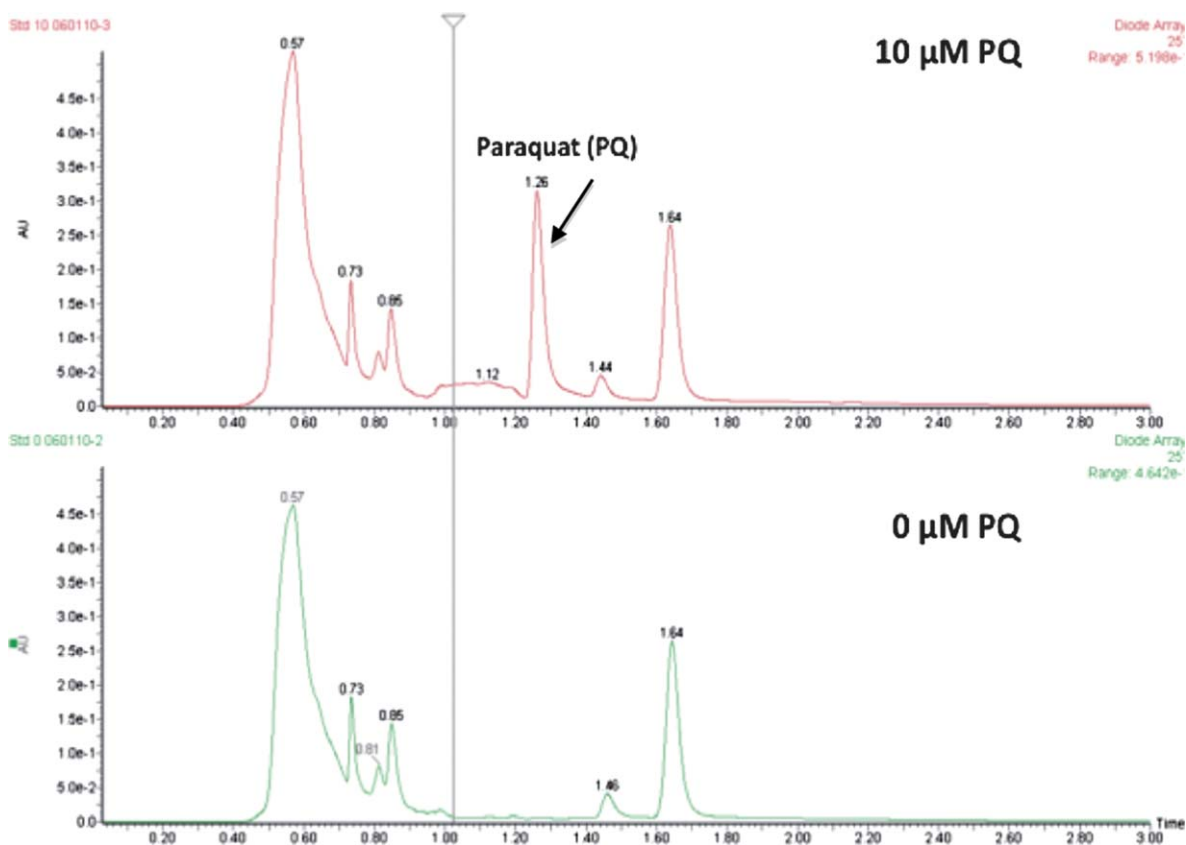


Fig. 2 Exemplar chromatograms (measured at 257 nm) from a PQ-free fly homogenate, and an aliquot of the same homogenate with added PQ to generate a final concentration of 10 μM .

flies permitted estimation of the concentration of PQ appropriate for use in the standard addition assay. For quantification of PQ in homogenate samples, each homogenate was divided into 3 aliquots. To these aliquots was added 0, 5 or 10 μL of standard PQ solution (100 μM), prior to centrifugation. Following analysis of each aliquot, the areas of integrated peaks corresponding to PQ were plotted *versus* volume of PQ standard added, and the resultant line was extrapolated to the horizontal axis (*i.e.*, zero peak area). Using classical standard addition methodology, the absolute value of the horizontal intercept (*i.e.*, $|V_{\text{int}}|$) corresponds to the equivalent volume of PQ standard that would have to be added to a PQ-free sample to generate the PQ peak area of the untreated aliquot. Concentrations of PQ in the untreated aliquots were then computed according to the formula:

$$\text{CPQ} = (3|V_{\text{int}}|C_{\text{std}})/500 \quad (1)$$

Exemplar chromatograms for three aliquots of a given PQ+ homogenate (*i.e.*, including the untreated and two PQ-treated aliquots) are shown in Fig. 3.

Using this method, analysis of 35 homogenate samples, including PQ– and PQ+ flies from each of 5 different genotypes, was undertaken. The computed mean PQ concentrations recovered in the homogenates of PQ+ flies of different genotypes ranging from 2.8 to 6.3 μM (corresponding to 1.2 to 2.6×10^{-12} mol PQ per mg of fly; Fig. 4). Paraquat was not detected in any of the PQ– homogenates.

Discussion

Benefits of standard addition approach based on UPLC-PDA

The data showed that it was possible to quantitatively measure recovered PQ in homogenates of *Drosophila* of various genotypes that have been exposed to PQ through feeding. Multiple genotypes were assayed to ensure that our results were not specific to any one line of flies, but a general feature of this system. In this work, individual homogenates of 5 flies were used to generate 3 aliquots for analysis by the standard addition method. The ability to quantify the amount of PQ consumed, not just in the sample treatment concentration, will allow for more accurate quantification of oxidative stress in biological samples and simplify comparison between treatments, experiments and laboratories.

The method of standard addition we have used is a classical method of analysis and one of the best methods to account for matrix effects in complex samples. While many analytical methods rely on the use of calibration based on external standards prepared in a matrix that is, ideally, matched to that of the unknown samples, the ability to properly conduct such “matrix-matching” is often limited. Although this limitation is typically dealt with through the addition of internal standards to samples and standards prior to sample preparation, the standard addition approach provides for the best possible correction for such matrix effects since the added standard is chemically identical to

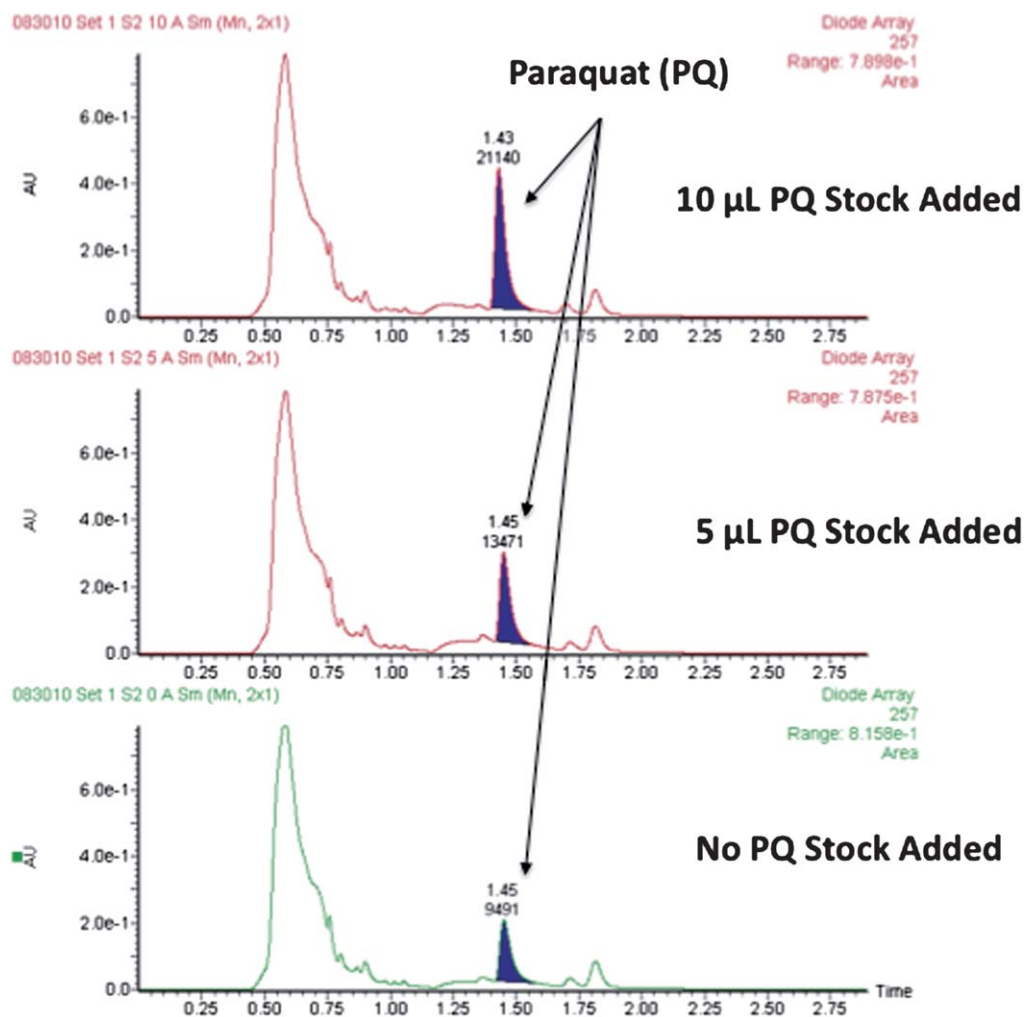


Fig. 3 Exemplar chromatograms (measured at 257 nm) from aliquots of a PQ+ fly homogenate, with 0, 5, or 10 μ L of a 100 μ M PQ solution added (from bottom to top).

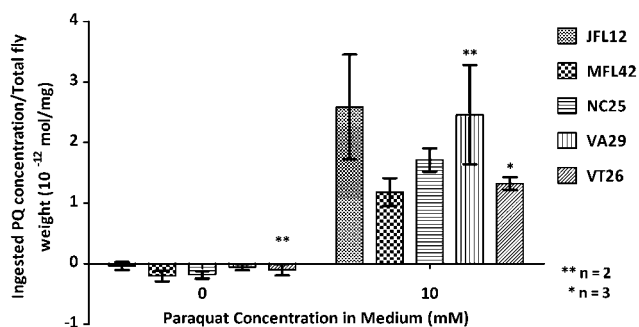


Fig. 4 Paraquat (PQ) concentration in fly samples fed for 24 h on either 0 or 10 mM PQ in 1% sucrose. Bars represent the average PQ concentration, expressed as mol PQ recovered per unit mass fly, across 5 replicates from each of 5 different genotypes, JFL12, MFL42, NC25, VA29, and VT26, after the 24 h exposure. Three replicates of the VT26 0 mM exposure, and JFL 10 mM exposure, were lost, and those bars represent the average across the remaining 2 samples. Two replicates of the VT26 10 mM exposure were lost and those bars represent the average across the remaining 3 samples. Error bars represent standard errors.

the analyte. While methods based on mass spectrometry can account for inter-sample differences in extraction recovery through the use of deuterated analogues of the analyte, methods based on other detection motifs cannot resolve deuterated and non-deuterated analogues, leaving a choice for internal standard that can be difficult. Moreover, accurate measurement of absolute recovery of PQ from the exposed flies is not possible using this methodology unless the total amount consumed could be properly accounted for. Such measurements would be best achieved by exposure of flies to a radiolabeled PQ analogue, and direct comparison of radioactivity from recovered supernatant with that of the pelleted tissue residue. As this is tedious for most experiments in the study of oxidative stress, we propose that the measurement of recovered PQ in the homogenate supernatant is a convenient and suitable alternative.

The standard addition approach also proved useful in correcting for retention time drift observed when numerous samples were assayed in a batch (typical oxidation stress experiments in the Merritt lab involve hundreds of samples). Such correction may also be achieved through the use of an internal standard, but when using optical detection (*i.e.*, photodiode array), the

requirement for a non-deuterated compound as internal standard implies some risk that the correction for retention time (*e.g.*, measurement of relative retention time) may be compromised. Over the course of hundreds of samples assayed, measured retention time values were observed to shift by as much as 40% relative to initial values using a new column, despite rigorous column conditioning. In such a case, the use of the standard addition approach provided verification of PQ peak identification.

Our detection method provided sufficient sensitivity to measure the PQ recovered in a homogenate of 5 flies, corresponding to roughly 5 mg of material. While it may be possible to adjust sample preparation methods in terms of the volume of homogenizing solution used, the amount used represented a convenient solution volume for handling, aliquot division, and for the autosampler used on the UPLC instrument. Sensitivity may be improved with other detection modes (*e.g.*, MS or MS/MS), but many laboratories may not have access to costly tandem MS instrumentation. The performance demonstrated here facilitates rapid analysis on an economical instrument platform with a minimum of sample preparation. Sensitivity may also be tuned within this method through monitoring of a longer wavelength (395 nm); the spectral features of PQ provide reasonable absorptivity at this wavelength, allowing for background reduction in cases where initial PQ levels were very low.

Conclusions

We describe a rapid method for quantitative analysis of paraquat in biological homogenates, for use in biochemical studies of oxidative stress. This method allows researchers to simply and accurately quantify the amount of PQ present in a biological sample, a much more biologically meaningful parameter than the concentration of PQ in an experimental treatment. Studies using PQ generally present only the treatment concentration, but different genotypes or species likely ingest these treatments differently resulting in different biological concentrations and complicating comparisons between studies or organisms. In such cases, quantification of PQ in a sample will allow meaningful comparison of PQ induced stress across species or genotypes. The method made use of classical standard addition

methodology to estimate recovered PQ in homogenates of *Drosophila* flies of various genotypes. Analysis was simple, using minimal sample preparation, and exploited the resolution and speed of UPLC to provide rapid (3 min) assays of homogenate samples containing paraquat in the range of 0–10 μM .

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