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FULL PAPER

Direct Determination of Malachite Green and Leucomalachite Green in Natural Waters by Exploiting Solid-phase Sorption and Digital Image

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Abstract:

Although malachite green (MG), a triphenylmethane dye, is banned for use in aquaculture in several countries, it is still widely employed to treat infections in fish and fish eggs. In living organisms, it is reduced to leucomalachite green (LMG) during physiological processes and can accumulate in adipose tissue. This work describes the development and verification of a simple and portable method, using preconcentration on an adsorbent surface and digital image analysis, for the determination of malachite green and leucomalachite green in natural waters. The optimum conditions of production and extraction in the film were evaluated univariate and the images were analyzed with the aid of Image J. The analytical curves were obtained from each color channel, using multiple linear regression (MLR) models for all parameters of the RGB system. Malachite green was adsorbed on a Florisil surface, followed by quantification using a calibration curve obtained with RGB image parameters, with the preconcentration factor was close to 10. Accuracy was assessed using recovery tests on river natural waters samples, showing no significant matrix effect or additive error. The technique is suitable for environmental monitoring purposes. Simple method, practical and versatile.

Keywords: Adsorption; aquaculture; solid-phase spectrophotometry

1. Introduction

Malachite green (MG) (IUPAC name: 4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethylaniline) is a synthetic cationic triphenylmethane dye that has been used at low concentrations (around 0.10 μg L^{-1}) as a fungicide and ectoparasiticide in aquaculture since around 1930 [1–5]. Malachite green shows monoprotic Brønsted acid-base comportment, with pKa between around 3.70 and 4.80. Leucomalachite green (LMG) also presents Brønsted acid-base comportment, with two aromatic amines whose pKa values are very close, with pK_1 of 4.70 ± 0.40 and pK_2 of 5.50 ± 0.40 [6], Figure S1 (supplementary material).

After being absorbed by fish organisms, malachite green is rapidly reduced to the leucomalachite green form and accumulated in adipose tissue, due to its neutral charge [1–3]. Both compounds are toxic, so their presence can present risks to human health and the environment. They have been associated with the development of liver tumors and other carcinogenic effects in fish. The malachite green acts as a toxic compound also in mammals. According to some clinical and experimental studies, causing toxic effects in multiple organs, among which renal alterations can be mentioned in rabbits; reduction in growth and fertility in rats; besides damage to the liver, spleen, kidney and heart; lesions of the skin, eyes, lungs and bones; and teratogenic and carcinogenic effects. Consequently, the use of MG as an antimicrobial

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has been banned in several countries, although it is still used illegally, due to its low cost and high antifungal effectiveness [1,3,7]. Therefore, in order to enable effective monitoring, sensitive and selective analytical methods are needed for the determination of this chemical in matrices such as water and fish tissues [2,4,5].

In 2002, the European Commission published a Directive 2002/657/CE [8], which deals with the performance of methods and interpretation of analytical results, and defines maximum residue limits (MRLs) and minimum required performance limits (LMDR) applicable to the determination of contaminants in foods. And the Directive 2004/25/CE [9] adds an MRL of 2.00 mg kg^{-1} for the sum of MG and LMG in aquaculture products to the previous Directive.

There are some methods reported in the literature using HPLC/Vis [5,10,11], spectrophotometry [4,12–15] and HPLC/MS [3] for determination of malachite green and leucomalachite green. Extraction techniques were also reported for determination of these compounds, such as extraction using polymethylmethacrylate matrix an analytical device for visual and spectrophotometric determination of malachite green in fish samples using a colour scale, with the limit of detection of 1.0 mg kg^{-1} [16], graphene-based solid-phase extraction coupled with ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) for the rapid determination of malachite green and leucomalachite green in fish tissues, with the limit of detection and quantification of 0.09 and 0.30 µg kg^{-1} respectively, and relative standard deviation of 2.29% - 10.68, the range of 0.25–50.00 µg kg^{-1} [2], using carbon nanotube as sorbents for selective extraction of malachite green from aquatic products that was determined by high performance liquid chromatography (HPLC), wich the limit of detection of 0.70 µg kg^{-1}, relative standard deviations of intra-day and inter-day were obtained in the range of 0.90% and 4.70%, and 3.40% and 9.80%, respectively and satisfied recoveries were in the range of 89.2% to 104.6% [11], and using polymer inclusion membrane, wich average percent extraction achieved for were > 96.00%, for wastewater samples of range 50.00 and 100.00 mg L^{-1} [14].

Although several studies have described the application of new analytical procedures for the detection and determination of malachite green in tissues of fish species, relatively few methods have been developed to monitor MG residues in fish culture water and fish tissue samples. In most cases, the procedures for the analysis utilize liquid chromatography and preconcentration techniques that are employed from fish samples from sequential extraction steps [4,10]. However, for complex matrices, extraction and stability is still a challenge, making pre-treatment necessary in case of residue analysis in food. In this way, the solid-phase extraction (SPE) has been widely used for preconcentration of analytes in several matrices due to its advantages: such as high enrichment factor, fast phase separation, low cost, low consumption of organic solvents and combining ability with different detection techniques [2,3,5].

The use of digital image has become increasingly important because of the ability to perform quick and inexpensive analyses, where images can be obtained from devices such as digital cameras, webcams, scanners, and even smartphones. Color is one of the most important characteristics of the image because it contains the elementary information of an image stored in the pixels. Color reproduction in digital systems can be performed by color systems, such as RGB, CYMK, HSI among others [17–19]. These are analyzed through *softwares* that provide various information not only of each pixel, but of the image as a whole [17,19].This work describes a simple method with a low limit of detection, achieved by preconcentration on an adsorbent surface, enabling the direct determination of malachite green and leucomalachite green in natural waters samples by digital image analysis.

2. Material and Methods

2.1. Reagents and chemicals

All solutions were prepared using analytical grade reagents and deionized water obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Stock solutions of malachite green (CAS-569-64-2) and leucomalachite green (CAS-129-73-7) (both from Sigma-Aldrich) were prepared at concentrations of 1.00 mmol L^{-1}. The citrate buffer solution was prepared using citric acid ($C_6H_8O_7$) (Vetec), with pH adjustment using solutions of

1.00 mol L^{-1} sodium hydroxide (Vetec). The volume was completed with deionized water.

The materials Florisil, silica 60, and XAD-7 resin (all from Merck) were tested for preparation of the adsorbent surface. Neutral transparent silicone adhesive (Tekved) and epoxy (Araldite) were used for adhesion of the adsorbent material on the support. The supports tested were polypropylene and cellulose acetate.

The reagents used in the leucomalachite green oxidation test were 2-hydroxy-3,5-dinitrobenzoic acid (CAS-138659-74-2), hydrogen peroxide and sodium hypochlorite. The potassium iodide (all from Merck), at concentrations of $1.00x10^{-2}$ mol L^{-1}, was used as a catalyst and the studies were performed in the presence and absence of the same.

For evaluation of the selectivity of the method, stock solutions were prepared of the dyes methyl violet (CAS-8004-87-3), bromothymol blue (CAS-76-59-5), and methyl orange (CAS-547-58-0) (all from Merck), at concentrations of 1.00 mmol L^{-1}, in the presence and absence of potassium iodide catalyst.

2.2. Apparatus

Absorption spectra of malachite green and leucomalachite green were acquired in the wavelength range 200-1100 nm, using a diode array spectrophotometer (Model 8453A, Agilent) and a quartz cuvette with an optical path length of 10.0 mm.

The images were produced using a simply closed cardboard chamber (13.0 cm high) fitted with a 15 W fluorescent lamp, Figure S2 (supplementary material). A smartphone Zenfone 2 (Asus); sensor Toshiba T4K37 (1.12µm, 1/3.07"); Numerical Aperture was F 2.0; focal distance 3.8 mm; equipped with camera an resolution 13 megapixels (with an 8-bit resolution for each RGB channel) was positioned over an aperture located at the top of the chamber.

2.3. General procedure

2.3.1. The behavior of malachite green in aqueous solution

The comportment of malachite green at 10.00 µmol L^{-1} in aqueous solution was evaluated in the pH range from 2.00 to 10.00 and times from 0 to 120.00 min. An analytical curve of malachite green was prepared at 618 nm using quartz cuvette with 10 mm optical light path and the pH was adjusted with citrate buffer.

2.3.2. Preparation of the adsorbent film used for preconcentration

For the adsorbent film production, the different substrates, adhesives, and adsorbents were evaluated. It was used cellulose acetate and polypropylene as substrate and acetic silicone and epoxy as adhesives. And florisil, silica 60, and XAD-7 resin as adsorbents.

The adsorbent film was prepared by weighing 0.50 g of the adsorbent and added to a beaker containing 100.00 mL of a solution of 40.00 µmol L^{-1} malachite green under stirring. During the time interval from 0 to 220.00 minutes, aliquots of 5.00 mL were removed, centrifuged (2.00 min), and analyzed in the spectrophotometer in the visible region and returned to the beaker.

The adsorbent film was prepared by applying a uniform layer of the adhesive on a resistant plastic substrate, followed by spreading a layer of the adsorbent on the adhesive and allowing the system to dry for 24.0 h. The excess adsorbent was removed and the film was washed, dried at room temperature, and cut into square pieces with areas of 2.00 cm^2.

2.4. Optimization of adsorption on the film

2.4.1. Influence of time

The effect of the adsorption time was evaluated using measurements of the malachite green present in aqueous solution during the time interval from 0 to 60.00 min. For this, 0.50 g of the adsorbent was weighed out and added to 100.00 mL of a 40.00 µmol L^{-1} solution of malachite green. The percentage adsorption of the malachite green was calculated according to Equation 1:

$$Adsorption\ (\%) = \frac{(C_i - C_f)}{C_i}\ x100 \qquad \text{Eq. (1)}$$

where C_i is the initial concentration of the dye in the solution and C_f is the final or equilibrium

concentration of the dye in the solution.

2.4.2. Influence of pH on the adsorption of the dye

Investigation of the effect of pH on the adsorption of malachite green on the film was performed in the pH range of 3.00 and 10.00. A volume of 100.00 mL of malachite green solution (0.50 µmol L^{-1}) at a specified pH was transferred to a beaker, followed by addition of adsorbent film with an area of 2.00 cm^2. The system was maintained under agitation for 24.0 h, after which the film was removed, dried, and placed in the photographic chamber for image acquisition.

2.4.3. Selection of oxidant

Solutions of leucomalachite green at concentrations in the range from 0.10 to 1.00 µmol L^{-1} were transferred to 100.00 mL beakers, followed by addition of 0.50 mL volumes of the oxidant solutions and leaving under agitation for 6.0 h on a shaker table. Subsequently, the samples were submitted to the adsorption procedure using 0.50 g of the Florisil film, followed by image acquisition.

2.5. Figures of Merit

Characterization and verification of the proposed method employed the following figures of merit: selectivity, analytical range, limits of detection (LoD) and quantification (LoQ), precision (repeatability), and accuracy (using recovery assays).

2.6 Data treatment

All the data were treated using electronic spreadsheets (Microsoft Excel 2003-2010) and OriginPro 8.0 *software* (OriginLab). The images were analyzed using the open-source ImageJ *software* developed by Wayne Rasband of the U. S. National Institute of Health (NIH), on a Java platform [20]. The images obtained with the smartphone camera were loaded into the program, the total area of the film was selected, and the command “Analyze – Histogram” was applied to obtain the average values for the RGB (red, green, and blue) and L (grey) channels. The value for the L channel was obtained using Equation 2.

$$L = \sqrt{R^2 + G^2 + B^2} \qquad \text{Eq. (2)}$$

2.7. Procedure at the optimized conditions

A 2.00 cm^2 film was produced with the surface coated with 0.50 g of the forisil adsorbent. This was prepared using a propylenepropylene support, in which an acetic silicone adhesive was uniformly deposited throughout the surface. A layer of the adsorbent was then spread thereon, allowing to dry for 24.0 h. Thereafter, the film was washed, dried at room temperature and trimmed. An adsorbent film of 2.00 cm^2, maintained under shaking for 24.0 h, was added to 100.00 mL of the pH-adjusted sample at 7.00. After this period the films were removed, dried and taken to the photographic booth where the images were produced using the total area of the film. In the oxidation step of the leucomalachite green, dinitrosalicylic acid was used as the oxidant.

The preconcentration factor was close to 10, estimated indirectly from the analytical bands of the MG in solution (1.00 to 10.00 µmol L^{-1}) and after adsorption (0.10 to 1.00 µmol L^{-1}).

Plates contaminated with MG were maintained in 2.00% (v/v) sodium hypochlorite solution for 24 h to ensure dye degradation. Subsequently, the plates were washed and discarded as chemical residue.

2.8. Determination of malachite green in natural waters samples

Natural waters samples were collected from two lakes on the campus of the Federal University of Viçosa, in the city of Viçosa (Minas Gerais State, Brazil). The samples were filtered through 0.45 µm filters and stored at 4 ºC, prior to analysis using the optimized method for the determination of malachite green in natural waters.

3. Results and Discussion

3.1. Determination of malachite green and leucomalachite green in aqueous solution

Selection of the wavelength for construction of the analytical curve using UV-Visible spectrophotometry was achieved using spectra obtained for malachite green and leucomalachite

green at concentrations of 20.00 and 40.00 µmol L^{-1}, respectively. The wavelengths of maximum absorbance in the UV-visible region were 618 and 256 nm for malachite green and leucomalachite green, respectively (Figures S3, Supplementary Material). These wavelengths were used to construct the analytical curves for the UV/Vis spectrophotometric method. The regressions for the MG and LMG analytical curves in maximum wavelength are shown in Equations 3 and 4, respectively. The quality of fit was evaluated by R^2 (0.999 (MG) and 0.997 (LMG)), and by residual standard deviation (s_{res}) with values of 0.035 and 0.062, respectively.

$$\hat{A} = (5.89 \pm 0.58).10^{-2}\, c/(\mu mol.L^{-1}) - (0.05 \pm 0.01)$$

Eq. (3)

$$\hat{A} = (2.69 \pm 0.05).10^{-2}\, c/(\mu mol.L^{-1}) - (0.09 \pm 0.03)$$

Eq. (4)

where *c* corresponds to the concentrations of analyte and Â, the estimate absorbance in maximum wavelength of analyte. The quality of fit is adequate to analyte with high molar absorptivity (ε).

The limits of detection (LoD) and quantification (LoQ) were 0.41 and 1.37 µmol L^{-1} for MG, and 1.92 and 6.41 µmol L^{-1} for LMG, calculated as described by Deming and Morgan [21]. The analytical ranges were 1.37 to 50.00 µmol L^{-1} (MG) and 6.41 to 100.00 µmol L^{-1} (LMG). These value of limits of quantification were not enough for environmental analysis. Besides, the use of ultraviolet region lower than 300 nm is complicated due interference of several substances present in waters.

The spectra of malachite green after 60.0 min solutions at different pH values was shown in Figure 1A. The kinetics behavior of MG was evaluated at 618 nm (Figure 1B).

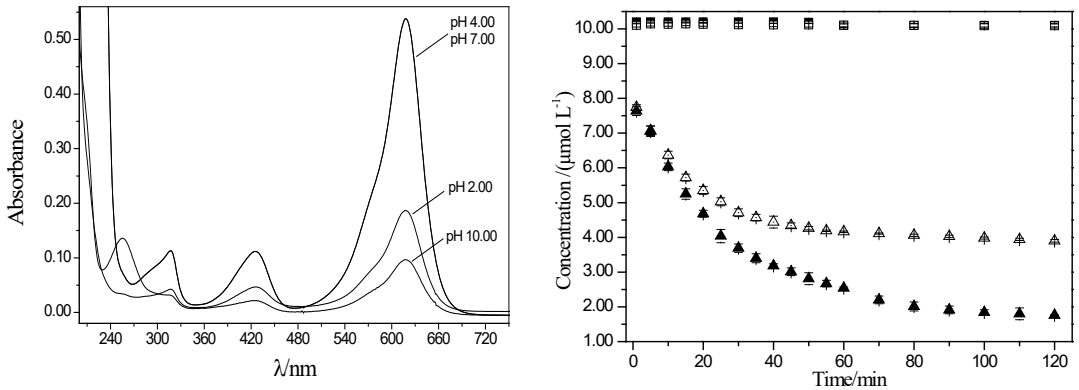

Figure 1. Effect of pH on the absorbance of malachite green in solution (10.00 µmol L^{-1}). (A) Spectra obtained after 60.0 min at different pH values. (B) Absorbance at 618 nm in function of time at different pH values:(Δ) 2.00, (□) 4.00 (■) 7.00, and (▲) 10.00.

The spectra obtained at pH 4.00 and 7.00 were very similar (Figure 1A). At pH 4.00 and 7.00, there were no significant changes in the MG concentration over time (Figure 1B).

The intensities of the characteristic bands of MG varied according to pH, without any wavelength shifts. The lower values at pH 10.00 could be attributed to changes in the MG structure, with the formation of carbinol by nucleophilic attack of hydroxyl on the central carbon atom [22].

At pH 2.00, there are the formation of a yellow-colored protonated species associated with the appearance of a new band at 250 nm. This band has been associated to MGH^{2+} [23], but Bronsted acid-base equilibrium are reached very fast and the observed low kinetics suggests the existence of other process such as the dimerization of system (charge transference complex). This kind of coupling is usual in plane cationic species, as example methylene blue [23]. This result suggests the proposed specie MGH^{2+} could not be formed in aqueous solution.

3.2. Optimization of film preparation

Cellulose acetate and polypropylene were evaluated as supports for preparation of the adsorbent films. The bluish color of the cellulose acetate hindered in the images obtained, so polypropylene was selected as the support for the films (Figure S4, Supplementary Material).

Acetic silicone and epoxy adhesives were tested for attachment of the adsorbent material. Use of the silicone adhesive resulted in a film with a uniform surface, while the use of the epoxy adhesive led to films with irregular areas. The silicone adhesive was therefore selected for the preparation of the films (Figure S5, Supplementary Material).

Florisil, silica 60, and XAD-7 resin were evaluated for use as the adsorbent. The results obtained for the absorption rates of malachite green, at a concentration of 40.00 μmol L^{-1} and pH 6.00, are shown in Figure 2.

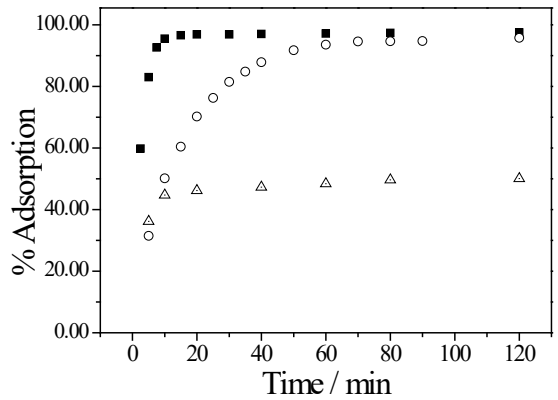

Figure 2. Absorption rates of malachite green on the adsorbent surfaces: silica 60 (Δ), XAD-7 resin (○), and Florisil (■). The adsorbent dosage of 5.00 g L^{-1}.

The MG adsorbed rapidly onto the silica 60, reaching equilibrium within 10 min (Figure 2). Even with the high kinetic adsorption, silica 60 presented the lowest adsorption of MG (maximum of 50.05%) in comparison with other two materials. It is due to saturation of the available sites. These values were low in relation to the values obtained by the other adsorbents studied. The XAD-7 resin presented slower adsorption rates, compared to the silica, but with maximum adsorption of around 95.75%. The slow kinetics could be explained by the need for diffusion of MG into the macropores of the resin. Despite the high adsorption rates, the adsorbent was not chosen due to slow kinetics. The Florisil showed fast adsorption rates, reaching equilibrium at around 97.52% adsorption after 20.0 min. Therefore, this material was selected as the adsorbent, due cited characteristics as well as the formation of a film with satisfactory homogeneity, due to the small particle size of this material.

3.3. Optimization of malachite green preconcentration

The adsorption process was followed using measurements of malachite green present in the solution. The Figure 3, the absorption rates on the Florisil film supported on polypropylene, using 100.00 mL of 40.00 μmol L^{-1} malachite green is shown. In this condition, the adsorption equilibrium was reached in approximately 10.0 min. However, to ensure complete adsorption of malachite green in the adsorbent films, the adsorption studies were performed in a longer time than 10 min.

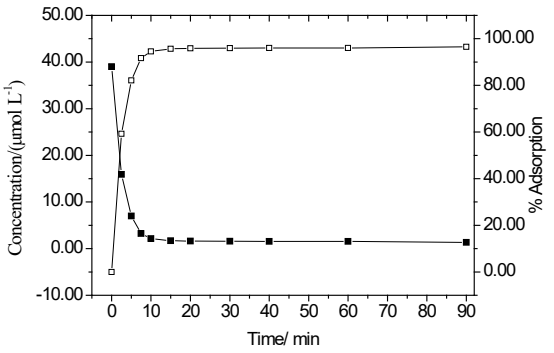

Figure 3. Adsorption of malachite green on the Florisil film supported on polypropylene. Florisil dosage: 5.00 g L^{-1}. (■) The concentration of malachite green; (□) relative adsorption (%).

3.4. Digital image analysis

The influence of pH on the adsorption of MG onto the Florisil film was evaluated at pH in the range 3.00-10.00, with the construction of analytical curves for MG at concentrations between 0.10 and 1.00 μmol L^{-1}. The results (Figure 4) showed that maximum adsorption occurred at pH 7.00, but satisfactory preconcentration of MG on the adsorbent was observed at pH 5.00-7.00, so this pH range was therefore selected in the subsequent experiments.

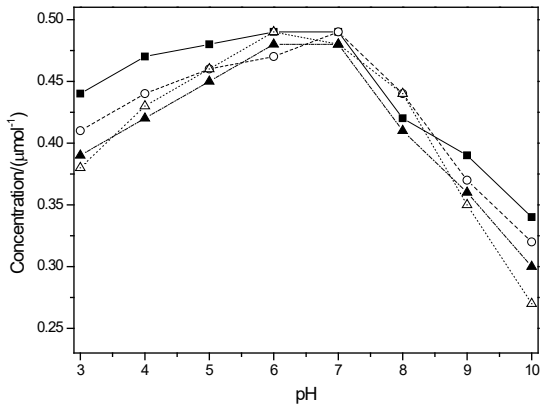

Figure 4. Effect of pH on the adsorption of MG onto the Florisil film. Color channels: R (■), G (○), B (Δ), and L (▲).

3.5. Analytical curves and linearity of the response

The results of adsorption tests with the films under optimized conditions were used to construct analytical curves for MG at concentrations from 0.10 to 1.50 μmol L^{-1}, at pH 7.00, obtaining the RGB and L values for each MG concentration. The linearity of the response was determined by linear regression applied to the analytical curves (Figure S6, Supplementary Material). The analytical curve parameters for the RGB and L channels are presented in Table 1.

Table 1. Analytical curve parameters obtained for MG measurements using channels RGB and L.

Channel	Regression model	R^2	Analytical range (μmol L^{-1})	S_{Res}*/bit
R	R/bit = -(92.88±1.48)c/(μmol.L^{-1}) + (141.40±1.01)	0.996	0.25-1.00	1.80
G	G/bit = -(31.07±0.52)c/(μmol.L^{-1}) + (76.17±0.47)	0.991	0.10-1.50	1.50
B	B/bit = -(22.72±0.90)c/(μmol.L^{-1}) + (179.02±1.42)	0.965	0.10-1.50	2.50
L	L/bit = -(52.26±0.85)c/(μmol.L^{-1}) + (151.92±0.75)	0.993	0.10-1.50	2.40

*S_{Res}/slope

The analytical curves for MG were linear, with R^2 values higher than 0.99 for channels R, G, and L. The analytical curve obtained using channel B presented a lower fit, with R^2 of 0.97. The highest sensitivity was shown by the analytical curve obtained using the R channel (around 44.00% higher than for the other channels).

It should be remembered that each detector (pixel) is formed by three wide band filters and the spectrum of malachite green show large bands in same region. This explains the good sensitivity not only in R channel but also observed for the other channels, although about half the sensitivity of the R channel. These channels can be used to validate the quality of the result, since the obtained concentration should be similar calculating from each channel. This will avoid false positives, that is, it allows to identify the presence of interferents.

The coefficient of determination (R^2) is a relative parameter, making it difficult to determine the effect of small variations on the quality of a regression model, especially in the case of high values (above 0.90). The residual standard deviation is a better parameter for this purpose, because the response has a dimension (in this case, bits). Evaluation of the residual standard deviations considered that the smallest measurement variation was 1.00 bit, with a total range of 256.00 bits. The residual standard deviations obtained for channels RGB and L were in the range 1.50-2.50 bits, indicating the high quality of the model.

The use of multiple linear regression (MLR) model was also evaluated. The model described by Equation 5 was obtained after removal of the terms (effects) corresponding to channels G and B, which were not significant [24].

$$c\ /(\mu mol\ L^{-1}) = -(7.22 \pm 0.42) \times 10^{-2}\ (R) + (3.64 \pm 0.35) \times 10^{-2}\ (L) + (0.91 \pm 0.07) \quad \text{Eq. (5)}$$

The estimated residual standard deviation (S_{res}) was 0.02 μmol L^{-1}, whereas the determination coefficient (R^2) was 0.998, which was five times smaller than the lower limits of the analytical ranges employed (0.10 μmol L^{-1}), with a *p-value* lower than 0.001 indicating a good fit of the model. The standard deviation of residues (s_{res}), is a better statistical parameter to resume the comportment observed, is the absolute value, with dimension of Y-results and so, s_{res} can be compared with full range of Y-results or estimative

of the standard deviation of replicates.

3.6. Determination of leucomalachite green

The determination of leucomalachite green (colorless) was by oxidation to malachite green, enabling its determination by the proposed method. In this case, the sum of concentration of each chemical species. This strategy has been used in other studies employing 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) [25], salicyclic acid, and hydroxybenzoic acids [26].

Only 2-hydroxy-3,5-dinitrobenzoic acid was able to oxidize, in the absence of a catalyst, leucomalachite green to malachite green. The oxidation yield was of around 98.00% achieved using the oxidant at a concentration of 50.00 µmol L^{-1}. This concentration was therefore used in the subsequent studies of the oxidation of LMG, which were performed with agitation on a shaker table for 6.0 h. The concentration of LMG was obtained as the difference between the concentrations of MG found in the procedures performed with and without the oxidation step.

The regression models fitted to the LMG analytical curves constructed using the different channels of the image analysis method are shown in Table 2.

Table 2. Parameters of the analytical curves for LMG, after the oxidation step, obtained using channels RGB and L.

Channel	Regression model	R^2	Analytical range (µmol L^{-1})	S_{Res}*/bit
R	R/bit = -(105.18±1.29)c/(µmol.L^{-1}) + (137.82±0.88)	0.999	0.25-1.00	1.02
G	G/bit = -(46.75±1.23)c/(µmol.L^{-1}) + (178.29±0.75)	0.995	0.10-1.00	1.27
B	B/bit = -(54.77±1.45)c/(µmol.L^{-1}) + (1786.88±0.89)	0.994	0.10-1.00	1.49
L	L/bit = -(67.50±1.26)c/(µmol.L^{-1}) + (165.92±0.76)	0.997	0.10-1.00	1.31

*S_{Res}/slope

Equation 6 shows the expression obtained for the MLR model, after removal of the effect of channel G.

$$c\ /(\mu mol\ L^{-1}) = -(2.00 \pm 0.40) \times 10^{-2}(B) + (8.60 \pm 3.10) \times 10^{-3}(R) - (0.87 \pm 0.43) \times 10^{-3}(L) + (2.34 \pm 0.36) \quad \text{Eq. (6)}$$

The residual standard deviation for the model was 1.40×10^{-2} µmol L^{-1}, which was three times smaller than the lower limit of the analytical ranges (0.10 µmol L^{-1}), while the R^2 value was 0.999.

The leucomalachite green oxidation step showed satisfactory results, with recoveries in the range 98.00 to 103.00% (Table S1, Supplementary Material).

3.7. Figures of merit

3.7.1. Selectivity

The selectivity of the method was evaluated by comparing the RGB and L values in the presence of different dyes at the same concentrations as those used for MG (0.25 and 0.75 µmol L^{-1}). The dyes tested were methyl violet, bromothymol blue, and methyl orange (Table 3).

The regressions for the different channels in the presence of the dyes showed that only methyl violet caused increases in the percentage recovery values for all the channels. This could be explained by the fact that methyl violet is a member of the triphenylmethane family of compounds and presents characteristics similar to those of malachite green, as shown by its adsorption onto the film and the similarity between the spectra for the two compounds (Table 3).

The figures of merits obtained for the proposed method were compared with other existing methods using other preconcentration techniques and analyzed water samples by UV/ Vis spectrophotometry, Table 4.

3.7.2. Limits of detection (LoD) and quantification (LoQ) of the method

The limits of detection and quantification were calculated from the parameters of the analytical curves for channels RGB and L, as well as for MLR (Table 5).

Table 3. Recovery percentages (% R) and coefficients of variation (CV, %) for channels RGB and L, and for MLR, in the tests with the dyes methyl violet, bromothymol blue, and methyl orange.

Channel	Methyl violet				Bromothymol blue				Methyl orange			
	0.25 μmol L^{-1}		0.75 μmol L^{-1}		0.25 μmol L^{-1}		0.75 μmol L^{-1}		0.25 μmol L^{-1}		0.75 μmol L^{-1}	
	% R	CV (%)	% R	CV (%)	% R	CV (%)	% R	CV (%)	% R	CV (%)	% R	CV (%)
R	144.41	2.0	128.21	1.10	89.78	3.14	98.62	0.36	93.11	4.42	99.96	0.54
G	280.08	1.52	224.76	5.29	97.40	15.42	101.13	6.73	96.44	6.42	97.38	4.92
B	221.61	16.67	141.30	1.33	92.68	9.91	101.36	7.46	99.50	9.87	94.11	2.86
L	187.30	14.17	162.95	1.24	99.29	8.84	95.77	4.20	101.06	14.66	97.12	0.32
MLR	211.71	9.23	164.56	2.52	95.34	4.27	98.97	5.04	98.25	0.75	96.55	2.17

Table 4. Comparison of figures of merits obtained for the proposed method with other existing methods using other preconcentration techniques.

Methods	Analyzed sample	Preconcentration	LoD/ (μmol L^{-1})	LoQ/ (μmol L^{-1})	Analytical range/ (μmol L^{-1})	%RSD	Concentration factor	Reference
Digital imaging	Natural waters	Florisil film	(2.30-4.90)x10^{-2}	(6.90-14.80) x10^{-2}	0.1-1.0	1.79-7.08	10.00	This study
UV–Vis spectrophotometer	Water and fish tissues	Fe_3O_4 nanoparticles	1.37 x10^{-1}	-	0.274 – 21.9	1.66	-	[4]
UV–Vis spectrophotometer	Water samples	Maghemite nanoparticles	7.67x10^{-4}	-	13.70x10^{-4}-6.85x10^{-1}	0.86-1.60	50.00	[12]
UV–Vis spectrophotometer	Water samples	pH-sensitive hydrogel	0.40 x10^{-2}	-	0.01-0.5	3.03	20.00	[13]
UV–Vis spectrophotometer	Water samples	Magnetic poly(acrylonitrile-***co***-acrylic acid) nanofibers	8.20 x10^{-2}	0.30	0.80-4.90	< 7.68	50.00	[15]
UV–Vis spectrophotometer	Fish farming water samples	Cloud point extraction	0.30 x10^{-2}	-	0.01-1.37	1.13	-	[27]
UV–Vis spectrophotometer	Aqueous samples	Micro-cloud Point extraction	1.12x10^{-3}	37.3x10^{-3}	0.16–1.64	8.39	29.30	[28]
UV–Vis spectrophotometer	Water samples	DLLME*	0.01	0.04	1.00-40.00	3.30-4.50	77.50	[29]

*Dispersive Liquid–Liquid Microextraction

Table 5. Limits of detection (LoD) and quantification (LoQ) determined from the analytical curves for channels RGB and L, as well as for MLR.

Channel	LoD (μmol L^{-1})	LoQ (μmol L^{-1})
R	0.03	0.09
G	0.05	0.14
B	0.05	0.15
L	0.03	0.10
MLR	0.02	0.07

It could be concluded from the LoD and LoQ values that only channels R and L provided satisfactory quantification limits for the working range considered (0.10 to 1.00 μmol L^{-1}), while the use of MLR resulted in small improvements of the parameters. However, the LoD and LoQ values obtained in the method were 10 times lower than the direct spectrophotometric analysis.

The values obtained for the R channel, although having the highest sensibility was close to the other channels, due to the LoD and LoQ values take into account the analytical sensibility and standard deviation of the residues.

Comparing the values of LoD and LoQ obtained in the proposed method, with values obtained in other work, Table 4 shows that very high values were obtained compared to other works using preconcentration techniques, Cloud point extraction [27], Micro-cloud Point extraction [28], Maghemite nanoparticles [12], pH-sensitive hydrogel [13] and DLLME [29]. These procedures for the analysis of these compounds use requires extraction steps using strategies more complex, require a greater number of steps, experience to analyse, for example preconcentration using DLLME [29] required blend, centrifugation when compared with the method proposed, the support is agitated for 24.0 h. Simple method, practical and versatile compared to these.

The values of the limits of detection obtained in the proposed method were lower than those obtained by preconcentration with Fe_3O_4 nanoparticles [4] and Magnetic poly (acrylonitrile-co-acrylic acid) nanofibers [15], although the preconcentration factor obtained in this study was lower in relation to the use of preconcentration with other materials (Table 4), highlighting that the method can be used to determine MG in water samples.

3.7.3. Precision and intermediate precision

The coefficients of variation (CV) obtained for the samples ranged from 1.79 to 7.08%, demonstrating high repeatability. MLR presented a standard deviation higher than that of the R channel, but lower than obtained for the other channels (Table S1, Supplementary Material).

The intermediate precision was determined considering the coefficients of variation (CV) for analyses performed on three different days (Table S2, Supplementary Material). The CV values obtained for the determination of malachite green in water samples were lower than 6.93%, indicating that the method provided good intermediate precision.

The values obtained for precision in the proposed method were comparable to those obtained in other studies (Table 4) the studies presented %RSD < 20.0%, as recommended [30], indicating that there is the agreement between the results of successive measurements by the same method under the same conditions of measurement.

3.8. Accuracy and determination of malachite green in environmental natural waters samples

The recovery values obtained for a spike in rivers natural waters sample ranged between 102.48 and 123.93 for channels, with coefficients of variation <15.11% (Table 6), as recommended [30]. These values were comparable to those obtained in other studies analyzing MG in water samples using pre-concentration techniques, pH-sensitive hydrogel [13] with recovery between 94.00 and 98.00, Fe_3O_4 nanoparticles [4] with recovery between 90.05 and 107.80, Magnetic poly (acrylonitrile-co-acrylic acid) nanofibers [15] with recovery of 95.00 and 103.00%, Micro-cloud Point extraction [28] 80.00 and 103.33 and DLLME [29] 77.50 and 100.70%.

The regression of experimental concentration and spike concentration showed no effect matrix (slope is not significative different than one) neither additive error (the constant term is not significative different than zero) indicating that the method provided satisfactory analytical accuracy.

Table 6. Evaluation of the accuracy of the method (percentage recoveries and coefficients of variation).

Channel	Concentration (μmol L^{-1})									
	0.10		0.25		0.50		0.75		1.00	
	% R	CV (%)	% R	CV (%)	% R	CV (%)	% R	CV (%)	% R	CV (%)
R	106.55	15.11%	104.50	3.03%	104.29	1.55%	102.48	0.34%	102.71	1.03%
G	116.98	6.71%	117.92	1.83%	121.52	1.24%	119.73	0.84%	118.01	0.14%
B	117.89	7.03%	116.31	2.60%	123.93	3.06%	115.20	0.65%	119.34	0.32%
L	117.27	8.63%	118.66	0.80%	121.76	0.77%	117.94	0.91%	111.41	0.74%
MLR	116.18	2.76%	115.42	1.49%	119.25	0.90%	114.70	0.64%	113.60	0.20%

The recovery of the fortified samples was satisfactory, confirmed by the standard addition method, indicating agreement between the quantities of analyte added and measured, confirming through the precision of the method. Thus, the method is suitable for preconcentration and determination of MG in conventional samples.

4. Conclusions

A novel simple method, practical and versatile was developed, optimized, and validated for the determination of malachite green and leucomalachite green in natural waters, employing digital image analysis of the analyte collected on an adsorbent surface. Adsorption onto a Florisil film provided satisfactory preconcentration, while the use of digital image greatly simplified the method and ensured its portability.

The figures of merit of the developed method confirmed its effectiveness for the preconcentration and determination of malachite green, as well as its reduced form, in samples of natural waters. The method provides a low limit of detection and is suitable for monitoring purposes, wich the preconcentration factor was close to 10. Malachite green was adsorbed on a Florisil surface, followed by quantification using a calibration curve obtained with RGB image parameters, wich the preconcentration factor was close to 10.

Supporting Information

Supporting Information is obtained in supplementary material.

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