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Contribution to the Molybdenum Blue Reaction and its Application in Soil Analysis

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This paper presents a study on the molybdenum blue reaction (MB) as a finishing detection step in soil analysis for quantification of plant available phosphorus. An ammonium acetate/calcium lactate reagent (pH = 4.2) was used for soil phosphorus extraction. The molybdenum blue color reaction using premixed Murphy-Riley reagent and ascorbic acid as a reductant was reinvestigated. UV-Vis characteristics of MB, optimal wavelength, reaction time and concentration of reductant were studied. The effect of ascorbic acid concentration and the reaction time on linearity, bias and uncertainty was discussed. The molybdenum blue reaction was found to obey Beer's law in the targeted concentration range of 0.04 – 1.0 mg L^{-1} PO_4^{3-}-P. The linearity was proved by "lack-of-fit" test. The uncertainty budget was made and the uncertainty was estimated by modelling approach, as well as single laboratory and quality control approach. The recovery and the expanded uncertainty were found to be, respectively, (95.7 ± 8.7) % (P = 95%, n = 3) and 9.2 mg PO_4^{3-}-P /kg dry soil (k = 2). The results showed that the soil sample inhomogeneity and the repeatability of extraction process were the main factors which contribute to the uncertainty of measurement in soil analysis.

Keywords: molybdenum blue reaction, uncertainty, soil analysis, plant available phosphorus

Nowadays, molybdenum blue (MB) reaction is widely used for phosphate determination in different solutions as: environmental waters [1], technological solutions or recommended as a detection finishing step in soil testing [2-4].

Phosphorus exists in soils as water-soluble (soils solution), exchangeable/available, fixed/non-exchangeable and mineral phosphorus (incorporated in the crystal lattice of mineral and inaccessible for plants). The extracting solution contains a mixture of various reagents that reacts with soil and releases specific parts of soil phosphorus into the solution. Many different extracting solutions have been used worldwide due to variable nature of soil and it has been well recognized that no universal soil testing method exists [5-8]. A given fertilization program should be well reasoned based on field studies in which crop yield response to P fertilizer addition was validated against the amount P extracted by a specific reagent mixture. Additionally, the instrumental technique for determination of P in soil extract also influenced the data [4]. Usually, fertilizer recommendations based on a soil test are developed for a particular extraction procedure and detection technique. Thus, the choice of detection technique appears to be of crucial importance. Although, ICP-OES has the great advantage for multicomponent determination, in the case of P determination this technique provides data about total phosphorus (organic and inorganic). Spectrophotometry is preferable in soil analysis as it offers the advantage to detect the agronomically important inorganic part of soil P [5].

Different extraction procedures using single or mixed extracting reagents have been developed in order to estimate the plant available phosphorus in arable soils [9-11]. Composition of extraction solutions added additional components to the liquid matrix, which impose examination of the analytical behavior of molybdenum blue reaction in a specific medium used for plant available phosphorus determination [4]. Moreover, having in mind an innovative sensor based on MB reaction which have been recently proposed by Heidari-Bafroui et al. [12] and its possible applications, the study of MB in routine soil testing conditions is of special interest. In soil analysis MB reaction has been applied in various experimental conditions as: procedures for color reagent preparation, reaction time, temperature, reducing agents, catalyzers [5, 10, 13-15].

The molybdenum blue method involved a reaction of ammonium molybdate with phosphate ions in acid media to form 12-molybdophosphoric acid, which was further reduced to molybdenum blue [1, 16]. Molybdenum blue presented not a single species, but a group of reduced molybdate compounds [1]. The experimental conditions for MB reaction allowed not

only phosphates to be determined, but other labile phosphorus containing compounds. The measurand frequently was noted as molybdenum blue reactive phosphorus (PO_4^{3-}-P) [17]. Organic phosphorus could also be determined after appropriate sample treatment [18]. A line of modification of the reaction conditions has been proposed in attempt to increase the sensitivity, decrease the reaction time and broader the working concentration range [1,6,10,11, 13-15]. The reaction time in water samples previously reported was between 5 and 20 min [1]. However, when the MB was applied for determination of soil extractable phosphates one-hour reaction time was recommended [13-16, 19]. Additionally, the procedure of preparation of MB color reagent could substantially differ. Different concentrations of reagents for color development (sulfuric acid, ammonium molybdate, ascorbic acid) were proposed as optimal, based on the highest sensitivity and stability of the MB species [15]. Total phosphorus standard operating procedure (SOP) recommended premixed color solution containing Mo(VI), Sb(III) in H_2SO_4 to be added to freshly prepared solution of ascorbic acid and the obtained mixture to be stirred ½ h before use [20]. FAO-SOP recommended each component of MB color reagent to be prepared as an individual solution and to be mixed just before adding to the sample extract [13, 14]. In the present study the method of Murphy and Riley [16] with ascorbic acid as reductant in the presence of Sb(III) was studied. The procedure described by Ivanov [19] was applied: ascorbic acid was dissolved in a premixed color reagent before each run. Although considered as a classical one, MB method still is in the focus of recent research due to the variety of experimental conditions and the characteristics of the final MB product formed [1,11,17]. These facts additionally supported our motivation to initiate the study on the effect of experimental conditions on the analytical characteristics of MB method applied for soil testing.

Nagul et al. presented a detailed review on the MB based methods for MB reactive phosphorus in water samples [1]. As the authors stated the studies have been focused mainly on water samples. The MB spectrophotometric method for reactive phosphorus determination in soil extracting solution still needs additional characterization [1, 4]. In 2021 FAO published standard operation procedures (SOP) for phosphorus determination in soil applying different extraction methods [13-15]. Moreover, estimation of uncertainty associated to the analytical method would contribute to the detailed understanding of the principle of methods and to the comparability of the obtained results [21-23].

The aim of this study was to reinvestigate Murphy-Riley method and to estimate its analytical behavior for determination of plant available phosphorus in arable soils after extraction by an ammonium acetate/calcium lactate reagent. Additionally, an attempt to clarify some of the optimal conditions of the method, to study the effect of components of the extracting solution on the determination of phosphates, to reveal the factors which influenced uncertainty of measurement was made. The extracting solution chosen for this study presented a mixture of ammonium acetate/acetic acid and calcium lactate/lactic acid buffers at pH 4.2, proposed by P. Ivanov [19]. The extractant was proved to be suitable for wide range of arable soil types encountered in Bulgaria for simultaneous determination of P and K. The buffer capacity of the extractant was high enough to maintain constant extraction conditions from acidic to calcareous soil samples at 1:25 solid-to-liquid ratio. Calcium, ammonium and hydronium ions contributed to potassium extraction by displacement mechanism, as well as enhanced desorption of soil P into solution [8, 24]. Lactate and acetate ions contributed to phosphorus extraction of readily soluble and exchangeable phosphates, as well as Ca phosphates from fertilizers [6, 8]. Combined with spectrophotometric determination by MB method and flame emission spectrometry, the extractant allowed determining plant available P and K. It was found to well correlate with plant response to P and K fertilization, respectively. The widely applied scale for estimating P and K fertility status of Bulgarian arable soil was based on the studied method. However, its analytical behavior and the uncertainty of measurement were not accessed in detail till now.

Materials and methods

Preparation of Murphy-Riley color reagent. Murphy-Riley solution was prepared following the procedure proposed by Ivanov [19]. 12 g of ammonium molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$ were dissolved in 500 mL of d. H_2O. 50 mL 10 M HCl were added and the solution was diluted to 1 L. Potassium antimonyl tartrate ($C_8H_4K_2O_{12}Sb_2.3H_2O$) 0.2928 g were dissolved in 500 mL d. H_2O. The obtained solutions were added to 1 L 2.5 M H_2SO_4 and diluted up to 2 L. The final concentration of the premixed Murphy-Reilly reagent was: 0.005 M ammonium molybdate, 0.012 M potassium antimonyl tartarate in 1.25 M H_2SO_4. Thus prepared, the solution was stored in dark place at room temperature (20 °C). The solution was found to be stable at least two weeks. Molybdenum blue color reagent was prepared daily by mixing 1.056 g ascorbic acid and 200 mL of premixed Murphy-Riley solution. All reagents were of p.a. grade, produced by Sigma-Aldrich.

Preparation of extracting solution. The used extractant [19] composition was: 0.05 M calcium lactate, 0.05 M lactic acid, 0.2 M ammonium acetate, 0.2 M CH_3COOH and 0.05 M $CaCl_2$. The solution pH 4.2 was controlled potentiometrically and adjusted by adding aqueous ammonia or acetic acid. The solution was prepared as followed: 154.15 g of $C_6H_{10}CaO_6.5H_2O$ were dissolved in about 800 mL of d. H_2O at gentle heating; after cooling, 50 mL of

10 M HCl were added and the solution was made up to 1 liter with d. H_2O; 77,10 g CH_3COONH_4 were dissolved in 500 mL of water, 60 mL of acetic acid was added and the solution was made up to 1 liter; both solutions were mixed and diluted to 5 L with deionized water. All reagents were of p.a. grade, produced by Sigma-Aldrich.

Calibration. A reference material (RM) containing 50 mg L^{-1} PO_4^{3-} (Hach-Lange, Lot A0080, traceable to NIST) was used for preparation of calibration standards by appropriate dilution in the concentration range: 0.04-0.87 mg L^{-1} PO_4^{3-}-P (corresponded to 0.1-2.0 mg L^{-1} as P_2O_5). Two mL of acetate/lactate extracting mixture were added to each standard solution to mimic soil extracting medium. The blank was prepared by adding 2 mL extracting reagent, 4 mL MB color reagent and diluted up to 25 mL with d. H_2O. The absorbance of each solution (λ_{max} = 880 nm) was measured in duplicate against the reagent blank and the mean value was used for standard curve construction. The analytical function was obtained applying linear regression by least squares method to the obtained data.

Stability study: calibration curves in the concentration range 0.04-0.40 mg L^{-1} PO_4^{3-}-P and MB reaction time of 60 min were daily prepared using freshly mixed MB color reagent, containing ascorbic acid and premixed Murphy-Riley solution. The absorbance of each solution was measured in duplicate and the mean value was calculated. A set of 40 calibration curves were obtained for one-year period. The mean value of absorbance of standard solutions and its standard deviation were used for construction of mean calibration curve. The stability of the calibration curve was monitored by analyzing standard solutions at two concentration levels (RM Hach-Lange) with each batch of samples.

Determination of plant available phosphorus in soil

PO_4^{3-}-P extraction by acetate/lactate method: Soil PO_4^{3-}-P extraction was carried out for 1 hour at soil-to-liquid ratio 1:25 (2 g accurately weighted soil was mixed with 50 mL extracting solution) at continuous agitation. The sample was filtered and MB reactive phosphorus content was determined spectrophotometrically by the molybdenum blue method. *Determination of soil phosphorus as extractable PO_4^{3-}:* 2 mL of soil extract were mixed with 4 mL of MB color reagent and the volume was made up to 25 mL in a measuring flask. After 1 h, the absorbance was measured at 880 nm against a blank sample by Hach Lange DR 3900 Spectrophotometer.

Uncertainty and trueness estimation. Method trueness was estimated as bias and relative spike recovery by standard addition of PO_4^{3-} solution (Hach-Lange, Lot A0080, traceable to NIST) to the soil and CRM sample before extraction. A CRM 090-100G Nutrients in clay soil (Lot LRAA3577, Sigma-Aldrich) was used. Soil CRM certified content was: total Kjeldahl nitrogen (2070 ± 137) mg kg^{-1}; total P (889 ± 133) mg kg^{-1}; ammonia as N (587 ± 50) mg kg^{-1}; total organic carbon (5430 ± 614) mg kg^{-1}; loss on ignition (550 °C) (6.06 ± 0.043) %. An aliquot of standard phosphate solution was added to 2 g of dry soil, homogenized and left to equilibrate for 6 days. Four replicates were made and the average concentration was used for calculations. Bias was calculated as a difference between content of PO_4^{3-} in the studied soil sample before and after spiking. Recovery was calculated as a relative ratio between content of PO_4^{3-} in the spike obtained after analysis and calculated from the volume of the spike and the concentration of standard solution of PO_4^{3-}. The uncertainty of bias was calculated from standard deviation of PO_4^{3-} in soil after spiking (4 replicates analyzed) according to Cuadros-Rodrıguez et al. [23].

Method uncertainty was estimated according to [21-23]: (1) mathematical modelling approach and (2) single laboratory validation and quality control approach.

Quality control test soil samples characterization. The soil samples used in this study for quality control monitoring were collected from two well developed agricultural regions in Bulgaria: the East region and the Thracian valley. The samples were collected from a depth of 0-30 cm by automatic soil sampler following the sampling plan recommended by Nikolova *et al.* [25]. Each sample was formed from 15-20 individual samples. The samples were air dried, grounded and sieved through 2 mm sieve. *Active acidity* (pH) of the soil samples was determined in water at 1:2 soil-to-liquid ratio after 1 h mixing. pH was measured using WTW pH-meter equipped with a combined glass electrode. *Soil organic carbon* was determined after oxidation with $K_2Cr_2O_7$ by back titration with standard solution of Mohr salt (Turin's method). *Mineral nitrog*en (NO_3-N and NH_3-N) was determined after diluted acid extraction at 1 : 5 soil-to-liquid ratio for 5 min followed by spectrophotometric determination of nitrates by cadmium reduction method (Method 8039, Hach Lange) and ammonium by Nessler reaction (Method 8038, Hach Lange). The main characteristics of the soil samples are presented in the Table 1.

Table 1. Characteristics of soil samples used as quality control samples in this study.

Control sample	pH	∑ NH_3- N + NO_3-N, mg/kg	P_2O_5, mg/100g	K_2O, mg/100g	Organic carbon, %
C1	7.24	14.8	4.6	20.9	2.96
C2	7.44	39.5	12.5	46.8	4.55

Results and discussion

1. Study of molybdenum blue reaction in the presence of ammonium acetate/calcium lactate extracting reagent

The UV-Vis spectra of molybdenum blue at different concentrations of MB reactive phosphorus denoted as PO_4^{3-}-P (0.04 – 0.44 mg L^{-1}) are presented on Figure 1. The spectra revealed two peaks: a sharp higher peak at 880 nm and a broader lower peak at 684 nm. The blank sample didn't show significant absorbance at 880 nm and this wavelength was chosen for the further study. The molar absorptivity of MB at 880 nm was $3.83 \cdot 10^3$ L/mol.cm.

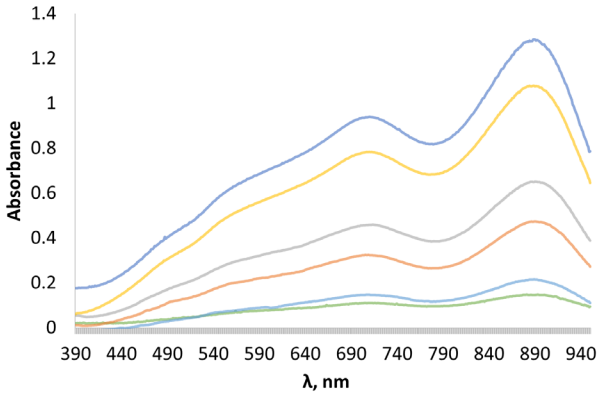

Figure 1. UV-Vis spectra of Molybdenum blue at pH = 1 in the presence of acetate/lactate extracting solution, at 5 cm optical length and concentration levels: (1) 0.04; (2) 0.1; (3) 0.2; (4) 0.25; (5) 0.35; (6) 0.44 mg L^{-1} as PO_4^{3-}-P and reaction time of 1 h. The final concentrations of color reagent components were: $4.8 \cdot 10^{-3}$ M ascorbic acid; 0.2 M H_2SO_4; $5.6 \cdot 10^{-3}$ M Mo(VI) and $3.8 \cdot 10^{-4}$ M Sb(III).

It is well known that the rate of molybdenum blue reaction was accelerated by ascorbic acid as reducing agent [1, 16], however different concentrations of ascorbic acid have been proposed in the literature. In contrast to water analysis where shorter reaction times (10-20 min) were found to be effective enough to assure appropriate working range, sensitivity and selectivity [1], in soil analysis – 60 min reaction time was generally proposed [13-15]. The variety of experimental conditions motivated the study on the reaction time and the effect of concentration of ascorbic acid, the components of acetate/lactate extracting reagent and the soil matrix.

Figure 2 shows the kinetics curves obtained in the concentration range 0.1-0.4 mg L^{-1} PO_4^{3-}-P in presence of acetate/lactate reagent.

It was observed that the time necessary to achieve stable absorbance (±0.002 absorbance units) varied with the phosphate concentration. The reaction time increased with increase of concentration. In the concentration range: 0.04-0.20 mg L^{-1} PO_4^{3-}P, a complete MB reaction was achieved after 10 min reaction time. However, at 0.35 mg L^{-1} at least 25 min were necessary. The reaction time increased up to 60 min at concentrations higher than 0.44 mg L^{-1}. In an attempt to shorten the reaction time, the influence of the ascorbic acid concentration on the MB reaction was studied in the presence of the components of extracting solution. The results showed that the concentration of ascorbic acid in the range: $2.4 \cdot 10^{-3}$ – $2 \cdot 10^{-2}$ M did not affect the reaction rate and originally proposed concentration $4.8 \cdot 10^{-3}$ M was used. Additionally, the results of study of ten arable soil samples demonstrated that the soil matrix did not affect the MB reaction rate and the same trend as in phosphate standard was observed.

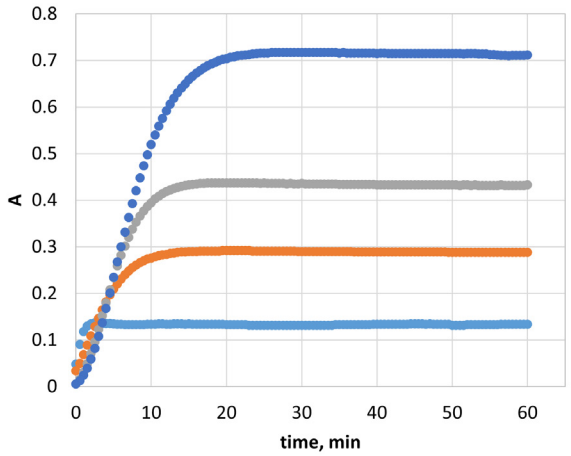

Figure 2. Absorbance as a function of time at different MB reactive phosphorus concentrations: (1) 0.04 mg L^{-1}; (2) 0.09 mg L^{-1}; (3) 0.18 mg L^{-1}; (5) 0.44 mg L^{-1}. The kinetic curves were acquired at 880 nm and 2 cm optical path in the presence of acetate/lactate extracting reagent and color reagent containing: $4.8 \cdot 10^{-3}$ M ascorbic acid; 0.2 M H_2SO_4; $5.6 \cdot 10^{-3}$ M Mo(VI) and $3.8 \cdot 10^{-4}$ M Sb(III).

Applying 60 min reaction time, the molybdenum blue was found to obey Beer's law at 880 nm in the studied concentration interval 0.04 - 1.0 mg L^{-1} PO_4^{3-}-P. Thus, based on the wider linear concentration range and the reproducibility of calibration curve, 60 min reaction time was recommended.

The stability of MB reagent was studied at 5.28 g L^{-1} concentration of ascorbic acid in the color MB reagent. The spectra of the color reagent of freshly prepared solution and after 4 h are presented on the Figure 3.

Although some changes of the color reagent with time could be observed on Figure 3, the reagent blank absorbance at 880 nm was practically insignificant. Additionally, the absorbance of MB at 0.35 mg L^{-1} PO_4^{3-}-P concentration level with freshly prepared reagent and 6 hours after coincided statistically. Thus, the MB color reagent was found to be stable at least 6 h stored at ambient conditions.

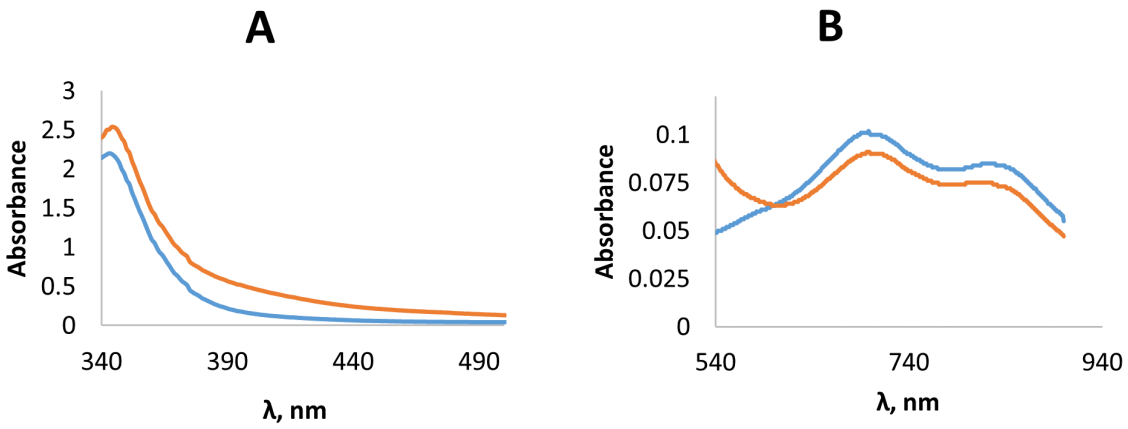

Figure 3. Spectra of MB reagent at 0 (blue line) and 4 h (red line) after preparation of the color reagent containing 5.28 g L^{-1} ascorbic acid, 0.035 M Mo(VI) and 0.024 M Sb(III) in 1.25 M H_2SO_4.

2. Analytical behavior of molybdenum blue method for determination of extractable phosphorus in soil

Linearity. Calibration curves were obtained in the concentration interval 0.04 – 1.0 mg L^{-1} PO_4^{3-}-P at reaction time 60 min and different length of optical path: 1, 2 and 5 cm. The measurements were run in duplicate for 9 concentration levels and the means were used for calibration curve construction. The regression analysis by the least squares method, plot of residuals and "lack-of-fit test" were applied to the obtained data according to [26, 27]. The plot of residuals revealed the homoscedasticity of the data. The following acceptance criteria for linearity were applied: correlation coefficient $r^2 \geq 0.995$ [28] and relative residuals < 20 % [29]. The intercept was statistically insignificant ($b < 2.s_b$) and the Beer's law was obeyed at the studied concentration range. Additionally, "lack-of-fit" test was performed to the linear model in the concentration range 0.03-0.65 mg L^{-1} PO_4^{3-}-P. The value of F_{cal} (0.264) was found below F_{tab} (2.37) and linear model was accepted in the further study. The results are presented in Table 2.

Limit of detection and limit of quantification. Limit of detection (LOD) and limit of quantification (LOQ) were estimated using two approaches: (1) calculation of LOD and LOQ based on 3.3 or 10 times, respectively, standard deviation of the absorbance of blank sample. The standard deviation (SD) was calculated in the repeatability conditions [21]. Four MB reagent blanks were prepared and their absorbance was measured against dist. H_2O, absorbance of each blank was measured in triplicate and the means were used to calculate the concentration from the calibration curve and corresponding standard deviation. The standard deviation (in mg/L PO_4^{3-}-P) was divided by the square root of the number of measurements. The obtained results were LOD = 3.7 µg L^{-1} PO_4^{3-}- P and LOQ = 11.3 µg L^{-1} PO_4^{3-}- P. (2) calculation based on 3.3 or 10 times the standard deviation of residuals divided by the slope of standard curve [26, 27]. The obtained results were instrumental LOD = 0.7 µg L^{-1} PO_4^{3-}- P and LOQ = 2.2 µg L^{-1} PO_4^{3-}-P. In both cases the calibration curves were prepared in the concentration range: 0.09-0.44 mg L^{-1} PO_4^{3-}-P (N=6, R^2=0.9986). Based on approach (1) the method LOD and LOQ (MLOQ) were estimated to be 0.09 and 0.28 mg PO_4^{3-}-P/kg dry soil. The presented results demonstrated that LOD and LOQ values differed depending on the calculation strategy, those based on SD of blank giving more realistic values: LOD 3.7 and LOQ 11.4 µg L^{-1} P. Thus, the procedure for color reagent preparation was proved to assure low reagent blanks and correspondingly lower detection limit. For comparison Wuenscher et al. [6] report a modified Murphy and Riley reagent and achieved LOD of 25 µg L^{-1} P in soil extracts.

Table 2. Calibration curves for PO_4^{3-}-P mg L^{-1} at different length of optical path (N = 9, P = 95 %).

Concentration range, mg L^{-1}	Length of optical path, cm	Slope	Slope stdev	Intercept	Intercept stdev	R^2
0.04-0.90	1	0.544	0.0090	- 0.0006	0.0054	0.9984
0.04-0.90	2	1.570	0.0093	+0.0068	0.0042	0.9998
0.09-0.45	5	2.994	0.1938	-0.0822	0.0680	0.9836

Stability of calibration function based on MB reaction. Stability of the calibration curve was studied during one year of routine laboratory work. The individual calibration curves were prepared daily. The data from 40 calibrations were averaged. Figure 4 demonstrate the mean calibration curve and corresponding variation intervals.

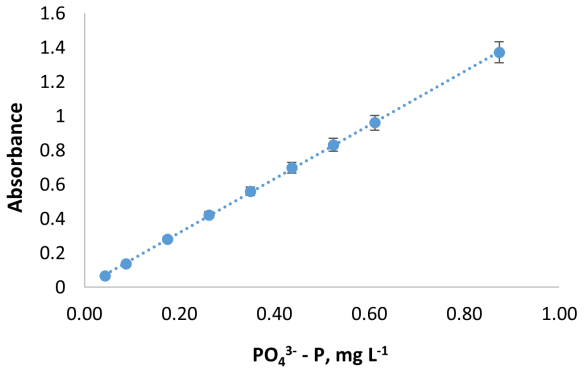

Figure 4. Mean calibration curve for MB reactive phosphorus in the presence of ammonium acetate/ calcium lactate reagent obtained in the one-year period (N = 40 individual calibrations); y = 1.571 x+0.0053, R^2 = 0.9998.

The mean calibration curve was used in the further work and regularly monitored by standard phosphate solutions used as quality control samples. The control charts were prepared for two levels of phosphate 0.09 and 0.26 mg L^{-1} PO_4^{3-}-P. The warning limits were set at 1 x s and the action limits at 2 x s. It should be pointed out that the standard deviation was estimated in conditions of intermediate precision: long period of time (one year), different analysts (6 people), different reagent solutions prepared by different analysts, different stock reagents. The results are presented on Figure 5.

In the case of exceeding warning limits, two freshly prepared quality control standards were reanalyzed to check the exceeding point. Usually, this overcame the problem and the work of the analyst was revised. No need to recalibrate the analytical function in the studied period which demonstrates the stability of MB method.

As the results presented above, the linearity and stability of calibration curve in the range 0.04-0.87 mg L^{-1} PO_4^{3-}-P at 60 min reaction time was proved. The chosen concentration range was appropriate for the majority of soil samples from Bulgarian arable soils encountered in our laboratory. It should be pointed out, that if shorter working range was considered (0.04 - 0.26 mg L^{-1} PO_4^{3-}-P) the reaction time could be shortened to 20 min still preserving linearity and stability of calibration curve, but appropriate dilution of the sample was needed.

Repeatability and intermediate precision

The precision of molybdenum blue method was estimated at 60 min reaction time and a length of optical path of 2 cm at the conditions of repeatability and intermediate precision [20]. The tests were designed to take into account the variations in operational conditions as: color reagent preparation, different analyst, changes in laboratory ambient, different instruments, different samples in order to estimate the variation during routine application of the method.

The repeatability was estimated on standard orthophosphate solutions, as well as clay soil CRM090 (Sigma-Aldrich, Lot LRAA3577) and real soil samples (Table 3). The phosphate in soil samples in duplicates was extracted by the acetate/lactate method and determined applying MB reaction. The experiments were made for a short period, by the same user with the same instrument and the same color reagent. The intermediate precision was estimated by analyzing phosphate standard solutions as well as the duplicates of the same real soil sample following the whole procedure during one-year period using different reagents, different analysts but the same instrument. For the concentration range 0.09 – 0.44 mg L^{-1} PO_4^{3-}-P standard solutions the intermediate precision estimated as RSD was between 4.8 and 2.8 %.

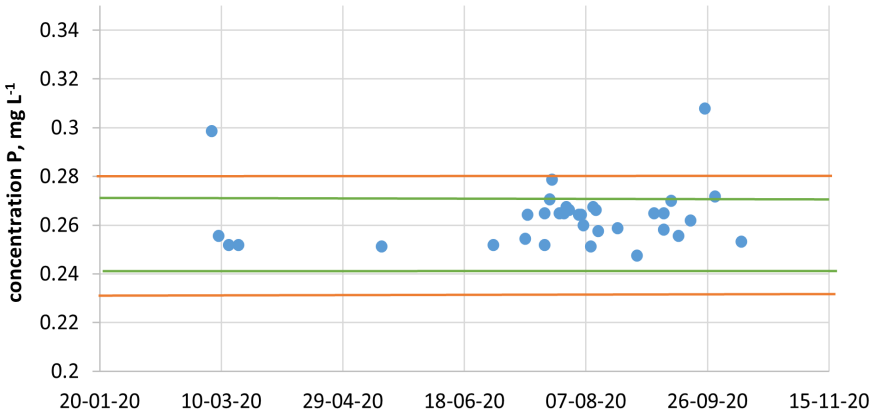

Figure 5. Control chart for 0.26 mg L^{-1} PO_4^{3-}-P applied for monitoring the mean calibration curve. Warning limits (green line) were set at ±1 x s and action limits (red line) – at ± 2 x s.

As can be seen from the results, the repeatability of the overall method for soil analysis ranged between RSD from 1.5 to 6.1 % in the concentration range 6 - 520 mg PO_4^{3-}-P/ kg, and corresponded to the target precision in repeatability conditions of between 7.3-5.3 % at 10-100 mg kg^{-1} [30]. It could be noted that repeatability as well as intermediate precision in soil CRM samples was 3 times higher (RSD 1.3 %) than in real soil samples (mean RSD 4 %). It could be supposed that the higher the RSD was mainly due to the real soil sample inhomogeneity and the variability of soil particle dimensions, and thus, the extraction conditions.

Bias. The method trueness was practically assessed by bias and relative spike recovery [21, 26-27]. A standard addition approach was applied as follows: (1) to assess method bias – the standard addition of PO_4^{3-} RM solution before extraction of real soil sample and soil CRM and (2) to assess measurement bias – standard addition of RM solution after extraction and before measurement. The advantage of the applied approach was that it allowed the bias to be evaluated in sample matrix routinely encountered in the laboratory. Soil matrix varied widely from sample to sample and the bias estimation could be widely effected by the soil type. As a drawback of standard addition approach, it should be noted that the spiked PO_4^{3-} hardly reach full equilibrium with the soil matrix and the obtained bias appeared approximate estimate of bias in extractable phosphate in soil [21, 26-27]. The defined volume of standard PO_4^{3-} solution was added to test soils as well as to soil CRM and let to equilibrate for six days. The spiked soils were subjected to overall extraction-determination procedure. The recovery of the spike was calculated by:

$$R', \% = \frac{\bar{x}' - \bar{x}}{x_{spike}} . 100,$$

where: $\bar{x}'$ was the mean of the spiked sample, $\bar{x}$ - the mean concentration of unspiked sample, x_{spike} was the concentration of spike.

The average bias was - 0.10 mg PO_4^{3-}-P/kg dry soil with standard deviation 0.01, corresponding to (95.5 ± 8.7) % recovery (P = 95 %, n = 3).

Uncertainty estimation. The uncertainty was estimated following two approaches recommended by EURACHEM guide (2014) [21-22]: a) modelling approach and b) single laboratory validation and quality control approach. The measurand was defined as the concentration of MB reactive phosphorus, extracted by the acetate/lactate reagent per kg dry soil, denoted as mg kg^{-1} PO_4^{3-}-P.

a) uncertainty estimation by modelling approach

The model equation applied in this study was:

$$C_{PO_4^{3-}-P} = \frac{\frac{A-a}{b} * f_{dil} * V_{ext}}{m_s}$$

where: A was the absorbance of sample solution; a and b were the intercept and the slope of the calibration curve; $f_{dil.}$ was the dilution factor as 2 mL aliquot of extracting solution was taken for color reaction, mixed with color reagent and diluted up to 25 mL in a measuring flask; $V_{ext.}$ was the volume of the extracting solution (L) and m_s was the weight of soil sample (kg). The sources of uncertainty and the equations used for the estimation of its contribution are presented in Table 4. The estimated expanded uncertainty (k = 2) at 58 mg kg^{-1} PO_4^{3-}-P was 4.3 mg kg^{-1} PO_4^{3-}-P which corresponded to a concentration level in extracting solution 0.17 mg L^{-1} PO_4^{3-}-P and uncertainty 0.013 mg L^{-1}. Intermediate precision standard deviation was used for estimation of uncertainty component due to repeatability. The data from a phosphate standard solution analyzed as a quality control sample and concentration calculated from mean calibration curve were used.

Table 3. Precision of the molybdenum blue method applied for determination of extractable phosphate in soil by acetate/lactate reagent.

soil sample mg PO_4^{3-}-P/ kg	number of replicates	Repeatability (RSD, %)	number of replicates	Intermediate precision (RSD, %)
6.3	2	1.5	-	-
54	2	6.1	18	6.2
519	2	4.0	-	-
110 (CRM)	4	1.3	9	1.7

Table 4. Sources of uncertainty and expressions used for its calculation.

Expressions used to calculate the uncertainty of the method	Sources of uncertainty
$u_c\ (CON) = C * \sqrt{u_{rel}^2(CA) + u_{rel}^2\ (m) + u_{rel}^2(V)}$	u_c (*CON*) – combined uncertainty; u_{rel} *(CA)* – standard uncertainty associated with calculation of analyte concentration from calibration curve; u_{rel} *(m)* – standard uncertainty associated with weighting the sample aliquot; u_{rel} *(V)* – standard uncertainty associated with measurement of the volume of the extracting solution all presented in terms of relative uncertainty;
$u_{rel}(CA) = \sqrt{u_{rel}^2(Stand) + u_{rel}^2(Cal) + u_{rel}^2(Repet)}$	u_{rel} *(Stand)* – standard uncertainty associated with preparation of calibration standards; u_{rel} *(Cal)* – standard uncertainty associated with calibration curve – linear regression; u_{rel} *(Repet)* – standard uncertainty obtained in repeatability conditions;
$u_{rel}(Stand) = \sqrt{u_{rel}^2(Prim) + u_{rel}^2\ (Dil)}$	u_{rel} *(Prim)* – uncertainty derived from the preparation of the primary standard solution; u_{rel} *(Dil)* – uncertainty derived from the preparation of the calibration curve at four concentration levels by diluting the standard solution;
$u(Cal) = \frac{1}{b}\sqrt{s_{resid}^2 * \frac{1}{n} + (Ci - Cm)^2 * s_b^2}$	*u (Cal)* – uncertainty derived from linear least squares calibration;
$u(Repet) = \frac{s_s}{\sqrt{n}}$	S_s is the standard deviation from the sample replicates; *n* is the number of replicates of each sample when analyzed in routine analysis;
$U = k * u_C$	*U* is expanded uncertainty calculated at coverage factor of 2 (*k* = 2) and 95 % confidence level;

Figure 6 illustrates the summary of the uncertainty budget and the contribution of each factor. As can be seen the main contributors to the uncertainty of MB method for reactive phosphorus determination were the standard solution preparation and calibration function.

b) Uncertainty estimation by single laboratory validation and quality control approach

Single laboratory validation and quality control data approach was applied for uncertainty estimation [22]. An uncertainty estimate was derived from results obtained during long period of time during the routine use of the method. The within-laboratory reproducibility was obtained from the quality control data at two concentration levels. The overall bias under within-laboratory reproducibility conditions was estimated by spiking of CRM and test sample and was used to evaluate the component of uncertainty associated with trueness.

$$u_c = \sqrt{u(s)^2 + u(bias)^2}$$

where: *u(s)* – within laboratory reproducibility (*u(s)* = 3.5 at 20 mg PO_4^{3-}-P/kg) and *u(s)* = 4.4 at 55 mg *κ*g^{-1}; *u(bias)* – standard uncertainty of the estimate of the laboratory and the procedure bias was 0.11 mg PO_4^{3-}-P/*κ*g dry soil.

The results demonstrated that the main factor which governs the uncertainty of the studied method was repeatability and it was studied in detail. The highest value of standard deviation was obtained when the sample followed the whole procedure and the main contribution to uncertainty could be assigned to the extraction step and inhomogeneity of the sample 2 mm sieved. In order to overcome these problems, the sample could be grinded to particle size less than 100 μm. However, standard procedure for soil analysis for determination of plant available nutrient recommended < 2 mm. Thus, further uncertainty estimation was based on tests samples with particles < 2 mm. It should be pointed out that the studied extraction procedure was designed to extract the only part of available phosphorus in soil and the efficiency of the extraction depended on the soil-to-

liquid ratio, time of the extraction, agitation, soil type, soil characteristics, particles dimension, etc. In order to estimate the repeatability of the extraction step, soil CRM sample with particle dimensions < 100 μm were analyzed, the relative standard deviation was found to be 1.3 %. At the same time the repeatability in real soil sample (2 mm sieved) was in average 4 %. Thus, it could be hypothesized that the main contribution to the uncertainty of the studied method for plant available phosphorus determination was the sample inhomogeneity sieved at 2 mm.

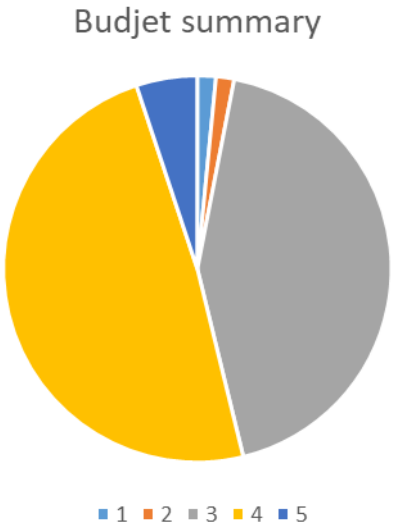

Figure 6. Uncertainty budget and contribution of the main factors: (1) uncertainty associated with sample concentration (1.5 %); (2) within lab precision (1.5 %); (3) uncertainty associated with standard solution preparation (43 %); (4) uncertainty of the linear model (49 %); (4) sample dilution (5 %).

The combined uncertainty estimated by analyzing clay soil CRM at 110 mg kg^{-1} extractable PO_4^{3-}-P was: 2.4 mg kg^{-1}. The expanded uncertainty was 4.8 mg kg^{-1} at 95 % confidence level (coverage factor k = 2). The target uncertainty was calculated following Eurochem/CITAC guide 2015 [31] and based on the soil fertility status classification according to Good practice in agriculture [25].

The target expanded uncertainty was calculated based on the compliance interval approach following the equation [28]:

$$U^{target} = \frac{Q^{max} - Q^{min}}{8} * 1.3$$

where the compliance interval Q_{max} and Q_{min} was defined to be at least 26 mg kg^{-1} [25]; the coefficient 1.3 was based on the recommendation of Eurachem guide that if target uncertainty was not defined in regulatory documents, an addition of 20-30 % was allowed to the variability of the estimation of the analytical process. Thus, target uncertainty was estimated to be 34 mg/kg PO_4^{3-}-P. The results presented above demonstrated that the expanded uncertainty was below the estimated target uncertainty. The obtained result motivated us to study the expended uncertainty based on the real soil sample analyzed during one year as a quality control. The combined uncertainty was 3.7 at 20 mg PO_4^{3-}-P/kg and 4.6 at 55 mg PO_4^{3-}-P/kg real soil samples. The values obtained demonstrate that the uncertainty slightly depended on the concentration at the studied concentration interval. The expended uncertainty was 9.2 mg PO_4^{3-}-P/kg at 55 mg PO_4^{3-}-P/kg dry soil obtained at 95 % confidence level (coverage factor k=2).

Internal Quality control

The performance of spectrophotometric measurement was monitored applying the following criteria: (1) the absorbance reading and corresponding concentration calculated by mean calibration curve within 5% of the standard concentration of a quality control standard solution (prepared by appropriate dilution of RM); (2) the regression coefficient for the calibration equation $R^2 \geq 0.99$; (3) the relative standard deviation of a set of three absorbance readings ≤ 0.6 %. If the criteria were not met, the calibration standards, MB reaction reagents and the calibration were renewed. The method and laboratory performance were monitored by analyzing test soil samples [19]. The soil samples containing: 20 and 55 mg PO_4^{3-}-P per kg dry soil, were used as internal quality control samples and were analyzed in duplicates with each batch of 12 soil samples for the period of one year. The control charts were constructed and the warning and action limits were set at ± 1 x s and ± 2 x s (s = 3.6 mg kg^{-1}, n= 41) as recommended by SOP [32].

Conclusion

This paper presents a study on the molybdenum blue reaction (MB) as a finishing detection step in soil analysis for quantification of plant available phosphorus. An acetate/lactate solution at pH 4.2 was used as an extracting reagent. The molybdenum blue color reaction by preliminary mixed Murphy reagent and ascorbic acid as a reductant was reinvestigated. The MB was found to obey Beer's law in the targeted concentration range of 0.04 – 1.0 mg L^{-1} PO_4^{3-}-P at 880 nm and 60 min reaction time. The linearity was proved by "lack-of-fit" test. One-year stability of calibration curve was demonstrated. The uncertainty was estimated by modelling approach, as well as single laboratory and quality control approach. The recovery and the expanded uncertainty were found to be (95.5 ± 8.7) % (P = 95 %, n = 3) and 9 mg PO_4^{3-}-P/kg at 55 mg PO_4^{3-}-P/kg dry soil at coverage factor 2. The results showed that the soil sample inhomogeneity and the repeatability of extraction chemistry were the main factors which contribute to the uncertainty of measurement in real soil samples. The obtained result would benefit further study on the comparison of different extracting solutions for plant available phosphorus determination, as well as field based experiments on phosphorus uptake by plants.

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