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Methylene Blue Extraction Employing NanoPak-C All-Carbon Microbeads-Packed 3 mL SPE Columns, & Quantification Using Visible Spectrophotometry

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Abstract.

Background. Methylene blue, a heterocyclic aromatic chemical compound, is a known medication and organic dye. It is a significant source of water contaminant. Herein, we report an optimal solid phase extraction method to extract methylene blue in milli-liter volumes, using an all-carbon reverse phase material.

Method Used. Methylene blue dissolved in deionized water was extracted using NanoPak-C graphite microbead (250 mg bed weight). It was packed into 3 ml SPE columns. A visible spectrophotometer was used to characterize and quantify mL volumes of methylene blue.

Results. The optimized methylene blue extraction protocol allowed 95% recovery efficiencies.

Conclusion. All carbon microbeads as reverse phase media efficiently extract methylene blue. The results suggest this media is suitable for solid-phase extraction of heterocyclic aromatic compounds.

Keywords. Methylene Blue, NanoPak-C, All Carbon Microbeads, Solid Phase Extraction, Visible Spectrophotometer.

1. Introduction.

Methylene blue, a member of the phenothiazines family, is a cationic tricyclic phenothiazine salt. It is a well-established medication and ubiquitous organic printing dye.[1,2] Above specific concentration, it is a known carcinogen and also toxic to aquatic organisms.[2] Thus, its continuous discharge into water bodies is considered a threat to the environment and human health.[2] Over the years, significant efforts have been made to develop effective, low-cost solutions to remove them.

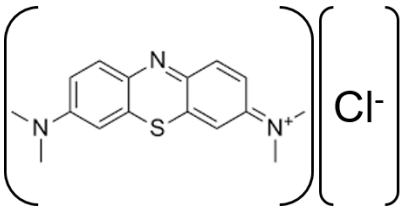


Figure 1: Structure of Methylene Blue.

Solid-phase extraction (SPE) is a widely employed sample preparation technique in chromatography.[3] It is used to purify and concentrate analytes before introducing them into more expensive gas- or liquid- chromatography instrumentation. This process is now gaining recognition as a method for rapid fractionation of phenothiazines.

In this study, we use methylene blue (**Figure 1**), a representative member of the phenothiazine family. We present an optimal method to extract methylene blue employing the NanoPak-C graphite microbead SPE columns. Further, we share a protocol to quantify mL volumes of methylene blue using a visible spectrophotometer.

2. Materials and Methods.

2.1 Chemicals. NanoPak-C graphite microbeads (average diameter = 40 µm, Particle Size Distribution 40% of Average Diameter, Catalog # MT-12-MG-40-RR-11, Millennial Scientific, Stony Brook, NY USA), HPLC grade methanol (>99.9%), ultra-pure water, methylene blue (ACS reagent grade) were used as received.

2.2 Methylene blue solution. Methylene stock solution (100 ppm) was prepared by adding 10 mg of methylene blue in 100ml of ultra-pure water. The working solution of methylene blue (2 ppm) was prepared from the stock solution by serial dilution. Additionally, calibration standards between 0 and 5 ppm of methylene blue were prepared.

2.3 SPE columns and Manifold. NanoPak-C graphite microbeads (bed weight = 250 mg) were manually packed into empty 3 mL straight barrel SPE columns. The microbeads were loaded between a lower and upper frit (average pore size = 20 µm). A 24-port vacuum manifold was used for the extraction process.

2.4 SPE Extraction. Each experiment was performed in triplicates. The SPE columns were first conditioned with 100% of the eluting solvent acetonitrile. Next, an equilibration step was performed by passing 2 mL of ultra-pure water. Fresh collection vials were kept under each SPE cartridge pre-equilibrated with ultra-pure water.

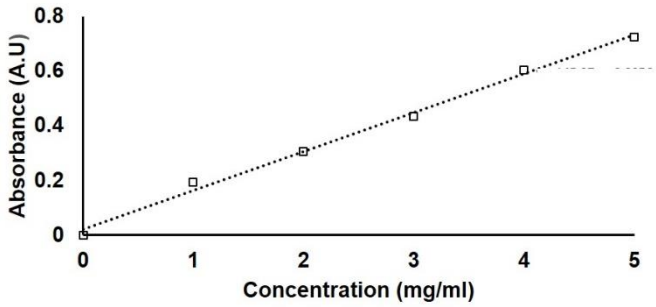
The sample loading step involved passing 2ml of Methylene Blue stock solution (1.5 ppm concentration) (labeled Solution 1) through each cartridge at a 1 mL/min flow rate. The eluted clear liquid (labeled Solution 2) was saved in the collection vial.

The sample recovery step involved passing 2ml of 100% Acetonitrile into the 3ml cartridge at 1 mL/min flow rate. The eluted methylene blue liquid was captured in a new collection vessel (labeled Solution 3).

2.5 Analysis. Vials with solutions 1, 2, and 3 were transferred in separate quartz cuvettes. Their absorbance was measured using a visible spectrophotometer.
The visible spectra of methylene blue solution in 100% ultrapure water were obtained between 350 nm – 700 nm wavelength to identify peak visible absorbance wavelength λ_{max}. Next, 2 mL samples (n=3) of 5 methylene calibration standards at concentrations between 0-10 ppm were prepared. A visible absorbance vs. concentration calibration plot was generated. The linear region of this plot was fit to beer lamberts equation using least-squares regression. Finally, the absorbance at λ_{max} of solution 1 and solution 3 collected during the SPE extraction was measured. Their concentration was determined using the calibration plot. Recovery efficiency (RE) was calculated with the equation RE%= $\frac{C0\ x\ 100}{C1}$ …(1); RE is the recovery efficiency, C0 is the concentration of methylene blue (mg) in solution 3 (100% acetonitrile), C1 is the concentration of methylene blue solution 1 (100% water), respectively. Substituting Beer Lambert's equation A = k x C/V where A is absorbance, k = extinction coefficient, and C is the concentration in mg and V is the volume in ml in equation 1, RE% = $\frac{A0\ x\ V0\ x\ k1\ 100}{A1\ x\ V1\ x\ k0}$.
We noted k0 = k1 from the standard curves. Additionally, V0 = V1. Thus, RE% = $\frac{A0\ x\ 100}{A1}$

3. Results and Discussion. This study's overall objective was: (a) to present an optimal method to extract methylene blue using 3 ml SPE columns loaded with NanoPak-C graphite microbead SPE columns (bed weight – 250 mg). (b) Introduce a protocol to characterize and quantify methylene's extraction efficiency loaded onto NanoPak-C graphite microbead SPE using a visible absorbance spectrophotometer. This setup is suitable for quantifying medium (>1.5 mL) to large volumes of the solution.

Our previous studies[4] showed 2 ppm is the breakthrough concentration of methylene blue loaded onto 250 mg bed weight all carbon microbead media packed into 3 ml SPE columns. Thus, in this study, sample concentrations lower than this upper limit were used to ensure



acceptable recovery. We first characterized the visible spectra of methylene dissolved in ultrapure water. The figure shows visible peak absorbance wavelength λ_{max} values of 668 nm. This value is similar to published λ_{max} values for methylene blue.[5]

Absorbance values at 668 nm were used for all calculations in each sample group. These concentration values were determined through the linear model $y = 0.142x + 0.038, R^2 = 0.9911$, where y = Absorbance and x = methylene blue concentration (ppm) fit to the prepared calibration standards. Similar results were obtained from visible spectra of methylene blue dissolved in acetonitrile.

Table 1. Average Methylene Blue Recovery	
Absorbance A0	0.23
Absorbance A1	0.22
Recovery Efficiency %	95.6%

The loaded (solution 1) and recovered (solution 2) concentrations were of methylene blue were 1.5 ppm and 1.43 ppm, respectively. Additionally, solution 2 did not show any absorbance indicating no loss of methylene after loading. The calculated recovery efficiency of methylene blue was 95.6%.

These results indicate that methylene blue can be retained on all-carbon microbeads. The results suggest this media is suitable for solid-phase extraction of heterocyclic aromatic chemical compound compounds such as phenothiazines. These results lay the foundation for the extraction and quantification of methylene blue from more complex matrices. Our procedure can be employed as an inexpensive method to remove methylene blue as a contaminant or isolate during *in vitro* assays.

4. Conclusions. Methylene dissolved in aqueous media in small microliter volumes could be

recovered efficiently and quantified with NanoPak-C All-Carbon Microbeads-Packed 3 mL SPE Columns. Taken together, the increased sample sizes without affecting recovery efficiency and reusability of these SPE columns could control analytical costs and processing times.

5. References

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