OPTIMISATION OF GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) METHOD FOR DETECTION OF PARAQUAT BY SODIUM BOROHYDRIDE-NICKEL CHLORIDE CHEMICAL REDUCTION

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ABSTRACT: Paraquat is an extremely toxic herbicide and is still extensively used in third world countries. Problems resulting from paraquat exposure are commonly due to intentional, accidental or occupational exposures. In this study, a Gas Chromatography-Mass Spectrometry (GC-MS) method for quantifying paraquat is described. The method involved an initial chemical reduction step for both paraquat and ethyl paraquat (used as internal standard) using sodium borohydride-nickel choride to their respective perhydrogenated products, followed by a GC-MS analysis. For optimisation of GC-MS parameters, the effects of varying injector and oven temperature programmes were investigated. Chromatographic separations of paraquat and ethyl paraquat was successfully achieved on a Varian VF-5ms capillary column (30 m x 0.25 mm I.D x 0.25 µm film thickness) with a total run time of less than 15 minutes.

Keywords: Paraquat, GC-MS, optimisation.

Introduction

Suicides or intentional self-poisoning is one of the major contributing factors for acute pesticide poisoning [1]. According to World Health Organisation [2], approximately 75.5% of the global suicides cases was occurring in the low- and middle-income countries in year 2012. Pesticide self-poisoning was one of the three most common suicide methods accounts for approximately 30% of the suicide cases [2].

Paraquat (PQ) is an effective contact herbicide used to control broad-leaved and grassy weeds and is frequently use in a wide variety of crops to increase the productivity of agriculture in both developed and developing world. Due to its popularity and ease of access, PQ is commonly used as a suicide agent particularly in developing countries [3, 4]. However, PQ is extremely toxic. Once administered into mammalian system through oral, dermal or inhalational, PQ is rapidly distributed *via* blood circulation to all organs but is selectively concentrated in the lung. Ingestion of high dosage of PQ may eventually lead to death due to multi-organ failure. To date, there are no clinically available proven antidotes for PQ.

There are many analytical methods available to measure PQ in biological samples (mainly in serum, plasma or urine), including thin layer chromatography (TLC), spectrophotometry, gas chromatography (GC) and high-performance liquid chromatography (HPLC). However, not many methods are available for the quantitative determination method for PQ in organ tissues by GC-MS. The objective of this study is to fill in this gap by developing a GC-MS method for quantifying PQ. A sequential experimental optimisation procedure to detect paraquat using gas chromatography is described. This method is applicable to detect PQ, particularly in postmortem tissue samples.

Materials and Methods

Materials

Paraquat dichloride (methyl viologen dichloride, 98%), ethyl paraquat (ethyl viologen dibromide, 99%), sodium borohydride (≥ 98.0%) and nickel (II) chloride hexahydrate (ReagentPlus®) were all obtained from Sigma-Aldrich, Inc. (St Louis, MO). Sodium hydroxide and ethyl acetate were of analytical grades and were obtained from Merck & Co.,Inc (KGaA, Darmstadt, Germany). Toluene and hexane were of liquid chromatography grades (LiChrosolv®) and were obtained from Merck. Stock solutions (500 µg/mL) of paraquat dichloride and ethyl paraquat (internal standard) were

prepared in deionised water and stored at 4ºC when not in use. Working solutions of the mixture of analytes were prepared by diluting stock solution with deionized water before analysis. Both sodium borohydride solution and nickel chloride solution were prepared fresh daily.

Sample preparation

The chemical reduction of PQ and its internal standard (ethyl paraquat, EPQ) to their perhydrogenated products involved a sodium borohydride-nickel chloride ($NaBH_4$-$NiCl_2$) reduction procedure adapted from Posecion *et al.* [5] with some modifications. A 50 µL of EPQ (500 µg/mL) was added to 0.5 mL of PQ working solution in screw-capped silanised test tubes. A 200 µL of toluene was added to the surface of the mixtures, followed by 400 µL of $NiCl_2$ (1% w/v). A 500 µL of $NaBH_4$ (40% w/v) was then added dropwise. The solution was mixed well and left to stand for 1.5 hours at room temperature for chemical reduction to take place. At the end of the chemical reduction step, 1 mL of sodium hydroxide (NaOH, 5 N) was added to the tube to stop the reaction.

The perhydrogenated products of PQ and EPQ were extracted by adding 3 mL of hexane:ethyl acetate (4:1 v/v) to the mixture. The tubes were capped tightly and tape-sealed followed by shaking at 450 rpm using a Barnstead/Thermolyne Bigger Bill Orbital Shaker (Dubuque, Iowa, USA). After 30 min, separation of the layers was achieved by centrifugation at 3200 g for an hour. The organic solvents layer was transferred to a silanised high recovery vial (Chromacol, Hertfordshire, UK) and evaporated to dryness under a gentle stream of nitrogen (99.99% purity) at room temperature. The sample vials were reconstituted in 1 mL hexane and subjected for GC-MS analysis.

Optimisation of GC-MS analysis

Analysis of reduction products of PQ and EPQ were performed using a Varian CP-3800 Gas Chromatograph equipped with a Varian Saturn 2200 ion trap MS. Sample injection (1 µL) were performed by a Varian CP-8410 Autosampler with standard on-column injection mode. The chromatographic column used was a Varian VF-5ms (5%-phenyl, 95%-dimethylpolysiloxane stationary phase; 30 m × 0.25 mm i.d., 0.25 µm film thickness) capillary column. Instrument system control and data acquisition was performed by the Varian MS Workstation software version 6.9.1 (Palo Alto, CA).

Injector port was initially operated in splitless mode and the split vent was opened after 0.75 min at split ratio 20:1. For optimisation of injection port temperature, the temperature investigated were at 160ºC, 180ºC, 200ºC, 220ºC and 250ºC, respectively. Helium gas (99.999% purity) as carrier gas was set at a constant flow rate (1 mL/min). The oven programme started at 100ºC and was immediately ramped at 25ºC/min to 180ºC, held for 5 min. Finally, the temperature was ramped at 40ºC/min and held at 300ºC for 5 min. The transfer line, manifold and trap temperature were set at 275ºC, 40ºC and 200ºC, respectively. All mass spectra were acquired in the full scan electron ionisation (EI) mode at 70 eV with emission current of 10 µA. The mass range was 40 – 650 m/z, with a scan rate of 5 µscan/s. Solvent delay time was set at 2.5 min. Chromatographic peak areas of perhydrogenated products of PQ and EPQ were determined by reconstructing the total ion chromatogram (TIC) using specific ions for each compound. The ions selected for each compound were of m/z 96, 181, 196 [molecular ion, M^+] for PQ and m/z 110, 195, 224 [M^+] for EPQ.

For optimisation of the column oven temperature programme, injector port temperature was set at 220ºC. All other GC-MS parameters, including the injection mode, flow rate and mass acquisition mode were kept unchanged. Four different column oven initial temperatures, xºC (60ºC, 80ºC, 100ºC and 120ºC) immediately ramped at 25ºC/min to yºC (160ºC, 180ºC, 200ºC, 220ºC and 250ºC), respectively were investigated.

Optimisation of chemical reduction process for sample preparation

In order to further increase the sensitivity of the GC-MS system towards detection of perhydrogenated products of PQ and EPQ, the effects of varying chemicals compositon during chemical reduction process were also investigated. The parameters tested include varying i) the percentage of $NaBH_4$, ii) the volume of $NaBH_4$ and iii) the percentage of $NiCl_2$. For this purpose, injector port temperature was set at 220ºC. The oven programme started at 80ºC and was immediately ramped at 25ºC/min to 180ºC and was held for 5 min. Finally, the temperature was ramped at 40ºC/min and held for 5 min. All other GC-MS parameters were kept unchanged.

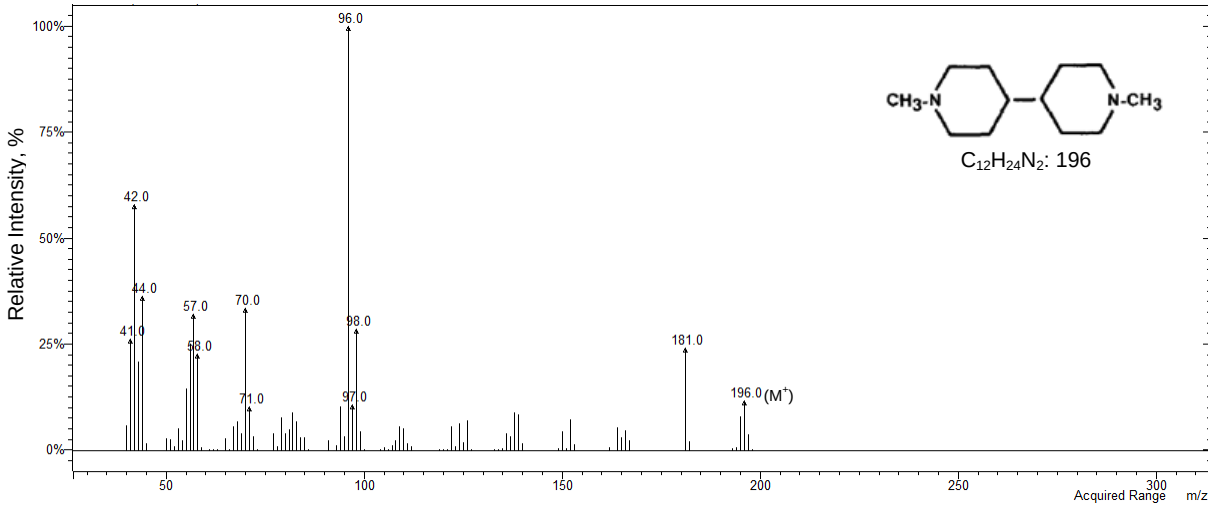
Results

Optimisation of GC-MS analysis

The fragmentation patterns of perhydrogenated product of both PQ and its internal standard, EPQ in EI-MS were shown in Figure 1. The fragmentation patterns of monoene by-product

derived from the chemical reduction system for both drug standards were shown in Figure 2.

(a)



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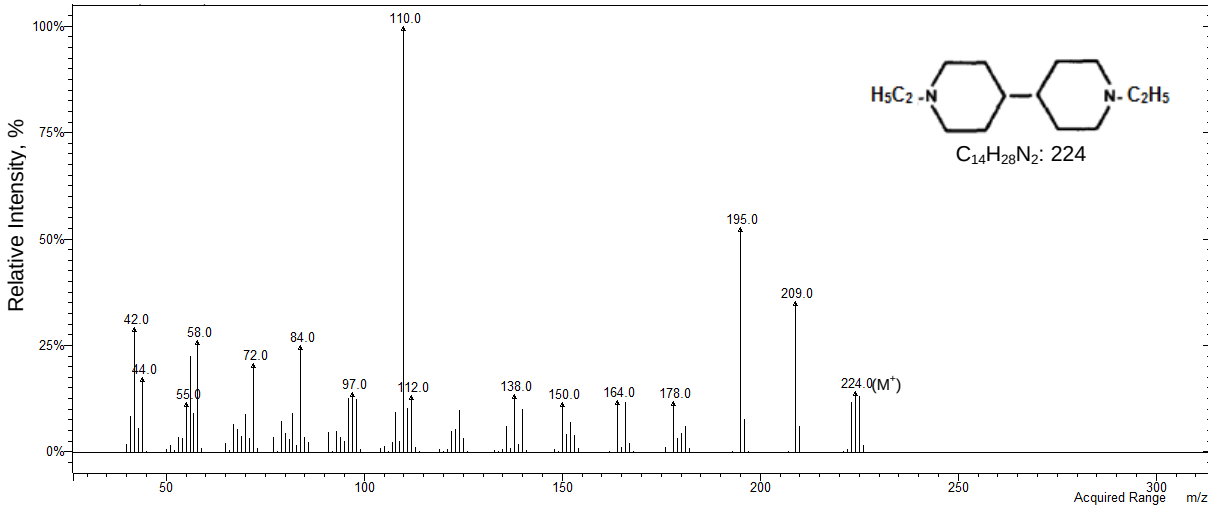
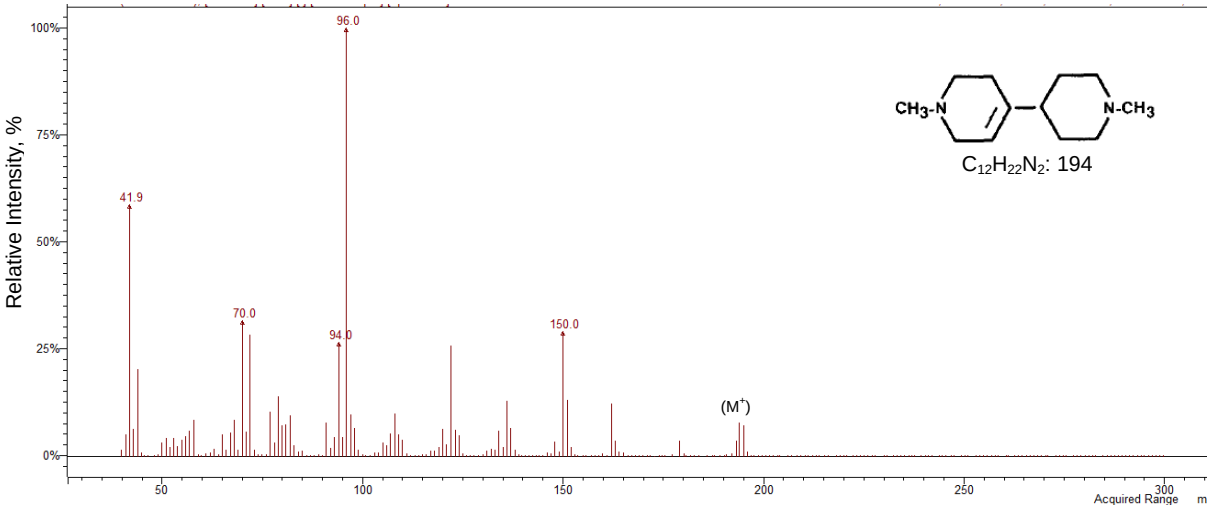


Figure 1: Full scan EI mass spectra of perhydrogenated product of (a) PQ, $C_{12}H_{24}N_2$ and (b) EPQ, $C_{14}H_{28}N_2$ after treatment with $NaBH_4$-$NiCl_2$

(a)



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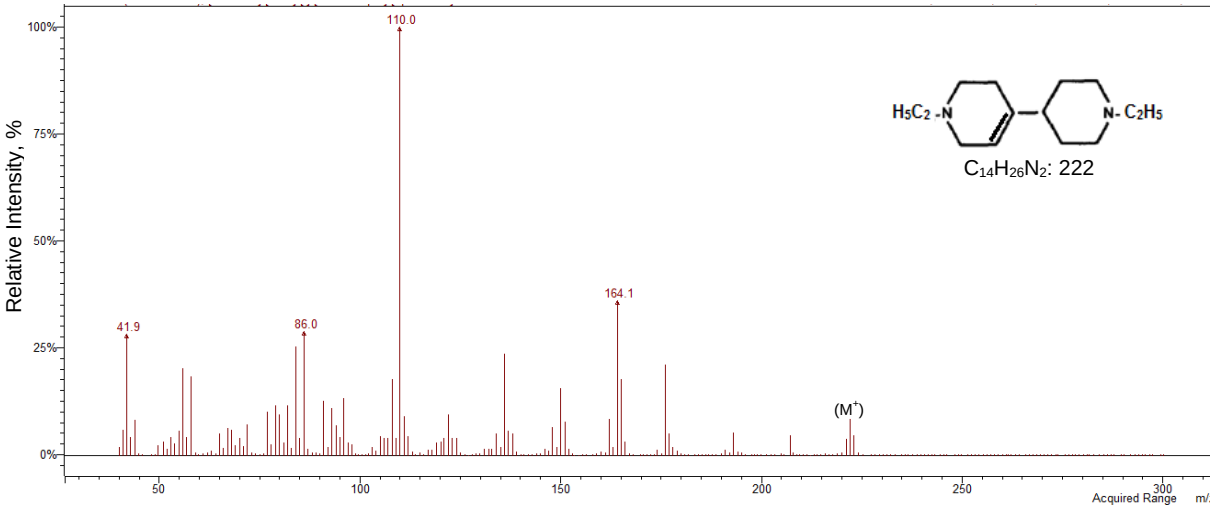


Figure 2: Full scan EI mass spectra of monoene by-product of (a) PQ, $C_{12}H_{22}N_2$ and (b) EPQ, $C_{14}H_{26}N_2$ after treatment with $NaBH_4$-$NiCl_2$

Optimisation of injector port temperature

The peak area of perhydrogenated PQ showed an increasing trend with increase in injector port temperature from 160ºC to 220ºC but started to decrease again after that (Figure 3). Hence, injector temperature of 220ºC which gave a maximum peak area was selected. The reconstructed ion chromatogram obtained (ions: 96 + 110 m/z) at injector temperature of 220ºC was shown in Figure 4.

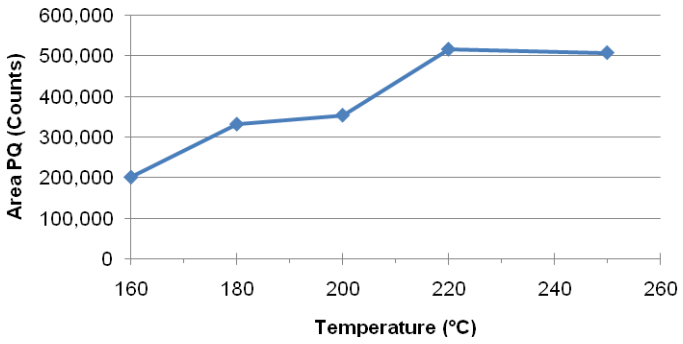


Figure 3: The effect of varying temperature of the injector ports on area of perhydrogenated PQ [PQ concentration, 100 ppm; reconstitution volume, 1 mL hexane; oven temperature, 100ºC ramped to 180ºC at 25ºC/min]

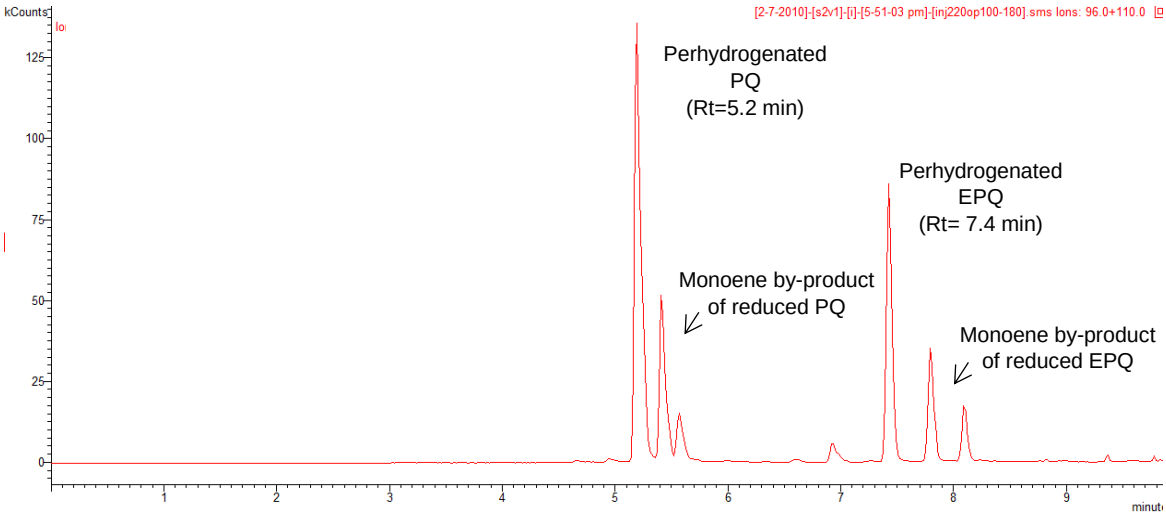


Figure 4: Reconstructed ion chromatogram obtained (ions: 96 + 110 m/z) after injection of 1 µL sample that have undergone a chemical reduction process. Retention time of perhydrogenated product of PQ and EPQ were 5.2 min and 7.4 min, respectively [PQ concentration, 100 ppm; reconstitution volume, 1 mL hexane; injector temperature, 220ºC; oven temperature, 100ºC ramped to 180ºC at 25ºC/min]

Optimisation of column oven temperature

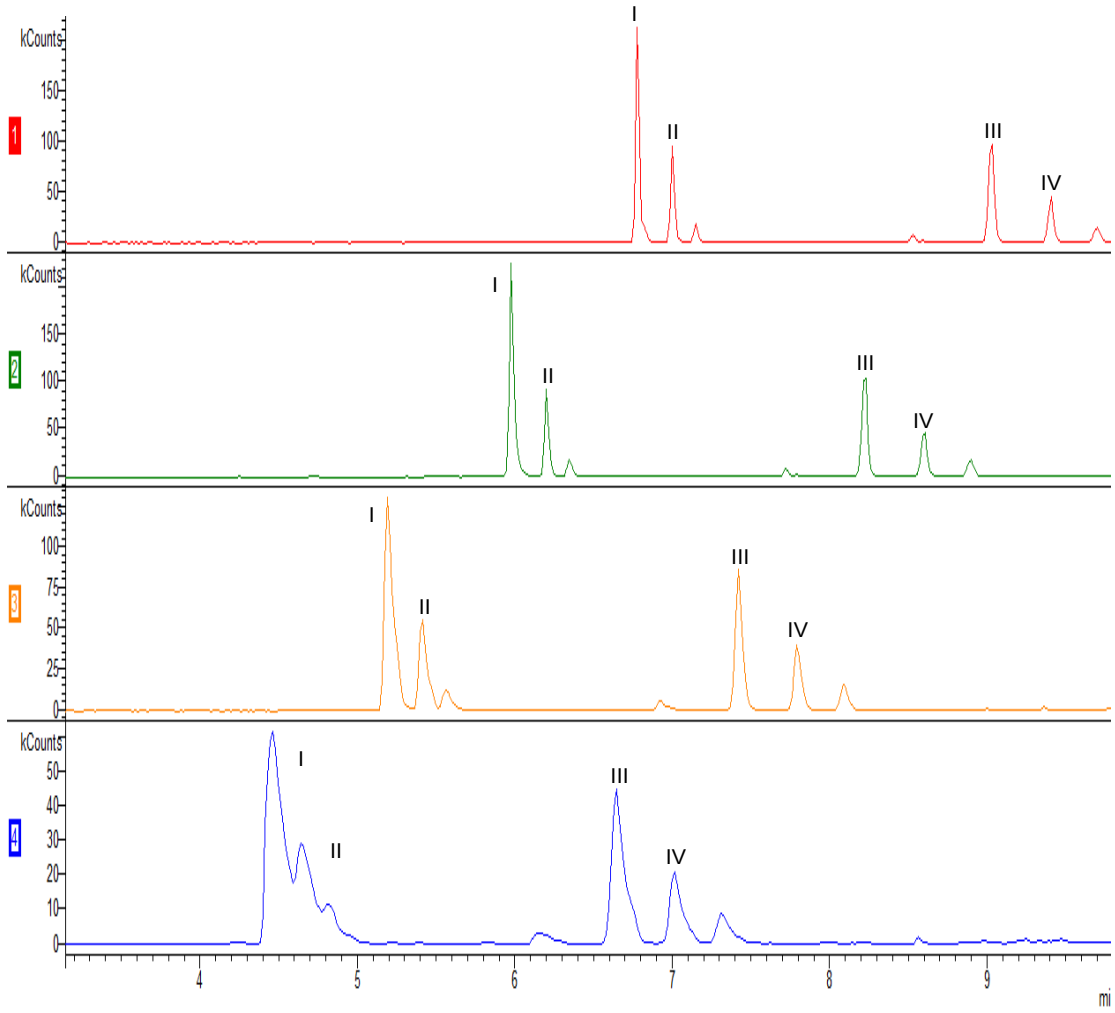
For optimisation of column oven temperature programme, a total of 20 conditions were tested to investigate their effects to the chromatographic separation of reduction products of PQ and EPQ. Parameters that were taken into consideration include the peak shape, resolution and their retention times. During this experiment, the injector temperature was set at 220ºC and all other parameters were kept constant as mentioned earlier (*i.e.* injection mode, flow rate, and mass acquisition mode). The final concentration of PQ and EPQ used were 100 ppm and 50 ppm, with final reconstitution in hexane (1 mL).

Overall, varying the initial column oven temperature (xºC) alone affects the analytes' retention time. The elution time for both drug standards reduced with increased in initial column oven temperature. The peaks of perhydrogenated product of PQ and EPQ are well resolved without co-elutions. However, the resolution between perhydrogenated PQ and by-products worsened with increased in initial column oven temperature. At initial oven temperature of 120ºC, the by-products started to co-elute with the peak of interest, perhydrogenated PQ. The effects of varying initial column oven temperature which were immediately ramped at 25ºC/min to reach 180ºC were shown in Figure 5. Similar trends were observed for other oven temperature programmes tested (yºC at 160ºC, 200ºC, 220ºC or 250ºC).

When comparing different oven temperature programmes with constant initial oven temperature

(varying the yºC), it is observed that at lower yºC temperature, the analytical condition are more favourable towards the internal standard. Both reduction products of PQ and EPQ tend to elute faster with increasing yºC and this was especially shown with the internal standard (Figure 6).

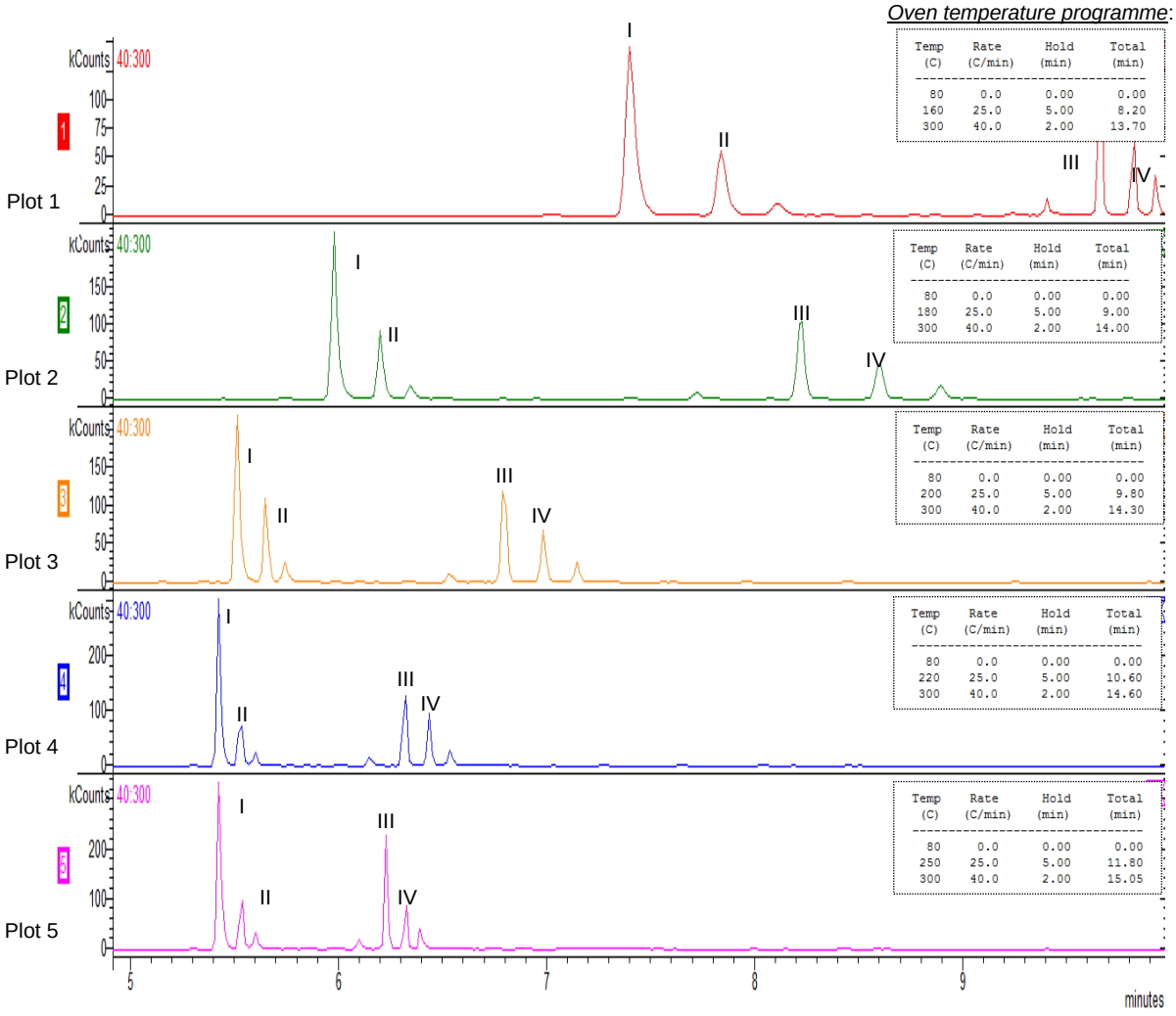
As a conclusion, column oven condition with an initial temperature 80ºC, immediately ramped at 25ºC/min to reach 180ºC, and held for 5 min was finally selected because it gave a reasonably short elution time with good peak area and shape (Figure 7).



Plot	Initial oven temperature	Retention time (minute)			
		I	II	III	IV
1	60ºC	6.8	7.0	9.0	9.4
2	80ºC	6.0	6.2	8.2	8.6
3	100ºC	5.2	5.4	7.4	7.8
4	120ºC	4.5	4.6	6.6	7.0

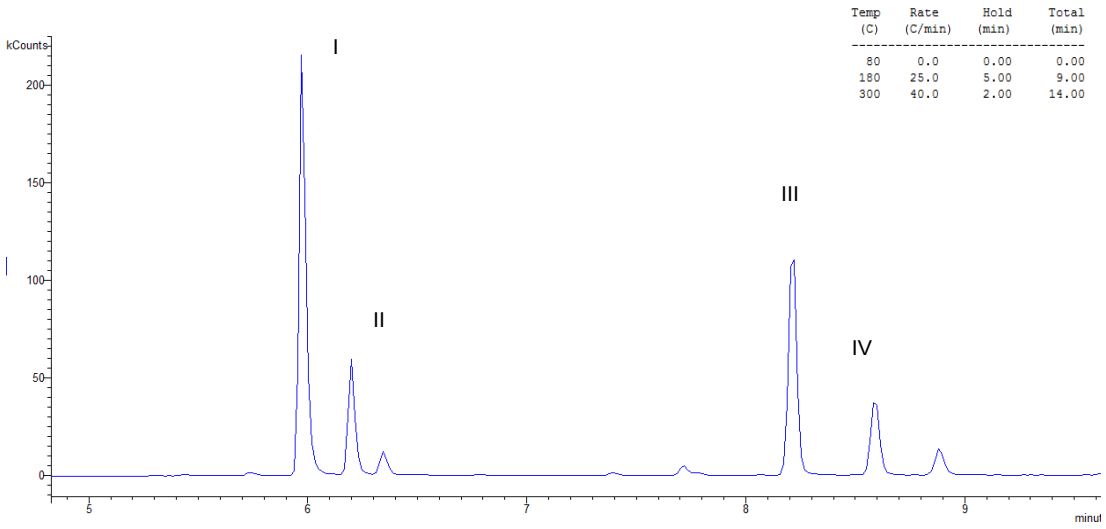
I: perhydrogenated PQ
II: monoene by-product of PQ
III: perhydrogenated EPQ
IV: monoene by-product of EPQ

Figure 5: The effects of varying initial column oven temperature ramped at 25ºC/min to 180ºC. TIC was reconstructed under ion 96 + 110 m/z. The retention times of the chemical reduction products of PQ and EPQ are as tabulated



I: perhydrogenated PQ
II: monoene by-product of PQ
III: perhydrogenated EPQ
IV: monoene by-product of EPQ

Figure 6: The effects of varying *y*ºC with initial column oven temperature (*x*ºC) 80ºC ramped at 25ºC/min to yºC. Chromatograms shown were reconstructed under ion 96 + 110 m/z



I: perhydrogenated PQ
II: monoene by-product of PQ
III: perhydrogenated EPQ
IV: monoene by-product of EPQ

Figure 7: Reconstructed ion chromatogram (ions: 96 + 110 m/z) obtained under column oven condition 80ºC to 180ºC (25ºC/min). The peak of perhydrogenated PQ (I) was well resolved from its monoene by-products (II) as well as the internal standard, perhydrogenated EPQ (III). Peak height of internal standard also correlated with the concentration used (Final concentration of PQ and EPQ were 100 ppm and 50 ppm respectively, final reconstitution in 1 mL hexane). Retention time of perhydrogenated PQ and EPQ were 6 min and 8.2 min, respectively

Optimisation of chemical reduction process during sample preparation

During the optimisation of chemical reduction process as below, PQ (0.5 mL, 0.5 ppm) and EPQ (50 µL, 500 ppm) were prepared and the final reconstitution with hexane was kept constant at 1 mL.

The effect of varying the percentage of $NaBH_4$

The percentages of $NaBH_4$ tested were 25%, 30%, 35%, 40% and 45% (w/v). During this experiment, 400 µL of $NiCl_2$ (1% w/v) was used with the volume of $NaBH_4$ fixed at 500 µL. $NaBH_4$ gave higher area count of perhydrogenated PQ at 25% compared to when 30% and 35% $NaBH_4$ were used. It was also observed that higher percentage of $NaBH_4$ (from 30% to 45% NaBH4) gave higher area count of perhydrogenated PQ (Figure 8). However, the area count did not differ much when the percentage of $NaBH_4$ was varied. Hence, only 25% $NaBH_4$ was selected as it was more cost effective and could preserve longer column life due to the possibility of excessive hydrogenation due to higher concentrations of $NaBH_4$.

The effect of varying the volume of $NaBH_4$

In this experiment, 400 µL of $NiCl_2$ (1% w/v) and 25% $NaBH_4$ were used while the volumes of $NaBH_4$ were varied (200 µL, 400 µL, 500 µL, 600 µL and 800 µL). The area count decreased as the volume of $NaBH_4$ increased from 200 µL to 500 µL, and only increased again up to 800 µL (Figure 9). Although higher volume of $NaBH_4$ tend to improve the area of perhydrogenated PQ, 200 µL of 25% NaBH4 was selected to avoid possible column damage and more cost effective.

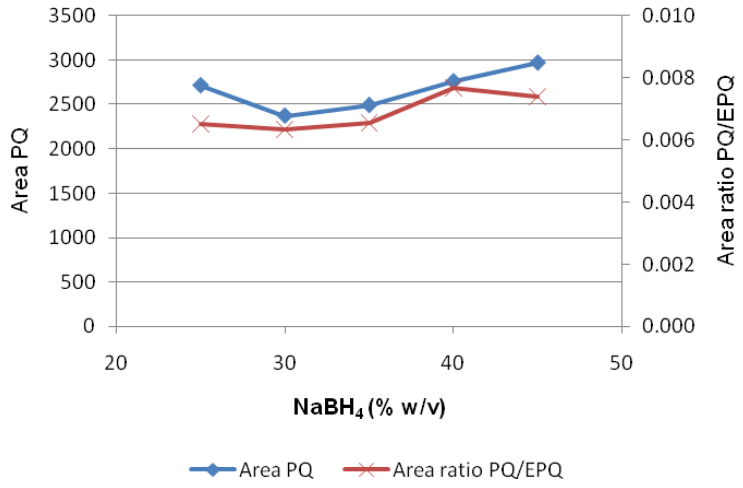


Figure 8: The effects of varying percentage of $NaBH_4$ on area of perhydrogenated PQ. The area ratio of perhydrogenated PQ to perhydrogenated EPQ (PQ/EPQ) were shown in secondary vertical axis

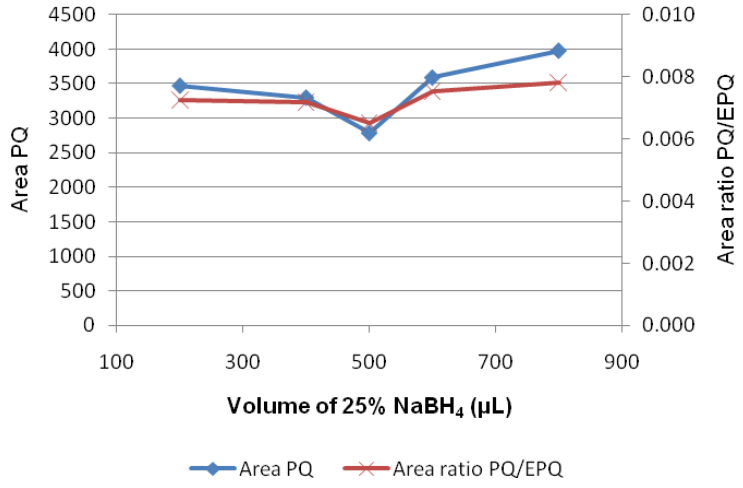


Figure 9: The effects of varying volume of 25% $NaBH_4$ on the area of perhydrogenated PQ and area ratio of perhydrogenated PQ to perhydrogenated EPQ

Discussion

PQ, a quaternary ammonium compound is characterised by its high polarity and low volatility. Therefore, conversion of these substances into a thermally-stable and volatile substance before GC analysis is necessary. One of the methods is by reduction of PQ through hydrogenation process, utilising $NaBH_4$-$NiCl_2$ chemical reduction reaction. $NaBH_4$ readily release hydrogen in water and react with various transition metals *e.g.* nickel salts to yield black precipitates which possess catalytic hydrogenation properties. $NiCl_2$ in this case acts as hydrogenation catalyst [6]. Chemical reduction products formed include volatile N, N-dimethylbipiperidine (formed after complete reduction occurs) or its mono- and diunsaturated derivatives (monoene or diene by-products formed after partial reduction occurs) [7, 8]. These reduction products are amenable for organic solvent extraction, which offer advantages of compound isolation by solvent partitioning and can be concentrated before GC-MS analysis [5, 7]. In this research, we have optimised the GC-MS analytical condition for detection of reduction products of PQ followed by optimisation of the parameters for chemical reduction.

A suitable internal standard is required to achieve reliable quantitative analysis. This is to avoid possible calculation error due to sample losses during sample preparation or variation in instrument response from run to run. Commercially-available EPQ was chosen as the

internal standard in this study due to its structural similarity with paraquat allowing it to undergo a similar reaction during chemical reduction process [9, 10]. Both drugs contain two connected pyridinium rings (where hydrogenation/ reductions occur) with different side chain: N-methyl for PQ and N-ethyl for EPQ.

During optimisation of GC-MS parameters, high concentration of PQ and its internal standard, EPQ were used (100 ppm and 50 ppm, respectively, with final reconstitute volume of 1 mL hexane). From the chromatogram obtained, it was observed that other than the two major peaks that represent perhydrogenated product of PQ and EPQ, there are two minor peaks that appeared adjacent to both major peaks, respectively. As mentioned earlier, the $NaBH_4$-$NiCl_2$ chemical reduction system yielded both complete (perhydrogenated) and partial reduced products (monoene or diene by-products).

Mass spectrum of perhydrogenated PQ was similar as previously reported [5, 8, 11], with parent ion at m/z 196 and base peak ion at m/z 96. Parent ion and base peak ion for EPQ were at m/z 224 and m/z 110, respectively. The base peak in the spectra of monoene by-products for both PQ (m/z 96) and EPQ (m/z 110) are the same, but there are some differences in the relative intensity of their fragments due to different fragmentation patterns of perhydrogenated product and monoene derivatives. In the fragmentation of perhydrogenated PQ, higher intensities of parent ion m/z 196 (M^+) and ion m/z 181 (= M^+ - CH_3) are detected [11]. For its monoene by-product, intensities of fragment ions m/z 150 (= M^+ - C_2H_6N) and m/z 122 (=M^+ - C_2H_6N – C_2H_4) [8] are relatively higher. In the fragmentation of internal standard EPQ, mass spectra of perhydrogenated product show higher intensities of m/z 209 and m/z 195 suggesting dissociation routes at M^+ - CH_3- CH_2 or M^+ - C_2H_5. Higher intensities at ions m/z 164 found in mass spectra of monoene by-product suggesting a molecular fragment of $C_{11}H_{18}N$.

In GC, temperature is an important operating parameter that must be carefully optimised to suit the polarity or boiling points of the components of the sample [12]. Typically, the GC column is fixed between an injector port and a detector. The control of the temperatures at which these components operated is important to achieve a good separation in a reasonable amount of time [13]. For instance, the injector port temperature should be high enough for rapid sample vapourisation so that it can be carried into the column by passing carrier gas and prevent loss in efficiency results from the injection technique. Meanwhile, the temperature should be low enough to prevent thermal decomposition or chemical rearrangement of the analytes which will affect quantitative accuracy [12, 13]. In the GC-MS analysis, injector temperature at 220 ºC gave better sensitivity compared to temperature setting at 250 ºC. Lower temperature setting could prevent analytes from thermal degradation.

Temperature programming accommodates mixtures with a wide boiling range or broad polarity span. It is now a commonly-practiced elution technique besides flow programming to accelerate the elution rate of the late peaks, and thus shorten the analysis time [12]. As the temperature increases, the retention times and retention factors also decreased. A decrease in temperature decreased the vapour pressure of the solute, subsequently resulted in a decrease in relative amount of solute in the mobile phase followed by an increase in the retention factor and an increase in retention times [13]. Reducing the initial column oven temperature helped to improve the resolution of the early peaks but increased the retention time and analysis time [12]. Similar trend was observed during optimisation of column oven temperature programme. Reduction products of PQ and EPQ eluted out faster by increasing column initial temperature. However, resolution between perhydrogenated PQ and its monoene by-product decreases at higher temperature (from 80ºC to 100ºC) and co-eluted at 120ºC.

Since there are relatively few methods utilising chemical reduction of PQ for GC-MS analysis, the present work described mainly the modification from recent methods reported by Posecion *et al.* [5] which involved GC-MS determination of PQ and internal standard dibenzyl in meconium samples by $NaBH_4$-$NiCl_2$ reduction. In our study, capillary column with film thickness of 0.25 µm instead of 1 µm was used. Stationary phase film thickness influenced both the phase ratio (the ratio of the volume of mobile phase in the column to the volume of stationary phase in the column) and resistance to mass transport in the column [14]. Thus, column film thickness affects directly on retention time and elution temperature. Retention time of perhydrogenated PQ is 6 min in current study (11.96 min as reported by Posecion *et al.* [5] and the total analysis time had been reduced. Retention of analytes increased when thicker film was applied while thinner films retained analytes less by minimising amount of time the analytes spend in the stationary phase. Besides, higher operating temperatures were required to elute compounds of interest from thicker film [13].

Another published report by de Almeida and Yonamine [10] involved GC-MS detection of PQ and diquat in human plasma and urine after chemical reduction of the analytes by $NaBH_4$ alone.

The reduction process yields both diene derivatives of PQ and EPQ due to incomplete hydrogenation. During optimisation of the conditions of chemical reduction reaction in their studies, quantity of $NaBH_4$ at 10, 20, 30, 50, 100 and 150 mg were investigated and no difference was observed in relation to the amount used [10]. Similar finding was observed in the present study where varying percentage and volume of $NaBH_4$ did not significantly affect area of perhydrogenated products.

Conclusion

The present work proposed a GC-MS method for the determination of chemical reduction products of paraquat. An optimised GC-MS condition and chemical reduction process was successfully developed.

Aknowledgements

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