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# The molybdenum blue reaction for the determination of orthophosphate revisited: Opening the black box

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# 5 **The molybdenum blue reaction for the determination of** 6 **orthophosphate revisited: Opening the black box**

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## 13 **Abstract**

14 The molybdenum blue reaction, used predominantly for the determination of orthophosphate in  
15 environmental waters, has been perpetually modified and re-optimised over the years, but this core  
16 reaction in analytical chemistry is usually treated as something of a 'black box' in the analytical  
17 literature. A large number of papers describe a wide variety of reaction conditions and apparently  
18 different products (as determined by UV-visible spectroscopy) but a discussion of the chemistry  
19 underlying this behaviour is often addressed superficially or not at all. This review aims to  
20 rationalise the findings of the many 'optimised' molybdenum blue methods in the literature, mainly  
21 for environmental waters, in terms of the underlying polyoxometallate chemistry and offers  
22 suggestions for the further enhancement of this time-honoured analytical reaction.

23 **Keywords:** Molybdenum blue reaction; orthophosphate; dissolved reactive phosphate;  
24 phosphomolybdate

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## 1 **List of Abbreviations**

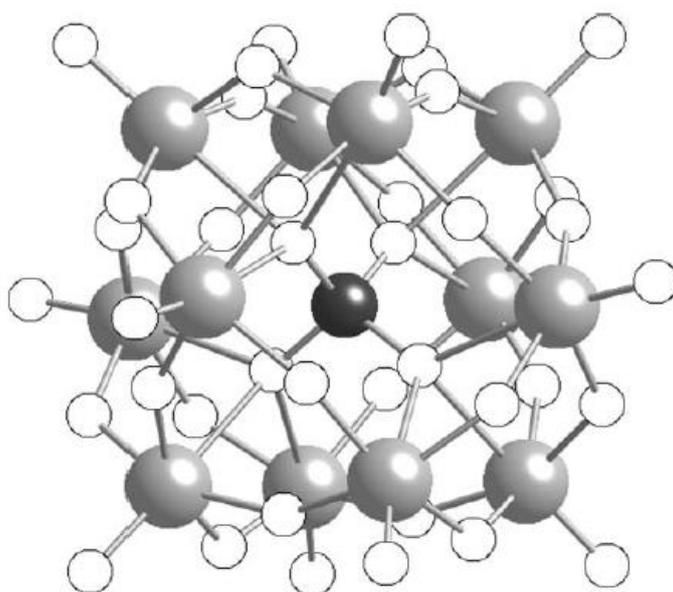
- 2 (br): Broad absorption band
- 3 (sh): Absorption shoulder
- 4 12-MPA: 12-Molybdophosphoric acid ( $\text{H}_3\text{PMo}_{12}\text{O}_{40}$ )
- 5 11-MPA: 11-Molybdophosphoric acid ( $\text{H}_3\text{PMo}_{11}\text{O}_{37}$ )
- 6 12-MSA: 12-Molybdosilicic acid ( $\text{H}_4\text{SiMo}_{12}\text{O}_{40}$ )
- 7 AA: Ascorbic acid
- 8 ANS: 1-Amino-2-naphthol-4-sulfonic acid
- 9 AsMB: Arsenomolybdenum blue
- 10 DA: Discrete analyser
- 11 DAPH: 2,4-Diaminophenol dihydrochloride
- 12 DOP: Dissolved organic phosphorus MB: Molybdenum blue
- 13 DRP: Dissolved reactive phosphorus
- 14 ESI-MS: Electrospray ionisation mass spectrometry
- 15 FIA: Flow injection analysis
- 16 HQ: Hydroquinone
- 17 HS: Hydrazine sulfate
- 18 IVCT: Intervalence charge transfer
- 19 LMCT: Ligand-metal charge transfer

- 1 Metol: 4-(Methylamino)phenol sulfate
- 2 MRP: Molybdate reactive phosphorus
- 3 PMB: Phosphomolybdenum blue
- 4 rFIA: Reverse flow injection analysis
- 5 SFA: Segmented continuous flow analysis
- 6 SIA: Sequential injection analysis
- 7 SiMB: Silicomolybdenum blue
- 8 TDP: Total dissolved phosphorus
- 9

## 1. Introduction

Orthophosphate is a key water quality parameter and spectrophotometric detection using the molybdenum blue (MB) reaction is the most common means of determination [1]. It can also be used for the spectrophotometric determination of silicate, arsenate and germanate. Strictly, this reaction determines the 'molybdate reactive phosphorus' (MRP) fraction which includes other labile phosphorus species in addition to orthophosphate [2] as discussed in Section 4.1.

The reaction involves the formation of a polyoxometallate species, a heteropoly acid, from orthophosphate and molybdate under acidic conditions, which is then reduced to form an intensely coloured phosphomolybdenum blue (PMB) species. This reaction was mentioned by Scheele in 1783, but is widely attributed to Berzelius (1826) [3]. It was not until 1934, however, that Keggin proposed the structures of a range of 12-heteropoly acids [4] (**Fig. 1**). 'Molybdenum blue' refers not to a single species, but rather to a family of reduced molybdate compounds, which may or may not contain a heteroatom, e.g. phosphorus. Distinction between heteropoly (containing a hetero-atom) and isopoly (containing no hetero-atom) molybdenum blue species is made in this review where necessary.



16

1 **Figure 1.** Structure of the Keggin ion  $[\text{PW}_{12}\text{O}_{40}]^{3-}$ , analogous to that of  $[\text{PMo}_{12}\text{O}_{40}]^{3-}$ . The black,  
2 grey and white spheres represent P, W and O respectively. Reproduced from Ref. [5] with  
3 permission from The Royal Society of Chemistry

4 A fundamental knowledge of the inorganic chemistry of the MB reaction is important for  
5 optimising its analytical application for the determination of phosphate. In particular, the  
6 concentrations of the reagents can be optimised to maximise the degree of product formation and  
7 product stability (for batch methods) and achieve good precision and accuracy.

8 This review systematically summarises the fundamental chemistry of the MB reaction and discusses  
9 the optimal conditions for the selective determination of MRP using batch methods under  
10 equilibrium conditions. The additional requirements for non-equilibrium, flow-based methods are  
11 also considered.

12

## 13 **2. Chemistry of the molybdenum blue reaction**

### 14 **2.1. Reaction overview**

15 The MB reaction occurs in two stages; the first stage involves the formation of a Keggin ion around  
16 the analyte anion and the second stage entails the reduction of this heteropoly acid to form a deeply  
17 blue-coloured product. These stages can be described in the simplified forms shown in Eqs. (1) and  
18 (2) [2].

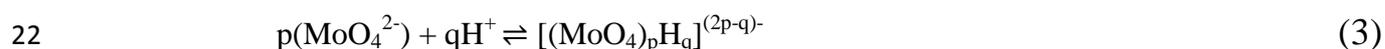


21 All MB methods require a strong acid, a source of Mo(VI) and a reductant, normally in aqueous  
22 solution. The concentrations of acid and molybdate are vital, not only for the formation of the  
23 heteropoly acid but also for controlling its reduction. It is well-known that orthophosphate ( $\text{PO}_4^{3-}$ ),

1 like other tetrahedral anions of the form  $XO_4$ , form Keggin ions of composition  $[X^{n+}Mo_{12}O_{40}]^{(8-n)-}$   
2 where X is the heteroatom. In the case of orthophosphate, this species is known as 12-  
3 molybdophosphoric acid (12-MPA). It is also well-established that procedures for orthophosphate  
4 determination are best carried out at pH 0 – 1; the reasons for this are seldom explained in terms of  
5 the molybdate chemistry but are empirically selected to give the best colour intensity. Many  
6 reductants and reaction conditions have been used in the MB reaction, typically giving rise to a  
7 mixture of reduced products as evidenced by the wide variety of absorption maxima and molar  
8 absorptivities reported.

## 9 **2.2. Mo(VI) speciation and 12-MPA formation**

10 An understanding of acidic molybdate speciation and the mechanism of heteropoly acid formation  
11 in the MB reaction is necessary to optimise the practical application of this reaction. Key factors are  
12 the acid and molybdate concentrations, sources of interference and the range of chemical conditions  
13 under which this reaction is useable. The MB reaction for orthophosphate is usually performed  
14 between pH 0 - 1, as this range appears to be necessary to form suitable amounts of stable reduced  
15 product without excessive direct reduction of Mo(VI). However, it is not pH alone that determines  
16 Mo(VI) speciation; rather, it is a combination of acid concentration and molybdate concentration.  
17 These parameters determine the ‘degree of protonation’, Z, which is defined as the average number  
18 of protons bound to molybdate in solution [6-9]. Z reflects the complex solution equilibria of  
19 Mo(VI); at a given Mo(VI) concentration, the Z value of a solution can be used to predict the  
20 predominant molybdate species present as a function of pH (Table 1). Z is defined as the ratio q / p  
21 in Eq. (3).



23

24

**Table 1:** Predicted pH values for maximum concentrations of Mo(VI) species in 10 mmol L<sup>-1</sup> Mo(VI) solution. Shorthand (dehydrated) forms for molybdic acid and the two cations are also shown.

Species	Name	Z	pH
MoO <sub>4</sub> <sup>2-</sup>	Molybdate	0.00	7.00
HMoO <sub>4</sub> <sup>-</sup>	H-Molybdate	1.00	4.58
Mo <sub>2</sub> O <sub>7</sub> <sup>2-</sup>	Dimolybdate	1.00	4.58
Mo <sub>7</sub> O <sub>24</sub> <sup>6-</sup>	Heptamolybdate	1.14	4.30
HMo <sub>7</sub> O <sub>24</sub> <sup>5-</sup>	H-Heptamolybdate	1.29	3.64
Mo <sub>3</sub> O <sub>10</sub> <sup>2-</sup>	Trimolybdate	1.33	3.52
H <sub>2</sub> Mo <sub>7</sub> O <sub>24</sub> <sup>4-</sup>	H <sub>2</sub> -Heptamolybdate	1.43	2.85
Mo <sub>4</sub> O <sub>13</sub> <sup>2-</sup>	Tetramolybdate	1.50	2.25
HMo <sub>2</sub> O <sub>7</sub> <sup>-</sup>	H-Dimolybdate	1.50	2.25
Mo <sub>8</sub> O <sub>26</sub> <sup>4-</sup>	Octamolybdate	1.50	2.25
HMo <sub>3</sub> O <sub>10</sub> <sup>-</sup>	H-Trimolybdate	1.67	1.48
Mo <sub>6</sub> O <sub>19</sub> <sup>2-</sup>	Hexamolybdate	1.67	1.48
HMo <sub>4</sub> O <sub>13</sub> <sup>-</sup>	H-Tetramolybdate	1.75	1.33
HMo <sub>5</sub> O <sub>16</sub> <sup>-</sup>	H-Pentamolybdate	1.80	1.28
Mo <sub>11</sub> O <sub>34</sub> <sup>2-</sup>	Undecamolybdate	1.82	1.27
HMo <sub>6</sub> O <sub>19</sub> <sup>-</sup>	H-Hexamolybdate	1.83	1.27
Mo <sub>12</sub> O <sub>37</sub> <sup>2-</sup>	Dodecamolybdate	1.83	1.25
MoO <sub>3</sub> or MoO <sub>3</sub> (OH) <sub>2</sub>	Molybdic acid	2.00	1.20
HMo <sub>2</sub> O <sub>6</sub> <sup>+</sup> or Mo <sub>2</sub> O(OH) <sub>9</sub> (OH <sub>2</sub> ) <sup>+</sup>	Molybdate dimer cation	2.50	< 1.20
HMoO <sub>3</sub> <sup>+</sup> or MoO <sub>2</sub> (OH)(OH <sub>2</sub> ) <sup>+</sup>	Molybdate monocation	3.00	< 1.20

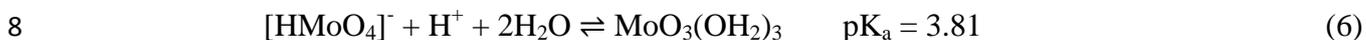
\* Species and Z values are drawn from Refs. [6-8, 10-20]

- 1 Using this ratio is not as simple as using the often cited  $[H^+]/[Mo(VI)]$  ratio, but the latter is
- 2 generally misleading as discussed in Section 3.1. Instead, the total proton concentration in solution

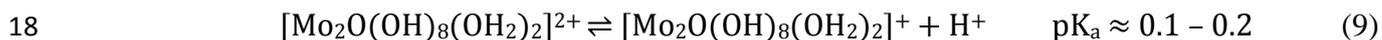
1  $([H]_{\text{total}}^+)$  involving both free  $H^+$  ( $[H]_{\text{free}}^+$ ) and  $H^+$  bound to protonated and/or condensed Mo(VI)  
 2 must be considered. In addition to the definition above,  $Z$  is also expressed in terms of the  
 3 concentrations of the relevant species in solution (Eq. (4)), and has been determined for a wide  
 4 range of solution compositions (Fig. 2).

$$5 \quad Z = \frac{[H]_{\text{total}}^+ - [H]_{\text{free}}^+}{[\text{Mo(VI)}]} \quad (4)$$

6 The molybdate anion can be protonated twice to form neutral molybdic acid (Eqs. (5) – (6)) [21].

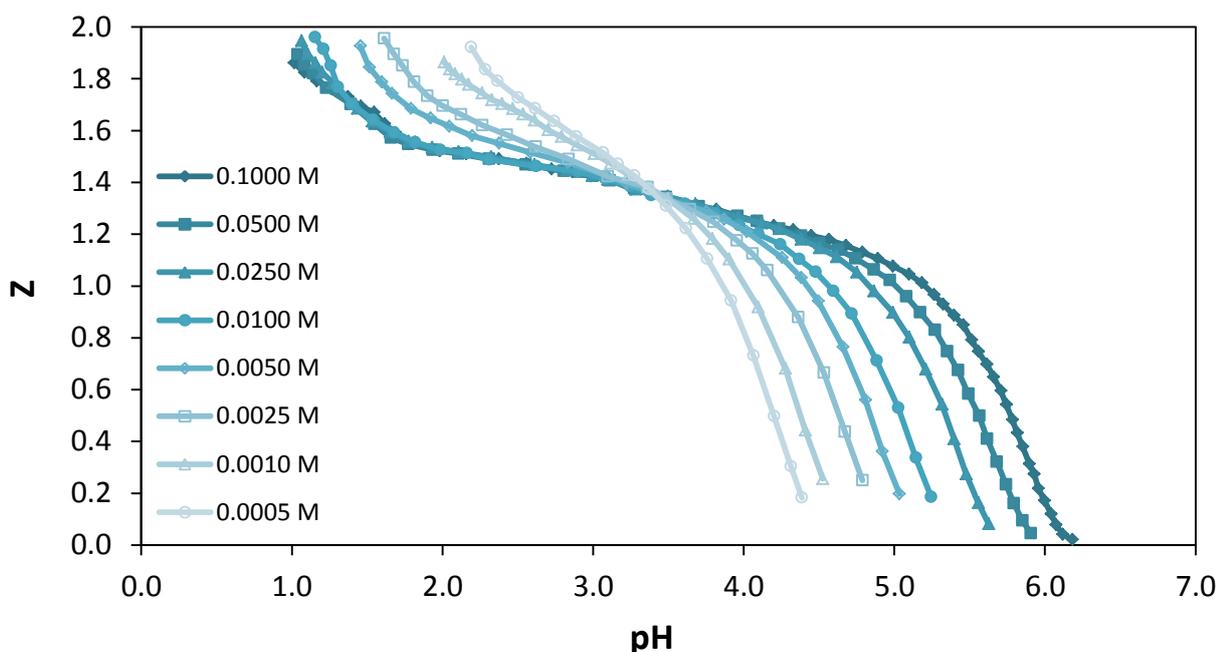


9 When the solution is sufficiently acidic, the second protonation also involves a change in the  
 10 coordination chemistry of Mo(VI) from the 4-coordinate  $\text{HMoO}_4^{2-}$  to the 6-coordinate hydrated  
 11 molybdic acid  $\text{MoO}_3(\text{OH}_2)_3$ , which significantly decreases both of its  $\text{pK}_a$  values [13, 22]. The  
 12 process of hydrating molybdic acid in the second protonation step requires that the acid  
 13 concentration be at least ten-fold in excess of that of Mo(VI), otherwise precipitation of poorly  
 14 soluble  $\text{MoO}_3$  may occur [23]. However, molybdic acid will begin to protonate and dimerise when  
 15  $Z > 2$  (Eqs. (7) – (9)) [8, 11, 16, 17, 24, 25], which is undesirable for 12-MPA formation.

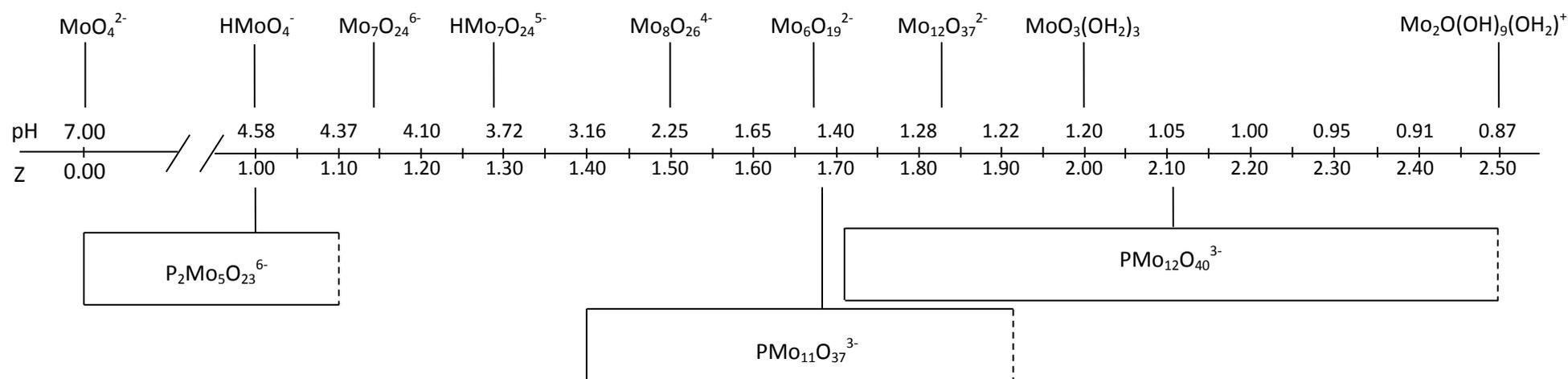


19 In order for acidified Mo(VI) to polymerise, which is a prerequisite for the formation of MB,  
 20 Mo(VI) concentrations of at least  $10^{-3} - 10^{-2} \text{ mol L}^{-1}$  are required [24]. The prevalent species in  
 21 solution at various  $Z$  values, assuming  $10 \text{ mmol L}^{-1}$  Mo(VI), are shown in Table 1, whilst the  
 22 relationship between pH,  $[\text{Mo(VI)}]$  and  $Z$  is shown in Fig. 2, and the Mo(VI) solution equilibria are

1 shown in Fig. 3. The low Mo(VI) concentrations and ionic strengths used in the MB reaction  
2 actually simplify matters, as higher polymolybdates tend to form when either of these parameters is  
3 considerably higher [6, 14, 26]. It should also be noted that the choice of  $\text{Na}_2\text{MoO}_4$  or  
4  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 7\text{H}_2\text{O}$  for MB methods is irrelevant due to the equilibria shown in Fig. 3, provided  
5 that sufficient time is allowed for the equilibration of Mo(VI) species before the solution is used.  
6 Whilst it has been reported that several hours are needed for this process in pure solutions of either  
7  $\text{Na}_2\text{MoO}_4$  or  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$  [27], equilibration appears to occur much more quickly if the  
8 solution is pre-mixed with acid, requiring only about 10 - 30 min [26, 28]. Furthermore,  $\text{Na}_2\text{MoO}_4$   
9 may be the favoured Mo(VI) salt in laboratories where P and  $\text{NH}_3$  determination are carried out  
10 simultaneously, in order to avoid cross-contamination.

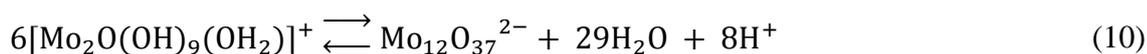


**Figure 2.** Plots of the degree of protonation,  $Z$ , vs.  $\text{pH}$  at different Mo(VI) concentrations in  $1 \text{ mol L}^{-1}$   $\text{NaCl}$  to reduce the influence of ionic strength variation. Reproduced from Ref. [6] with permission from Elsevier.



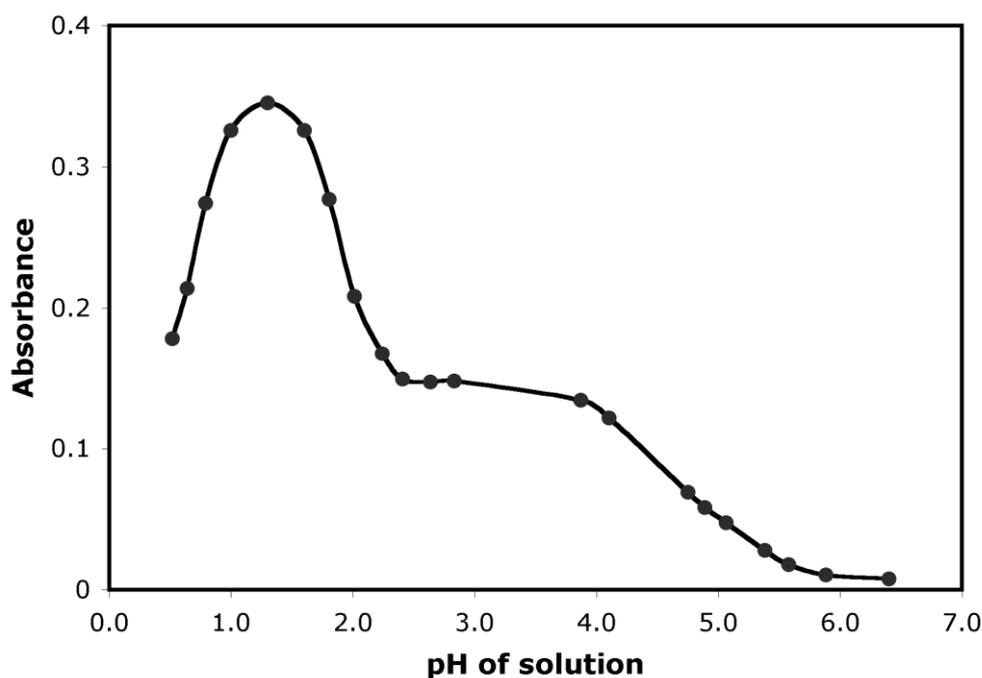
**Figure 3.** Mo(VI) speciation in aqueous solution containing  $10 \text{ mmol L}^{-1}$  Mo(VI) as a function of Z (bottom), or as a function of pH (top), and the phosphomolybdate species which may form under these conditions (deprotonated forms shown for clarity). The optimal Z value for the formation of each phosphomolybdate and Mo(VI) species is indicated by a vertical line. The width of the box for each phosphomolybdate denotes the Z range in which it has been observed; a dashed line indicates that the prevalence of the complex at higher Z values has not been clearly characterised. Note that pH values for  $Z > 2$  have been extrapolated from literature data (Fig. 2) [6], and represent approximations only. Phosphomolybdate speciation data adapted from Ref. [8] with permission from Elsevier.

1 It is important to consider how 12-MPA is formed from its precursors, as a number of studies  
 2 addressing this question are based on several early conclusions made before more detailed data  
 3 about Mo(VI) equilibria were available in the literature. For example, explanations of both pH-  
 4 related behaviour [29] and the observed rate laws for 12-MPA formation [23, 27, 30, 31] were  
 5 initially based on conclusions about Mo(VI) and H<sup>+</sup> stoichiometry obtained under highly acidic  
 6 conditions [27] which favoured monomeric and dimeric Mo(VI) cations (Table 1), and were not  
 7 optimal for 12-MPA formation. It was suggested in these studies that the assembly of 12-MPA  
 8 occurred via the reaction of cationic Mo(VI) dimers with H<sub>3</sub>PO<sub>4</sub> and the release of H<sup>+</sup> [23, 27, 30,  
 9 31], although these authors also allowed for the possibility of a pre-equilibrium between the dimers  
 10 and a larger Mo(VI) ion which constituted the actual reactive species. More recent UV-visible  
 11 spectrophotometry and Raman spectroscopy studies [7, 8, 10, 32] have shown that this latter  
 12 consideration was in fact much more accurate than the theory of cationic dimers as the reactive  
 13 species, as it accounts for the decomposition of 12-MPA as well as the inhibition of its formation at  
 14 high acidities where dimeric Mo(VI) cations actually predominate. In fact, dodecamolybdate  
 15 (Mo<sub>12</sub>O<sub>37</sub><sup>2-</sup>) appears to be the main precursor Mo(VI) species in equilibrium with 12-MPA [7, 8,  
 16 10], and a detailed mechanism of 12-MPA formation is given later in this section. The prevalence of  
 17 the dodecamolybdate anion at very low pH values is minimal since the condensation of cationic  
 18 Mo(VI) species into larger ions such as Mo<sub>12</sub>O<sub>37</sub><sup>2-</sup> requires the release of H<sup>+</sup> into an already highly  
 19 acidic solution, as per Eq. (10). Since high Z solutions are unfavourable for the existence of the 12-  
 20 MPA precursor species, 12-MPA dissociates under these conditions.



21 Another important consideration is the [Mo(VI)] / [P] ratio used in some studies of  
 22 phosphomolybdate speciation. When [Mo(VI)] / [P] < 12, the speciation of phosphomolybdates as a  
 23 function of Z changes markedly, yet it has previously been presumed that speciation under these  
 24 conditions is true of all P – Mo(VI) systems [33]. In MB methods, Mo(VI) is present in large excess  
 25 over P, and these Mo(VI)-deficient conditions do not apply.

1 It has been shown via Raman spectroscopy that each anionic molybdate species in the presence of  
2 orthophosphate favours the formation of one particular molybdophosphoric acid [8] (Fig. 3) where  
3  $[\text{Mo(VI)}] / [\text{P}] \geq 12$ , with mixtures of Mo(VI) species leading to mixtures of heteropoly acids. For  
4 example, both 12-MPA and 11-MPA are formed effectively where molybdic acid,  
5 dodecamolybdate and hexamolybdate are prevalent [32] (Fig. 4). It is therefore important to use  
6 reaction mixtures with  $Z \approx 2$ , or  $\text{pH} \leq 0.9$  assuming a Mo(VI) concentration of  $10 \text{ mmol L}^{-1}$ , to  
7 ensure orthophosphate is present only as 12-MPA and not as a mixture of 12-MPA and its  
8 hydrolysis product, 11-MPA. 11-MPA is very difficult to reduce [33], and the molar absorptivity of  
9 its  $4e^-$ -reduced form is probably quite low owing to its low molecular symmetry [34].

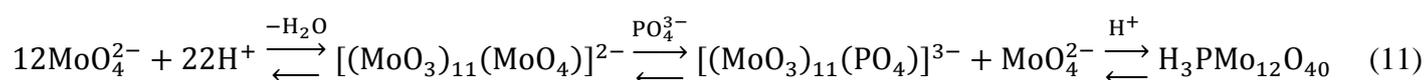


10

11 **Figure 4.** Influence of pH on the formation of phosphomolybdate species in aqueous solution;  $[\text{P}] =$   
12  $1 \text{ mmol L}^{-1}$ ,  $[\text{Mo(VI)}] = 12 \text{ mmol L}^{-1}$ ,  $\lambda = 420 \text{ nm}$ . Whilst the absorbance above approximately pH  
13 1 arises from a combination of phosphomolybdate species, note the sharp decline in absorbance  
14 towards lower pH values. Redrawn from Ref. [32] with permission from Elsevier.

15 Two mechanisms for 12-MPA formation have been proposed based on the known equilibria of  
16 Mo(VI) and time-resolved Raman spectroscopy measurements of individual Mo(VI) species [10].

1 The first mechanism (Eq. (11)), known as the 'displacement' mechanism, occurs when  
 2 orthophosphate is added to Mo(VI) pre-acidified to pH 1, in which orthophosphate displaces the  
 3 central labile molybdate unit from pre-existing dodecamolybdate to form 11-MPA. This is clearly  
 4 shown by the existence of an isosbestic point between the two latter species [10]. 11-MPA then  
 5 reacts with the displaced (and now acidified) molybdic acid unit to form 12-MPA; the rate-limiting  
 6 step is the final formation of 12-MPA from 11-MPA.



7 The displacement mechanism (Eq. (11)) best matches the conditions normally used in MB methods,  
 8 as the sample is typically introduced into a pre-mixed reagent solution in both batch-wise and flow-  
 9 based methods. Furthermore, in MB methods, the ratio of [Mo(VI)] / [P] in solution is high, thus  
 10 accelerating the reaction. For example, a method that uses 10 mmol L<sup>-1</sup> Mo(VI) to determine 1 mg  
 11 L<sup>-1</sup> P (32 μmol L<sup>-1</sup>) as orthophosphate will exhibit a [Mo(VI)] / [P] ratio of over 300, whereas  
 12 Murata and Ikeda in their Raman spectroscopy work used only the stoichiometric ratio of 12 [10]. It  
 13 should be noted that if conditions change such that [Mo(VI)] / [P] ≤ 14, 12-MPA will decompose  
 14 into 11-MPA, 9-MPA and ultimately [P<sub>2</sub>Mo<sub>5</sub>O<sub>23</sub>]<sup>6-</sup> [8, 35]. It is therefore expected that a large  
 15 excess of Mo(VI) over P will drive the phosphomolybdate equilibria to form 12-MPA almost  
 16 exclusively, rather than leaving significant residual amounts of 11-MPA.

17 The alternative mechanism, known as the 'ground-up' mechanism, occurs when acid is added to a  
 18 pre-mixed solution of orthophosphate and Mo(VI) and is much more rapid than the displacement  
 19 mechanism. This mechanism involves the assembly of 12-MPA around an orthophosphate ion.  
 20 Raman spectroscopy has indicated that only MoO<sub>4</sub><sup>2-</sup> appears to exist in solution before acidification  
 21 [10], yet it has previously been reported that orthophosphate binds so strongly to molybdate in  
 22 solution that it cannot exist as the free ion in the presence of the latter [32]. Indeed, interactions  
 23 between orthophosphate and several condensed molybdates have been reported, each leading to the

1 formation of a different phosphomolybdate species [8, 28]. It is reasonable to conclude, then, that  
2 this 'ground-up' mechanism proceeds via initial reaction of orthophosphate with a low-Z molybdate  
3 species [10] to form a smaller phosphomolybdate, followed by rapid equilibration to 12-MPA upon  
4 acidification via the condensation of Mo(VI) (Fig. 3).

5 When 12-MPA is finally formed, it is inevitably protonated to some extent, contrary to early  
6 opinion [36]. Its  $pK_a$  values in aqueous solutions have been determined as 2.4, 4.31 and 5.46 [37];  
7 12-MPA should therefore exist mainly as  $H_3PMoO_{40}$  in MB reaction mixtures. At pH values where  
8 the trianion is prevalent, the complex decomposes in aqueous solutions [32].

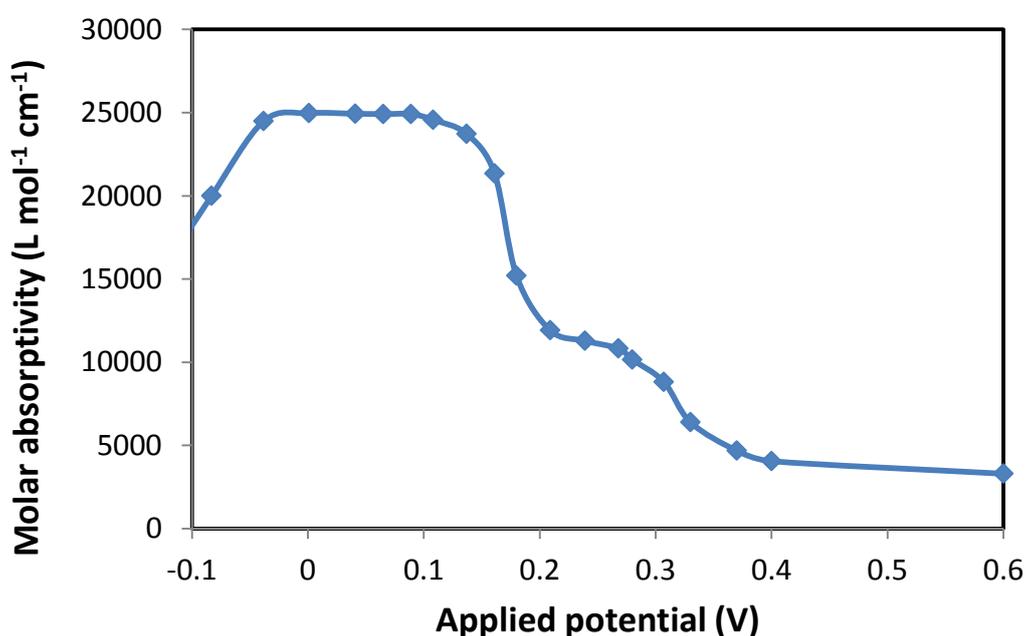
## 9 **2.3. Redox chemistry of PMB**

### 10 **2.3.1. Reduction of 12-MPA**

11 The reduction of 12-MPA to form PMB is not a trivial process, as the nature of the product(s) is  
12 determined by a number of factors such as the reductant, pH, reaction time, temperature, and  
13 presence of interfering ions. Knowledge of these factors is therefore of great importance in  
14 developing MB methodology. In this discussion, the degree of reduction of the PMB species is  
15 denoted using the number of electrons they are reduced by, i.e. PMB( $2e^-$ ) refers to the two-electron  
16 reduced species, and PMB( $4e^-$ ) refers to the four-electron reduced species.

17 It is well-established that the redox behaviour of 12-MPA is highly acid-dependent; the reduction of  
18 aqueous 12-MPA occurs in single electron steps at higher pH values, whilst this behaviour changes  
19 to two-electron steps under acidic conditions [38-42] via proton-induced disproportionation of the  
20 odd-electron reduced species [43]. Each two-electron reduction step of 12-MPA is accompanied by  
21 the addition of two protons [38, 39], and increasing solution acidity shifts its reduction potential to  
22 more positive values [38, 39] since the overall negative charge of the complex is decreased as it  
23 protonates. In fact, the correlation between the anionic charge of 12-MPA and its first reduction  
24 potential is linear [38, 43]. Voltammetric analysis of 12-MPA in aqueous  $0.2 \text{ mol L}^{-1} \text{ H}_2\text{SO}_4$

1 solutions ( $\text{pH} \approx 0.78$ ) has shown that 12-MPA may undergo two successive and reversible two-  
2 electron reductions at +0.31 V and +0.17 V [42], which decrease to +0.27 V and + 0.13 V at pH 1  
3 [44]. A third two-electron reduction at about -0.08 V is possible but this renders the product highly  
4 unstable, resulting in immediate decomposition [42] (Fig. 5). It must be noted that reduced  
5 heteropoly acids are unstable with respect to re-oxidation by dissolved  $\text{O}_2$  [45]; their continued  
6 existence in aqueous solutions depends on an excess of reductant to react preferentially with  $\text{O}_2$ , an  
7 aspect of reaction optimisation which is discussed in Section 3.3.



8

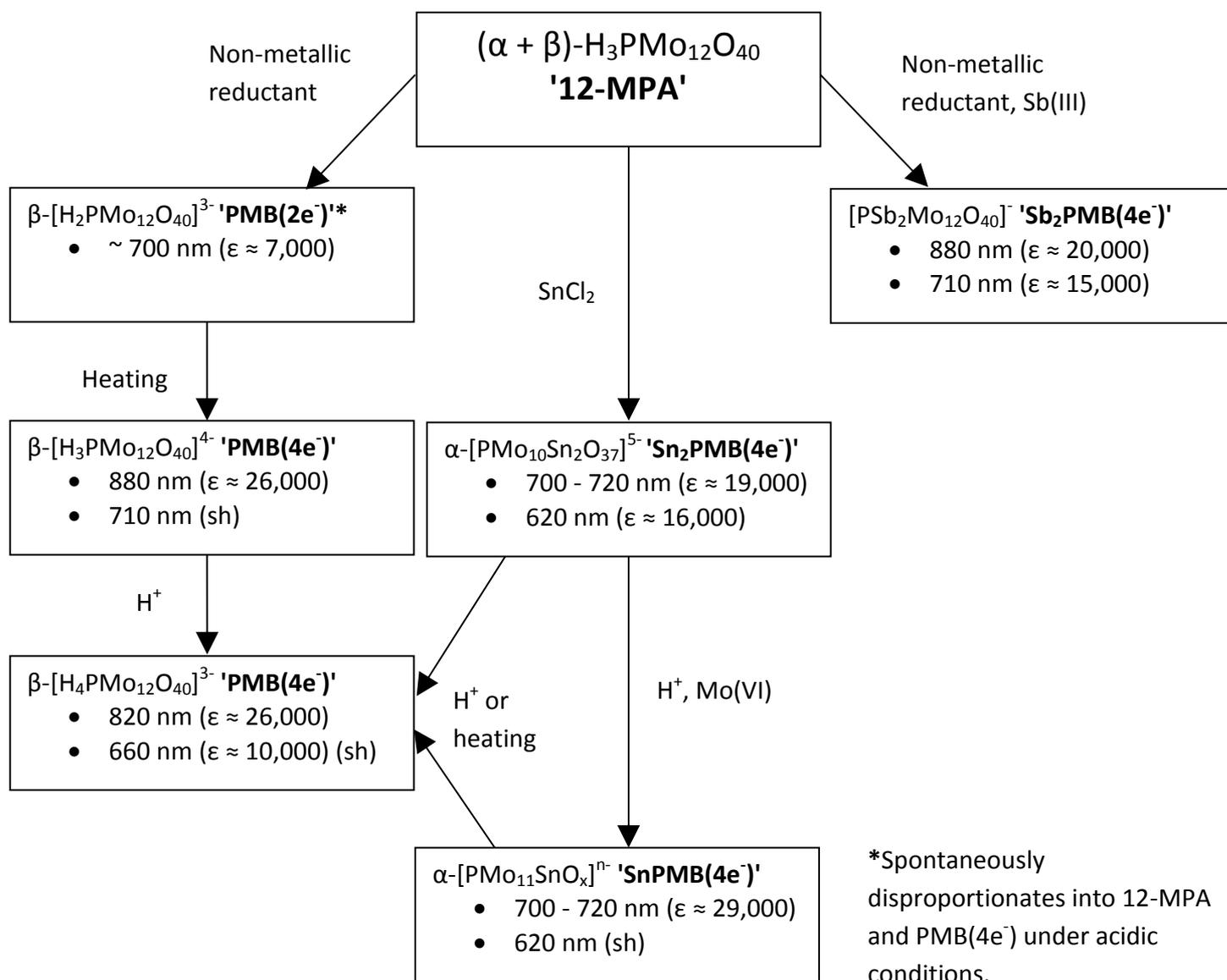
9 **Figure 5.** Molar absorptivity at 830 nm of a 12-MPA solution as a function of applied potential  
10 during its electrochemical reduction.  $[\text{H}_2\text{SO}_4] = 0.2 \text{ mol L}^{-1}$ ,  $[\text{12-MPA}] = 2 \text{ mmol L}^{-1}$ . Adapted with  
11 permission from Tanaka et al. [42]. Copyright 2015 American Chemical Society.

12 Furthermore, the ability of Keggin-type structures to delocalise metal d-electrons influences their  
13 redox behaviour in an unusual way; it is now well-established that each added electron pair is  
14 delocalised across all twelve Mo atoms [46-48], thus avoiding multiple reduction of any individual  
15 Mo centre. In general, the reduction of 12-MPA by  $n$  electrons (omitting further protonation) can be  
16 described by Eq. (12).



2 **2.3.2. Spectral features of PMB**

3 It has frequently been observed that the use of different reductants – or even the same reductant  
4 under different conditions – yields products with different UV-visible spectra and various apparent  
5 molar absorptivities, thus generating considerable confusion as to the ‘optimal’ reaction conditions  
6 and measurement wavelengths [40, 49-51]. However, the nature of the absorbing species produced  
7 by the reduction step is rarely described in any detail, and generalisations have instead been made  
8 about the stoichiometric behaviour of a given reductant, rather than the actual MB species in  
9 solution [27, 52]. The reaction conditions required to form each different heteropoly blue, as well as  
10 their spectral properties, are discussed in the remainder of Section 2 and are summarised in Fig. 6.



1

2 **Figure 6.** Spectrophotometric properties of the various PMB species and the conditions under  
3 which they form. Molar absorptivities ( $\epsilon$ ) are in units of L mol<sup>-1</sup> cm<sup>-1</sup>. (sh) = absorption shoulder.

4 The UV-visible-NIR spectra of PMB species contain seven individual absorption bands, each of  
5 which corresponds to a particular structural feature (**Table 2**), and the spectra of the heteropoly  
6 molybdenum blue species (phosphate, arsenate and silicate species) all behave in a similar manner.  
7 However, only two of these bands are of interest for MB methodology; namely, the strong inter-  
8 valence charge transfer bands which arise due to electron exchange between Mo(V) and Mo(VI)  
9 centres. Contrary to some earlier claims, it is not the d-d bands of Mo(V) which are responsible for  
10 its intense absorption [53]; their nature as symmetry-forbidden transitions marks them as orders of  
11 magnitude weaker than charge transfer bands, and they appear as little more than faint shoulders on

1 typical MB spectra if they are discernible at all (Table 2).

2 **Table 2.** UV/visible spectral features of PMB(4e<sup>-</sup>) species.

Wavelength (nm)	Type of transition	Assignment	Band intensity
220	LMCT	Mo-O	Strong, decreases with further
315	LMCT	Mo-O-Mo bridges	reduction. Not specific to reduced species.
550	d-d	Mo(V)	Very weak, often obscured
760	d-d	Mo(V)	Very weak, often obscured
600 - 700	IVCT	Mo(V) → Mo(VI)	Moderate, $\epsilon \approx 10,000 \text{ L mol}^{-1}$ $\text{cm}^{-1}$
700 - 900	IVCT	Mo(V) → Mo(VI)	Strong, $\epsilon \approx 26,000 - 34,000 \text{ L}$ $\text{mol}^{-1} \text{ cm}^{-1}$
1500	IVCT	Mo(V) → Mo(VI)	Very weak, obscured

IVCT denotes an inter-valence charge transfer transition. LMCT denotes a ligand-metal charge transfer transition. Data obtained from refs. [34, 48, 54-56].

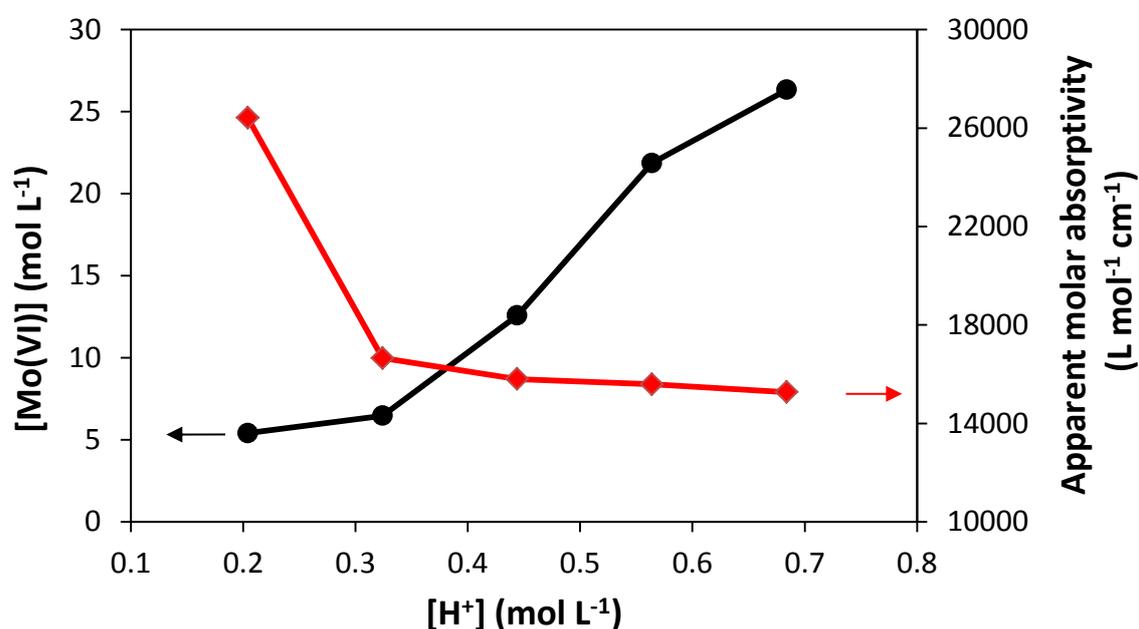
3 The molar absorptivities of the strong IVCT bands are significantly higher for PMB(4e<sup>-</sup>) than  
4 PMB(2e<sup>-</sup>) [54, 55, 57], but excessive reduction yields PMB(6e<sup>-</sup>) which immediately decomposes in  
5 acid [40, 42, 58]. This demonstrates that whilst a higher degree of reduction is desirable for  
6 improving analytical sensitivity, overwhelming reducing power should be avoided. The positions of  
7 the intense IVCT absorption bands are dependent on a number of factors discussed below.  
8 Molar absorptivity values of PMB in various reported methods have been used as a means of  
9 comparing analytical sensitivity between methods. However, usage of this term as a comparative  
10 measure is often incorrect. All methods which use non-metallic reductants ultimately produce the

1 same product with the same innate molar absorptivity,  $[\text{H}_4\text{PMo}_{12}\text{O}_{40}]^{3-}$ , or the  $2e^-$  reduced species as  
2 an intermediate when heating is not used (Fig. 6); differences in apparent absorptivity are simply  
3 due to the extent of the reduction process at the time of measurement or the influence of organic  
4 solvents [42, 56, 59]. The molar absorptivities of PMB species can be difficult to determine since  
5 quantitative formation of a single PMB species is necessary. The difficulty lies in the nature of MB  
6 chemistry as one complex equilibrium system; rather than stoichiometric reactions occurring,  
7 sufficient reagent must be introduced to perturb the equilibria to a desirable extent. Thus, if one  
8 attempts to simply dissolve solid 12-MPA in acid and reduce it without adding Mo(VI), very low  
9 apparent absorptivities will be obtained [40], as most of the 12-MPA will in fact decompose in  
10 accordance with its formation equilibria (Fig. 3) [40, 60]. Thus, sufficient Mo(VI) must be present  
11 to stabilise 12-MPA against hydrolytic decomposition.

12 According to Tanaka et al. [42], electrochemically formed  $\text{PMB}(4e^-)$  exhibits an absorptivity of  
13 approximately  $25,000 \text{ L mol}^{-1} \text{ cm}^{-1}$  at 830 nm (Fig. 5). This study also indicates a molar  
14 absorptivity of  $\sim 11,000 \text{ L mol}^{-1} \text{ cm}^{-1}$  at potentials where  $\text{PMB}(2e^-)$  should be the main product.  
15 However, given that  $\text{PMB}(2e^-)$  is expected to undergo acid-induced disproportionation to 12-MPA  
16 and  $\text{PMB}(4e^-)$  [58, 61] (Fig. 6), and that the UV-visible spectrum of the product obtained at these  
17 potentials was identical to that of  $\text{PMB}(4e^-)$ , it can be concluded that the species observed here was  
18 not  $\text{PMB}(2e^-)$  at all, but  $\text{PMB}(4e^-)$  formed in approximately 50% yield. The molar absorptivity of  
19  $\text{PMB}(2e^-)$  is likely to be around  $7,000 \text{ L mol}^{-1} \text{ cm}^{-1}$  at its absorption maximum of  $\sim 700 \text{ nm}$ , based  
20 on the ratio between peak intensities of similar heteropoly blue species [58].

21 The more Mo(VI) is added to the reaction, the more the formation of 12-MPA is favoured. The  
22 obvious question then is how much Mo(VI) can be added without the resulting gains in sensitivity  
23 being offset by direct Mo(VI) reduction, which is the phenomenon responsible for the reagent blank  
24 in MB methods. In keeping with the equilibria discussed at length above, the concentration of  
25 Mo(VI) needed to maintain constant Z is a non-linear function of acidity, whilst the acidity itself  
26 strongly affects the stability of 12-MPA. At a given acid concentration, the amount of 12-MPA

1 formation and reduction increases sharply with increasing Mo(VI) concentration, until a certain  
2 critical Mo(VI) concentration is reached. At this point, the further formation of PMB tapers off and  
3 the direct reduction of Mo(VI) to form isopolymolybdenum blues begins [40]. Thus, it can be seen  
4 that whilst the Mo(VI) concentration can be easily optimised for each acidity, sensitivity is quickly  
5 lost at higher acidities (Fig. 7) (i.e. Z values) due to 12-MPA decomposition into cations (Fig. 3).  
6 Note that heating the reaction allows this effect on 12-MPA formation to be overcome to a great  
7 extent (Table 3), but at the expense of sampling rate.



8  
9 **Figure 7.** Plot of acidity against the maximum tolerable Mo(VI) concentration (●) before direct  
10 reduction of Mo(VI) to isopoly MB begins, and the apparent molar absorptivity of the SnPMB(4e<sup>-</sup>)  
11 product at 700 nm (◆) under these conditions (Data obtained from El-Shamy and Iskander [40].  
12 [12-MPA] = 40 μmol L<sup>-1</sup> for molar absorptivity data, [SnCl<sub>2</sub>] = 880 μmol L<sup>-1</sup>).

13

Table 3. Comparison of chemical and spectrophotometric parameters of batch MB methods.

Authors (Year)	Acid	[Acid] (mmol L <sup>-1</sup> )	[Mo(VI)] (mmol L <sup>-1</sup> )	[Reductant] (mmol L <sup>-1</sup> )	Temperature (°C), (reaction time)	$\lambda_{\max}$ (nm)	$\epsilon$ (L mol <sup>-1</sup> cm <sup>-1</sup> )	Notes	Ref
Tanaka (1982)	H <sub>2</sub> SO <sub>4</sub>	200	2 (12-MPA)	Electroreduction	-	830	25,000	Optical path length uncertain	[42]
Fontaine (1942)	H <sub>2</sub> SO <sub>4</sub>	1000	36	SnCl <sub>2</sub> : 210	100 (20 min)	820	28,000		[62]
Sims (1961)	HCl	650	17	SnCl <sub>2</sub> : 2.43	-	815	26,400		[63, 64]
El Sayed (2001)	H <sub>2</sub> SO <sub>4</sub>	50	1.3	SnCl <sub>2</sub> : 1.04	-	700	23,000	Broad peak, shoulder at ~ 820 nm	[65]
Kriss (1971)	H <sub>2</sub> SO <sub>4</sub>	300	5	SnCl <sub>2</sub> : 5	(< 3 h)	680	20,400		[66]
Levine (1955)	HCl	600	11	SnCl <sub>2</sub> : 0.16	-	735	19,000		[67]
El-Shamy	HCl	840	20 & 0.08	SnCl <sub>2</sub> : 1.93	-	810	17,000		[40]

(1973)			(12-MPA)					Peaks in equilibrium	
	HCl	360	20 & 0.08	SnCl <sub>2</sub> : 1.93	-	720	16,000	via isosbestic point at	[40]
			(12-MPA)					780 nm	
	HCl	450	0.165	SnCl <sub>2</sub> : 0.66	-	810	4,400	Broad shoulder around	[40]
			(12-MPA)					720 nm	
	HCl	280	0.24	SnCl <sub>2</sub> : 1.44	-	700	1,200	Shoulder at 820 nm	
			(12-MPA)						[40]
Hesse	H <sub>2</sub> SO <sub>4</sub>	300	31	SnCl <sub>2</sub> : 0.13	(20 min)	730	25,500		[68]
(1968)				HS: 2.31					
Drummond	H <sub>2</sub> SO <sub>4</sub>	127	2.4	AA: 9.6	(12 h)	820	26,760		[29]
(1995)									
Han	H <sub>2</sub> SO <sub>4</sub> ,	330,	14	AA: 54	45 (20 min)	825	26,000		[69]
(1995)	HCl	170							
Chen	H <sub>2</sub> SO <sub>4</sub>	300	14	AA: 57	37 (90 min)	820	25,000		[70]
(1956)									
Lowry	pH 4		5.7	AA: 5.7	(5 min)	860	4,600	Very similar	[71]

								absorbance from 700 –	
								900 nm	
(1946)	Katewa	H <sub>2</sub> SO <sub>4</sub>	500	14	AA: 11.4	(60 min)	820	26,100	[49]
(2003)					HS: 15.4				
Sims (1961)	H <sub>2</sub> SO <sub>4</sub>	500	10.3	HS: 0.46	100	815	32,300	[63]	
					(unspecified)				
Ganesh	H <sub>2</sub> SO <sub>4</sub>	500	39	HS: 9.61	60 (30 min)	830	29,000	[72]	
(2012)									
Boltz	H <sub>2</sub> SO <sub>4</sub>	500	10.3	HS: 0.46	93 (10 min)	830	26,400	[73]	
(1947)									
Huey	HClO <sub>4</sub>	1200	24	HS: 1.8	100 (15 min)	805	14,000	[60]	
(1967)									
Burton	H <sub>2</sub> SO <sub>4</sub>	30	2.95	Metol: 2.9	100 (2 h)	860	29,900		
(1956)				Na <sub>2</sub> SO <sub>3</sub> : 87					
	H <sub>2</sub> SO <sub>4</sub>	200	2.95	Metol: 2.9	100 (2 h)	820	29,300	[50]	
				Na <sub>2</sub> SO <sub>3</sub> : 87					
	H <sub>2</sub> SO <sub>4</sub>	30	2.95	Metol: 1.5	(2 h)	720,	3,900	Extremely broad	

				Na <sub>2</sub> SO <sub>3</sub> : 44		830		absorbance peak
						(sh)		
Sims (1961)	HClO <sub>4</sub>	920	143	Metol: 0.33	100 (10 min)	820	18,500	[63]
				NaHSO <sub>3</sub> : 30				
				Na <sub>2</sub> SO <sub>3</sub> : 4.0				
Harris (1954)	HClO <sub>4</sub>	480	11.3	Metol: 1.16	-	820	3,900	[63, 74]
				NaHSO <sub>3</sub> : 96				
				Na <sub>2</sub> SO <sub>3</sub> : 15				
El Sayed (2001)	H <sub>2</sub> SO <sub>4</sub>	30	11	ANS: 0.11	90 (30 min)	830	32,000	[65]
Sims (1961)	HClO <sub>4</sub>	920	143	ANS: 0.33	100 (10 min)	820	28,100	[63]
				NaHSO <sub>3</sub> : 30				
				Na <sub>2</sub> SO <sub>3</sub> : 4.0				
Griswold (1951)	H <sub>2</sub> SO <sub>4</sub>	500	14.2	ANS: 0.42	100 (10 min)	820	27,000	[75]
				NaHSO <sub>3</sub> : 570				
				Na <sub>2</sub> SO <sub>3</sub> : 16				
Fiske	H <sub>2</sub> SO <sub>4</sub>	250	14	ANS: 0.42	-	730	3,900	[76, 77]

(1925)				NaHSO <sub>3</sub> : 57.7					
Sims	HClO <sub>4</sub>	920	143	DAPH: 0.33	100 (10 min)	820	34,500		[63]
(1961)				NaHSO <sub>3</sub> : 30					
				Na <sub>2</sub> SO <sub>3</sub> : 4.0					
Huo	H <sub>2</sub> SO <sub>4</sub>	300	10.3	Thiamazole: 0.245	-	710	1,000		[78]
(2012)									
Kriss	H <sub>2</sub> SO <sub>4</sub>	300	5	Fe(II): 5	(< 3 h)	700	12,800		[66]
(1971)									
Kriss	H <sub>2</sub> SO <sub>4</sub>	30	5	HQ: 5	(< 3 h)	700	12,800		[66]
(1971)									
Salem	H <sub>2</sub> SO <sub>4</sub>	76	2.8	Oxalyldihydrazide:	100 (10 min)	880	33,000	Shoulder at 820 nm	[79]
(1991)				0.017					
Gupta	H <sub>2</sub> SO <sub>4</sub>	200	5.4	AA: 4.8	(10 min)	882	25,670		[80]
(1981)				Sb(III): 0.066					
Going	H <sub>2</sub> SO <sub>4</sub>	150	3.5	AA: 4.5	(10 min)	880	22,400	$\epsilon = 26400$ (840 nm)	[81]
(1974)				Sb(III): 0.8				without Sb.	

Pai (1990)	H <sub>2</sub> SO <sub>4</sub>	200	5.4	AA: 4.8 Sb(III): 0.066	(30 min)	880	22,400	[82]
Harwood (1969)	H <sub>2</sub> SO <sub>4</sub>	200	2.7	AA: 23 Sb(III): 0.32	(10 min)	890	20,600	[83]
Edwards (1965)	H <sub>2</sub> SO <sub>4</sub>	57	1.5	AA: 1.4 Sb(III): 0.15	(10 min)	880	20,400	About 4% EtOH required to dissolve Sb-PMB precipitate for P > 1 mg L <sup>-1</sup> [84]
Murphy (1962)	H <sub>2</sub> SO <sub>4</sub>	200	5.4	AA: 4.8 Sb(III): 0.066	(10 min)	882	22,400	[85]
Drummond (1995)	H <sub>2</sub> SO <sub>4</sub>	127	2.4	AA: 9.6 Sb(III): 0.058	(1.5 min)	880	21,680	[29]

---

Table abbreviations: AA; ascorbic acid, HS; hydrazinium sulfate, HQ; hydroquinone, ANS; 1-amino-4-naphthol-2-sulfonic acid, DAPH; 2,4-diaminophenol dihydrochloride, 12-MPA; 12-molybdophosphoric acid, EtOH; ethanol, Metol; 4-(methylamino)phenol sulfate, PMB; phosphomolybdenum blue.

### 14 2.3.3. Nature of the reduced products

15 It is well-known that the UV-visible spectrum of a particular method's PMB product(s) is modified,  
16 potentially dramatically, if the concentrations and proportions of reagents are altered (Table 3). The  
17 question we seek to resolve here, then, is what these absorbing species actually are and how their  
18 equilibria can be controlled in order to best optimise a MB method. These data are summarised in  
19 Fig. 6. Furthermore, since the spectral behaviour of the various 12-heteropoly blues is very similar,  
20 studies on arseno- and silico-molybdate species are useful for understanding their phosphorus  
21 counterparts.

22 The positions of the two intensely absorbing IVCT bands are determined by the degree of reduction,  
23 the protonation state of the absorbing species, and whether or not a metallic reductant is used. The  
24 separation between the two bands is also dependent on these parameters, but more so on the degree  
25 of reduction; the IVCT bands of PMB(2e<sup>-</sup>) are much more widely separated than those of PMB(4e<sup>-</sup>).

26 **Table 4.** Effect of protonation on IVCT absorption maxima and approximate molar absorptivities  
27 for  $\beta$ -[X<sup>n+</sup>Mo<sub>12</sub>O<sub>40</sub>]<sup>(12-n)-</sup>. Roman numerals denote the number of electrons the compound is reduced  
28 by.

---

**Protonation and reduction state**      **Absorption maxima (nm) in aqueous solution. Approximate molar absorptivities (L mol<sup>-1</sup> cm<sup>-1</sup>) and peak shapes are given in parentheses where possible; (br) = broad absorption peak, (sh) = absorption shoulder.**

	X = As <sup>5+</sup>	X = Si <sup>4+</sup>	X = P <sup>5+</sup>
IV	1110 (6600), 741(8400)		1050, 765
HIV	990 (9600),	1000 (br), 760 (br)	

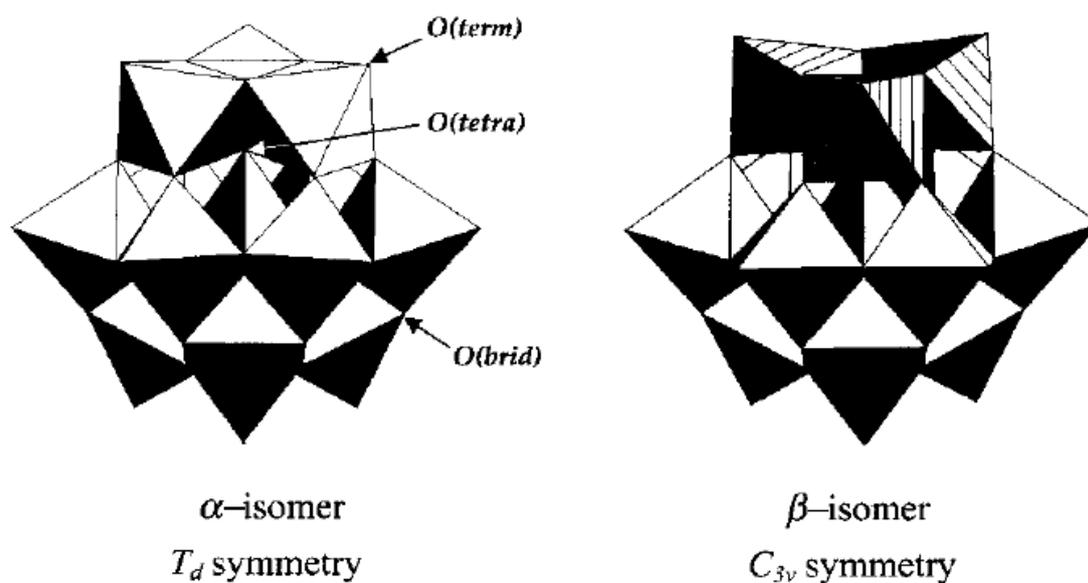
	752 (9000)		
H <sub>2</sub> IV	935 (16000), 719 (sh)	925, 720 (sh)	
H <sub>3</sub> IV	885 (25000), 704 (sh)	880, 700 (sh)	880 (26000), 700-710 (sh)
H <sub>4</sub> IV	840 (25000), 667 (sh)	820, 660-680 (sh)	820 (26000), 660 (sh)
II		1120 (br), 720	1030, 760
H <sub>2</sub> II		1050, 680	~ 1000, 730 (7000, br)

---

Data obtained from refs. [42, 50, 54-56, 58, 61, 76, 79, 86]

29 As can be seen in Table 4, protonation has a profound effect on the absorptivities and positions of  
30 the PMB(4e<sup>-</sup>) IVCT bands. It is now established that for four-electron reduced heteropoly acids, the  
31 first three protons are strongly acidic, whereas the remainder are weakly acidic [58, 61, 87]. For  
32 example, the weak pK<sub>a</sub> values for SiMB(4e<sup>-</sup>) are 2.8, 3.8, 7.1 and 9.5 [58], whilst those for  
33 AsMB(4e<sup>-</sup>) are ~ 3, 4.5, 7.2 and 9.5 [61]. Given that orthophosphate MB methods are generally  
34 performed at pH < 1, it can be expected that PMB(4e<sup>-</sup>) will almost always be present as  
35 [H<sub>4</sub>PMo<sub>12</sub>O<sub>40</sub>]<sup>3-</sup>, with the possibility of [H<sub>3</sub>PMo<sub>12</sub>O<sub>40</sub>]<sup>4-</sup> existing at very low acid concentrations.  
36 This assignment is confirmed by the spectra of electrochemically formed PMB(4e<sup>-</sup>) [42],  
37 characterisation of pure PMB(4e<sup>-</sup>) at pH 1 [86], and ion-pair HPLC [56]. These two highly  
38 protonated forms of PMB(4e<sup>-</sup>) are amongst the most intensely absorbing of any PMB species, with  
39 virtually identical absorptivities at their respective wavelength maxima (Fig. 6).

40 On the other hand, PMB( $2e^-$ ) is rarely desirable or even observed; it has considerably lower molar  
41 absorptivities at its peak wavelengths compared with those of PMB( $4e^-$ ) which renders it an inferior  
42 chromophore, and its oxidising ability will readily continue the reduction to PMB( $4e^-$ ) in the  
43 presence of further reductant. PMB( $2e^-$ ), like its As and Si analogues, can be presumed to also be  
44 unstable with respect to acid-induced disproportionation, spontaneously forming PMB( $4e^-$ ) and 12-  
45 MPA [58, 61]. Therefore, this species is generally only obtained via electrochemical means in  
46 organic solvents [54] or transiently with a kinetically slow reductant [74, 77]; on the rare occasions  
47 when it is encountered, its IVCT bands are distinctively separated by 350 - 400 nm, such that only  
48 the broad  $\sim 700$  nm band is observed in the visible region [54, 56].



49

50 **Figure 8.** Polyhedral representations of the  $\alpha$ - and  $\beta$ -isomers of the Keggin structure. Point group  
51 symmetries are indicated, and the labels in parentheses distinguish between the three types of O  
52 atoms (terminal, bridging, or part of the central tetrahedron). The  $\beta$ -isomer is obtained from the  $\alpha$ -  
53 isomer by a  $60^\circ$  rotation of one  $MO_3O_{13}$  subgroup. Reprinted with permission from [88]. Copyright  
54 2015 American Chemical Society.

#### 55 2.3.4. Isomerism of 12-MPA and its reduced forms

56 12-MPA is typically encountered as one of two isomers; the  $\alpha$ -isomer is the nominal Keggin  
57 structure and is the stable form of unreduced 12-MPA, whereas the  $\beta$ -isomer, obtained by a  $60^\circ$   
58 rotation of a  $\text{Mo}_3\text{O}_{13}$  group, is the more stable form of the reduced species (Fig. 8) [88, 89]. The  
59 optical and redox properties of the two isomers differ, in that the  $\beta$ -isomer is reduced at more  
60 positive potentials and exhibits more intense IVCT bands [34, 55, 89]. This phenomenon is well-  
61 known in silicomolybdate chemistry where the two isomers can be isolated in aqueous solution  
62 under distinctly different conditions [90-92], but has been overlooked in analytical methods based  
63 on phosphomolybdate. This is probably because  $\beta$ -MPA is less stable than its silicon counterpart  
64 [88], and whilst evidence suggests that the two isomers form simultaneously from different  
65 molybdate precursors in fresh 12-MPA solutions [89, 91, 93],  $\beta$ -MPA isomerises to  $\alpha$ -MPA within  
66 minutes [89]. However, isomerisation from  $\alpha \rightarrow \beta$  appears to begin in earnest once the reduction  
67 process begins, and voltammetric data for aqueous 12-MPA indicate that nearly 10 min after the  
68 first reduction of pure  $\alpha$ -MPA, the ratio of  $\alpha$ - and  $\beta$ -isomers is nearly 1:1 [42]. Because the  $\beta$ -isomer  
69 is the thermodynamically favoured form of the reduced species, heating will accelerate the  $\alpha \rightarrow \beta$   
70 transformation of PMB [94]. It should be noted, however, that the isomerism of PMB applies  
71 strictly only to  $[\text{H}_n\text{PMo}_{12}\text{O}_{40}]^{(7-n)-}$ , which is generally only obtained from the heating of organic  
72 reductants. Metallic reductants are capable of altering the composition of PMB and locking it into  
73 one particular isomer, a phenomenon discussed below.

### 74 **2.3.5. Organic reductants**

75 MB methods which use only organic reductants or hydrazine sulfate (the ‘non-metallic’ reductants)  
76 in combination with heating demonstrate a clear absorption maximum at 820 nm (Table 3), with a  
77 discernible shoulder at 660 nm. Electrochemical reduction of 12-MPA also demonstrates these  
78 features [42], which are attributed to the species  $\beta\text{-}[\text{H}_4\text{PMo}_{12}\text{O}_{40}]^{3-}$  (Table 4). An exception to this is  
79 seen in the method of Salem [79] where the unusually low acid concentration encourages the  
80 predominance of  $\beta\text{-}[\text{H}_3\text{PMo}_{12}\text{O}_{40}]^{4-}$  instead, with an absorption maximum at 880 nm instead of the  
81 usual 820 nm.

82 A heating step is necessary with these reductants due to the slow kinetics of 12-MPA reduction  
83 when using them, possibly because of the complex reaction steps required for their oxidation [27]  
84 or, more likely, their weak reducing power in acidic solutions (Eq. (13)) [95].



86 It should be pointed out that whilst virtually all methods which use these reductants exhibit molar  
87 absorptivities of at least  $25,000 \text{ L mol}^{-1} \text{ cm}^{-1}$ , several of them reportedly exhibit absorptivities of  
88  $32,000 - 35,000 \text{ L mol}^{-1} \text{ cm}^{-1}$  (Table 3), the highest such values for PMB reported in the analytical  
89 chemistry literature. However, there exists considerable variation in the molar absorptivity data  
90 reported in the literature, which is of some concern. For example, two studies using the Murphy and  
91 Riley method [80, 85] obtained apparent molar absorptivities differing by  $3,000 \text{ L mol}^{-1} \text{ cm}^{-1}$  (Table  
92 3), whilst the hydrazine sulfate methods of Sims and Boltz [63, 73] reported absorptivities  $6,000 \text{ L}$   
93  $\text{mol}^{-1} \text{ cm}^{-1}$  apart despite using very similar conditions and wavelengths which should have yielded  
94 very similar absorptivities (Table 3). These significant differences are noteworthy, since there is of  
95 course only one actual 'molar absorptivity' for a particular compound at a given wavelength. The  
96 differences in reported absorptivities are attributed to variations in handling procedures, extent of  
97 reaction, uncorrected blank formation, the spectrophotometric instrumentation used, and  
98 assumptions about apparent absorptivity versus actual molar absorptivity.

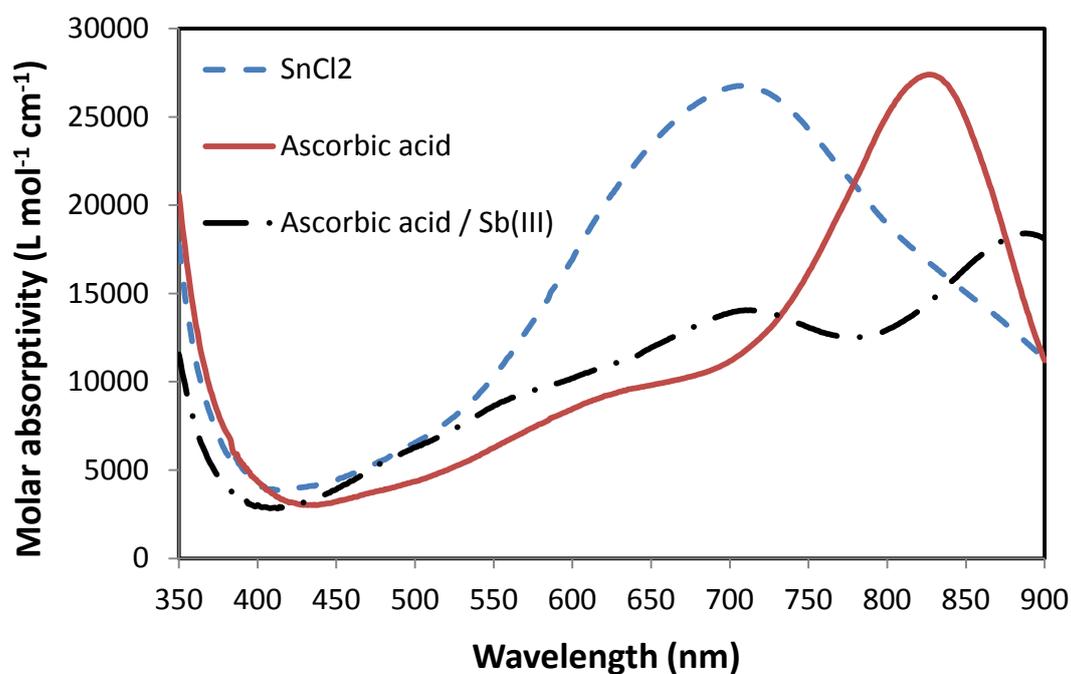
99 Aside from methods using  $\text{SnCl}_2$  as a reductant (discussed below), those which report an  
100 absorbance maximum at ca.  $720 \text{ nm}$  show vastly inferior molar absorptivities (Table 3). These  
101 methods do not use heating; as a result, the predominant reduction product is the two-electron  
102 reduced species  $[\text{H}_2\text{PMo}_{12}\text{O}_{40}]^{3-}$ , and the actual extent of  $12\text{-MPA} \rightarrow \text{PMB}(2\text{e}^-)$  reduction is  
103 expected to be quite low as well. Furthermore, this species is only transient, and will exhibit an  
104 unstable UV-visible spectrum over time as it disproportionates to  $\text{PMB}(4\text{e}^-)$  and 12-MPA.

### 105 **2.3.6. Metallic reductants**

106 The use of metallic reductants has a significant effect on MB chemistry, as the metals themselves

107 may be incorporated into the reduced product. Sn(II) and Sb(III) are the main species used in this  
108 regard; the former reduces 12-MPA very rapidly, whereas the latter is used to accelerate the  
109 reduction by an otherwise kinetically slow reductant such as ascorbic acid.

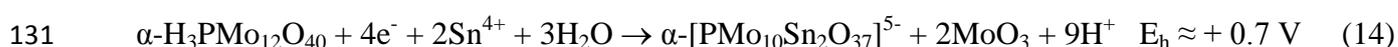
110 SnCl<sub>2</sub> is very widely used due to its fast reduction kinetics which obviates the need for heating.  
111 However, it is quite clear that 12-MPA reduction with SnCl<sub>2</sub> yields a distinctly different visible  
112 spectrum to that of [H<sub>4</sub>PMoO<sub>12</sub>O<sub>40</sub>]<sup>3-</sup> (Fig. 9) which cannot be accounted for via (de)protonation. In  
113 fact, instead of the conventional four-electron reduction of 12-MPA, the presence of newly-formed  
114 Sn(IV) ions permits a different and much more favourable reaction in which the four-electron  
115 reduced product, still in the  $\alpha$ -form, is immediately reacts with two Sn(IV) ions which substitute for  
116 Mo(VI) [94, 96]; whilst this behaviour has only been studied on 12-MSA, it appears analogous to  
117 that of 12-MPA. The vastly more positive reduction potential of 12-MPA in the presence of Sn(IV)  
118 has been discussed as the likely cause of the fast reduction kinetics observed with SnCl<sub>2</sub> [97]. The  
119 Sn(IV) substitution process is not observed with 12-MPA before reduction, nor does Sn(II)  
120 substitute for Mo after reduction.



121  
122 **Figure 9.** Comparison of product spectra for the three most commonly used reductants in MB  
123 methods. Ascorbic acid / Sb(III); prepared according to the procedure described in Standard

124 Methods for the Examination of Water and Wastewater [98]. Ascorbic acid; prepared as for  
125 ascorbic acid / Sb(III) procedure without the addition of Sb(III) and with heating at 100 °C for  
126 10 min. SnCl<sub>2</sub>; prepared using the working concentrations reported in [99]. All solutions contained  
127 500 µg L<sup>-1</sup> P as orthophosphate.

128 Thus, upon four-electron reduction of 12-MPA by SnCl<sub>2</sub>, the immediate product appears to be α-  
129 [PMo<sub>10</sub>Sn<sub>2</sub>O<sub>37</sub>]<sup>5-</sup> [94, 96], denoted as Sn<sub>2</sub>PMB(4e<sup>-</sup>), with an absorption peak between 700 - 720 nm  
130 (ε ≈ 19,000 L mol<sup>-1</sup> cm<sup>-1</sup>) and a pronounced shoulder at 620 nm.



132 The rapid kinetics of this reduction process are attributed to a combined reduction/substitution step  
133 with a much more positive reduction potential than for the direct reduction of 12-MPA alone (Eq.  
134 (14)) [97]. Over time, this species spontaneously undergoes the loss of one Sn(IV) ion to form the  
135 incompletely characterised SnPMB(4e<sup>-</sup>) species, as has been observed with its silicate analogue  
136 [94]. SnPMB(4e<sup>-</sup>) absorbs more intensely at 720 nm than Sn<sub>2</sub>PMB(4e<sup>-</sup>) whilst the absorptivity of  
137 the 620 nm band hardly changes, becoming a barely noticeable shoulder (Fig. 9) [96], and this  
138 mono-tin species is the main reduction product monitored in methods using SnCl<sub>2</sub> reduction. The  
139 spectral features of both products are reported in the analytical literature, i.e. Sn<sub>2</sub>PMB(4e<sup>-</sup>) [85, 100]  
140 and SnPMB(4e<sup>-</sup>) [66, 99]. The nature of the transformation of Sn<sub>2</sub>PMB(4e<sup>-</sup>) to the more intensely  
141 absorbing SnPMB(4e<sup>-</sup>) remains unclear, although it is not a redox process since Sn<sub>2</sub>PMB(4e<sup>-</sup>)  
142 should be stable with respect to oxidation [96]. However, this process is spontaneous, and is likely  
143 the result of attack by acidified Mo(VI) species [97].

144 SnPMB(4e<sup>-</sup>) may ultimately be hydrolysed to [H<sub>4</sub>PMo<sub>12</sub>O<sub>40</sub>]<sup>3-</sup> over time, although this process is  
145 accelerated with either heating [62, 94] or further acidification [40], indicated by the emergence of  
146 the 820 nm peak (Fig. 6). This process therefore appears to involve the displacement of Sn(IV) by  
147 molybdic acid, which seems to occur in the conversion of Sn<sub>2</sub>PMB(4e<sup>-</sup>) to SnPMB(4e<sup>-</sup>) as well.

148 These reactions are summarised in Fig. 6.

149 It should be pointed out that  $\text{SnCl}_2$  only undergoes the chemistry discussed above when it reduces  
150  $\alpha$ -isomers of heteropoly acids, and all of the stannomolybdate species discussed above are  
151 themselves  $\alpha$ -isomers [96]; the reduction of  $\beta$ -isomers yields  $\text{PMB}(4e^-)$  directly without  
152 incorporation of  $\text{Sn(IV)}$ . Given the tendency for  $\alpha$ - and  $\beta$ -MPA to form concurrently from  
153 orthophosphate and acidified molybdate [89], and the equilibrium between  $\text{SnPMB}(4e^-)$  and  
154  $\text{PMB}(4e^-)$ , it is to be expected that methods using  $\text{SnCl}_2$  will form a mixture of  $\text{PMB}(4e^-)$ ,  
155  $\text{SnPMB}(4e^-)$  and  $\text{Sn}_2\text{PMB}(4e^-)$  giving absorbing species with wavelength maxima 100 nm apart.  
156 This effect is clearly shown in the spectra recorded by a number of authors [40, 65, 85]. If an  
157 undesirable shoulder is seen at 820 nm, or the absorption peak is unusually broad, the acidity should  
158 be decreased to favour the formation of only the Sn-substituted products [40].

159 Antimony (Sb) has been of great importance in MB methodology since the work of Murphy and  
160 Riley in 1962 [85]. The presence of  $\text{Sb(III)}$  was originally found to greatly accelerate the reduction  
161 of 12-MPA by ascorbic acid, with the reaction going to completion in approximately 10 min  
162 without the need for heating [85, 101] and yielding a product which remains stable for hours. With  
163 sufficient ascorbic acid, this time can even be decreased to 1.5 min [29]. The development of  
164 methods using  $\text{Sb(III)}$  was of great practical significance, as it enabled the comparatively rapid  
165 determination of orthophosphate without lengthy heating steps. Whilst other organic reductants  
166 such as hydroxylamine salts [102] behave in the same manner as ascorbic acid in the presence of  
167  $\text{Sb(III)}$ , ascorbic acid's popularity as a reductant predates even Murphy and Riley's work by  
168 decades [103], presumably due to its ready availability and non-hazardous nature. Just as for the  
169 case of  $\text{SnCl}_2$ , the presence of  $\text{Sb(III)}$  has a significant effect on the visible IVCT bands of the PMB  
170 product, countering the early view that  $\text{Sb(III)}$  served only as a catalyst. 12-MPA reduced in the  
171 presence of  $\text{Sb(III)}$  exhibits an absorption maximum at ca. 880 nm, with a distinct second peak at  
172 710 nm and a shoulder in the vicinity of 550 nm (Fig. 9) [80, 85, 104]. These features are similar to  
173 the  $[\text{H}_3\text{PMo}_{12}\text{O}_{40}]^{4-}$  species (Table 4), except that the 710 nm band in the  $\text{Sb(III)}$  product is a  
174 distinct peak.

175 It has long been known that antimony becomes part of the reduced complex and a central element in  
176 the stoichiometry of the reaction. However, the original 1:1 Sb:P ratio determined by Murphy and  
177 Riley [85] has been called into question by more recent and reliable results that show a 2:1 ratio of  
178 Sb:P in the reduced species [80, 81, 105] instead. This conclusion is in excellent agreement with  
179 observations about method linearity; a comparison of Sb(III) MB methods has shown that the linear  
180 range is limited by antimony concentration, and has a cut-off precisely where the Sb:P ratio  
181 becomes less than 2:1 [83]. This phenomenon has been exploited to extend the linear range to more  
182 than three times that of Murphy and Riley's method [83], although adding excessive amounts of  
183 Sb(III) results in solubility problems, presumably due to hydroxide salts [83, 85]. Of course, this  
184 linear range is only with respect to absorbance at 880 nm; the further addition of orthophosphate  
185 results in the additional formation of the familiar  $[\text{H}_4\text{PMo}_{12}\text{O}_{40}]^{3-}$  species with IVCT bands at 820  
186 and 680 nm, giving the false impression that the 880 nm band blue-shifts with added  
187 orthophosphate due to the rising 820 nm band [51, 81, 83].

188 The structure of the reduced phosphoantimonymolybdate species  $\text{Sb}_2\text{PMB}(4e^-)$ , and the cause of its  
189 accelerating effect on the reduction kinetics, are not clearly established. The molar absorptivity of  
190  $\text{Sb}_2\text{PMB}(4e^-)$  at 880 nm compared with the conventional  $[\text{H}_4\text{PMo}_{12}\text{O}_{40}]^{3-}$  product at 820 nm under  
191 the same conditions is actually lower by 20 - 30% [29, 81, 85]; this trend is clearly confirmed in  
192 Table 3 and in Fig. 9. Nevertheless, molar absorptivities in the vicinity of  $20,000 \text{ L mol}^{-1} \text{ cm}^{-1}$  still  
193 suggest a four-electron reduced product. Aside from the Sb:P ratio of 2:1, the stoichiometry of the  
194 entire  $\text{Sb}_2\text{PMB}(4e^-)$  complex has never been established with certainty. Whilst it has been held for  
195 some time that the complex is probably  $[\text{PSb}_2\text{Mo}_{10}\text{O}_x]^{n-}$ , wherein Sb(III) is substituted for Mo(VI),  
196 this conclusion appears to be based only on the findings of Going and Eisenreich in 1974 [81],  
197 which were derived from an average measured Mo:P ratio of 11.4:1 and the observed stoichiometry  
198 of other heteropoly species. A recent ESI-MS study addressing this question found no evidence of  
199  $[\text{PSb}_2\text{Mo}_{10}\text{O}_4]^{n-}$  or protonated derivatives under any conditions, but did observe peaks in aqueous  
200 solutions of  $\text{pH} \approx 0.8$  corresponding to  $[\text{PSb}_2\text{Mo}_{12}\text{O}_{40}]^{n-}$  and species with one or both antimony

201 atoms removed in organic extracts of this solution [106]. Whilst these results have not yet been  
202 addressed by other studies, they nonetheless suggest that Sb does not replace Mo in the Keggin  
203 structure as originally thought; the twelve Mo atoms remain regardless of Sb addition.

204 Going and Eisenreich originally suggested that the dramatic increase in the reduction rate of 12-  
205 MPA in the presence of Sb(III) may be due to either a structural change in the heteropoly acid  
206 which facilitates reduction, or to Sb(III) acting as an electron relay by first reducing 12-MPA and  
207 forming Sb(V) which is then immediately reduced by ascorbic acid to Sb(III) [81]. An electron  
208 relay mechanism would require Sb(III) to act as a reductant before being subsequently reduced by  
209 ascorbic acid; Sb(III) capable of reducing 12-MPA directly but only at high temperatures [81, 107,  
210 108], and no reaction is observed under the conditions expected for the MB reaction [106]. This  
211 suggests, therefore, that Sb(III) does not act as an electron relay.

212 Sb(III) does not interact with acidified molybdate, nor does it alter the IVCT bands of pre-formed  
213 PMB [81]; it does not even appear to form an adduct with unreduced 12-MPA at ambient  
214 temperature [106], contrary to earlier assumptions [109]. However, it does redshift the characteristic  
215 UV charge transfer bands of 12-MPA, as do a number of other metals [81], suggesting at least weak  
216 coordination to the oxygen atoms of 12-MPA. The full  $\text{Sb}_2\text{PMB}(4e^-)$  adduct seems to form only  
217 during 12-MPA reduction [106]. Heteropoly complexes which contain metals as counter-cations are  
218 known to exist as charge-transfer complexes, in which the metal acts as the electron donor and 12-  
219 MPA is the electron acceptor; isolation of antimony salts in the solid phase has shown that Sb(III)  
220 can indeed partially reduce 12-MPA when in this configuration, although again only after a heating  
221 step [107, 110]. However, the observation that Sb(III) does not bind to PMB after reduction would  
222 seem to discount it acting only as a countercation or loosely coordinating species. Taken together,  
223 all of the above evidence suggests that much like in the case of  $\text{SnCl}_2$ , Sb(III)-facilitated reduction  
224 is a reaction distinct from the normal four-electron reduction of 12-MPA. Interestingly, in the  
225 presence of both Sb(III) and Sn(II), only the  $\text{SnPMB}(4e^-)$  product is observed, probably due to the  
226 much faster reduction process involving substitution of Sn(IV) into the product structure [111].

227 Despite these observations, the precise mechanism of  $\text{Sb}_2\text{PMB}(4\text{e}^-)$  formation and the redox  
228 potential for the  $\text{Sb}(\text{III})$ -facilitated reduction remain unknown.

229 Heating is sometimes applied in methods using  $\text{Sb}(\text{III})$  to further enhance the rate of reduction [82,  
230 112]. However, heating of this reaction can be problematic. Not only does it increase the impact of  
231 silicate interference [82, 109] as would be expected of any heated method (see Section 4.1.2), but  
232 higher reaction temperatures also appear to degrade the  $\text{Sb}_2\text{PMB}(4\text{e}^-)$  product [109, 112]. If the  
233 product is already fully formed and is exposed to successively higher temperatures, the UV-visible  
234 spectrum of the product exhibits lower apparent molar absorptivities at 710 and 880 nm with an  
235 isosbestic point at 935 nm [112]. This evidence suggests that at high temperatures, the  $\text{Sb}_2\text{PMB}(4\text{e}^-)$   
236 product equilibrates with one or more species with an absorption maximum at wavelengths longer  
237 than 950 nm (see Section 2.3.2). However, this spectral change was found to be almost entirely  
238 reversible upon cooling the reaction mixture again. In contrast, if the reaction itself is performed at  
239 elevated temperatures, the concentration of  $\text{Sb}_2\text{PMB}(4\text{e}^-)$  appears to decrease irreversibly, with  
240 considerably instability in absorbance at 880 nm during the reaction [112]. Pai et al. recommend  
241 that MB methods using  $\text{Sb}(\text{III})$  should not exceed a reaction temperature of  $35^\circ\text{C}$ .

### 242 **3. MB method optimisation**

243 The parameters of the MB reaction have traditionally been optimised for batch methods according  
244 to the following goals:

- 245 • Maximal sensitivity to orthophosphate
- 246 • Minimal blank absorbance
- 247 • Broad linear calibration range
- 248 • Stability of colour (i.e. absorbance) during the measurement interval
- 249 • Fast PMB formation

250 However, the goals of flow-based techniques such as flow injection analysis (FIA), reverse flow  
251 injection analysis (rFIA) and sequential injection analysis (SIA) differ considerably from those of  
252 batch, automated discrete analyser (DA) and air-segmented continuous flow analyser (SFA)  
253 methodology [113]. Whilst the demands for sensitivity, low blank absorbance and a broad linear  
254 range remain, the temporal stability of the product is no longer of particular importance since  
255 sample detection occurs at a fixed point during the reaction, i.e. under non-equilibrium conditions.  
256 However, reaction kinetics are still important in flow systems; whilst the extent of reaction is  
257 inherently reproducible in these systems, it is obviously advantageous to maximise the extent of  
258 reaction in a short timeframe to enhance sensitivity. It is therefore unsurprising that the SnCl<sub>2</sub> and  
259 Sb(III) methods now dominate the literature on both flow-based and batch orthophosphate detection  
260 using MB, as the reduction reaction occurs almost instantaneously with the former reagent, and  
261 within a few minutes with the latter. Furthermore, whilst the low and zero dispersion of SFA and  
262 DA methods respectively allow for the exact replication of optimised solution conditions from  
263 batch methods, the much higher degree of dispersion encountered using FIA, rFIA or SIA makes  
264 the same task impossible using these methods. Rather than dealing with discrete volumes of  
265 reaction mixture, such systems inherently create concentration gradients between neighbouring  
266 liquid zones, which are further subjected to a parabolic flow profile arising from friction with the  
267 walls of the manifold tubing.

### 268 **3.1. Reagent concentrations**

269 The optimisation and re-optimisation of MB methods has largely been based on the widespread and  
270 erroneous assumption that MB chemistry can be adequately described by the ratio of  
271  $[H^+]/[Mo(VI)]$ , at which the behaviour of the reaction supposedly remains constant regardless of the  
272 actual reagent concentrations used. The use of this ratio appears to have originated in the work of  
273 Strickland [114, 115], and has been used ever since as an attempt to summarise the optimal  
274 conditions for both the formation and reduction of 12-MPA, as well as blank minimisation.  
275 However, this ratio does not describe 12-MPA formation in solution due to the speciation of

276 Mo(VI) and the non-linear variation of Z with  $[H^+]$  and  $[Mo(VI)]$  discussed in Section 2. Pai et al.  
277 [82] have clearly demonstrated this by showing that PMB formation is not consistent in solutions  
278 where  $[H^+]$  and  $[Mo(VI)]$  are varied but  $[H^+]/[Mo(VI)]$  remains constant. If the  $[H^+]/[Mo(VI)]$  ratio  
279 were an accurate means of describing molybdate chemistry, either the pH should be invariant across  
280 all solutions of the same  $[H^+]/[Mo(VI)]$  ratio or one of the two variables should not affect the  
281 system at all [114]. Truesdale and Smith clearly showed that the pH varies considerably between  
282 solutions of various compositions with the same  $[H^+]/[Mo(VI)]$  ratio [114], and it is self-evident  
283 from any study optimising the MB method that both parameters strongly affect the system. In  
284 Strickland's work, however, the narrow range of Mo(VI) concentrations used did not affect product  
285 formation significantly, and the  $[H^+]/[Mo(VI)]$  ratio was thus effectively developed as an alternative  
286 acidity scale within a strict range of Mo(VI) concentrations [114, 115]. In fact, Truesdale and Smith  
287 commented that since the  $[H^+]/[Mo(VI)]$  ratio is an inappropriate fundamental framework for  
288 describing MB chemistry, its adoption has served to mislead further investigation of the system  
289 [91]. The use of this ratio has also been inconsistent between methods using monoprotic and  
290 diprotic acids; older studies using the normality scale for acid concentration clearly assume that  
291 both protons of  $H_2SO_4$  fully dissociate in the reagent solution, when in fact the second  $pK_a$  of  
292  $H_2SO_4$  is only 1.99 [116].

293 No single optimised combination of  $H^+$  and Mo(VI) concentrations is clearly evident in the  
294 literature. Methods involving heating tend to dramatically reduce the influence of reagent  
295 concentrations on the apparent absorptivity due to faster reaction kinetics.  $SnCl_2$  methods raise the  
296 question of a mixture of absorbing products and the reported absorptivities for Sb(III) methods  
297 using the same reaction conditions are inconsistent (Table 3). It is therefore of much greater  
298 practical use to recommend a procedure for the optimisation of the MB method based on the  
299 principles discussed in this review.

300 Whilst heating the MB reaction typically renders the yield of PMB rather insensitive to the acid and  
301 Mo concentrations used (Table 3), it is more practical to perform the reaction at room temperature.

302 Furthermore, prolonged heating appears to decompose both PMB(4e<sup>-</sup>) and Sb<sub>2</sub>PMB(4e<sup>-</sup>) [60, 112],  
 303 and any heating at all accelerates the spontaneous hydrolysis of the SnPMB(4e<sup>-</sup>) product [62, 94]  
 304 obtained from methods using SnCl<sub>2</sub> as the reductant. Without heating, however, the effects of  
 305 reagent concentrations on reaction kinetics and equilibrium positions become more important. At  
 306 this point, it is important to highlight the trend shown in Fig. 7; a greater Mo concentration will  
 307 always require a greater acidity to mitigate the reagent blank (discussed in Section 3.2), but the loss  
 308 of sensitivity due to 12-MPA decomposition at these higher acidities is significant. Thus, whilst the  
 309 acidity can easily be optimised for any given Mo concentration, method sensitivity will necessarily  
 310 decrease as [Mo(VI)] is increased. Therefore, the concentration of Mo(VI) must be sufficiently high  
 311 to stabilise 12-MPA at P concentrations appropriate for the analysis, but not so high as to warrant  
 312 deleterious concentrations of acid. Going and Eisenreich originally recommended that Mo(VI) be  
 313 used in at least a 40-fold molar excess over the maximum P concentration expected [81] although  
 314 their experiments focused on the [H<sup>+</sup>]/[Mo(VI)] ratio at different Mo(VI) concentrations.  
 315 Nevertheless, this recommendation broadly agrees with other studies which have found that Mo(VI)  
 316 concentrations as low as 3 – 5 mmol L<sup>-1</sup> are sufficient for extensive 12-MPA formation from  
 317 approximately 1 mg L<sup>-1</sup> P (Table 3). The lowest possible acidity should be used, such that PMB  
 318 decomposition is minimised but direct Mo(VI) reduction is still prevented or mitigated. El-Shamy  
 319 and Iskander found that at 5 mmol L<sup>-1</sup> Mo(VI), an acidity of only 0.20 mol L<sup>-1</sup> HCl was effective in  
 320 preventing isopoly MB formation [40]. However, whilst higher acidities at this Mo concentration  
 321 drastically inhibited PMB formation, no acidities lower than this value were examined.

322 **Table 5.** Chemical parameters and linear ranges of Sb(III) MB methods.

---

**Reagent concentrations at linear range maximum**

Author (Year)	[H <sup>+</sup> ] (mmol L <sup>-1</sup> )	[Mo(VI)] (mmol L <sup>-1</sup> )	[AA] (mmol L <sup>-1</sup> )	[Sb] (μmol L <sup>-1</sup> )	[P] (μmol L <sup>-1</sup> )	[Sb]/ [P]	[Mo(VI)]/ [P]	Ref.
Harwood	200	2.7	23	329	> 97	Excess	< 28	[83]

---

---

(1969)								
Edwards	57	1.5	1.4	149	71	2.1	21	[84]
(1965)								
Murphy	200	5.4	4.8	66	32	2.0	167	[85]
(1962)								
Drummond	127	2.4	96	58	~ 26	2.2	93	[29]
(1995)								

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323

324 Reductant concentrations vary widely across the literature (Table 3) depending on the species  
325 chosen, and typically tolerate much more variation without deleterious consequences compared  
326 with the acid and molybdate concentrations. For example, ascorbic acid has traditionally been used  
327 in large excess [81] for kinetic reasons; there is evidence to suggest that the reduction of 12-MPA  
328 by ascorbic acid is first order in the latter [27], and given its weak reducing ability in acidic  
329 solutions [95], a high concentration of ascorbic acid is indeed expected to be of some practical  
330 benefit. Whilst this approach is effective in Sb(III) methods [29] where complete 12-MPA reduction  
331 within a few minutes is desirable, it is probably unjustified when heating is used. In heated MB  
332 methods, sub-millimolar concentrations of an organic reductant are sufficient for extensive PMB  
333 formation in a similar timeframe to the Sb(III) methods (Table 3). Even at room temperature, SnCl<sub>2</sub>  
334 or Sb(III) methods require only low millimolar concentrations of reductant for effective use; most  
335 SnCl<sub>2</sub> studies report reductant concentrations of 1.0 – 2.5 mmol L<sup>-1</sup> SnCl<sub>2</sub>, and Sb(III) / ascorbic  
336 acid methods are effective with around 5 mmol L<sup>-1</sup> ascorbic acid. Even a 1 mmol L<sup>-1</sup> reductant  
337 concentration represents an approximately 30-fold molar excess over 1 mg L<sup>-1</sup> P. Clearly, the  
338 question is not one of stoichiometry, but of reduction potential and kinetics. Excessive reductant  
339 concentrations have long been known to induce direct Mo(VI) reduction after 12-MPA reduction is  
340 apparently complete [67, 73, 117], and to ‘over-reduce’ 12-MPA to colourless decomposition  
341 products [40, 42, 79].

### 342 3.2. The reagent blank: isopoly molybdenum blue species

343 Care must be taken in any MB method to minimise the reagent blank absorbance, which arises from  
344 the direct reduction of Mo(VI). In contrast to the comparatively small Keggin structure, reduction of  
345 Mo(VI) alone yields a variety of giant isopolymolybdates with proportions of Mo(V) varying from  
346 15 - 20% [118], such as the 'Big Wheel' ( $H_xMo_{154}O_{462}^{n-}$ ) and the 'Blue Lemon'  
347  $[H_xMo_{368}O_{1032}(H_2O)_{240}(SO_4)_{48}]^{(x-48)-}$ . The latter species forms only in the presence of sulfate ions,  
348 and is likely to be a major contributor to many MB reagent blanks given the widespread use of  
349 sulfuric acid in MB methods. These isopoly species are interferents for two reasons. Firstly, since  
350 they possess some of the same structural motifs as reduced 12-MPA, they also possess intense  
351 IVCT absorption bands in the same region of the visible-NIR spectrum, generally around 750 –  
352 780 nm and 1050 - 1100 nm [3, 118, 119]. Wheel-type structures have a comparatively well-defined  
353 peak in this region, whereas other isopoly MB species have exceptionally broad and ill-defined  
354 absorption bands. Since the molar absorptivity of MB species scales linearly with the number of  
355 Mo(V) centres [3], these isopoly MBs are very intensely coloured with molar absorptivities in the  
356 order of  $10^5 \text{ L mol}^{-1} \text{ cm}^{-1}$  [118]. Interestingly, one study has exploited the apparent catalytic effect  
357 of nanomolar P concentrations on the rate of isopoly MB formation when Mo(VI) concentrations  
358 are much larger and acidities are lower than in conventional PMB methods [111]. However, this  
359 effect was only apparent with the use of high concentrations of ascorbic acid; hydrazine sulfate and  
360  $SnCl_2$  did not yield the same results.

361 Secondly, isopoly MB species are capable of aggregating in solution to form colloids, with  
362 aggregate sizes of at least tens of nanometres [3, 120], as well as adsorbing to surfaces due to their  
363 very large surface area [120]. This is obviously of concern for spectrophotometric methods due to  
364 light scattering, but the process of aggregation is a slow one and is generally only problematic  
365 where long reaction times are used. Of greater importance is the formation of coatings and deposits  
366 on the inside surfaces of flow analysis systems where automated MB methods are implemented.  
367 However, the principles underlying many modern methods (short reaction times, blank

368 minimisation, avoidance of heating) are effective in mitigating isopoly MB formation. Coating  
369 problems are typically due to the limited solubility of PMB species instead, which is discussed in  
370 more detail in Section 5.

371 Just as for the reduction of 12-MPA, the reduction of Mo(VI) depends strongly on the acidity, Mo  
372 concentration and reductant concentration. As a general rule, 12-MPA can be effectively reduced  
373 under conditions where Mo(VI) alone cannot; isopoly MB species are easily formed between pH  
374 1.0 - 2.5 [3, 118], whereas the formation and reduction of 12-MPA can tolerate higher acidities and  
375 much lower Mo concentrations. Isopoly MB formation is favoured with higher Mo concentrations,  
376 lower acidity, and higher reductant concentrations; MB method optimisation is thus a compromise  
377 between maximising 12-MPA formation and reduction and minimising isopoly MB formation (Fig.  
378 7).

379 It is also interesting to note that in methods where a combined reagent solution containing acid,  
380 molybdate and reductant is prepared, such as those reported in Standard Methods for the  
381 Examination of Water and Wastewater [98], the reagent solution tends to exhibit a yellow colour  
382 [91] due to absorption bands at 490 and 385 nm. These bands can also be observed in some aged  
383 PMB products, manifesting as a green hue, and reflect the d-d transitions of dimeric Mo(V) present  
384 as  $[\text{Mo}_2\text{O}_4(\text{OH}_2)_6]^{2+}$  [121, 122]. The presence of this species indicates the direct reduction of  
385 Mo(VI) under conditions where isopoly species cannot form, due to either the higher acidity of a  
386 combined reagent solution or the low Mo(VI) concentration used in some methods. Mo(V) halide  
387 complexes with a similar yellow colour have also been reported to form in marine sample matrices  
388 [123]. Whether or not the  $[\text{Mo}_2\text{O}_4]^{2+}$  species itself acts as a reductant or is directly involved in  
389 forming PMB is currently unknown, but it does not interfere with the spectrophotometric  
390 determination of P. 12-MPA itself is also attributed a faint yellow colour, although this appears to  
391 arise from the tail of the 315 nm LMCT band extending into the visible region in concentrated 12-  
392 MPA solutions.

### 393 3.3. Product stability

394 The stability of the colour in MB chemistry is attributable to three factors; the extent of reduction at  
395 the time a measurement is taken, the stability of the product and the availability of excess reductant  
396 to protect PMB from re-oxidation by dissolved O<sub>2</sub>. For example, it is frequently noted in studies  
397 which make use of organic reductants without heating that the absorbance of the product  
398 continually increases for periods up to several hours [49, 63, 70, 74]; given the slow reduction  
399 kinetics of these species, it is now clear that the reported ‘instability’ of the colour arises from the  
400 slow, ongoing disproportionation of PMB(2e<sup>-</sup>) to 12-MPA and PMB(4e<sup>-</sup>), which exhibits a different  
401 absorption spectrum. However, once formation of PMB(4e<sup>-</sup>) using organic reductants is complete,  
402 the product appears to be stable for at least several hours with respect to oxidation by dissolved O<sub>2</sub>  
403 [49, 60, 72, 73, 124]. Sb(III) methods behave in the same manner; once the reduction finishes after  
404 several minutes, the Sb<sub>2</sub>PMB(4e<sup>-</sup>) adduct appears stable for many hours with respect to oxidation  
405 [51, 85]. By contrast, a recent study on the use of UV photo-reduction of 12-MPA found that  
406 PMB(4e<sup>-</sup>) immediately began to re-oxidise once irradiation ceased, as no reducing reagents other  
407 than PMB existed in the absence of UV light [125].

408 SnCl<sub>2</sub> methods are well known to yield comparatively short-lived products. However, this  
409 unusually short lifespan cannot be attributed solely to re-oxidation by O<sub>2</sub>, since electrochemical  
410 measurements of Sn-substituted PMB complexes show that these products are in fact more stable  
411 toward oxidation than PMB(4e<sup>-</sup>) [96]. Instead, it has been found that the Sn-substituted products are  
412 hydrolytically unstable and are degraded in a stepwise fashion, presumably by acidified Mo(VI)  
413 [96], which may proceed to ultimately form PMB(4e<sup>-</sup>). However, in comparison to heated and  
414 Sb(III)-containing ascorbic acid methods, the reductant concentration in SnCl<sub>2</sub> methods is normally  
415 much lower, which presents a greater risk of untimely product re-oxidation. Therefore, SnCl<sub>2</sub>  
416 methods are often augmented with a sacrificial co-reductant such as hydrazine sulfate to extend the  
417 oxidative stability of the product to 30 – 60 min [49, 68, 126].

### 418 3.4. Method linearity

419 The linear range of a given MB method is the range of P concentrations over which 12-MPA  
420 formation and reduction occur to the same degree, and in which no deviation from the Bouguer-  
421 Beer-Lambert law is observed. MB method linear ranges, particularly those based on the Murphy  
422 and Riley method [85], typically extend up to around 1 mg L<sup>-1</sup> P. In practice, modern methods  
423 typically achieve quantitative, or at least substantial reduction, even in the short timeframes of FIA  
424 measurements; SnCl<sub>2</sub> is well-known to reduce 12-MPA almost instantly, and Sb(III) methods can  
425 achieve full reduction within one minute if the chemical conditions are carefully chosen [29].

426 The actual extent of the linear range is governed by the presence of sufficient acidified Mo(VI) to  
427 stabilise 12-MPA, sufficient reductant, and in the case of antimony methods, sufficient Sb(III).

428 Furthermore, linear ranges reported for FIA/SIA and batch/DA/SFA versions of the same method  
429 are not typically comparable, as they treat the sample volume in a fundamentally different way.

430 Whereas sample aliquots are diluted to a fixed volume in batch procedures, they undergo dispersion  
431 in flow methods. Since the practical dispersion coefficient of a flow system describing the extent of  
432 sample dilution is unknown unless specifically characterised, it is easier to discuss method linearity  
433 in the context of batch methods.

434 Antimony-based methods are the simplest case. As discussed above, these form a complex in which  
435 the ratio of Sb:P is 2:1, and it is clear from a number of investigations [29, 83-85] that the upper end  
436 of the linear calibration range of each method occurs at P concentrations where the Sb:P ratio  
437 becomes less than 2, with the graph suddenly adopting a shallower gradient (Table 5). At this point,  
438 the [H<sub>4</sub>PMo<sub>12</sub>O<sub>40</sub>]<sup>3-</sup> product forms which absorbs at 820 nm, contributing only slightly to the  
439 absorbance increase at 880 nm. By increasing the Sb(III) concentration used in the Murphy and  
440 Riley method fivefold, Harwood et al. were able to greatly extend the linear range of the former  
441 [83], whilst simultaneously halving the unnecessarily high Mo(VI) concentration. It has been found  
442 that even with a [Mo(VI)]/[P] ratio as low as 21, Sb(III) still acts as the limiting reagent (Table 5)

443 [84], suggesting that a 21-fold [Mo(VI)]/[P] ratio is sufficient for complete 12-MPA formation.

444 Ascorbic acid concentrations of 5 – 100 mmol L<sup>-1</sup> are reported (Table 3), with higher concentrations

445 accelerating the reduction process somewhat [29, 81]; it has been suggested that ascorbic acid must

446 be present in a 20-fold excess over 12-MPA to completely reduce it, with or without Sb(III) [81].

447 The addition of further Sb(III) does not accelerate the reduction process, nor does it appear to

448 interfere with the reduction chemistry, but the extension of the linear range to arbitrarily high P

449 concentrations by adding more Sb(III) is limited by solubility problems, which have been reported

450 at working concentrations of ca. 10<sup>-4</sup> mol L<sup>-1</sup> Sb(III) [83, 85]. A similar phenomenon is well-known

451 during the preparation of the mixed reagent solution in methods derived from that of Murphy and

452 Riley [85], in which turbidity may become evident once both Sb(III) and Mo(VI) have been added

453 to the solution [84, 98], and for which the general recommendation is to keep mixing the solution

454 until the precipitate re-dissolves. This phenomenon is curious given much greater solubility of

455 potassium antimonyl tartrate in pure water than in combined reagent solutions. It seems likely that

456 the insoluble species being formed is in fact a salt of Sb(III) and molybdate [127] which forms at

457 low [Mo(VI)] / [Sb(III)] ratios, rather than a basic salt as suggested by Murphy and Riley [85]. If a

458 salt of Sb(III) and molybdate is responsible for the Sb(III) solubility issues, it is expected that pH

459 manipulation will be ineffective in dissolving it [127]; dilution should be the preferred course of

460 action.

461 Data on the linear ranges of MB methods other than those utilising Sb(III) have seldom been

462 reported; many authors claim that their method demonstrates a linear response up to at least a given

463 P concentration, rather than examining the maximum tolerable P concentration. As an

464 approximation, non-Sb(III) methods appear to be limited by reductant concentration as long as

465 [Mo(VI)]/[P] ≥ 21. For SnCl<sub>2</sub> methods, it appears that an approximately 8-fold SnCl<sub>2</sub>:12-MPA

466 excess is required for complete product formation; the standard SnCl<sub>2</sub> method deviates from

467 linearity around this point, even though Mo(VI) is present in more than an 80-fold molar excess

468 over orthophosphate [98]. Similarly, El-Shamy and Iskander found that the reduction of 12-MPA

469 continued up to a 5- to 6-fold molar excess of  $\text{SnCl}_2$  over 12-MPA [40]. In methods using heating  
470 with organic reductants, 10-fold excesses of hydrazine sulfate and 1-amino-2-naphthol-4-sulfonic  
471 acid (ANS) have also been reported as sufficient for complete reduction of 12-MPA [65, 73], as the  
472 reduction inherently becomes much more favourable and rapid at higher temperatures.

473 The ionic strength of the sample matrix is not typically problematic for the linearity of MB  
474 methods, as reagent concentrations are normally sufficiently high to mask any activity effects. For  
475 example, Murphy and Riley observed deviations in the analytical signal of less than 1% when their  
476 method was performed in a matrix of approx.  $0.44 \text{ mol L}^{-1} \text{ Cl}^-$  [85] as opposed to distilled water. A  
477 more extreme case has been reported by Zhang et al. [128] in which a sample matrix containing  $1$   
478  $\text{mol L}^{-1} \text{ NaCl}$  after a sequential extraction procedure did attenuate the linear range, but this  
479 vulnerability can likely be attributed to the very low working concentrations of all reagents used.

### 480 **3.5. Choice of acid**

481 The MB reaction requires a strong acid, with reported pH values generally below 1 to ensure  
482 appropriate Mo(VI) speciation and inhibition of direct Mo(VI) reduction. However, the choice of  
483 acid can negatively impact the reaction; oxidising acids interfere with the reduction process, and  
484 anion interactions with Mo(VI) species can perturb the formation of 12-MPA.

485 Neither nitrate nor perchlorate appear to coordinate to Mo(VI) species to any significant degree,  
486 even in  $0.5 \text{ mol L}^{-1} \text{ Mo(VI)}$  solutions in  $2.0 \text{ mol L}^{-1} \text{ HNO}_3$  or  $\text{HClO}_4$ , as determined by  $^{95}\text{Mo}$  NMR  
487 and Raman spectroscopic measurements [129]. However, the oxidising ability of nitric and  
488 perchloric acids should discourage their use in MB methods; even  $0.1 \text{ mol L}^{-1} \text{ HNO}_3$  interferes with  
489  $\text{SnCl}_2$  reduction of heteropoly acids [114] and  $\text{HClO}_4$  has been reported to form a precipitate with  
490  $\text{SnCl}_2$  and partially oxidise hydrazine sulfate [74].

491 Literature reports of interactions of sulfate with heteropoly acid systems are varied [130, 131],  
492 including one 1915 report of a molybdosulfate species co-existing with 12-MPA, apparently  
493 precipitated as  $[(\text{NH}_4)_2\text{SO}_4 \cdot 5\text{MoO}_3]$  [132]. The use of sulfuric acid significantly decreases the rate

494 constant for 12-MPA formation compared with nitric or perchloric acids [30]. It is also clear from  
495 UV-visible spectroscopic comparisons that at equilibrium, 12-MPA in H<sub>2</sub>SO<sub>4</sub> is markedly more  
496 dissociated into its precursor molybdates than in HNO<sub>3</sub>[27] or HClO<sub>4</sub>[30], particularly at pH < 1.0,  
497 with the effect also induced by addition of sulfate or bisulfate [27]. Spectrophotometric  
498 measurements of dilute, monomeric Mo(VI) solutions have shown that an equilibrium system  
499 between molybdic acid and hydrogen sulfate does indeed exist, involving a 1:2 reaction between  
500 Mo(VI) and HSO<sub>4</sub><sup>-</sup> (pK<sub>a</sub> = 1.9) [133] to form a deprotonated product. Only the 1:2 complex was  
501 reported, although ESI-MS data have suggested that a number of other complexes also exist at  
502 various Mo(VI) concentrations [20, 106].

503 The formation of 12-MPA is therefore inhibited to some extent in methods using H<sub>2</sub>SO<sub>4</sub> (or  
504 acidified sample matrices containing SO<sub>4</sub><sup>2-</sup>) since this reaction must involve the release of HSO<sub>4</sub><sup>-</sup>  
505 from molybdosulfate species into a solution already containing a high concentration of HSO<sub>4</sub><sup>-</sup>. This  
506 conclusion correlates very well with the early observations of Crouch and Malmstadt [27], whereby  
507 the extent of 12-MPA formation in H<sub>2</sub>SO<sub>4</sub> was seen to be far less than that observed with HNO<sub>3</sub> at a  
508 range of pH values, and Linares et al. [134] have reported that even using HNO<sub>3</sub> instead of H<sub>2</sub>SO<sub>4</sub>  
509 results in a significant enhancement in PMB(4e<sup>-</sup>) formation, despite the oxidising power of HNO<sub>3</sub>.  
510 A more detailed investigation of the impact of H<sub>2</sub>SO<sub>4</sub> on MB method sensitivity is warranted.

511 It is striking, then, that the vast majority of published MB methods utilise sulfuric acid. This can  
512 potentially be attributed to following in the footsteps of Murphy and Riley's seminal contribution  
513 published in 1962 [85], which itself may have used sulfuric acid as a means of avoiding the  
514 formation of iron chloro-complexes in hydrochloric acid, as did a number of its predecessors [93]. It  
515 is also likely that this acid's popularity is due to consistently low levels of PO<sub>4</sub><sup>3-</sup> contamination even  
516 in analytical reagent grade, thus eliminating the need for more expensive ultra-pure acid. In some  
517 samples such as peroxydisulfate digests, SO<sub>4</sub><sup>2-</sup> and HSO<sub>4</sub><sup>-</sup> are unavoidable in any case. Furthermore,  
518 the 'salt error' encountered in SnCl<sub>2</sub> methods appears to have discouraged the use of hydrochloric

519 acid as an alternative to sulfuric acid, even in cases where the  $\text{Cl}^-$  ion should not cause any  
520 interference.

521 Like sulfate, chloride is also known to form complexes with molybdate cations, and the complexes  
522  $\text{MoO}_2\text{Cl}_2(\text{H}_2\text{O})_2$  and  $\text{MoOCl}_3(\text{OH})(\text{H}_2\text{O})$  have been identified [25, 129]. However, these complexes  
523 are much weaker than similar sulfate complexes, with formation constants of 0.29 and 0.036  
524 respectively. Additionally, the formation of chloro-complexes with molybdate cations is only seen  
525 at HCl concentrations higher than  $0.4 \text{ mol L}^{-1}$ , and approximately  $0.8 \text{ mol L}^{-1}$  HCl is required for  
526 10% of the molybdate species to form the dichloro-complex. Whilst the effect of this species on 12-  
527 MPA formation is unclear, it can be assumed that due to the low formation constant of this  
528 complex, combined with the high HCl concentration required for it to form, the use of hydrochloric  
529 acid in the MB method should not perturb 12-MPA formation in any practical sense and further  
530 investigation of HCl as an alternative to  $\text{H}_2\text{SO}_4$  should be performed. However, if  $\text{SnCl}_2$  is preferred  
531 as the reductant, sulfuric acid should be used as the acid instead of hydrochloric acid due to the salt  
532 error, which is discussed in Section 4.2.4.

## 533 **4. Interferences**

### 534 **4.1. Additive interferences**

#### 535 **4.1.1. Arsenate**

536 Just as  $\text{PO}_4^{3-}$  forms a reducible heteropoly acid, so too does  $\text{AsO}_4^{3-}$ . The spectral profiles, molar  
537 absorptivities, chemical properties and formation conditions of arsenomolybdenum blue species are  
538 very similar to their orthophosphate counterparts (Table 4). As a result, resolution of the two  
539 compounds in the same solution, even using multiple wavelength spectrophotometry, is all but  
540 impossible with satisfactory accuracy. As(V) interference is generally countered either by  
541 exploiting subtle kinetic and spectral differences as the two MB species form [135], or by reducing  
542 As(V) to As(III) [136-138] which cannot form a heteropoly acid due to its lack of tetrahedral

543 geometry. The reduction of As(V) to As(III) is typically performed using sulfur-containing  
544 reductants such as dithionite ( $S_2O_4^{2-}$ ), thiourea ( $(NH_2)_2CS$ ) or thiosulfate ( $S_2O_3^{2-}$ ), but even so, the  
545 reduction is a slow process which requires heating if completion within tens of minutes is to be  
546 achieved [136, 138]. The use of thiosulfate also requires a source of  $SO_2$  such as acidified  
547 metabisulfite ( $S_2O_5^{2-}$ ), and the loss of this toxic gas from solution results in precipitation of  
548 colloidal sulfur [138] which is particularly troublesome for MB methods, since it depends on  
549 spectrophotometric detection.

550 Multiple authors have observed a 'synergistic' effect between As(V) and P in solution, such that  
551 solutions containing both appear to form a molybdenum blue species much more quickly than either  
552 of them in isolation [135, 137, 139, 140]. In methods using Sb(III), the absorption maximum of the  
553 new product appears to be redshifted by 10 nm compared with the superposition of AsMB and  
554 PMB spectra in isolation [137]. Johnson and Pilson demonstrated that at their measurement  
555 wavelength of 865 nm, the final absorbance of a mixed As(V) / P solution was lower, but attained  
556 much more rapidly, than the same solution without added P [137]. These authors also showed  
557 evidence that the species responsible for this synergistic behaviour was a molybdenum blue species  
558 containing a 1:1 ratio of As(V) : P. Dhar et al. [140] have since provided further evidence for a  
559 combined As(V) / P product in their As(V) method; in the presence of sufficient P, the reduction  
560 process was complete in 10 min, but in natural waters containing  $< 2 \mu\text{mol L}^{-1}$  P, the reaction  
561 required about 45 min for complete reduction to  $Sb_2AsMB$  to occur. Aside from the substantial  
562 kinetic effect of this phenomenon, however, the effect on the final absorbance of the solution  
563 typically gives rise to an error of only  $\sim 5\%$ . Furthermore, these authors made the intriguing  
564 observation that increasing the concentration of reductant (ascorbic acid) served to drastically  
565 decrease the impact of this combined product on the final absorbance [140]. López Carreto et al.  
566 observed similar synergistic phenomena with the standard PMB( $4e^-$ ) product [135]. If a discrete  
567 species containing both As and P is indeed formed under the conditions used by any of the  
568 aforementioned authors, its composition is unclear, but it may be a P-substituted derivative of an

569 arsenomolybdate complex, given the diverse range of As : Mo stoichiometries encountered in these  
570 compounds [35]. Whether or not the formation of combined As(V) / P species occurs in SnCl<sub>2</sub>  
571 reduction methods is unknown.

#### 572 **4.1.2. Silicate**

573 SiO<sub>4</sub><sup>4-</sup> has long been considered one of the main interferents in PMB methods as it also forms  
574 heteropoly acids ( $\alpha$ - and  $\beta$ -12-MSA) [114] reducible to molybdenum blues. Silicate interference is  
575 well-known to become problematic when the reaction acidity is too low and when heating is  
576 employed [29, 109]. This is due to two separate phenomena; 12-MSA is only stable at lower Z  
577 values (higher pH) than 12-MPA [73], and the speciation of silicic acid itself is dependent on both  
578 temperature and acidity. At low pH, orthosilicic acid (H<sub>4</sub>SiO<sub>4</sub>) exists in equilibrium with polysilicic  
579 acids [141, 142] which form 12-MSA much more slowly than orthosilicic acid alone [142],  
580 presumably because only orthosilicate possesses the appropriate molecular geometry to form 12-  
581 MSA. Of note is a very recent ESI-MS study [20] suggesting that the use of H<sub>2</sub>SO<sub>4</sub> actually  
582 decomposes polysilicic acids into a monosilicate complex with HSO<sub>4</sub><sup>-</sup> which may still react to form  
583 12-MSA, implying that silicate interference is therefore increased when H<sub>2</sub>SO<sub>4</sub> is used.  
584 Unfortunately, no more data on this phenomenon are available at this time.

585 Higher reaction temperatures favour the decomposition of polysilicic acids into orthosilicic acid  
586 [142], a phenomenon which probably gave rise to silicate's reputation as a major interferent in PMB  
587 methods due to the frequent use of heating employed in older literature methods (Table 3). Higher  
588 reaction temperatures also broaden the Z range in which both isomers of 12-MSA will form,  
589 exacerbating the problem even further [114]. However, it has been reported that heated acid  
590 digestion procedures are capable of re-polymerising silicic acid into unreactive species through  
591 dehydration [143].

592 Due to the phenomena described above, silicate interference can be effectively controlled by using a  
593 sufficiently high acidity [114, 141] in addition to avoiding heating, which is rarely used in more

594 modern PMB methods (Table 3). The rate of 12-MSA formation decreases sharply when  $Z > 2$  [90],  
595 which can be approximated as  $> 0.2 \text{ mol L}^{-1} \text{ H}^+$  for low millimolar Mo(VI) concentrations and is  
596 within the optimal range for many PMB methods (Table 3). For example, in a previously reported  
597 flow injection method where working concentrations of  $5 \text{ mmol L}^{-1} \text{ Mo(VI)}$  and  $0.2 \text{ mol L}^{-1} \text{ H}_2\text{SO}_4$   
598 were used,  $50 \text{ mg L}^{-1} \text{ Si}$  did not interfere at all and  $100 \text{ mg L}^{-1} \text{ Si}$  generated only a + 2.6% error in  
599 the timeframe of the method [125]. Since acidity controls silicate interference by slowing the  
600 kinetics of 12-MSA formation, it therefore follows that the more rapidly a measurement is made,  
601 the more silicate can be tolerated in a sample without detectable interference [109].

602 An alternative approach to inhibiting 12-MSA formation is the use of organic acids. These species  
603 function as ligands, sequestering Mo(VI) and thus slowing the formation of 12-MSA, a  
604 phenomenon further discussed in Section 4.2.1. This approach exploits the observation that 12-  
605 MSA's formation kinetics can be greatly slowed by the presence of organic acids in comparison to  
606 12-MPA which forms much more rapidly. However, this approach can be a double-edged sword  
607 since 12-MPA is decomposed by such species at higher concentrations, whereas 12-MSA is  
608 unaffected by organic acids if they are added after its formation is complete, a phenomenon often  
609 exploited in silicate determination [144]. For example, 12-MSA formation can be effectively  
610 suppressed with dilute oxalic acid whilst leaving 12-MPA mostly intact, whereas fully formed 12-  
611 MPA can be easily destroyed with more concentrated oxalic acid, which is ineffective in  
612 decomposing fully formed 12-MSA [145]. Tartaric acid ( $\text{C}_4\text{H}_6\text{O}_6$ ) is also often used for inhibiting  
613 silicate interference in orthophosphate determinations [145, 146], yet it is reported to interfere with  
614 this determination quite substantially in its own right [145, 147], more so than oxalic acid [145].  
615 Interestingly, since methods using Sb(III) almost invariably utilise potassium antimonyl tartrate  
616 ( $\text{K}_2\text{Sb}_2(\text{C}_4\text{H}_2\text{O}_6)_2 \cdot 3\text{H}_2\text{O}$ ) as the source of Sb(III), it may be expected that the tartaric acid acts to  
617 suppress silicate interference, even in micromolar quantities. Zhang et al. have reported that the  
618 addition of this salt can reduce silicate interference by almost 50% when the method is heated to

619 70<sup>0</sup>C [109], although whether this result can be attributed to the presence of tartaric acid or the  
620 formation of Sb-containing heteropoly acids is uncertain.

### 621 **4.1.3. Organic and inorganic P hydrolysis**

622 Concern is frequently voiced about the undesirable tendency of MB methods to hydrolyse other  
623 fractions of total dissolved phosphorus (TDP), thereby providing an overestimate of  
624 orthophosphate. This is the basis for labelling the phosphorus fraction determined by the MB  
625 method as 'dissolved reactive phosphorus' (DRP) or, more correctly, 'molybdate reactive  
626 phosphorus' (MRP) [2], which is assumed to also include some proportion of inorganic  
627 polyphosphates, labile organic P compounds and colloidal P species [2]. Hydrolytic degradation of  
628 phosphates is quite distinct from oxidative processes, which are only introduced in methods for  
629 TDP determination. Whilst (thermal) acid hydrolysis is effective for conversion of polyphosphates  
630 and labile organic P compounds, oxidative processes are much more effective for the total dissolved  
631 organic phosphorus (DOP) pool [50, 148-153]; as such, additive MB interference is concerned  
632 mainly with the former.

633 Hydrolysis and desorption of other P-containing fractions in TDP is widely considered to be acid-  
634 induced. However, a number of studies have shown that both acid and Mo(VI) act to hydrolyse  
635 these species [71, 154-156], and that the influence of Mo(VI) in such cases can be pronounced  
636 [154]. It has been tentatively suggested that Mo(VI) acts by binding to phosphate groups and then  
637 removing them from the parent compound [154, 156], which is supported by the extremely high  
638 affinity of Mo(VI) for orthophosphate discussed earlier [32].

## 639 **4.2. Subtractive interferences**

### 640 **4.2.1. Organic acids**

641 The presence of organic acids is known to inhibit the MB reaction, particularly in the context of  
642 masking one oxoanion in order to more accurately determine another. The nature of organic acid

643 interference in PMB methods is the formation of coordination complexes with Mo(VI) [144], acting  
644 to both sequester Mo(VI) and destroy 12-MPA [145]. However, this can only occur if the species in  
645 question is able to coordinate in a bidentate manner to form a stable 5- or 6-membered coordination  
646 ring with Mo(VI) [157]. A number of Mo(VI) complexes with common organic anions have been  
647 characterised, such as tartrate [158], oxalate [159-161], malate [162], and citrate [163, 164], and  
648 these complexes persist at the acidities typically used in MB methods. Formic and maleic acids do  
649 not appear to interfere with phosphate determination [165]; these species lack the necessary atom(s)  
650 on an  $\alpha$ -carbon to create a 5- or 6-membered coordination ring with Mo(VI), and thus coordinate  
651 too weakly to be problematic. In fact, organic acids are sometimes used in MB methods to prevent  
652 the reduction of excess Mo(VI), particularly in silicate methods where the requisite acidity is lower  
653 than for orthophosphate [166, 167]. By the same token, interference from organic ligands in PMB  
654 methods can be eliminated by complexing these species with excess Mo(VI) [147].

#### 655 4.2.2. Fluoride

656  $F^-$  is a strong negative interferent in the MB reaction [68, 147, 168, 169]. In a similar manner to that  
657 of organic acids, its mode of interference is binding to Mo(VI) to form discrete ions which cannot  
658 condense to form larger structures such as 12-MPA [170, 171]. This has the effect of slowing the  
659 MB reaction without necessarily changing the extent of the reaction, giving the appearance of a  
660 lower analytical signal if insufficient time is allowed for the inhibited reaction to reach completion  
661 [172].  $H_3BO_3$  is very effective at sequestering  $F^-$  as  $BF_4^-$  without negatively impacting the MB  
662 reaction [170, 173]. An interesting point is that silicate also complexes to  $F^-$  in solution, and can  
663 mitigate the inhibitory effect of  $F^-$  on the MB reaction in its own right [101]. Of course, this  
664 phenomenon interferes with the determination of silicate itself in waters containing sufficient  $F^-$ .  
665 Interestingly,  $F^-$  can also alter the redox potentials of metallic reductants. The use of Fe(II) as a  
666 reductant combined with NaF has been reported, wherein the complexation of  $F^-$  to Fe(II) enhanced  
667 the latter's reducing power [174]. NaF has also been used to enhance the reducing power of  $SnCl_2$

668 by converting its oxidation product to the more stable  $\text{SnF}_6^{2-}$  [175]. However, due to the formation  
669 of HF at the acidities used in the MB reaction, this approach cannot be recommended on practical  
670 grounds.

#### 671 **4.2.3. Chloride (salt error)**

672 A major source of subtractive interference in MB methods is the 'salt error' [50, 85, 97, 105, 176],  
673 which manifests as a decrease in analytical signal when  $\text{SnCl}_2$  is used as the reductant in assays of  
674 marine and estuarine waters or other matrices containing elevated chloride concentrations. Whilst a  
675 small (approx. 1 – 4%) decrease in analytical signal is generally expected at high ionic strengths  
676 due to the decrease in activity coefficients [50, 176], the decrease in signal is usually reported to be  
677 between 15 – 20% in marine samples [50, 64, 85, 176] and even higher in some cases [68, 97],  
678 implying a dependence on method conditions as well. The degree of analytical signal suppression  
679 varies with the  $\text{Cl}^-$  concentration in the matrix [68, 97] as well as the orthophosphate concentration  
680 [97], and method linear range is attenuated with increasing  $\text{Cl}^-$  concentration as well. As such, the  
681 salt error is troublesome not only because of the loss of method sensitivity, but because this loss  
682 varies in magnitude between sample matrices of different salinities and P concentrations.

683 The nature of the salt error is the decreased formation of  $\text{SnPMB}(4e^-)$  due to the disruptive effect of  
684  $\text{Cl}^-$  on the chemistry of Sn(IV). Chloro-complexes of Sn(II) and Sn(IV) readily form in the presence  
685 of  $\text{Cl}^-$  [105, 177-179], and their stability dramatically impedes the ability of Sn(IV) to substitute into  
686  $\text{PMB}(4e^-)$  to form  $\text{Sn}_2\text{PMB}(4e^-)$  [97, 105]. As a result, the highly favourable combined reduction  
687 and substitution step responsible for producing  $\text{Sn}_2\text{PMB}(4e^-)$  is impeded due to the depletion of  
688 uncomplexed Sn(IV). Thus, higher concentrations of  $\text{Cl}^-$  limit the extent to which  $\text{SnPMB}(4e^-)$  can  
689 form, and force the system to reduce 12-MPA via the slower and much less favourable direct  
690 reduction process [97]. This is clearly shown in UV-visible spectra of products suffering from the  
691 salt error, in which the 710 nm  $\text{SnPMB}(4e^-)$  peak is diminished and a distinct shoulder at 820 nm  
692 emerges due to the increased presence of  $\text{PMB}(4e^-)$ . Even though the two reduction products

693 possess absorption maxima 100 nm apart, dual-wavelength spectrophotometry cannot be used to  
694 counter the salt error since the total concentration of reduced products also decreases with higher  
695  $\text{Cl}^-$  concentrations [97].

696 Three strategies for countering the salt error have been proposed. Firstly, the reaction can be  
697 performed using reagents which already contain a sufficiently concentrated  $\text{Cl}^-$  'buffer', such that  
698 the sample matrix does not perturb the  $\text{Cl}^-$  concentration at the time of reduction [180]. This  
699 approach does not eliminate the sensitivity loss caused by the salt error but it does buffer against  
700 variations in the magnitude of the salt error between samples, allowing a single calibration curve to  
701 be used for analysis. Secondly, if a large number of samples in similar matrices are to be analysed,  
702 the method can be calibrated in that matrix (e.g. seawater) or a solution with matching salinity. The  
703 third option is to heat the reaction to near-boiling for several minutes in an analogous way to heated  
704 ascorbic acid methods; this results in the exclusive formation of  $\text{PMB}(4e^-)$  by accelerating both its  
705 direct formation and the hydrolysis of  $\text{SnPMB}(4e^-)$  [62], thus eliminating the role of  $\text{Sn(IV)}$  in the  
706 reaction entirely [97].

### 707 **4.3. Multifunctional interferents**

#### 708 **4.3.1. Sulfide**

709 Sulfide, present as  $\text{H}_2\text{S}$  under acidic conditions, is capable of both additive and subtractive  
710 interference in the MB reaction [181].  $\text{H}_2\text{S}$  can act as a reductant for 12-MPA in its own right [182,  
711 183] and causes distinct additive interference at low P concentrations as a result [181], particularly  
712 in terms of direct  $\text{Mo(VI)}$  reduction. At larger P concentrations,  $\text{H}_2\text{S}$  causes subtractive interference  
713 instead, apparently by complexing with  $\text{Mo(VI)}$  [181]. In  $\text{Sb(III)}$  methods,  $\text{H}_2\text{S}$  will also sequester  
714  $\text{Sb(III)}$  to form insoluble antimony sulfides; not only is the precipitate problematic for  
715 spectrophotometry, but the loss of dissolved  $\text{Sb(III)}$  also forces the formation of the  $\text{PMB}(4e^-)$   
716 species, thus altering the spectral properties of the product [184].  $\text{H}_2\text{S}$  can be conveniently oxidised  
717 by  $\text{KMnO}_4$  prior to the MB reaction [183], provided that excess reductant is subsequently used, or it

718 can be removed by acidification and subsequent degassing [181]. Concerning the former approach,  
719 a working  $\text{KMnO}_4$  concentration of  $5.3 \text{ mmol L}^{-1}$  can be reduced by a working ascorbic acid  
720 concentration of  $26.9 \text{ mmol L}^{-1}$  with no impact on method sensitivity [183].

#### 721 **4.3.2. Iron**

722  $\text{Fe(II)}$ , much like  $\text{H}_2\text{S}$ , can reduce 12-MPA. In fact,  $\text{Fe(II)}$  has been used as the main reductant in  
723 several MB methods [66, 185] as well as the analyte by virtue of its reducing ability [186].  
724 However, this is only troublesome in terms of method interference if the reductant concentration is  
725 too low or the acid – molybdate balance favours isopolymolybdenum blue formation.  $\text{Fe(III)}$  is  
726 more problematic as it appears to consume the reductant, reducing sensitivity and/or product  
727 stability [64, 187-190]. It has also been reported that  $\text{Fe(III)}$  might precipitate orthophosphate from  
728 solution [191].  $\text{Fe(III)}$  interference is effectively controlled with a sufficient excess of reductant or  
729 the presence of an organic acid to complex with  $\text{Fe(III)}$  [188, 189, 192].

#### 730 **4.3.3. Surfactants**

731 It has been reported that different types of surfactants interact with the MB reaction when using  
732 either ascorbic acid /  $\text{Sb(III)}$  or  $\text{SnCl}_2$  reduction [193]. In this study, cationic surfactants such as  
733 tetraalkylammonium chlorides were shown to cause severe negative interference even at low  $\text{mg L}^{-1}$   
734 concentrations;  $5 \text{ mg L}^{-1}$  surfactant caused 15% sensitivity loss for both reduction methods, and  
735 higher surfactant concentrations caused turbidity. A neutral detergent species, nonylphenol  
736 ethoxylate, also induced turbidity at similar concentrations; however, its effect on the absorbance  
737 resulting from ascorbic acid /  $\text{Sb(III)}$  reduction was variable, whilst the  $\text{SnCl}_2$  procedure seemed  
738 largely unaffected despite the turbidity. By contrast, commonly used anionic surfactants such as  
739 linear alkyl sulfonates did not interfere at any of the tested concentrations for either reduction  
740 method (up to  $2.5 \text{ g L}^{-1}$  for ascorbic acid /  $\text{Sb(III)}$  reduction), and branched sulfonates such as  
741 sodium dioctylsulfosuccinate only interfered above  $25 \text{ mg L}^{-1}$ . Neither of these anionic species  
742 caused any turbidity. Industrial detergent formulations were found to cause interference through

743 other components of the mixture in addition to the surfactants themselves, though again only above  
744 25 mg L<sup>-1</sup>.

745 Interference from neutral surfactants can be completely eliminated in either reduction method by  
746 adding a linear alkyl sulfonate in a 10-fold excess over the interfering species [193]. However,  
747 interference from cationic surfactants was only able to be eliminated in the SnCl<sub>2</sub> procedure,  
748 requiring a 5-fold excess of linear alkyl sulfonate over the cationic surfactant. In light of the above,  
749 it is probable that the severe interference and precipitation caused by cationic surfactants is due to  
750 the formation of ion-pairs with molybdate or PMB anions, whereas the interference from the other  
751 types of surfactants may be due to aggregation behaviour. The use of linear alkyl sulfonates  
752 evidently sequesters cationic surfactants and/or disrupts their interactions with the reacting species.  
753 In fact, the use of anionic surfactants in MB methods has previously been reported for different  
754 reasons; sodium dodecyl sulfate has frequently been used in automated methods to minimise the  
755 deposition of MB species on the surfaces of cuvettes or flow cells [99], an application discussed at  
756 length in Section 5.

## 757 **5. MB chemistry in flow methods**

758 The MB reaction is finding increasing use in flow analysis methods for P determination, taking  
759 advantage of the superior sampling rate and reproducibility inherent to these techniques when  
760 compared with batch methodologies [2, 194, 195]. However, beyond the inherent benefits of  
761 automation, flow analysis is often used as a platform for coupling P determination with more  
762 sophisticated operations which would be tedious or impossible to perform reproducibly under batch  
763 conditions. These include the determination of different P fractions using UV photo-oxidation or  
764 acid hydrolysis [148-150, 196], the use of reaction kinetics for P determination [197], independent  
765 determination of both P and As fractions [134], preconcentration [99, 198] and UV photo-reduction  
766 of 12-MPA using ethanol, allowing the use of a single long-lived reagent solution [125].

767 Flow analysis manifolds with spectrophotometric detection can incorporate liquid-core waveguide  
768 flow cells, which increase the optical path length (up to 500 cm) by the use of total internal  
769 reflection without significantly attenuating the light beam [199, 200]. This technology potentially  
770 allows for significant improvements in MB method sensitivity, although several practical issues  
771 such as back-pressure, bubble formation, the Schlieren effect and signal noise must be carefully  
772 managed [146, 199, 201, 202]. The Schlieren effect is of particular concern in saline samples, which  
773 often demand the greatest sensitivity of any P determination method. To this end, reverse FIA has  
774 been used for the analysis of samples with variable salinities to effectively counter the Schlieren  
775 effect [203].

776 In terms of actual reaction chemistry, the Murphy and Riley method [85] using ascorbic acid /  
777 Sb(III) is by far the most popular of any MB method in flow analysis owing to its relatively fast  
778 reaction kinetics at room temperature, insensitivity to  $\text{Cl}^-$  interference and low susceptibility to  
779 silicate interference, since heating is not required [2].  $\text{SnCl}_2$  methods also have considerable utility  
780 in a flow analysis setting; their very rapid reduction kinetics and more intensely absorbing product  
781 [97] allow for greater sensitivity and sample throughput than ascorbic acid / Sb(III) methods, since  
782 their limited stability after several minutes is typically of little concern. However, the salt error has  
783 led to the diminished popularity of such methods, particularly as many flow analysis applications  
784 are aimed at ultra-trace P determination in marine or estuarine waters.

785 An important phenomenon prevalent in flow-based, batch and discrete analyser formats alike is that  
786 of 'coating', or the deposition of reaction products on the walls of the reaction vessel or the interior  
787 of the flow manifold. This effect often manifests as a long tail on a signal peak as the deposited  
788 product is washed out of the photometer flow cell, a return to a non-zero absorbance baseline after  
789 the passage of an analyte peak, or an irregular, positive y-intercept in batch or DA analysis formats,  
790 an occurrence described as sample 'carryover' [109, 204]. Coating reduces the sample throughput  
791 of a method due to the extra time required for washing the system, and compromises the precision  
792 of subsequent analyses if sample carryover is still present in the system.

793 The coating phenomenon is dependent on the pH of the reaction mixture and the concentration of  
794 PMB product formed. The general observation that coating causes a smooth, elongated tail on FIA  
795 peaks suggests that the coating species exhibits a similar or identical spectral profile to the PMB  
796 product being measured. Combined with the observation that higher concentrations of PMB product  
797 increase sample carryover [109], it is apparent that the coating compound, which appears blue [109,  
798 126], is the main reduction product and not the isopoly MB species formed in the reagent blank.  
799 The effect of acidity on sample carryover is interesting; for  $\text{PMB}(4e^-)$ , the amount of sample  
800 carryover appears to reach a minimum at pH 0.5 and varies little at higher pH, but is greatly  
801 increased below pH 0.5 [109, 195]. By contrast,  $\text{Sb}_2\text{PMB}(4e^-)$  exhibits increased coating at pH  
802 values both above and below 0.5 [195], with precipitation of Sb(III) salts above pH 1.50 [109]. The  
803 coating phenomenon has previously been attributed to the colloidal behaviour of PMB species [109,  
804 195] caused by their limited water solubility. This explanation is reasonable given the clear  
805 decrease in coating incidence at higher temperatures, and suggests that the solubilities of both  
806  $\text{PMB}(4e^-)$  and  $\text{Sb}_2\text{PMB}(4e^-)$  decrease dramatically below pH 0.5, whilst  $\text{Sb}_2\text{PMB}(4e^-)$  also  
807 experiences solubility problems at higher pH values. The actual reductant used, so long as the  
808 reduced product is the same, has no inherent effect on coating; assertions to the contrary are very  
809 likely due to differing extents of reduction between methods [195].

810 The lower solubility of the  $\text{Sb}_2\text{PMB}(4e^-)$  complex than that of  $\text{PMB}(4e^-)$  has been investigated by  
811 Zhang et al. in two studies [109, 195]. These authors showed that between pH 0.5 – 1.5,  
812  $\text{Sb}_2\text{PMB}(4e^-)$  gave rise to a carryover percentage of between 1 - 2% at room temperature, roughly  
813 double that of  $\text{PMB}(4e^-)$  [109]. Curiously, these results differ greatly from those obtained in a  
814 subsequent study by the same authors in which carryover coefficients were significantly larger  
815 [195]. However, this later study also employed a commercial anionic surfactant formulation with  
816 unknown additives; the potential precipitation problems associated with such formulations have  
817 been previously discussed in Section 4.3.3.

818 Fortunately, the coating of PMB products is straightforward to control. It is recommended that  
819 either a low working concentration of glycerol (3.5 - 5.0% v/v) [126, 134] or sodium dodecyl  
820 sulfate (0.05% m/v) [99] should be added to the reaction mixture to suppress the deposition of  
821 reduction product. The coating behaviour of SnPMB(4e<sup>-</sup>) has not been studied, but the  
822 precautionary use of sodium dodecyl sulfate (0.05% m/v) has been effective in suppressing coating  
823 caused by this product as well [99]. The critical micelle concentration of sodium dodecyl sulfate  
824 varies considerably depending on solution composition [205], and a concentration of 0.05 - 0.20 %  
825 (m/v) of this surfactant in the final reaction mixture is recommended to ensure that the critical  
826 micelle concentration is exceeded. The effect of high acidity on PMB solubility is generally not  
827 problematic since a pH < 0.5 is expected to be highly detrimental to MB method sensitivity in any  
828 case, as discussed earlier in this review. Coating can be decreased by using a higher reaction  
829 temperature, as would be expected, although any such benefits for systems producing PMB(4e<sup>-</sup>) are  
830 typically small [109].

## 831 **6. Conclusions and recommendations**

832 The MB reaction consists of multiple interacting equilibria based on the complex speciation of  
833 aqueous Mo(VI), the formation of phosphomolybdic heteropoly acids and the reduction of 12-MPA  
834 by various organic or metallic species. Several long-standing assumptions about this reaction have  
835 been shown to be incorrect and counterproductive for method optimisation. In particular, it is  
836 demonstrated that the concept of the [H<sup>+</sup>]/[Mo(VI)] ratio, first introduced by Strickland [115] and  
837 widely adopted since, is a parameter which fails to define any chemical property of the MB system  
838 and is an entirely misleading framework with which to approach MB method optimisation.

839 Several possible 'molybdenum blue' species are identified as end products of the reaction  
840 depending on the conditions used. [H<sub>4</sub>PMo<sub>12</sub>O<sub>40</sub>]<sup>3-</sup> is the reduction product in methods which use  
841 organic reductants and/or heating, which may coexist with [H<sub>3</sub>PMo<sub>12</sub>O<sub>40</sub>]<sup>4-</sup> in methods using very  
842 low acidities. In contrast, the use of Sn(II) or Sb(III) in the reduction step yields MB species

843 incorporating these metals. Each of these MB species discussed above can be identified by its own  
844 distinctive Visible-NIR spectra. A mixture of giant isopolymolybdenum blues constitutes the blank  
845 signal of any MB method, which develops more readily with higher Mo(VI) concentrations and  
846 lower acidities.

847 The following practices are recommended in the use of the MB reaction for P determination:

### 848 **6.1. Recommended reductants**

849 In general, ascorbic acid and Sb(III) should be used since the  $\text{Sb}_2\text{PMB}(4e^-)$  reduction product forms  
850 within minutes, is stable for hours and is insensitive to chloride interference. This is particularly the  
851 case in batch, DA and SFA methods, where the temporal stability of the product is of greater  
852 importance. However, when maximum sensitivity is desirable and sample matrices contain  
853 negligible chloride concentrations,  $\text{SnCl}_2$  in combination with a sacrificial co-reductant (typically  
854  $\text{N}_2\text{H}_6\text{SO}_4$ ) should be used in preference to ascorbic acid and Sb(III) due to the faster reduction  
855 kinetics and significantly (~30%) higher molar absorptivity of  $\text{SnPMB}(4e^-)$ .

### 856 **6.2. Recommended acids**

857 Since both  $\text{HNO}_3$  and  $\text{HClO}_4$  are oxidising acids and interfere to a considerable extent with the  
858 reduction process,  $\text{HCl}$  and  $\text{H}_2\text{SO}_4$  are recommended as the strong acids of choice for MB  
859 procedures. However, there is some evidence to suggest that  $\text{H}_2\text{SO}_4$  inhibits the reaction, and a  
860 comparative study of  $\text{H}_2\text{SO}_4$  and  $\text{HCl}$  is warranted for MB procedures. If  $\text{SnCl}_2$  is used as the  
861 reductant,  $\text{H}_2\text{SO}_4$  should be used due to salt error interference from the  $\text{Cl}^-$  ion unless it is desirable  
862 to use  $\text{HCl}$  as a means of buffering the  $\text{Cl}^-$  concentration. If converting acid concentrations between  
863  $\text{H}_2\text{SO}_4$  and  $\text{HCl}$ , or when consulting older literature, care must be taken to accurately calculate  
864 acidity, since the older normality scale of acidity is an inaccurate measure of  $[\text{H}^+]$  when a diprotic  
865 acid such as  $\text{H}_2\text{SO}_4$  is used.

### 866 **6.3. Recommended optimisation procedure**

867 Any MB method for P determination should be optimised by first selecting the lowest Mo(VI)  
868 concentration which will be effective in the desired P concentration range (a 21-fold [Mo(VI)]/[P]  
869 excess at the highest predicted P concentration is sufficient). Using much larger Mo(VI)  
870 concentrations than are necessary increases the acidity needed to suppress blank formation, which  
871 decreases sensitivity. The acidity should then be varied such that the blank is minimised but  
872 sensitivity is not reduced. As an example, it has been previously shown that 3 – 5 mmol L<sup>-1</sup> Mo(VI)  
873 is more than sufficient (and potentially excessive) for complete 12-MPA formation from 1 mg L<sup>-1</sup>  
874 (32 µmol L<sup>-1</sup>) P, and that an acid concentration of around 0.20 mol L<sup>-1</sup> H<sup>+</sup> is an effective match for  
875 this Mo(VI) concentration range. Whether Na<sub>2</sub>MoO<sub>4</sub> or (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O is used is of no  
876 consequence for the reaction due to the equilibration of Mo(VI) species in acidic solutions within  
877 tens of minutes. If Sb(III) is used, the Sb concentration must be at least twice the highest expected P  
878 concentration in order to ensure that the method's linear range is sufficient; excess Sb(III) does not  
879 cause interference but high working concentrations may result in precipitation. Potassium antimonyl  
880 tartrate is a suitable source of Sb(III), and at typical working concentrations of ca. 10<sup>-5</sup> mol L<sup>-1</sup>, the  
881 interference of tartaric acid should be insignificant. The optimal reductant concentration will vary  
882 depending on the type of reducing system used (organic reductant with heating, organic reductant in  
883 the presence of Sb(III), or Sn(II)) and can be simply optimised for a given pair of H<sup>+</sup> and Mo(VI)  
884 concentrations by determining the concentration of reductant at which sensitivity is maximal but  
885 blank formation does not occur. Typical reductant concentrations are approximately 1 mmol L<sup>-1</sup>  
886 SnCl<sub>2</sub> or 5 - 20 mmol L<sup>-1</sup> ascorbic acid (Table 3). Silicate interference is easily controlled with  
887 sufficient acidity (pH < 1), short reaction times and the avoidance of heating. All MB methods  
888 should use a means of minimising product coating; working concentrations of glycerol (3.5 - 5.0%  
889 (m/v)) or sodium dodecyl sulfate (0.05% (m/v) or higher) have proven effective. This approach is  
890 recommended for flow and batch methods alike.

891 The authors of the present work recommend against the use of the [H<sup>+</sup>]/[Mo(VI)] ratio in method  
892 optimisation as it is a chemically unjustifiable and entirely empirical variable which is no good

893 indicator of MB reaction chemistry, the use of which can create considerable confusion. Amongst  
894 the literature methods using Sb(III) and ascorbic acid, the apparently optimal  $[H^+]/[Mo(VI)]$  ratio  
895 varies between 37 and 74 (Table 3), even after correcting for the common yet erroneous assumption  
896 that  $H_2SO_4$  fully dissociates twice at  $pH < 1$ . Several further points must be considered:

897 a) The  $[H^+]/[Mo(VI)]$  ratio does not inherently prescribe actual reagent concentrations, nor can it.  
898 Therefore, MB reaction chemistry may differ substantially between two methods using the same  
899 ratio but different reagent concentrations [82], which themselves must be determined by trial and  
900 error. Reagent concentrations can reportedly be scaled up or down for methods using Sb(III) at a  
901 fixed  $[H^+]/[Mo(VI)]$  ratio of 35, but only between Mo(VI) concentrations of 0.84 - 8.40  $mmol L^{-1}$   
902 [81]. However, this lower limit appears to be due to the P concentration used in these experiments,  
903 whilst at the upper limit where  $[H^+] = 0.588 mol L^{-1}$ , Z is sufficiently high for 12-MPA to begin  
904 decomposing. The linear range will also be limited by the Mo(VI) concentration used.

905 b) If the reaction is heated, silicate interference increases significantly at lower acidities, even if the  
906  $[H^+]/[Mo(VI)]$  ratio remains fixed.

907 c) Reaction time varies significantly, and in a complex manner, with both  $[H^+]$  and  $[Mo(VI)]$  [82].  
908 At a fixed  $[H^+]/[Mo(VI)]$  ratio, the reaction time invariably increases as the acid concentration  
909 increases.

910 It is considerably more complicated to optimise a method based on the trial-and-error  
911  $[H^+]/[Mo(VI)]$  framework as opposed to the method described above of selecting an appropriate  
912 Mo(VI) concentration, optimising the acidity on this basis, and then optimising the reductant  
913 concentration. As such, significantly greater clarity in the literature could be achieved if MB  
914 methods were to report their working concentrations of  $H^+$ , Mo(VI) and Sb(III) where appropriate,  
915 such as in Table 3, thus enabling at-a-glance comparison of the meaningful parameters which  
916 underlie Mo(VI) speciation, blank formation, silicate interference, linear range and reaction rate.

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921 **References**

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