PharmTech

International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.10, No.4, pp 246-255, 2017

# Optimization of Liquid Chromatography-Tandem Mass Spectrometry for Paraquat Detection in Biological Tissue Samples

**Sp Tang[1]*, Sa Sulaiman[1], Sc Tan[2], Sh Gan[3]**

[1]Department of Pharmacology, School of Medical Sciences, Health Campus, UniversitiSains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[2]UsainsBiomicsSdn. Bhd, Suite 124, EUREKA Complex, Main Campus, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.
[3]Human Genome Centre, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.

**Abstract :** A simple liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantifying paraquat(PQ) level was described. Ethyl paraquat (EPQ) was used as an internal standard. The effects of varying the mobile phase composition, flow rate and splitter ratio were investigated. Analytes detection was conducted using a tandem mass spectrometer in multiple reactions monitoring (MRM) modes with the electrospray ionization (ESI) source operated in a positive ion mode. To optimize the MS-MS condition, the effects of varying instrumental parameters such as capillary voltage, collision energy, drying gas and spray chamber temperature as well as nebulizing gas pressure were investigated. Initial tissue sample preparation involved a simple one-step protein precipitation using acetonitrile. Chromatographic separations of PQ and EPQ were successfully performed on an HILIC column (3 μm; 150 × 4.6 mm) with mobile phase 250 mM ammonium formate/ acetonitrile (6:4 v/v). The flow rate was 1 ml/min and reduced to 0.1 ml/min for MS detection using splitter. The MRM transitions (precursor ion/product ion) for quantitation were m/z 186/171 for PQ and m/z 107/185 for EPQ. The optimized condition for MS-MS detection include: drying gas, 375 °C, 15 psi; nebulizing gas pressure, 50 psi.Overall, the method provides a simple and direct analysis for detection of PQ with a total run time of less than 10 minutes and is applicable for quantification of PQ in biological tissue sample with good recovery and precision achieved.
**Keywords :** paraquat, ethyl paraquat, LC-MS/MS, HILIC.

## Introduction

Paraquat(PQ) or 1,1'-dimethyl-4,4'-bipyridinium dichloride is a synthetic quaternary ammonium compound. It is an effective contact herbicide used to control broad-leaved and grassy weeds. Its frequent use in a wide variety of crops has helped to increase the productivity of agriculture in both developed and developing



http://dx.doi.org/10.20902/IJPTR.2017.10431

world, being marketed in more than 100 countries[1-2].Despite its wide usage around the world, exposure to PQ is extremely harmful. Problems resulting from exposure to PQ are common around the world, mainly by intentional swallowing, accidental or occupational exposure[3]. PQ, irrespective of its route of administration into mammalian systems, whether oral, dermal, or by inhalation, is rapidly distributed via blood circulation to most tissues, with preferentially damage the lung.Ingestion of high dosage of PQ may eventually lead to death due to multi-organ failure[4-5]. To date, there are no clinically available proven antidotes for PQ[6].

Due to it widespread usage, PQ may be a potential pollutant and is present as residues in environmental, food and biological samples. There are many analytical procedures for isolation and determination of PQ in various sample matrixes i.e soil, agriculture products, water, biological samples (mainly in serum, plasma or urine) which include thin layer chromatography (TLC), capillary electrophoresis, spectrophotometry, gas chromatography (GC) and high performance liquid chromatography (HPLC)[7-11]. Due to the non-volatile nature of quaternary ammonium compounds, a chemical reduction steps to yield a more volatile compound is required for gas chromatographic analysis of such compounds[12].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) which utilized electrospray ionization (ESI) is another method of choice[13]. However, quaternary ammonium compounds such as PQ are ionic species and are highly soluble in water. Analysis of these polar and hydrophilic analytes become challenging due to their poor retention using conventional reverse phase liquid chromatography technique. The use of ion pairing reagents, modification of mobile phase's pH and highly aqueous mobile phases may not be favourable to MS detection, hence leading to reduced sensitivity. Hydrophilic interaction liquid chromatography (HILIC) is a technique designed for separation of polar-charged compounds[14]. It is a variant of the normal phase liquid chromatography where the polar compound is better retained in polar stationary phase with the non-aqueous mobile phase substituted with aqueous-organic mixture[15]. In addition, not many methods are available for quantitative determination of PQ in organ tissues[16]. In this study, a simple sequential experimental optimization procedure to detect PQ in tissue sample using LC-MS/MS method with HILIC column is described. Ethylviologen, also known as ethyl PQ (EPQ) was used as internal standard due to its structural similarity with PQ. Both drugs contain two connected pyridinium rings with different side chain (N-methyl for PQ and N-ethyl for EPQ; Fig. 1).

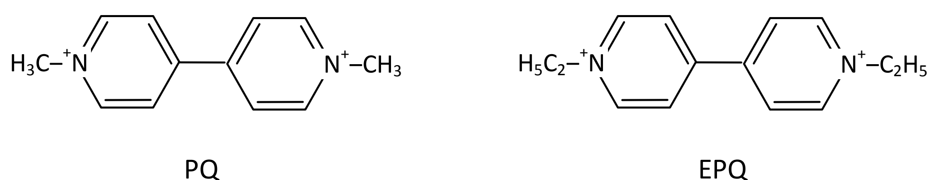


**Fig. 1: Chemical structures of paraquat (PQ) and ethyl paraquat (EPQ)**

## Experimental

### Chemicals and reagents

Methyl viologen dichloride (PQ, 98 %) andethyl viologendibromide (EPQ, 99 %) werepurchased from Sigma-Aldrich, Inc. (St Louis, MO, USA). Ammonium formate ($HCO_2NH_4$) and acetonitrile (ACN, LiChrosolv®) were purchased from Merck & Co., Inc. (KGaA, Darmstadt, Germany). Formic acid (Optima™) was purchased from Fisher Chemical (UK).

### Instrumentation

The LC-MS/MS system comprised of a Gilson 234 Autoinjector, a Varian 212-LC Chromatography Pump and a Shimadzu CTO-6A column oven coupled with Varian 320-MS triple quadrupole mass spectrometer. Instrument system control and data acquisition was performed using a Varian MS Workstation software version 6.9.1 (Palo Alto, CA, USA).

### Optimization of chromatographic and mass spectrometric conditions

Chromatographic separations of PQ and EPQ were performed on an Alltima™ HP HILIC column (150 × 4.6 mm; 3 µm). The mobile phase consisted of ACN and 250 mM$HCO_2NH_4$ (adjusted to pH 3.7 with formic

acid). PQ and its internal standard, EPQ detections were carried out using a tandem mass spectrometer in a multiple reactions monitoring (MRM) mode with electrospray ionization (ESI) source operated in a positive ion mode. The initial full scan to yield PQ and EPQ mass spectra was made by the direct injection of each analyte (1 µg/ml)into the electrospray source. From these spectra, the ideal MRM parameters for PQ and EPQ were selected. To optimize chromatographic condition, the effects of varying the mobile phase composition, flow rate and splitter ratio as well as injection volume were investigated. To optimize MS-MS condition, the effects of varying instrument parameters such as the temperatures of the drying gas and spray chamber as well as nebulizer gas pressure were investigated. Only a single parameter was varied for each analysis at one time.

**Preparation of calibration standards and quality control (QC) samples**

Stock solutions (500 µg/ml) of PQ and EPQ were prepared in deionized water and were stored in plastic tubes at 4ºC when not in use. Ten PQ calibration standards with concentrations ranging from 0.05 to 50.00 µg/ml were prepared by serial dilution of the stock solution with deionized water. EPQ working standard (5 µg/ml) and QC samples at three concentrations (0.75, 15.00 and 45.00 µg/ml) were prepared in the same manner. A calibration curve for PQ was constructed by plotting the peak area ratio of PQ to the internal standard EPQ against the concentration of the standard. Inter-assay accuracy and precision were evaluated using three QC samples analyzed in three different days. Accuracy was expressed as percentage of mean calculated PQ concentration versus actual PQ concentration. Precision was expressed as percentage of coefficient of variation (%CV).

**Analysis of PQ in tissue samples**

Tissue sample from healthy rats that were not administered with PQ served as a blank. To prepare the tissue homogenates, rat liver (0.5 g) was spiked with 25 µl PQ working standards (with a final tissue PQ concentration ranging from 0.1 to 45.0 µg/ml). EPQ (25 µl, 100 µg/ml) was added and the tissue was homogenized(LABSONIC® P, Germany)in 2 ml deionized water in plastic tubes. The homogenate was then centrifuged at 4000 × *g* for 5 min. The supernatant was collected into new plastic tubes and was re-centrifuged at 9500 × *g* for 10 min. The collected supernatant was stored at -20 °C until further analysis.

For analysis of PQ in tissue samples, protein precipitation was performed by adding 200 µl of ACN to 100 µl of the spiked supernatant. The mixture was vortexed for 1 min and was left to stand for another 15 min at room temperature before further centrifugation at 9500 × *g* for 15 min. The supernatant was collected into plastic tubes and was dried under a gentle stream of nitrogen. The samples were reconstituted in 100 µl of mobile phase before being subjected to LC-MS/MS analysis. Inter-day accuracies and precisions were analyzed on three different days and the CV was calculated.

## Results and Discussion

### Optimization of Chromatographic and Mass Spectrometric Conditions

***MRM parameters***

The selected MRM parameters for PQ and EPQ detections were outlined in Table 1. The MRM transitions (precursor ion/product ion) for quantitation were m/z 186/171 for PQ and m/z 107/185 for EPQ, respectively.

**Table 1: MRM parameters for PQ and EPQ**

| Analyte | Precursor ion (m/z) | Product ion (m/z) | Capillary voltage (V) | Collision energy (V) |
|---|---|---|---|---|
| PQ (m/z = 186) | 186 $[M]^+$ | <u>171</u> | 60 | 15 |
| | | 155 | 60 | 35 |
| EPQ (m/z = 214) | 107 $[M]^{2+}$ | <u>185</u> | 50 | 10 |
| | | 157 | 50 | 15 |

*Quantification ions are underlined.

**LC-MS/MS conditions**

The total ion chromatogram (TIC) obtained from using different mobile phase composition isshown in Fig 2. No peaks were observed within 30 min analysis time at ACN:$HCO_2NH_4$(30:70). This may be contributed by the fact that high aqueous buffer concentration may act as strong eluent for polar compounds[17]. The retention times for PQ and EPQ tend to increase with increase in ACN percentage. At a higher ACN percentage orACN:$HCO_2NH_4$(60:40), the analytes eluted after 30 min and was carried over to the next sample run. Therefore, a mobile phase composition of ACN:$HCO_2NH_4$(40:60) was selected where the retention times for PQ and EPQ were 16.0 and 19.5 min, respectively. Higher flow rate and split ratios tend to improve peak shape while reducing the retention time (Fig. 3). In addition, higher split ratio is preferred since it can prevent buffer salt saturation at ion source.

The effect of varying the drying gas and spray chamber temperatures as well as nebulizer gas pressure on PQ and EPQ peak areas are showed in Fig. 4. The peak area increased in proportion to the temperature of drying gas and spray chamber (Fig. 4a & b) and was inversely proportional to the nebulizer gas pressure (Fig. 4c). Higher drying gas and spray chamber temperatures may improve the desolvation of charge droplets to produce free, charged analytes. In addition, higher temperature may reduce the possibility of ion source contamination from undesolvated droplets and salt deposits formation which may influence the overall analytical robustness and therefore minimize the need for ion source maintenance[18].

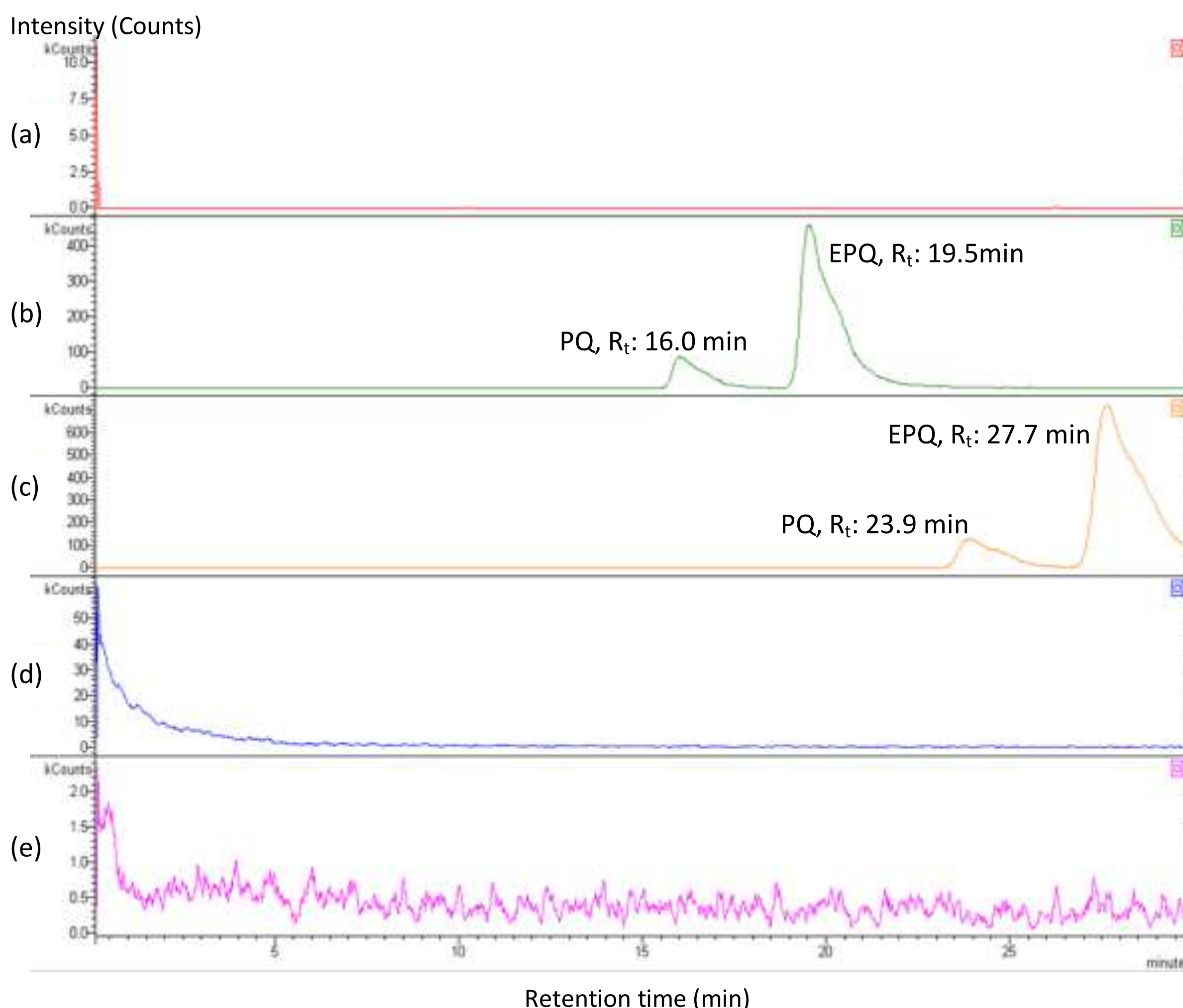


**Fig. 2: The effect of varying mobile phase composition on retention time ($R_t$) of PQ and EPQ (TIC shown). The mobile phase composed of ACN and 250 mM $HCO_2NH_4$ (pH 3.7) at different compositions: (a) 30:70, (b) 40:60, (c) 50:50, (d) 60:40 and (e) 70:30. [Flow rate: 0.4 ml/min]**

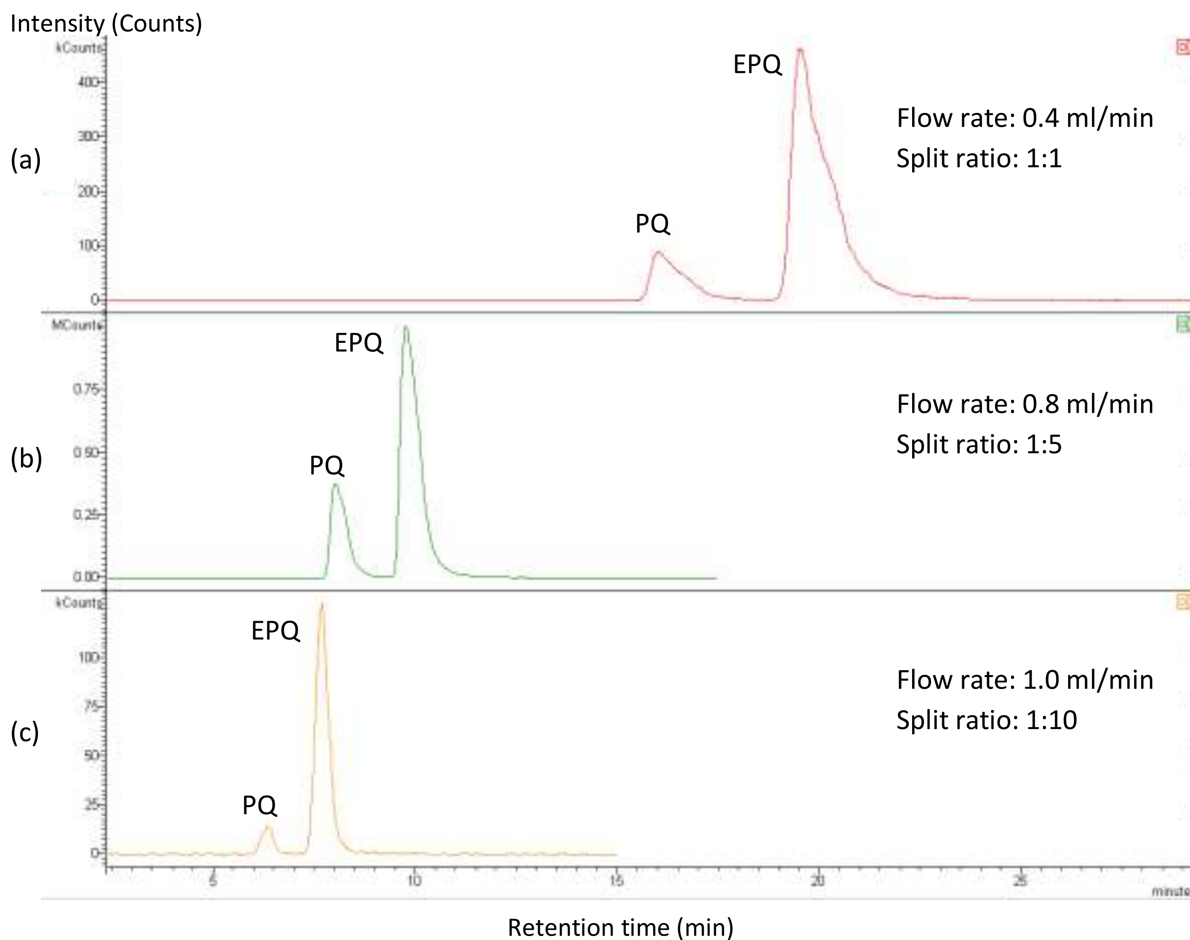


**Fig. 3: The effect of mobile phase flow rate and effluent split ratio (TIC shown). The higher split ratio is preferable when higher flow (lower $R_t$) was used to prevent effluent saturation at MS spray chamber. [Mobile phase consisted of ACN: $HCO_2NH_4$ (40:60)]**

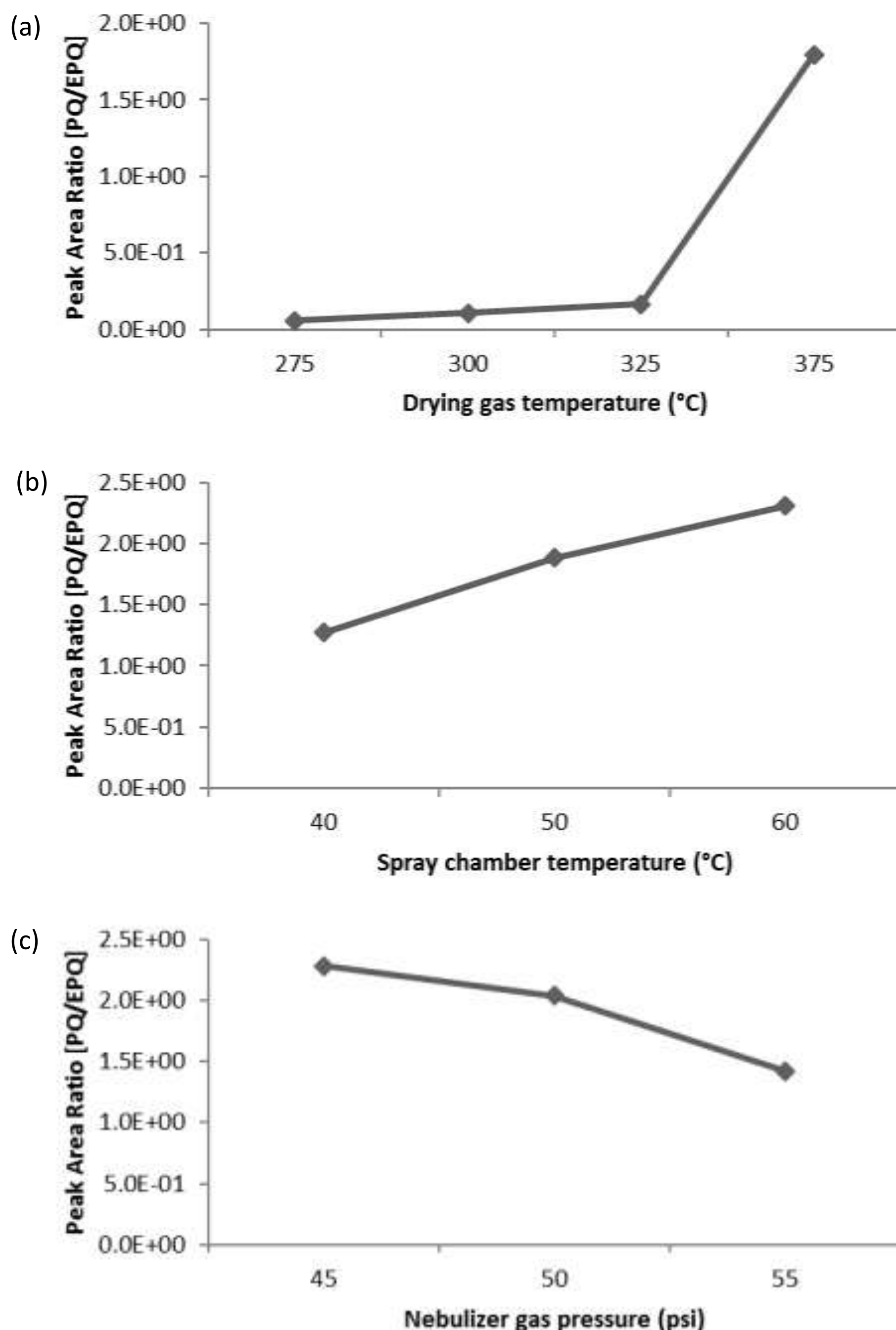


**Fig. 4: The effect of varying mass detector's (a) drying gas temperature, (b) spray chamber temperature and (c) nebulizer gas pressure on PQ intensity (MRM transition: 186/171)**

### Injection volumes

Usher *et al*. [19]previously showed that poor precision at low injection volumes can occur even when an internal standard was used. They recommended that improved precision may be achieved by increasing the volume of injection without affecting the method. In this study, injection precision was evaluated by injecting 10 replicates at three different injection volumes (Table 2). Poor precision was observed at lower injection volumes (16 and 36 µl) with % CV exceeding 15 %. In contrast, higher injection volume (46 µl) yielded good precision with % CV lower than 15 % for both analytes (Table 2).Therefore, 46 µl injection volume was selected as the most suitable volume based on similar optimized chromatographic and mass detection parameters.

**Table 2:Injection precision for PQ and EPQ (n = 10 for each volumes)**

| Injection volume (µl) | Peak Area (%CV) | | |
|---|---|---|---|
| | PQ | EPQ | Area Ratio |
| 16 | 29.80 | 26.50 | 16.51 |
| 36 | 22.34 | 11.29 | 17.34 |
| 46 | 14.84 | 7.74 | 9.89 |

**Analytical performance of the final optimized conditions**

Non-linear calibration curve with a quadratic fit was used to determine PQ concentration. A ten-point calibration curve constructed at three different days yielded a coefficient of determination ($R^2$) of higher than 0.99 for both standard (Fig. 5) and spiked samples (Fig. 6). There was good inter-day accuracy and precision (Table 3) which complies with the FDA guideline[20]. The accuracies were within ±20% of the actual concentration while the percentage CV was lower than 10% which complies with the FDA recommendation. The extraction recovery from repeated analysis of blank rat liver tissue spiked at three concentration levels (0.75, 15.00 and 37.50 µg/g) were also within ±10 % from the actual concentrations with inter-day precision of less than 15 % (Table 4) indicating that the method is accurate.

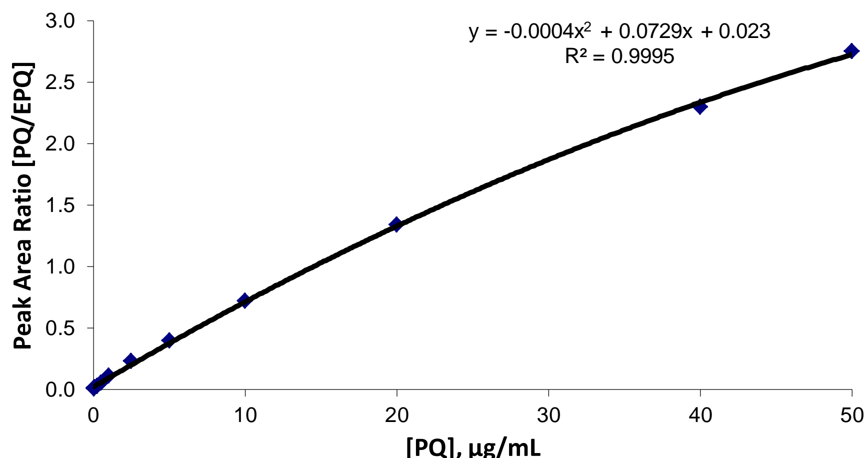


**Fig. 5: Calibration curve for PQ standards ranging from 0.05-50 µg/ml (MRM transition: 186/171)**

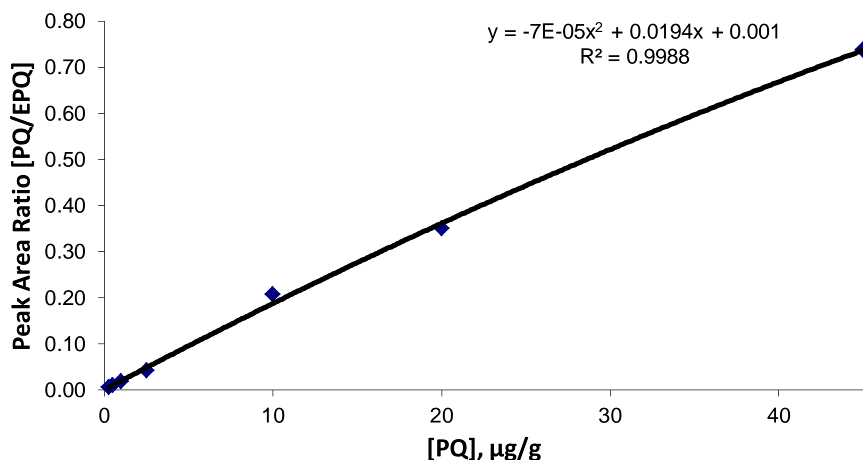


**Fig. 6: Calibration curve for PQ in tissue samples between 0.25 and 45.00 µg/g (MRM transition: 186/171)**

**Table 3: Analytical performance using a PQ drug standard**

| [PQ], µg/ml | Mean concentration (µg/ml) | Accuracy (%) | Precision (%) |
|---|---|---|---|
| 0.75 | 0.85 ± 0.04 | 112.74 | 4.70 |
| 15.00 | 13.96 ± 1.12 | 93.09 | 8.05 |
| 45.00 | 44.51 ± 3.32 | 98.91 | 7.45 |

**Table 4: Analytical performance in spiked tissue samples.**

| [PQ], µg/g | Mean concentration (µg/g) | Recovery (%) | Precision (%) |
|---|---|---|---|
| 0.75 | 0.78 ± 0.08 | 104.04 | 10.42 |
| 15.00 | 15.11 ± 2.08 | 100.70 | 13.76 |
| 37.50 | 35.10 ± 3.38 | 93.59 | 9.62 |

## Conclusion

Overall, the optimized LC-MS/MS conditions provide a simple and direct analysis for the determination of PQ in tissue sample with a short run time (less than 10 min) with good recovery and precision. The method can be applied to routine screening of PQ in biological samples.

## Acknowledgement

We would like to thank Universiti Sains Malaysia for providing the financial support under the USM-RU-PRGS grant (1001/PPSP/8142002). Special thanks to members from Usains Biomics for all the technical guidance and support.

## References

1. B. Carlile, Pesticide selectivity, health and the environment. Cambridge, UK: Cambridge University Press; 2006.
2. M. Eddleston and D.N. Bateman, *Pesticides Medicine*, **35**, 646 (2007).
3. IPCS, Paraquat health and safety guide. Geneva: World Health Organization; 1991. 36 p.
4. R.J. Dinis-Oliveira, J.A. Duarte, A. Sanchez-Navarro, F. Remiao, M.L. Bastos and F. Carvalho, *Crit. Rev. Toxicol.*, **38**, 13 (2008); doi:10.1080/10408440701669959.
5. J.A. Vale, T.J. Meredith and B.M. Buckley, *Hum. Exp. Toxicol.*, **6**, 41 (1987); doi:10.1177/096032718700600107.
6. I.B. Gawarammana and N.A. Buckley, *Br. J. Clin. Pharmacol.*, **72**, 745 (2011); doi:10.1111/j.1365-2125.2011.04026.x.
7. R.M. de Almeida and M. Yonamine, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **853**, 260 (2007); doi:10.1016/j.jchromb.2007.03.026.
8. I.S. Sellero, M. López-Rivadulla, A. Cruz, A. Bermejo and P. Fernández, *Anal. Lett.*, **26**, 1891 (1993); doi:10.1080/00032719308017438.
9. R. Lanaro, J.L. Costa, L.C. Fernandes, R.R. Resende and M.F. Tavares, *J. Anal. Toxicol.*, **35**, 274 (2011); doi:10.1093/anatox/35.5.274.
10. Y. Ouyang, R.S. Mansell and P. Nkedi-Kizza, *J. Environ. Qual.*, **33**, 406 (2004); doi:10.2134/jeq2004.4060.
11. B. Sun and Y. Chen, *Int. J. Clin. Exp. Med.*, **8**, 17067 (2015).
12. N.C. Posecion, E.M. Ostrea and D.M. Bielawski, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **862**, 93 (2008); doi:10.1016/j.jchromb.2007.11.002.

13. C. Hao, X. Zhao, D. Morse, P. Yang, V. Taguchi and F. Morra, *J. Chromatogr. A*, **1304**, 169 (2013); doi:10.1016/j.chroma.2013.07.033.
14. B. Buszewski and S. Noga, *Anal. Bioanal. Chem.*, **402**, 231 (2012); doi:10.1007/s00216-011-5308-5.
15. Y. Guo and S. Gaiki, *J. Chromatogr. A*, **1074**, 71 (2005); doi:10.1016/j.chroma.2005.03.058.
16. Y.C. Tsao, Y.C. Lai, H.C. Liu, R.H. Liu and D.L. Lin, *J. Anal. Toxicol.*, **40**, 427 (2016); doi:10.1093/jat/bkw042.
17. M. Liu, E.X. Chen, R. Ji and D. Semin, *J. Chromatogr. A*, **1188**, 255 (2008); doi:10.1016/j.chroma.2008.02.071.
18. Banerjee S, Mazumdar S., *Int. J. Anal. Chem.*, Article ID 282574 (2012).
19. K.M. Usher, S.W. Hansen, J.S. Amoo, A.P. Bernstein and M.E.P. McNally, *LC GC N. Am.*, **33**, 40 (2015).
20. Guidance for industry, Bioanalytical method validation [database on the Internet]. FDA. 2001. Available from: http://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf.


***** *****

**Extra page –not to be printed.**

**For your Research References Requirements , always log on to=**

**www.sphinxsai.com**

**International Journal of PharmTech Research**

*********