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P-chromophore stability of Mehlich-1 and Mehlich-3 under Braga & Defelipo or Murphy & Riley dosing methods

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ABSTRACT: The performance of colorimetric methods for P quantification has been overlooked by researchers for decades. We investigated the performance of two blue colorimetric methods for P quantification, Braga and Defelipo (B&D) and Murphy and Riley (M&R), using two commonly soil P extractants, Mehlich-1 (M1) and Mehlich-3 (M3). Specifically, we evaluated the color development time and its stability in different soil extractant solution proportions (1:1 or 1:4 v/v), the optimum wavelength, limits of detection, and element quantification. Our results indicate that M3 leads to lower limits of detection and quantification for both colorimetric methods, particularly for B&D. For M1, the volumetric ratio (1:1 or 1:4) did not influence color development and both B&D and M&R methods showed a fast color development. However, B&D showed greater color stability (from 5 to 600 min) and an optimum wavelength of 711 nm, while M&R was stable from 27 to 600 min an optimum wavelength of 889 nm. For M3 soil extractant, there are important issues, such as M&R being unstable and B&D presenting slow color development. In addition, the spectral profile obtained from soil extractant was different from the one obtained by the calibration curve without soil for both M&R and B&D. Therefore, the adoption of original methods on P quantification in M3 extracting solution represents a potential source of error, leading to wrong P fertilizer recommendations. Thus, the best option for P quantification in M3 extracts seems to be the use of Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES).

Keywords: accuracy, available-P, colorimetric approach, soil testing.

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INTRODUCTION

Phosphorus (P) is an essential element for plants and is required in high amounts by crops. Therefore, suitable content of available-P in soil is crucial to achieve high crop yields. Predictive P-soil tests are used to measure available P in the soils. In these methods, P is extracted under different extracting conditions in terms of acidity, buffer capacity, ionic force, and complexing compounds, including, as most common P extractors, Mehlich-1 (M1), Mehlich-3 (M3), Bray-1, Olsen, Anionic resin and mixed resin (Silva and van Raij, 1999; van Raij et al., 2001).

In the tropical and usually acidic soils of Brazil, mixed resin (van Raij et al., 1986) and M1 are the two official P-extractants (Mehlich, 1953). However, M3 has been proposed as a universal soil extractant (Valadares et al., 2017) that would work similarly in alkaline and acidic soils (Mehlich, 1984), which would allow it to be used everywhere in the world, simplifying P evaluation and fertilization.

Colorimetric methods are the most common way of quantifying P in extractant solutions (Bell and Doisy, 1920; Briggs, 1924; Murphy and Riley, 1962; Braga and Defelipo, 1974) since they are relatively inexpensive and have good sensitivity, especially for the orthophosphate species. There are several P colorimetric methods based on the phosphomolybdenum (PMB) blue reaction (Nagul et al., 2015), such as phosphate determination in water and detergents (Baird et al., 2017; Ismail, 2023) and phosphorous determination in soil extracts (Adesanwo et al., 2013; Angelova et al., 2022) and its quantification by spectrophotometric technique (UV-Vis). A good colorimetric method should show high sensitivity to orthophosphate concentration, a wide range of linear calibration, minimum blank absorbance, fast PMB formation, and temporal stability. However, PMB reaction depends on many factors, including the pH of the medium and ionic interaction (interfering ions) (Nagul et al., 2015). Moreover, P soil extracting solutions have different acidity, ionic force, and complexing agents, among others (Miller and Arai, 2016), which may affect the medium conditions and PMB reaction (Cai et al., 1994; Kowalenko and Babuin, 2007). Therefore, it is essential to understand the effects of P-extracting solutions on the formation and absorption spectrum of PMB species.

Murphy and Riley (1962) colorimetric method (M&R) is well-known by the international scientific community. This method was originally developed for P measurements in seawater, but it has also been widely used in soil test-extracting solutions (Miller and Arai, 2016; Wuenscher et al., 2016). However, issues of using M&R for P quantification in M3 solutions had already been shown (Mumbach et al., 2018). On the other hand, in Brazil, most routine labs for soil and plant materials use Bi-based PMB proposed by Braga and Defelipo (1974) (B&D) for P quantification, which was developed for plant and soil analysis using different soil extracting solutions, including Bray-1 and M1. One of the advantages of this method is the use of Bi, which accelerates the reduction rate of phosphomolybdic complex. When in an acidic medium, the formation of phosphomolybdenum complex, followed by its reduction, gives rise to the blue molybdenum complex, which presents at 725 nm, the maximum absorbance (Braga and Defelipo, 1974). However, despite being widely used in routine laboratories in Brazil, the B&D method is still internationally unknown. There are no reports about the performance of B&D for M3, therefore if it performs well with M3, it will be an alternative to improve P colorimetric quantification by M3 worldwide, allowing scientists and commercial labs to still rely on simple and cheap colorimetric methods rather than ICP for P quantification.

Our goal with this study was to evaluate the performance of Braga & Defelipo with Murphy & Riley as colorimetric methods for P quantification in extracts obtained from M1 and M3 extractors. Specifically, we aimed to evaluate: (i) the time for color development and its stability in different proportions of M1 and M3; (ii) optimum wavelength for P measurement content in different extractors and dilutions; (iii) limits of detection and element quantification.

MATERIALS AND METHODS

Soil test extracting solutions

Mehlich-1 (M1) and Mehlich-3 (M3) extractants were initially prepared with the double of their original concentrations aiming to build the calibration curve: HCl 0.10 mol L^{-1} and H_2SO_4 0.025 mol L^{-1} for M1 and CH_3COOH 0.40 mol L^{-1}, NH_4NO_3 0.50 mol L^{-1}, NH_4F 0.03 mol L^{-1}, HNO_3 0.026 mol L^{-1} and EDTA 2 mmol L^{-1} for M3 (Mehlich, 1953, 1984). Then, these solutions were diluted 2-fold to perform the P extraction from the soil.

Colorimetric methods

M&R solution was prepared as described in the original method (Murphy and Riley, 1962). For stock solution 1, we added 0.70 mL of H_2SO_4 in 200 mL of deionized water, and the final volume was brought to 500 mL. Stock solution 2 was prepared by dissolving 20 g of ammonium molybdate in 500 mL of deionized water. For stock solution 3, 1.32 g of ascorbic acid was dissolved in 75 mL of deionized water. Stock solution 4 was prepared by dissolving 0.2743 g of potassium antimony tartrate in 100 mL of deionized water. M&R reagent (100 mL) was obtained from the mixture of 40, 12, 24, 4 and 20 mL of the solutions 1, 2, 3, 4 and deionized water, respectively. The final concentrations of all reagents are described in table 1. Lastly, the M&R method recommends that absorbance should be taken at the wavelength of 882 nm and after not less than 10 min of M&R reagent addition.

For preparing the B&D colorimetric reagent, firstly we made the “725 stock solution” (Braga and Defelipo, 1974). Bismuth sub carbonate (1.00 g) was added to 500 mL of distillated water, and then 138.8 mL of concentrated H_2SO_4 plus 20 g of ammonium molybdate were added. After the solution cooled down close to room temperature, the volume was completed to 1 L (“725 solution”). For B&D colorimetric reagent, 0.4 g of ascorbic acid was added to 50 mL of “725 solution” and the final volume brought to 100 mL with water. The final concentration of the B&D solution is also described in table 1. In addition, this method recommends that absorbance should be taken at a wavelength of 725 nm and after not less than 5 min of B&D reagent addition. The shelf life is short for both colorimetric reagents (B&D and M&R) (~ 24 h), requiring daily preparation in the lab.

Formation and stability of PMB species

Phosphorus was added to both M1 and M3 solutions to reach a concentration of 1 mg L^{-1}. M&R and B&D colorimetric solutions were added, and the spectra of the PMB species generated were scanned in the range of 400-1100 nm and collected every 5 min for 600 min using a Uv/Vis Spectrometer (PerkinElmer lambda 25) (glass cuvette with path length of 1 cm). The first spectrum was taken at the time the colorimetric reagent was added. Furthermore, spectra were collected in solutions containing volume ratios of 1:1 or 1:4 (colorimetric reagent: extract) to verify possible spectral changes due to the extract dilution. Usually, these volumetric ratios are adopted in P-soil quantification, especially the 1:4 volume ratio in P-poor tropical soils. Lastly, this procedure simulates a spectrum obtained from a specific point on the calibration curve (1 mg L^{-1} P), which is useful to evaluate the performance of the methods related purely to P concentration in the extractants without the influence of the soil matrix. However, we also evaluated a real soil extract to understand if the influence of soil matrix is important for soil P extractions. We used an *Argilosso Vermelho-Amarelo* (Ultisol) (Santos et al., 2018) with pH(H_2O) 6.02 (1:2.5 v/v), 460 g kg^{-1} of clay content, and 2.4 % of organic matter.

Table 1. Reagent concentrations used in both M&R and B&D phosphomolybdenum (PMB) methods

Method	[H_2SO_4]	[Mo (VI)]	[Ascorbic acid]	[Catalyst]
	mmol L^{-1}			
Murphy and Riley (1962)	200	5.40	4.8	[a]0.066
Braga and Defelipo (1974)	250	11.33	4.5	[b]0.390

[a]Sb (III); [b]Bi (III).

Linear range and sensitivity of the methods

To investigate the linear range (Beer Lambert law) of the methods (B&D and M&R) in M1 and M3 extracts, a calibration curve (triplicate) was obtained at concentrations of 0.00, 0.25, 0.50, 1.00, 1.50, 2.00, 2.50, 5.00 and 10.00 mg L^{-1} P. Calibration curve was obtained only at 1:4 ratio (colorimetric reagent: extract), the commonly used ratio for tropical soils, and absorbance was measured after a reaction time of 30 min. The linear range and sensitivity of colorimetric methods are well known (Miller and Arai, 2016; Nagul et al., 2015), but not in 1:4 ratio (colorimetric reagent: extract) solutions. From the calibration curve, the limit of detection (LOD) and limit of quantification (LOQ) in the different extractants (M1 or M3) were calculated by equations 1 and 2 (Miller and Arai, 2016).

$$\mathrm{LOD} = 3.3s/S \qquad \text{Eq. 1}$$

$$\mathrm{LOQ} = 10s/S \qquad \text{Eq. 2}$$

in which: “s” is the standard deviation of the blank response (n = 10) and “S” is the slope of the calibration curve.

RESULTS

Formation and stability of PMB species

Absorption spectrum of B&D in M1 at 1:4 ratio (Figure 1a) presents a band around 711 nm that stabilized after 5 min, showing a fast development of blue color. Besides, absorbance showed to be very stable and increased only 0.014 (2.8 %) units in the interval from 5 to 600 min, which is a highly desirable characteristic.

Spectrum from M&R in M1 at 1:4 ratio (Figure 1b) displayed two absorption bands, one centered at 889 nm and a smaller one at 711 nm with a shoulder at 550 nm. The blue-purple color was developed after 5 min, but the formation of PMB species stabilized only after 27 min. In terms of color stability, the absorbance of the band at 889 nm decreased 0.026 (3.7 %) units in the time interval of 27-600 min, showing also a high stability. Therefore, B&D and M&R methods in M1 solution showed fast PMB formation and high stability over time, allowing the quantification to be done over a long period after the preparation of the colorimetric extract. Similar results were obtained using 1:1 volume ratio (data not shown).

Unlike what was observed for M1, M&R and B&D methods for M3 (Figures 1c, 1d, 1e and 1f) were highly unstable in terms of spectral development over time. The M&R method in M3 at both 1:1 and 1:4 ratios (Figures 1c and 1d) exhibited a wide absorption band centered at 737 nm and full PMB formation took a little longer (~15 min). However, spectrum stability was drastically affected, remaining stable for a very short time (15-48 min – Figures 1c and 1d), which is attributed to the precipitation of PMB species (Cai et al., 1994), even when the volume of M3 was lower (1:1 volume ratio).

The spectrum of B&D in M3 at 1:1 ratio (Figure 1e) showed two absorption bands, at 710 (bigger) and 825 nm (minor), instead of a single band at ~711 nm as obtained in M1. In addition, the time of PMB formation was slower (~60 min, Figure e) than M1 (~ 5 min, Figure 1a). At 710 nm, the absorbance (0.45 absorbance units) remained stable from 90 to 150 min, then absorbance dropped to 0.40 units (11 %) and gradually increased 0.007 units (1.75 %) from 150 to 588 min, demonstrating spectral stability to taken absorbance readings.

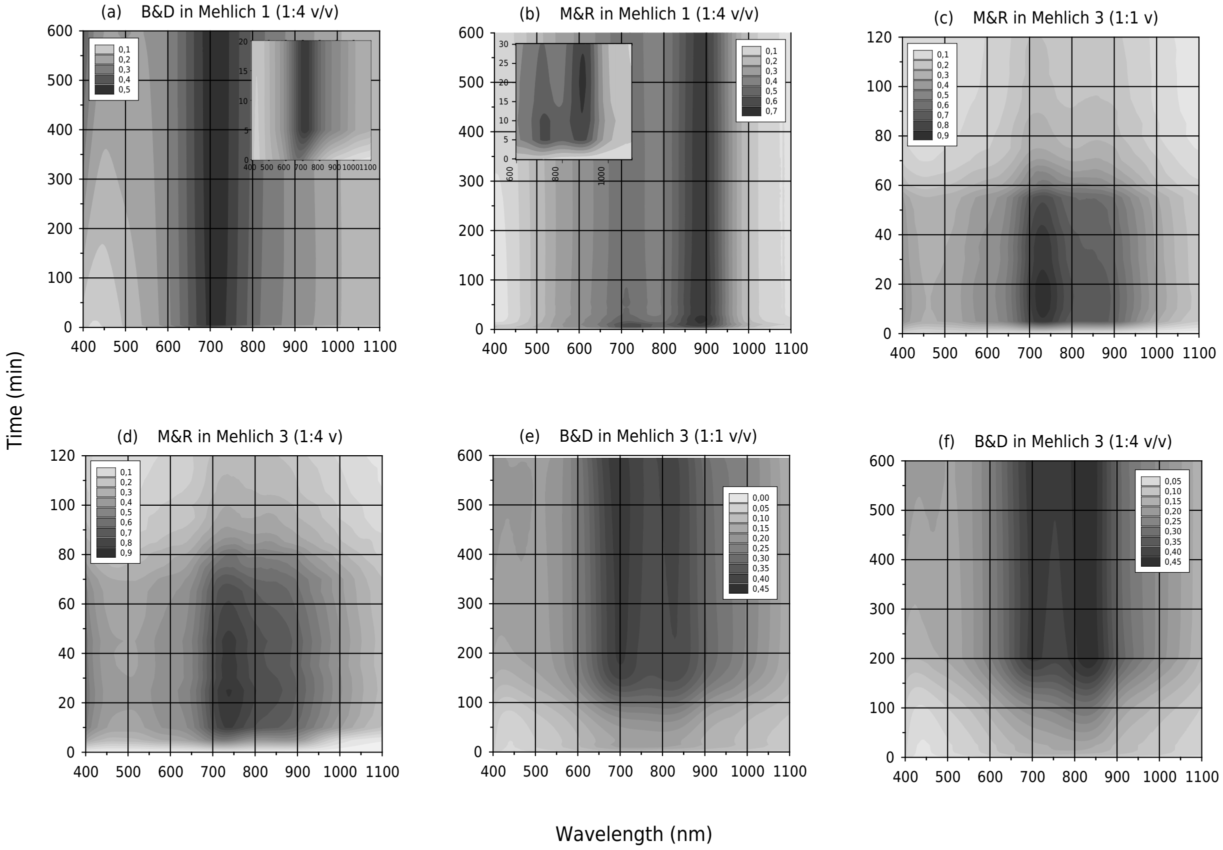

Figure 1. Absorbance spectra of molybdenum blue species (1 mg L^{-1} P) obtained from Braga & Defelipo (B&D) (a, e and f) and Murphy & Riley (M&R) (b, c and d) colorimetric methods as a function of time and background of Mehlich-1 (a and b) and Mehlich-3 (c, d, e and f) at different volume ratios (1:1 or 1:4).

The B&D method in M3 extract and 1:4 ratio (Figure 1f) displayed a smaller absorption band at 710 nm and a bigger one at 825 nm, opposed to that obtained at the 1:1 ratio. The PMB formation was complete at 180 min and remained stable up to 600 min, with no precipitation occurring for this method.

Comparing the spectra after 30 min, when all the color development is completed and stable, the spectral features of the calibration curve and the soil extract for M1 were virtually the same for both colorimetric methods and volumetric ratios (1:1 or 1:4) (Figures 2a and 2c), demonstrating that soil matrix does not affect the spectral behavior of P quantification for M1.

However, this is not the case for M3. The spectra of both colorimetric methods (B&D and M&R) for M3 have different characteristics when comparing ratios (1:1 or 1:4) and soil matrix effect (calibration curve vs soil extract). For B&D, the biggest difference is related to the solution ratio (Figure 2b). The different ratios changed the shape of the spectra. In the presence of more P (1:4), used to improve the quantification in low P soils, the overall spectra ranging from 500 to 1000 nm was flattened and showed a lower maximum intensity. The same way, the presence of the soil matrix also changed the spectra, but this time mostly the intensity and not really the shape (Figure 2b). The presence of soil matrix (solid line) led to a constant increase in absorbance compared to the P in the solution without soil (dashed line).

For M&R in M3, the ratio (1:1 vs 1:4) was not a problem, and the spectra were virtually the same for both ratios (Figure 2d). However, this was not the case for the soil matrix effect. When comparing the spectra of the solutions containing (soil extract) or not (calibration curve) the soil matrix effect, M&R showed a major difference, both in terms of intensity and wavelength of maximum absorption.

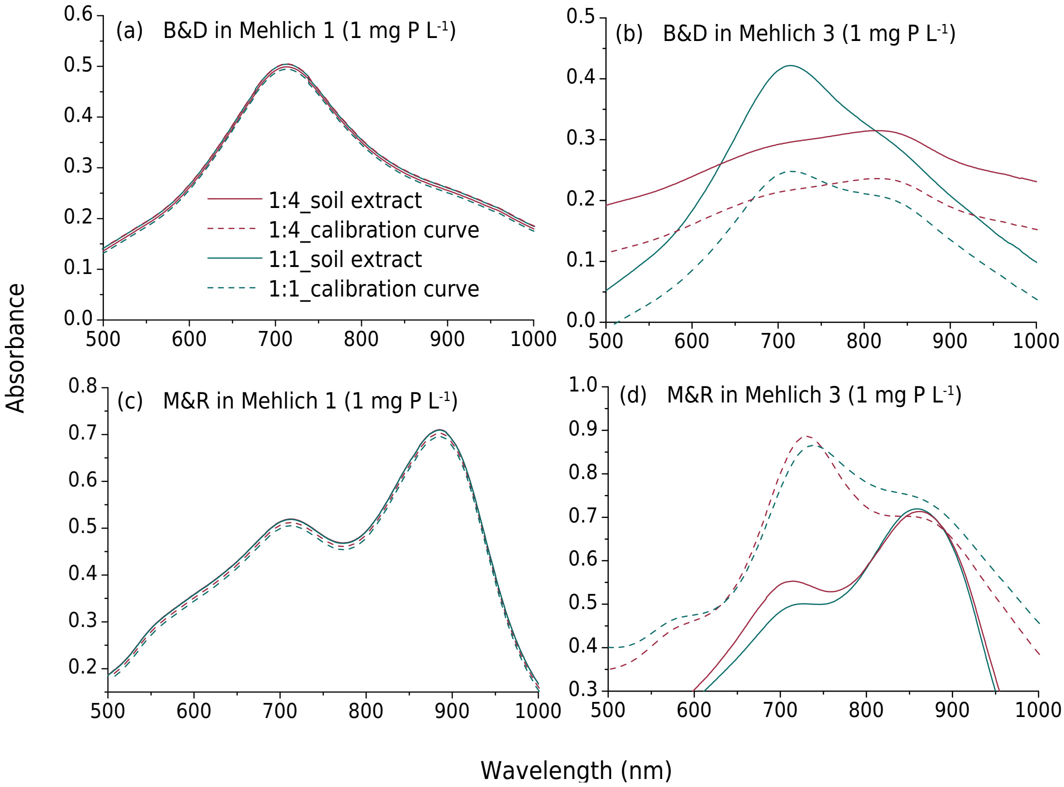

Figure 2. Absorbance spectra for the (a and b) Braga & Defelipo and (c and d) Murphy & Riley methods in Mehlich-1 (M1) or Mehlich-3 (M3) extract from soil and calibration curve in volume ratios 1:1 or 1:4 (colorimetric reagent:extract). Absorbance taken 30 min after reagent addition, and the soil provided a concentration close to 1 mg L-1 in the reading solution.

Linear range and sensitivity of the methods

Murphy & Riley method presented a strong linear fit in the range 0-1 mg L^{-1} P for both M1 and M3 extracting solutions, R^2 = 0.999 and 0.997, respectively. The B&D method showed a linear interval greater than the M&R method, ranging from 0.0-2.5 mg L^{-1} P in M1 extracting solutions (R^2 = 0.999), and even going up to 5 mg L^{-1} P (R^2 = 0.996). Apparently, B&D method presents a breakpoint of linearity between 2.5-5.0 mg L^{-1} of P. In addition, B&D method in M3 extract displays an exceptional linear fit at the range of 0-10 mg L^{-1} P (R^2 = 0.999).

The LOD and LOQ obtained for M1 extract were similar between the methods (Table 2). However, for M3 extract B&D method showed a higher LOQ (75.9 µg L^{-1} P) than M&R method (40.2 µg L^{-1} P). Furthermore, there was an increase in the blank standard deviation at M3 extract for both methods (data not shown), which corroborates with sensitivity reduction. Murphy & Riley method in M3 extract shows a slight increase in LOQ compared with M1 (Table 2) because the optimum wavelength changed to ~ 737 nm (Figure 2d), which promotes a reduction in the calibration curve slope.

Table 2. Limit of detection (LOD) and limit of quantification (LOQ) of P by Murphy & Riley (M&R) and Braga & Defelipo (B&D) colorimetric methods in Mehlich-1 or Mehlich-3 extracting solutions at 1:4 ratio (colorimetric reagent: extract)

Method	Wavelength	Extractant	LOD	LOQ
			µ g L^{-1}	
M & R	λ 882 nm	Mehlich 1	9.8	29.6
		Mehlich 3	12.3	40.2
B & D	λ 725 nm	Mehlich 1	10.6	32.0
		Mehlich 3	25.1	75.9

Absorbance taken at 30 min after reagent addition.

DISCUSSION

The PMB species from B&D method are formed rapidly due to the simultaneous catalytic action of Bi, becoming part of the reduced product (BiPMo) (Mihajlović et al., 2007). The absorbance gradual increase over time is due to Bi-based PMB specie (BiPMo) stability, and the slow reductive kinetics of ascorbic acid that allows a long reduction action (Nagul et al., 2015). Interactions among these factors promote a stable absorption spectrum over time, providing a large interval to perform the absorbance readings. In fact, the original work reports stability of the formed color from 5 min up to 24 h and optimum wavelength around 725 nm (Braga and Defelipo, 1974). Usually, absorption band of Bi-based PMB species occurs around 720 nm (Kobayashi and Nakamura, 2007; Mihajlović et al., 2007). Likewise, molybdophosphoric acid (MPA) in M&R method is rapidly reduced by the Sb catalyst, which is part of the reduced product (SbPMo) (Nagul et al., 2015). However, after Sb-based PMB formation, the absorbance gradually decreases, which indicates a lower stability of these species compared to Bi-based PMB (Goldman and Hargis, 1969; Mihajlović et al., 2003, 2007). In fact, the magnitude of absorbance decreases 0.026 units over 10 h does not expressively affect the spectrum stability.

Both colorimetric methods in M1 extracting solution lead to a stable spectrum and a short formation time of PMB species. This occurs because the M1 is weakly buffered, consisting of two strong acids at low concentrations (HCl 0.05 mol L^{-1} and H_2SO_4 0.0125 mol L^{-1}, pH ~1.2), which does not substantially change medium conditions that optimize PMB reaction (color development).

Our results strongly indicated that in M3 extract, when original protocols for quantifying P using both M&R or B&D methods are used, potential error sources occur (Cai et al., 1994), which may lead to mistakes in P-soil estimation. Indeed, the Sb-based PMB from M&R method precipitates rapidly, and Bi-based PMB from B&D presents a reduced formation rate in the presence of M3. The ethylenediaminetetraacetic acid (EDTA) contained in M3 is one of the main players that affects the formation rate and stability of PMB species (Alexander and Robertson, 1972; Cai et al., 1994; Kowalenko and Babuin, 2007). Nevertheless, absorbance spectra collected from soil extract did not demonstrate that EDTA negatively affected the PMB reaction, except for Bi-based PMB in volume ratio 1:4. Probably, the soil reduces EDTA activity in solution, due to interactions with the other soil components as metal ions in solution or associated to the solid phase. These interactions involving EDTA and soil components in the PMB reaction are still unclear. The solubility of Sb-based PMB reduces in the presence of EDTA, resulting in precipitation (Cai et al., 1994). Furthermore, EDTA can form complexes with colorimetric reagent compounds, such as Mo, Sb and Bi (Chan et al., 1964), promoting fading color, i.e., reducing PMB formation rate (Alexander and Robertson, 1972; Cai et al., 1994; Kowalenko and Babuin, 2007) as observed in our data for Bi-based PMB.

Probably, the slow rate of Bi-based PMB formation is more pronounced due to its stoichiometric ratio (1:1:18, Bi:P:Mo, respectively), requiring a larger amount of Mo compared to Sb-based PMB (1:1:12) (Going and Eisenreich, 1974). Thus, if the Mo is complexed by EDTA, there will be less Mo to form the MPA, consequently, there will be less Bi-based PMB synthesis. This fact agrees with the lower rate of PMB formation and the lower absorbance level in M3 extract.

Despite the discrepancy between the absorbance values obtained both in the calibration curve and in the soil extract, the M&R and B&D methods are widely used for this extractor (M3), what might be worrisome once available P can be overestimated, which, depending on the magnitude of inaccuracy, could underestimate rates of P-fertilizers, compromising crop yields.

Some studies proposed modifications of M&R method to overcome the EDTA interference (Alexander and Robertson, 1972; Nnadi and Tabatabai, 1973), however, to date, there are no studies that propose to modify B & D method to accelerate the stage of PMB formation in M3 extracting solution. In this sense, we suggest that increasing Mo and Bi concentrations may accelerate color development because it would supplant EDTA activity.

Linear range of the methods

A wide linear range allows the use of colorimetric methods in samples with higher concentrations of P, avoiding possible dilutions and minimizing pipetting errors. Usually, Sb-based methods similar to M&R exhibit linearity up to 1 mg L^{-1} P (Edwards et al., 1965; Drummond and Maher, 1995), this breakpoint in linearity occurs when Sb:P molar ratio is approximately 2:1 (Nagul et al., 2015). Lambert Beer's law is not satisfied when P concentration increases and reduces the ratio to less than 2:1. However, Harwood et al. (1969) observed that linearity range was extended when Sb concentration was increased and Mo was decreased. In this line, authors proposed a modification of M&R method, which allowed to extend the linear range by threefold, this improvement is related to the increase of Sb concentration (0.329 mmol L^{-1}) and reduced the excess of Mo (2.7 mmol L^{-1}) (Nagul et al., 2015). Braga & Defelipo method also uses the catalyst excess (0.39 mmol L^{-1} of Bi), which satisfactorily explains its greater linear range compared to M&R. Therefore, taking the absorbance reading at 737 nm, in the interval of 15-48 min (Figure 1f) would provide a greater sensitivity to M&R method.

CONCLUSIONS

Phosphorus Chromophores (PC) from Murphy & Riley in Mehlich-1 extract are stable for up to 600 min, with optimum wavelength at 899 nm; linear range of 0.0-1.0 mg L^{-1} P, limit of detection of 9.8 μg L^{-1} P and limit of quantification of 29.6 μg L^{-1} P. Already from Braga & Defelipo in Mehlich-1 extract, there is stability for up to 600 min, with optimum wavelength at 711 m, linear range of 0.0 - 2.5 mg L^{-1} P, limit of detection of 10.6 μg L^{-1} P and limit of quantification of 32.0 μg L^{-1} P.

Phosphorus chromophores from Murphy & Riley and Braga & Defelipo in Mehlich-3 are highly unstable. The optimum wavelength changes depending on the volumetric ratio of the colorimetric reagent:extract and the effect of the soil matrix on the Mehlich-3 extract. Murphy & Riley and Braga & Defelipo methods in Mehlich-3 showed lower sensitivity, with detection limits of 13.3-25.1 μg L^{-1} P and limits of quantification of 40.2 and 75.9 μg L^{-1} P, respectively.

For Murphy & Riley and Braga & Defelipo in Mehlich-3, PC are highly unstable. The optimum wavelength changes depending on the volumetric ratio of the colorimetric reagent:extract and the soil matrix on the extractor, limits of detection of 13.3-25.1 μg L^{-1} P, and limits of quantification of 40.2 and 75.9 μg L^{-1} P, respectively.

Our data support that both colorimetric methods should not be considered for measuring P from Mehlich-3 extracting solution.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://www.rbcsjournal.org/wp-content/uploads/articles_xml/1806-9657-rbcs-48-e0230093/1806-9657-rbcs-48-e0230093-suppl01.pdf.

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