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Simple procedures for analyzing and purifying methylene green

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Abstract

Methylene green is a versatile dye that can be used in a wide range of technical applications, most of which require the dye to be pure. Because commercial lots of methylene green are known to be heterogeneous, we report a thin layer chromatographic method for checking purity. We also describe a simple and effective flash chromatographic purification procedure for subsequent purification. The identity and purity of the dye can be checked easily using UV-visible absorption spectrum measurements or by more sophisticated procedures if necessary.

Key words: dye purity, flash chromatography, methylene green, phenothiazines dyes, thin layer chromatography

Phenothiazines have been used for antimicrobial research for nearly 80 years (Wainwright et al. 2007). The best known dye of this group, methylene blue, was the first synthetic dye to be used as an antiseptic (Wainwright et al. 2002). Recently, there has been a revival of interest in applications of phenothiazine dyes for various biological and technical applications. Perhaps the most widespread uses are as biosensors, in which dyes such as methylene green are used as electron transfer mediators while immobilized on the surface of electrodes (e.g., de Lucca et al. 2002, Svoboda et al. 2007, Upadhyay et al. 2009). Applications of these dyes also include their use as photodynamic therapy agents (e.g., Dai et al. 2009, Tardivo et al. 2005, Wong et al. 2005) and for photosensitization of chemical processes including photo-oxidation and photo-reduction of several substrates, and photo-initiation of polymerization (e.g., Jackusch et al. 1996, Lin and Chang 2007, Shailaga et al. 2007). Methylene blue continues to be applied widely as a stain for microscopy, and methylene green is used also in this way, for example, in histological investigations (Chaurasia et al. 2005) and as a green nuclear counterstain for histochemical staining (McNulty et al. 2004) and immunostaining (Mokrý et al. 2005).

Methylene green, whose chemical structure is shown in Fig. 1, is a cationic dye that is obtained by nitration of methylene blue. Alternative names for methylene green include 7-(dimethylamino)-*N*,*N*-dimethyl-4-nitro-3*H*-phenothiazin-3-iminium chloride (IUPAC terminology) and C.I. 52020/basic green 5 (Colour Index terms). Methylene green shares a core structure with methylene blue, but with a nitro group as a substituent in position 4 on the heterocyclic ring. In the solid state, methylene green has a dark green color; when dissolved in water or organic solvents, the dye displays green to green-blue colors (absorption maximum between 550 and 690 nm).

The impurity of commercial lots of many methylene blue-derived phenothiazine dyes has been acknowledged for many years, for example in the cases of azure B (Roe et al. 1940, Nerenberg and Fischer 1963, Marshall et al. 1975) and methylene green (Bellin and Ronayne 1966). In many cases, the discrepancies in the photo-physical/photochemical properties of dyes reported in the literature may be due to different degrees of purification of the samples studied. For example, Wainwright et al. (2007) reviewed the work of other investigators and reported that these implied that the quantum yield of singlet oxygen formation (Φ_Δ) for methylene green is half the value for methylene blue (0.52;

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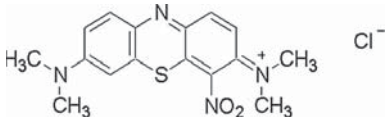

Fig. 1. Chemical structure of methylene green.

Fernandez et al. 1997). Glusko et al. (2011), however, found that the triplet quantum yields (Φ_T) of methylene green were very small (< 0.01) in both protic and non-protic media, and that singlet oxygen quantum yield should be $\leq \Phi_T$. A plausible explanation for this discrepancy is that contamination of the commercial methylene green with other dyes, particularly methylene blue, had occurred. Although Glusko et al. (2011) had purified their dye, the investigators whose work was cited by Wainwright et al. (2007) had not. This is consistent with the remark by Wainwright et al. (2007) that for such studies "chemical and isomeric purity are essential."

We present here details of simple and effective chromatographic methods for analyzing and purifying commercial lots of methylene green. The procedures start with a heterogeneous dye mixture of nominal dye content (65%) and yield chromatographically pure methylene green.

Materials and methods

Methylene green was purchased from Aldrich, Buenos Aires, Argentina as the chloride salt. The vendors stated that the content of colored material was approximately 65%. Methylene blue for use as a chromatographic standard was purchased from Fluka, Buenos Aires, Argentina. This material had a stated dye content of $\geq 95\%$ and was used as purchased. All solvents employed were HPLC grade and used as purchased. Silica gel (230–400 mesh) was provided by Analtech, Buenos Aires, Argentina.

UV/Vis absorption spectra

Spectra were obtained using a Shimadzu UV-2401 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Steady-state fluorescence measurements were made using a Fluoromax Spex Spectrofluorometer (HORIBA Jobin Yvon Inc. Edison, New Jersey). Fluorescence-lifetimes were measured using an Edinburgh Instruments OB 900 time-correlated single-photon counting fluorimeter (Edinburgh Instruments Ltd., Livingston, UK). The excitation was carried out using a diode PicoQuant PLS600 with emission centered at 600 nm (PicoQuant GmbH, Berlin, Germany).

Thin layer chromatography (TLC)

Analysis of a commercial lot of methylene green was carried out in the conventional way (Vogel et al. 1989) using silica gel as the stationary phase. A 0.02% w/v ethanolic solution of the dye was applied to the plates. Chromatographic elution was carried out using various mobile phases. The best chromatographic resolution was achieved by successive elutions with methanol followed by water:glacial acetic acid:concentrated hydrochloric acid (50:40:10, v/v). A series of two-dimensional TLC experiments provided both a simple method for analyzing the commercial lot and a way to establish the solvent system required for purification using flash chromatography. Initially, the dye solution was applied to the left corner of a 5 x 5 cm plate and eluted with methanol. The plate was dried, rotated 90° and eluted with the water:glacial acetic acid:concentrated hydrochloric acid solution.

Flash chromatography

Subsequent purification was carried out using a conventional flash chromatography technique, (Vogel et al. 1989) using an 80 X 4 cm column packed with 500 g of silica gel. Nitrogen was used to pressurize the system. Five milliliters of a 0.2 g/l aqueous solution of commercial dye was applied to the column. The column was eluted initially with approximately 700 ml of methanol at a flow rate of 6 ml/min, which allowed the complete elimination of low polarity impurities (*cf.* the TLC experiments). Subsequent elution with approximately 1 l of the water:glacial acetic acid:concentrated hydrochloric acid solvent at a flow rate of 4 ml/min produced separation of the remaining dyes. The major fractions corresponding to methylene green and methylene blue (see below) were neutralized with sodium carbonate and extracted with dichloromethane. The organic phases were concentrated under reduced pressure and the residual solid dyes were dried overnight at 25° C in a vaccum oven. The dyes were extracted as their acetate salts, as shown by FT-IR spectroscopy (approximately 0.1 mg of dye/100 mg KBr). The presence of the acetate ion was apparent from its characteristic absorptions near 1580 and 1400 cm^{-1}. The efficacy of the purification procedure was verified by repeating the two-dimensional TLC analysis on the final products. Both the methylene green and methylene blue fractions showed single spots.

Results

TLC

The initial elution step produced separation of three highly colored mobile spots; other colored material remained at the bottom of the plate. The second elution separated the initially immobile material into three colored spots. Methylene green ran fastest, followed by methylene blue and finally by an unidentified impurity (see below). Alumina plates also were investigated as a stationary phase, but these produced poor separations.

Flash chromatography

The colored bands eluting from the column were collected and identified (see below). The six fractions separated are shown in Fig. 2. A single flash chromatographic run produced approximately 0.40 mg of pure methylene green from 1.00 mg of commercial methylene green.

Identification of the fractions

Figure 3 shows the spectrum of a commercial sample of methylene blue (purity > 95 %) and that of the fraction collected after our purification procedure; the spectra are nearly indistinguishable. The absorption spectra of the purified and commercial methylene green, however, showed significant differences. The absorption spectra of the purified lots of methylene green and methylene blue were consistent with those reported in the literature (Gurr 1971).

For both dyes, we observed two absorption bands in the visible region of the spectrum. These correspond to allowed singlet-singlet transitions, $S_0 \rightarrow S_1$ at approximately 17000 cm^{-1}, and a less intense blue-shifted $S_0 \rightarrow S_1$ transition near 20500 cm^{-1} (Glusko et al. 2011). The ratio of intensities at the maximum of the absorption bands corresponding to transitions $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_1$ (I_2/I_1) is a simple and effective way to verify the purity of the dye. Although the value of this ratio depends on the solvent, it still is valid as a purity criterion. The value of the ratio I_2/I_1 for methylene green in water at pH 5.8 is 0.86 (Gurr 1971), which agrees exactly with the value obtained for the dye purified according to our procedure described above. The value of I_2/I_1 calculated for the commercial dye in water at pH 5.8, however, is 0.72. As shown in Fig. 3, I_2/I_1 values in dichloromethane are 0.70 ± 0.01 and 0.50 ± 0.01 for the methylene green purified by flash chromatography and commercial methylene green, respectively. These data are clear indicators of contamination of commercial methylene green with methylene blue.

Another procedure for confirming the purity of a dye is to verify that the fluorescence decay can be fitted to a mono-exponential function. In such cases, the fluorescence lifetime (τ_F) of the dye can be calculated. The experimental details and results obtained for methylene green in a wide variety of solvents have been reported by Glusko et al. (2011), e.g., $\tau_F = 0.60 \pm 0.02$ nsec and 0.33 ± 0.02 nsec in dichloromethane and water, respectively. On the other hand, the time-resolved fluorescence of samples from commercial methylene green can be fitted only to multi-exponential decay functions, which is a clear indication of the heterogeneity of the commercial lot.

Fig. 2. Fractions collected during flash chromatography of a commercial lot of methylene green. The extreme left-hand sample corresponds to the purified methylene green; the extreme right-hand sample corresponds to methylene blue. The four center tubes correspond to unidentified impurities.

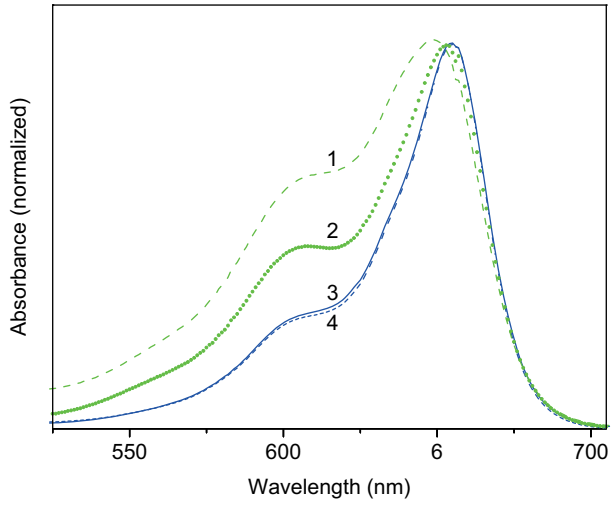

Fig. 3. Absorption spectra in dichloromethane: purified methylene green (1, ---), methylene green, commercial lot (2, …), methylene blue commercial lot (3, —) and methylene blue eluted using our purification procedure (4, ---).

Discussion

Rapid and convenient assessment of the number of colored components present in a commercial lot of methylene green was achieved using two-dimensional TLC on silica gel after eluting initially with methanol followed by an aqueous acetic acid-hydrochloric acid solution. Two major components (one green, one blue) plus four minor components were observed.

Flash chromatography using silica gel and the same eluents as for the TLC method provided an effective procedure for obtaining methylene green free of the five impurities found in the commercial lot. Each flash chromatographic run produced approximately 0.40 mg of chromatographically pure methylene green from 1 mg of commercial dye.

The identities of the two major components separated from the commercial dye lot were established by comparing their absorption spectra with those reported in the literature. These were confirmed in the case of methylene blue by comparison with a pure standard. Further confirmation involved other spectroscopic properties of the separated major fractions. The identities of the four minor components were not investigated; however, manufacture of methylene green involves exposure of methylene blue to an oxidizing nitration mixture (Colour Index (1975) and it is likely that these are demethylated methylene blue homologues, such as azure B.

Commercial methylene green has been shown to contain several colored impurities. Inexpensive, simple and rapid chromatographic procedures permit analysis (TLC) and purification (flash chromatography) that yield methylene green of high purity.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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