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

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Abstract

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

Keywords: Molybdenum blue reaction; orthophosphate; dissolved reactive phosphate; phosphomolybdate

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1

1. Introduction

2. Chemistry of the phosphomolybdenum blue (PMB) reaction

2.1. Reaction overview

2.2. Mo(VI) speciation and 12-molybdophosphoric acid (12-MPA) formation

2.3. Redox chemistry of PMB

2.3.1. Reduction of 12-MPA

2.3.2. Spectral features of PMB

2.3.3. Nature of the reduced products

2.3.4. Isomerism of 12-MPA and its reduced forms

2.3.5. Organic reductants

2.3.6. Metallic reductants

3. PMB method optimisation

3.1. Reagent concentrations

3.2. The reagent blank: isopolymolybdenum blue species

3.3. Product stability

3.4. Method linearity

3.5. Choice of acid

4. Interferences

4.1. Additive interferences

4.1.1. Arsenate

4.1.2. Silicate

4.1.3. Organic and inorganic P hydrolysis

4.2. Subtractive interferences

4.2.1. Organic acids

4.2.2. Fluoride

4.2.3. Chloride (salt error)

4.3. Multifunctional interferents

4.3.1. Sulfide

4.3.2. Iron

4.3.3. Surfactants

5. PMB chemistry in flow methods

6. Conclusions and recommendations

6.1. Recommended reductants

6.2. Recommended acids

6.3. Recommended optimisation procedure

Acknowledgements

References

List of Abbreviations

(br): Broad absorption band

(sh): Absorption shoulder

12-MPA: 12-Molybdophosphoric acid ($H_3PMo_{12}O_{40}$)

11-MPA: 11-Molybdophosphoric acid ($H_3PMo_{11}O_{37}$)

12-MSA: 12-Molybdosilicic acid ($H_4SiMo_{12}O_{40}$)

AA: Ascorbic acid

ANS: 1-Amino-2-naphthol-4-sulfonic acid

AsMB: Arsenomolybdenum blue

DA: Discrete analyser

DAPH: 2,4-Diaminophenol dihydrochloride

DOP: Dissolved organic phosphorus MB: Molybdenum blue

DRP: Dissolved reactive phosphorus

ESI-MS: Electrospray ionisation mass spectrometry

FIA: Flow injection analysis

HQ: Hydroquinone

HS: Hydrazine sulfate

IVCT: Intervalence charge transfer

LMCT: Ligand-metal charge transfer

Metol: 4-(Methylamino)phenol sulfate

MRP: Molybdate reactive phosphorus

PMB: Phosphomolybdenum blue

rFIA: Reverse flow injection analysis

SFA: Segmented continuous flow analysis

SIA: Sequential injection analysis

SiMB: Silicomolybdenum blue

TDP: Total dissolved phosphorus

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.

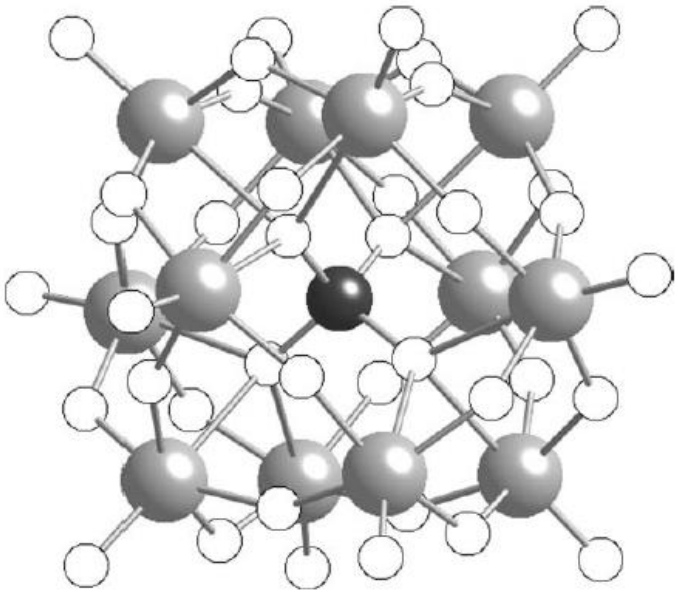

Figure 1. Structure of the Keggin ion $[PW_{12}O_{40}]^{3-}$, analogous to that of $[PMo_{12}O_{40}]^{3-}$. The black, grey and white spheres represent P, W and O respectively. Reproduced from Ref. [5] with permission from The Royal Society of Chemistry

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

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

2. Chemistry of the molybdenum blue reaction

2.1. Reaction overview

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

$$PO_4^{3-} + 12MoO_4^{2-} + 27H^+ \rightarrow H_3PO_4(MoO_3)_{12} + 12H_2O \quad (1)$$

$$H_3PMo(VI)_{12}O_{40} + \text{Reductant} \rightarrow [H_4PMo(VI)_8Mo(V)_4O_{40}]^{3-} \quad (2)$$

All MB methods require a strong acid, a source of Mo(VI) and a reductant, normally in aqueous solution. The concentrations of acid and molybdate are vital, not only for the formation of the heteropoly acid but also for controlling its reduction. It is well-known that orthophosphate (PO_4^{3-}),

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

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

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

$$p(MoO_4^{2-}) + qH^+ \rightleftharpoons [(MoO_4)_pH_q]^{(2p-q)-} \quad (3)$$

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

Species	Name	Z	pH
MoO_4^{2-}	Molybdate	0.00	7.00
$HMoO_4^-$	H-Molybdate	1.00	4.58
$Mo_2O_7^{2-}$	Dimolybdate	1.00	4.58
$Mo_7O_{24}^{6-}$	Heptamolybdate	1.14	4.30
$HMo_7O_{24}^{5-}$	H-Heptamolybdate	1.29	3.64
$Mo_3O_{10}^{2-}$	Trimolybdate	1.33	3.52
$H_2Mo_7O_{24}^{4-}$	H_2-Heptamolybdate	1.43	2.85
$Mo_4O_{13}^{2-}$	Tetramolybdate	1.50	2.25
$HMo_2O_7^-$	H-Dimolybdate	1.50	2.25
$Mo_8O_{26}^{4-}$	Octamolybdate	1.50	2.25
$HMo_3O_{10}^-$	H-Trimolybdate	1.67	1.48
$Mo_6O_{19}^{2-}$	Hexamolybdate	1.67	1.48
$HMo_4O_{13}^-$	H-Tetramolybdate	1.75	1.33
$HMo_5O_{16}^-$	H-Pentamolybdate	1.80	1.28
$Mo_{11}O_{34}^{2-}$	Undecamolybdate	1.82	1.27
$HMo_6O_{19}^-$	H-Hexamolybdate	1.83	1.27
$Mo_{12}O_{37}^{2-}$	Dodecamolybdate	1.83	1.25
MoO_3 or $MoO_3(OH_2)_3$	Molybdic acid	2.00	1.20
$HMo_2O_6^+$ or $Mo_2O(OH)_9(OH_2)^+$	Molybdate dimer cation	2.50	< 1.20
$HMoO_3^+$ or $MoO_2(OH)(OH_2)_3^+$	Molybdate monocation	3.00	< 1.20

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

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

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

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

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

$$MoO_4^{2-} + H^+ \rightleftharpoons [HMoO_4]^- \qquad pK_a = 3.66 \quad (5)$$

$$[HMoO_4]^- + H^+ + 2H_2O \rightleftharpoons MoO_3(OH_2)_3 \qquad pK_a = 3.81 \quad (6)$$

When the solution is sufficiently acidic, the second protonation also involves a change in the coordination chemistry of Mo(VI) from the 4-coordinate $HMoO_4^{2-}$ to the 6-coordinate hydrated molybdic acid $MoO_3(OH_2)_3$, which significantly decreases both of its pK_a values [13, 22]. The process of hydrating molybdic acid in the second protonation step requires that the acid concentration be at least ten-fold in excess of that of Mo(VI), otherwise precipitation of poorly soluble MoO_3 may occur [23]. However, molybdic acid will begin to protonate and dimerise when Z > 2 (Eqs. (7) – (9)**)** [8, 11, 16, 17, 24, 25], which is undesirable for 12-MPA formation.

$$MoO_3(OH_2)_3 + H^+ \rightleftharpoons [MoO_2(OH)(OH_2)_3]^+ \qquad pK_a \approx 0.9 \quad (7)$$

$$2[MoO_2(OH)(OH_2)_3]^+ \rightleftharpoons [Mo_2O(OH)_8(OH_2)_2]^{2+} + H_2O \qquad K_d = 100 \quad (8)$$

$$[Mo_2O(OH)_8(OH_2)_2]^{2+} \rightleftharpoons [Mo_2O(OH)_8(OH_2)_2]^+ + H^+ \qquad pK_a \approx 0.1 - 0.2 \quad (9)$$

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

shown in Fig. 3. The low Mo(VI) concentrations and ionic strengths used in the MB reaction actually simplify matters, as higher polymolybdates tend to form when either of these parameters is considerably higher [6, 14, 26]. It should also be noted that the choice of Na_2MoO_4 or $(NH_4)_6Mo_7O_{24}.7H_2O$ for MB methods is irrelevant due to the equilibria shown in Fig. 3, provided that sufficient time is allowed for the equilibration of Mo(VI) species before the solution is used. Whilst it has been reported that several hours are needed for this process in pure solutions of either Na_2MoO_4 or $(NH_4)_6Mo_7O_{24}.4H_2O$ [27], equilibration appears to occur much more quickly if the solution is pre-mixed with acid, requiring only about 10 - 30 min [26, 28]. Furthermore, Na_2MoO_4 may be the favoured Mo(VI) salt in laboratories where P and NH_3 determination are carried out simultaneously, in order to avoid cross-contamination.

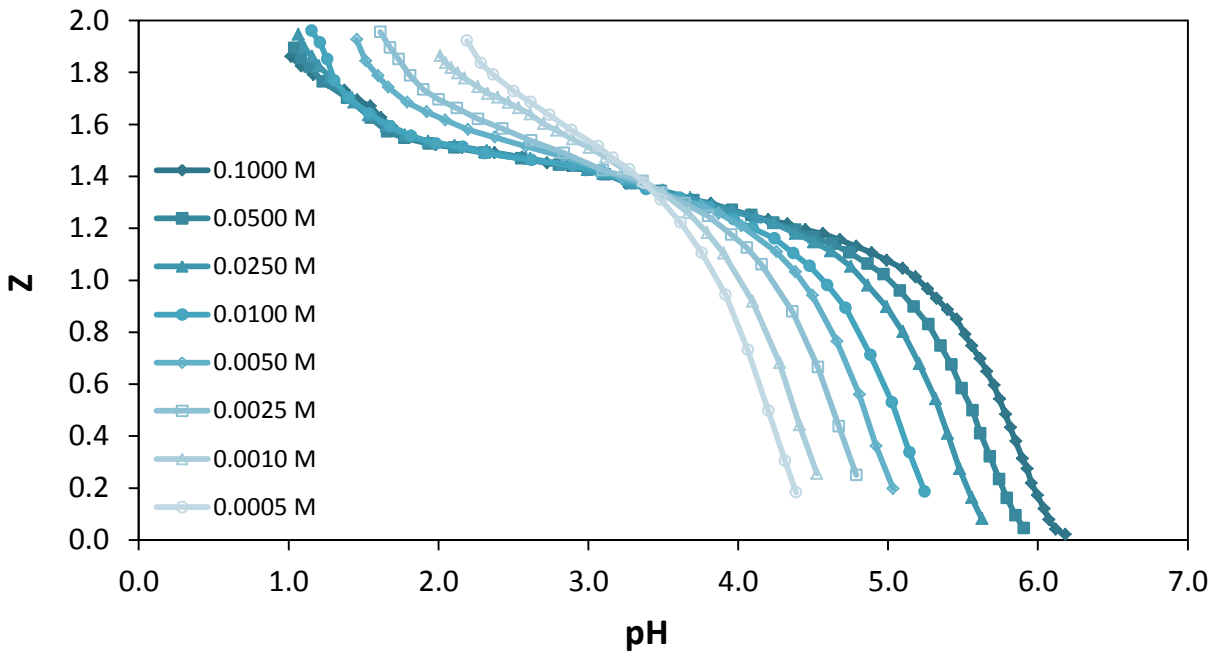

Figure 2. Plots of the degree of protonation, Z, vs. pH at different Mo(VI) concentrations in 1 mol L^{-1} NaCl to reduce the influence of ionic strength variation. Reproduced from Ref. [6] with permission from Elsevier.

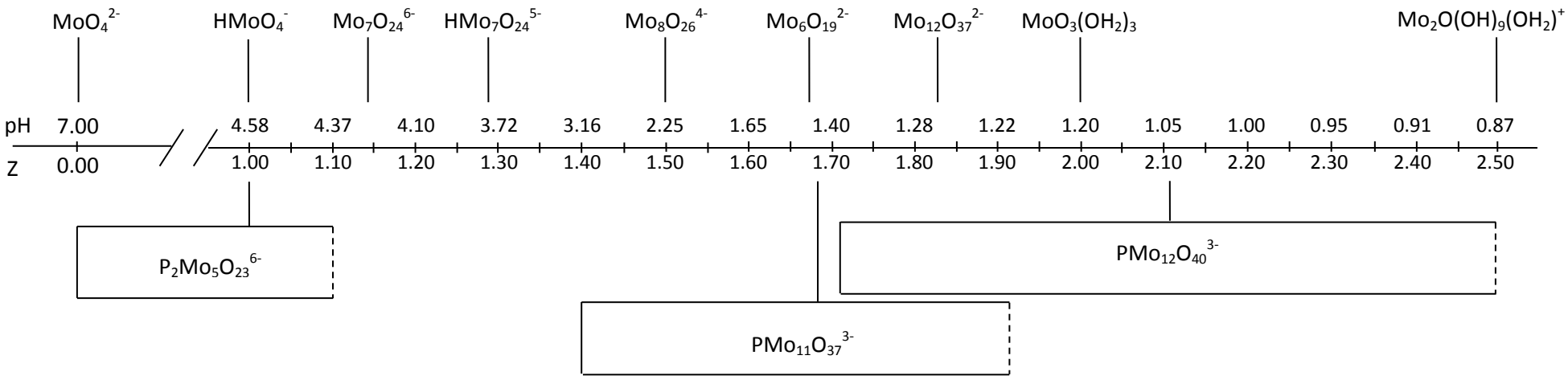

Figure 3. Mo(VI) speciation in aqueous solution containing 10 mmol L^{-1} Mo(VI) as a function of Z (bottom), or as a function of pH (top), and the phosphomolybdate species which may form under these conditions (deprotonated forms shown for clarity). The optimal Z value for the formation of each phosphomolybdate 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.

It is important to consider how 12-MPA is formed from its precursors, as a number of studies addressing this question are based on several early conclusions made before more detailed data about Mo(VI) equilibria were available in the literature. For example, explanations of both pH-related behaviour [29] and the observed rate laws for 12-MPA formation [23, 27, 30, 31] were initially based on conclusions about Mo(VI) and H^+ stoichiometry obtained under highly acidic conditions [27] which favoured monomeric and dimeric Mo(VI) cations (Table 1), and were not optimal for 12-MPA formation. It was suggested in these studies that the assembly of 12-MPA occurred via the reaction of cationic Mo(VI) dimers with H_3PO_4 and the release of H^+ [23, 27, 30, 31], although these authors also allowed for the possibility of a pre-equilibrium between the dimers and a larger Mo(VI) ion which constituted the actual reactive species. More recent UV-visible spectrophotometry and Raman spectroscopy studies [7, 8, 10, 32] have shown that this latter consideration was in fact much more accurate than the theory of cationic dimers as the reactive species, as it accounts for the decomposition of 12-MPA as well as the inhibition of its formation at high acidities where dimeric Mo(VI) cations actually predominate. In fact, dodecamolybdate ($Mo_{12}O_{37}^{2-}$) appears to be the main precursor Mo(VI) species in equilibrium with 12-MPA [7, 8, 10], and a detailed mechanism of 12-MPA formation is given later in this section. The prevalence of the dodecamolybdate anion at very low pH values is minimal since the condensation of cationic Mo(VI) species into larger ions such as $Mo_{12}O_{37}^{2-}$ requires the release of H^+ into an already highly acidic solution, as per Eq. (10). Since high Z solutions are unfavourable for the existence of the 12-MPA precursor species, 12-MPA dissociates under these conditions.

$$6[Mo_2O(OH)_9(OH_2)]^+ \rightleftarrows Mo_{12}O_{37}^{2-} + 29H_2O + 8H^+ \quad (10)$$

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

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

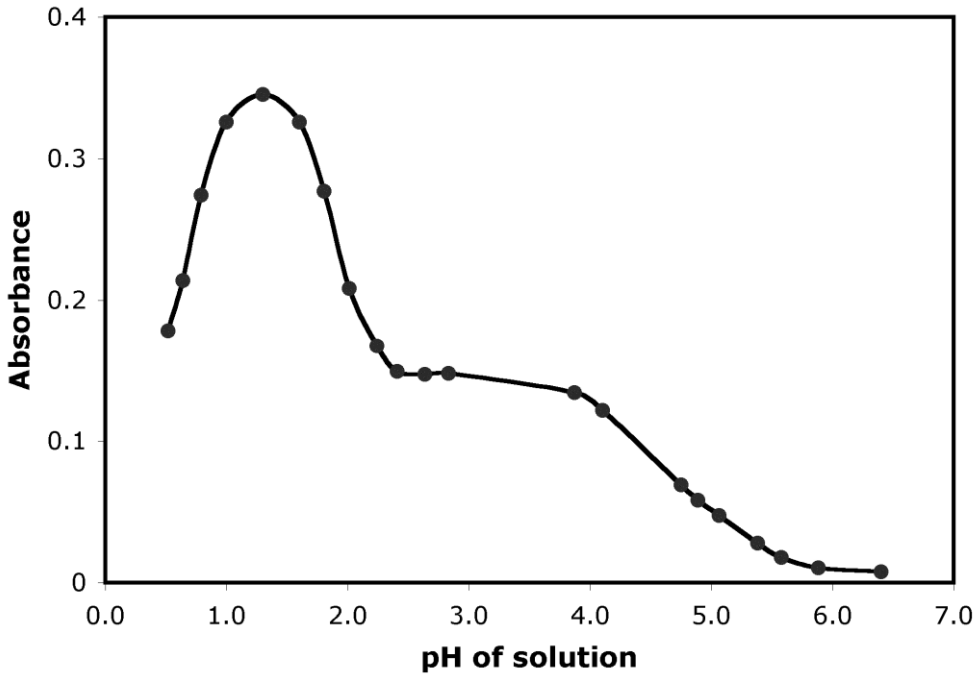

Figure 4. Influence of pH on the formation of phosphomolybdate species in aqueous solution; [P] = 1 mmol L^{-1}, [Mo(VI)] = 12 mmol L^{-1}, λ = 420 nm. Whilst the absorbance above approximately pH 1 arises from a combination of phosphomolybdate species, note the sharp decline in absorbance towards lower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

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

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

$$12MoO_4^{2-} + 22H^+ \overset{-H_2O}{\underset{\longleftarrow}{\longrightarrow}} [(MoO_3)_{11}(MoO_4)]^{2-} \overset{PO_4^{3-}}{\underset{\longleftarrow}{\longrightarrow}} [(MoO_3)_{11}(PO_4)]^{3-} + MoO_4^{2-} \overset{H^+}{\underset{\longleftarrow}{\longrightarrow}} H_3PMo_{12}O_{40} \quad (11)$$

The displacement mechanism (Eq. (11)) best matches the conditions normally used in MB methods, as the sample is typically introduced into a pre-mixed reagent solution in both batch-wise and flow-based methods. Furthermore, in MB methods, the ratio of [Mo(VI)] / [P] in solution is high, thus accelerating the reaction. For example, a method that uses 10 mmol L^{-1} Mo(VI) to determine 1 mg L^{-1} P (32 µmol L^{-1}) as orthophosphate will exhibit a [Mo(VI)] / [P] ratio of over 300, whereas Murata and Ikeda in their Raman spectroscopy work used only the stoichiometric ratio of 12 [10]. It should be noted that if conditions change such that [Mo(VI)] / [P] ≤ 14, 12-MPA will decompose into 11-MPA, 9-MPA and ultimately $[P_2Mo_5O_{23}]^{6-}$ [8, 35]. It is therefore expected that a large excess of Mo(VI) over P will drive the phosphomolybdate equilibria to form 12-MPA almost exclusively, rather than leaving significant residual amounts of 11-MPA.

The alternative mechanism, known as the 'ground-up' mechanism, occurs when acid is added to a pre-mixed solution of orthophosphate and Mo(VI) and is much more rapid than the displacement mechanism. This mechanism involves the assembly of 12-MPA around an orthophosphate ion. Raman spectroscopy has indicated that only MoO_4^{2-} appears to exist in solution before acidification [10], yet it has previously been reported that orthophosphate binds so strongly to molybdate in solution that it cannot exist as the free ion in the presence of the latter [32]. Indeed, interactions between orthophosphate and several condensed molybdates have been reported, each leading to the

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

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

2.3. Redox chemistry of PMB

2.3.1. Reduction of 12-MPA

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

It is well-established that the redox behaviour of 12-MPA is highly acid-dependent; the reduction of aqueous 12-MPA occurs in single electron steps at higher pH values, whilst this behaviour changes to two-electron steps under acidic conditions [38-42] via proton-induced disproportionation of the odd-electron reduced species [43]. Each two-electron reduction step of 12-MPA is accompanied by the addition of two protons [38, 39], and increasing solution acidity shifts its reduction potential to more positive values [38, 39] since the overall negative charge of the complex is decreased as it protonates. In fact, the correlation between the anionic charge of 12-MPA and its first reduction potential is linear [38, 43]. Voltammetric analysis of 12-MPA in aqueous 0.2 mol L^{-1} H_2SO_4

solutions (pH ≈ 0.78) has shown that 12-MPA may undergo two successive and reversible two-electron reductions at +0.31 V and +0.17 V [42], which decrease to +0.27 V and + 0.13 V at pH 1 [44]. A third two-electron reduction at about -0.08 V is possible but this renders the product highly unstable, resulting in immediate decomposition [42] (Fig. 5). It must be noted that reduced heteropoly acids are unstable with respect to re-oxidation by dissolved O_2 [45]; their continued existence in aqueous solutions depends on an excess of reductant to react preferentially with O_2, an aspect of reaction optimisation which is discussed in Section 3.3.

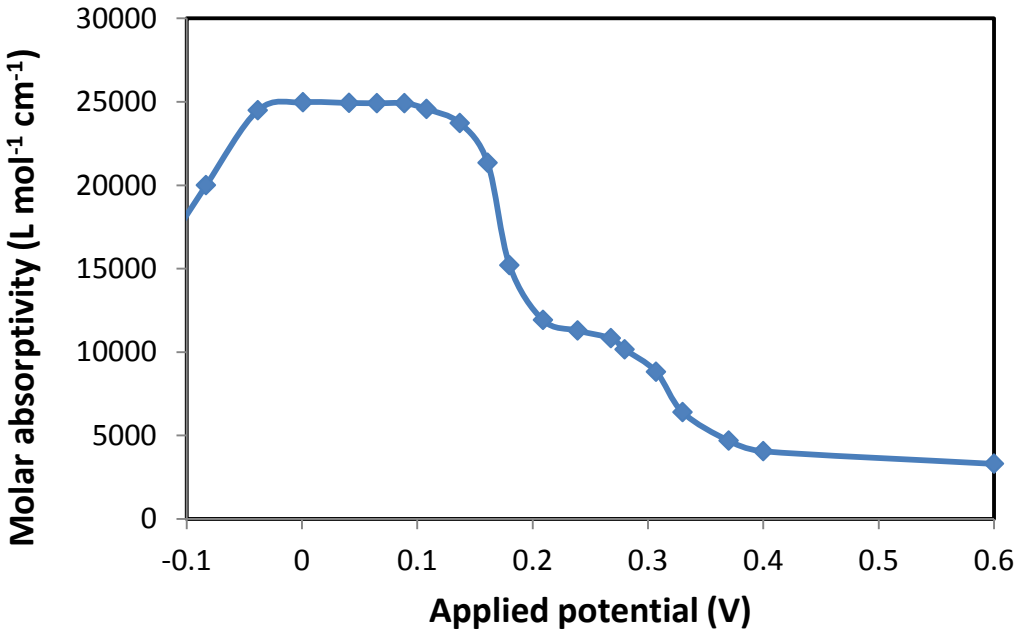

Figure 5. Molar absorptivity at 830 nm of a 12-MPA solution as a function of applied potential during its electrochemical reduction. [H_2SO_4] = 0.2 mol L^{-1}, [12-MPA] = 2 mmol L^{-1}. Adapted with permission from Tanaka et al. [42]. Copyright 2015 American Chemical Society.

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

$$H_3PMo(VI)_{12}O_{40} + ne^- \rightarrow [H_3PMo(VI)_{12-n}Mo(V)_n]^{n-} \qquad n = 2, 4 \qquad (12)$$

2.3.2. Spectral features of PMB

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

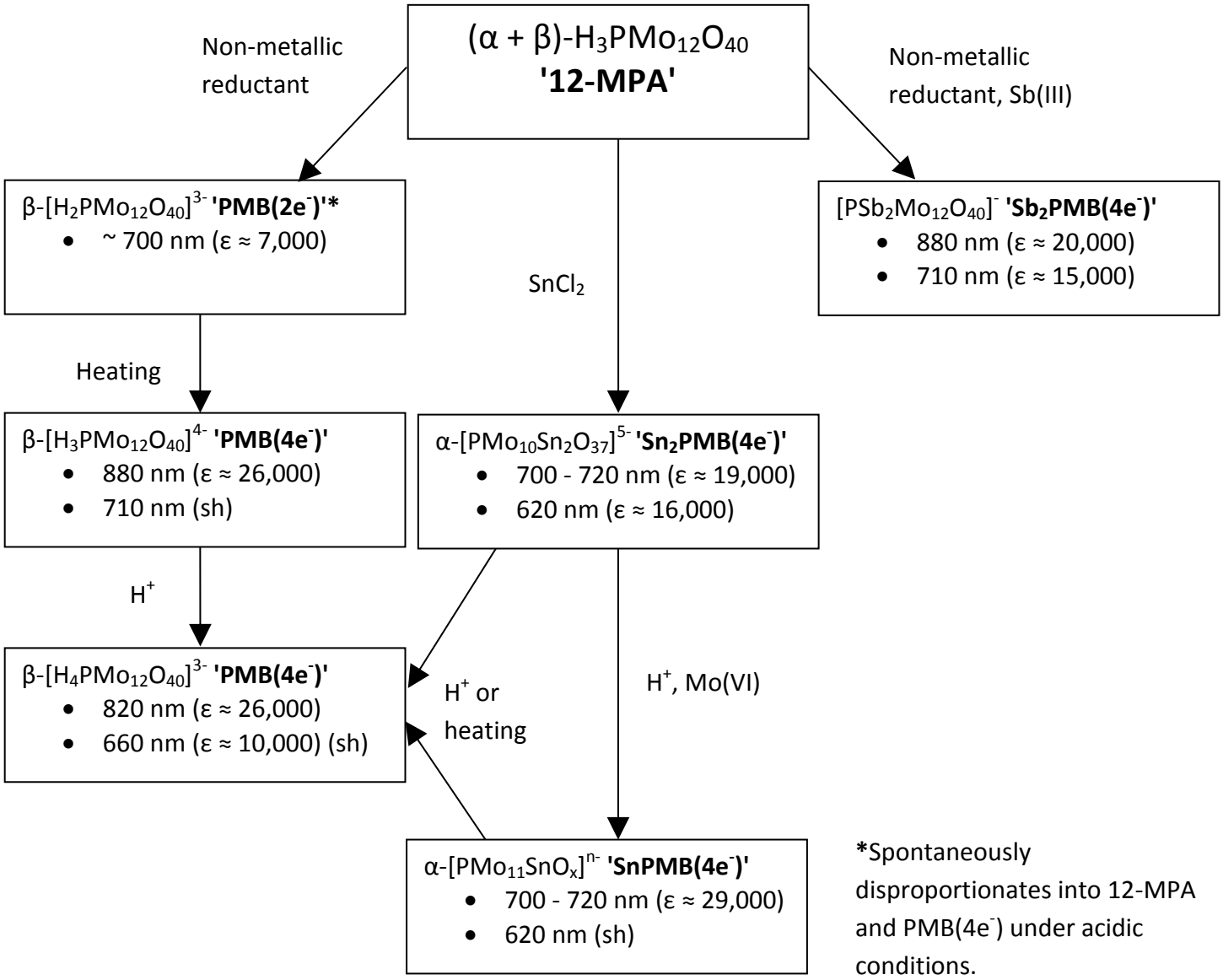

Figure 6. Spectrophotometric properties of the various PMB species and the conditions under which they form. Molar absorptivities (ε) are in units of L mol^{-1} cm^{-1}. (sh) = absorption shoulder.

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

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

Table 2. UV/visible spectral features of PMB($4e^-$) species.

Wavelength (nm)	Type of transition	Assignment	Band intensity
220	LMCT	Mo-O	Strong, decreases with further reduction. Not specific to reduced species.
315	LMCT	Mo-O-Mo bridges	
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, $\varepsilon \approx 10{,}000$ L mol^{-1} cm^{-1}
700 - 900	IVCT	Mo(V) → Mo(VI)	Strong, $\varepsilon \approx 26{,}000 – 34{,}000$ L mol^{-1} 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].

The molar absorptivities of the strong IVCT bands are significantly higher for PMB($4e^-$) than PMB($2e^-$) [54, 55, 57], but excessive reduction yields PMB($6e^-$) which immediately decomposes in acid [40, 42, 58]. This demonstrates that whilst a higher degree of reduction is desirable for improving analytical sensitivity, overwhelming reducing power should be avoided. The positions of the intense IVCT absorption bands are dependent on a number of factors discussed below.

Molar absorptivity values of PMB in various reported methods have been used as a means of comparing analytical sensitivity between methods. However, usage of this term as a comparative measure is often incorrect. All methods which use non-metallic reductants ultimately produce the

same product with the same innate molar absorptivity, $[H_4PMo_{12}O_{40}]^{3-}$, or the $2e^-$ reduced species as an intermediate when heating is not used (Fig. 6); differences in apparent absorptivity are simply due to the extent of the reduction process at the time of measurement or the influence of organic solvents [42, 56, 59]. The molar absorptivities of PMB species can be difficult to determine since quantitative formation of a single PMB species is necessary. The difficulty lies in the nature of MB chemistry as one complex equilibrium system; rather than stoichiometric reactions occurring, sufficient reagent must be introduced to perturb the equilibria to a desirable extent. Thus, if one attempts to simply dissolve solid 12-MPA in acid and reduce it without adding Mo(VI), very low apparent absorptivities will be obtained [40], as most of the 12-MPA will in fact decompose in accordance with its formation equilibria (Fig. 3) [40, 60]. Thus, sufficient Mo(VI) must be present to stabilise 12-MPA against hydrolytic decomposition.

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

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

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

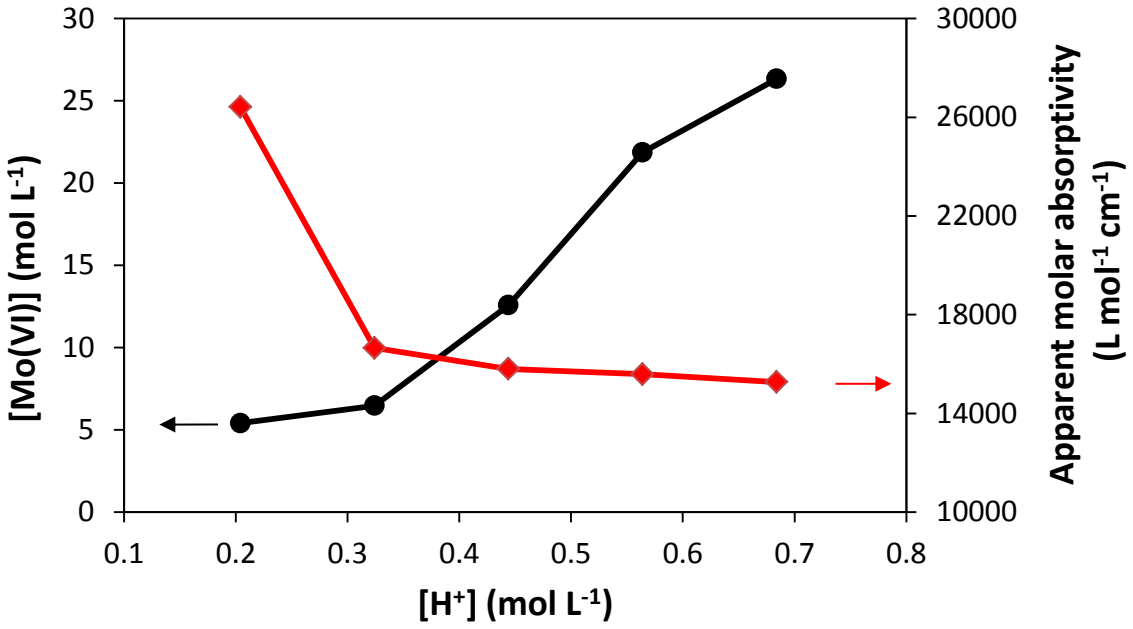

Figure 7. Plot of acidity against the maximum tolerable Mo(VI) concentration (●) before direct reduction of Mo(VI) to isopoly MB begins, and the apparent molar absorptivity of the SnPMB($4e^-$) product at 700 nm (◆) under these conditions (Data obtained from El-Shamy and Iskander [40]. [12-MPA] = 40 μmol L^{-1} for molar absorptivity data, [$SnCl_2$] = 880 μmol L^{-1}).

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

Authors (Year)	Acid	[Acid] (mmol L^{-1})	[Mo(VI)] (mmol L^{-1})	[Reductant] (mmol L^{-1})	Temperature (^{0}C), (reaction time)	λ_{max} (nm)	ε (L mol^{-1} cm^{-1})	Notes	Ref
Tanaka (1982)	H_2SO_4	200	2 (12-MPA)	Electroreduction	-	830	25,000	Optical path length uncertain	[42]
Fontaine (1942)	H_2SO_4	1000	36	$SnCl_2$: 210	100 (20 min)	820	28,000		[62]
Sims (1961)	HCl	650	17	$SnCl_2$: 2.43	-	815	26,400		[63, 64]
El Sayed (2001)	H_2SO_4	50	1.3	$SnCl_2$: 1.04	-	700	23,000	Broad peak, shoulder at ~ 820 nm	[65]
Kriss (1971)	H_2SO_4	300	5	$SnCl_2$: 5	(< 3 h)	680	20,400		[66]
Levine (1955)	HCl	600	11	$SnCl_2$: 0.16	-	735	19,000		[67]
El-Shamy	HCl	840	20 & 0.08	$SnCl_2$: 1.93	-	810	17,000		[40]

(1973)			(12-MPA)					Peaks in equilibrium	
	HCl	360	20 & 0.08 (12-MPA)	$SnCl_2$: 1.93	-	720	16,000	via isosbestic point at 780 nm	[40]
	HCl	450	0.165 (12-MPA)	$SnCl_2$: 0.66	-	810	4,400	Broad shoulder around 720 nm	[40]
	HCl	280	0.24 (12-MPA)	$SnCl_2$: 1.44	-	700	1,200	Shoulder at 820 nm	[40]
Hesse (1968)	H_2SO_4	300	31	$SnCl_2$: 0.13 HS: 2.31	(20 min)	730	25,500		[68]
Drummond (1995)	H_2SO_4	127	2.4	AA: 9.6	(12 h)	820	26,760		[29]
Han (1995)	H_2SO_4, HCl	330, 170	14	AA: 54	45 (20 min)	825	26,000		[69]
Chen (1956)	H_2SO_4	300	14	AA: 57	37 (90 min)	820	25,000		[70]
Lowry	pH 4		5.7	AA: 5.7	(5 min)	860	4,600	Very similar	[71]

(1946)								absorbance from 700 – 900 nm	
Katewa (2003)	H_2SO_4	500	14	AA: 11.4 HS: 15.4	(60 min)	820	26,100		[49]
Sims (1961)	H_2SO_4	500	10.3	HS: 0.46	100 (unspecified)	815	32,300		[63]
Ganesh (2012)	H_2SO_4	500	39	HS: 9.61	60 (30 min)	830	29,000		[72]
Boltz (1947)	H_2SO_4	500	10.3	HS: 0.46	93 (10 min)	830	26,400		[73]
Huey (1967)	$HClO_4$	1200	24	HS: 1.8	100 (15 min)	805	14,000		[60]
Burton (1956)	H_2SO_4	30	2.95	Metol: 2.9 Na_2SO_3: 87	100 (2 h)	860	29,900		
	H_2SO_4	200	2.95	Metol: 2.9 Na_2SO_3: 87	100 (2 h)	820	29,300		[50]
	H_2SO_4	30	2.95	Metol: 1.5	(2 h)	720,	3,900	Extremely broad	

				Na_2SO_3: 44		830 (sh)		absorbance peak	
Sims (1961)	$HClO_4$	920	143	Metol: 0.33 $NaHSO_3$: 30 Na_2SO_3: 4.0	100 (10 min)	820	18,500		[63]
Harris (1954)	$HClO_4$	480	11.3	Metol: 1.16 $NaHSO_3$: 96 Na_2SO_3:15	-	820	3,900		[63, 74]
El Sayed (2001)	H_2SO_4	30	11	ANS: 0.11	90 (30 min)	830	32,000		[65]
Sims (1961)	$HClO_4$	920	143	ANS: 0.33 $NaHSO_3$: 30 Na_2SO_3: 4.0	100 (10 min)	820	28,100		[63]
Griswold (1951)	H_2SO_4	500	14.2	ANS: 0.42 $NaHSO_3$: 570 Na_2SO_3: 16	100 (10 min)	820	27,000		[75]
Fiske	H_2SO_4	250	14	ANS: 0.42	-	730	3,900		[76, 77]

(1925)				$NaHSO_3$: 57.7					
Sims	$HClO_4$	920	143	DAPH: 0.33	100 (10 min)	820	34,500		[63]
(1961)				$NaHSO_3$: 30					
				Na_2SO_3: 4.0					
Huo	H_2SO_4	300	10.3	Thiamazole: 0.245	-	710	1,000		[78]
(2012)									
Kriss	H_2SO_4	300	5	Fe(II): 5	(< 3 h)	700	12,800		[66]
(1971)									
Kriss	H_2SO_4	30	5	HQ: 5	(< 3 h)	700	12,800		[66]
(1971)									
Salem	H_2SO_4	76	2.8	Oxalyldihydrazide:	100 (10 min)	880	33,000	Shoulder at 820 nm	[79]
(1991)				0.017					
Gupta	H_2SO_4	200	5.4	AA: 4.8	(10 min)	882	25,670		[80]
(1981)				Sb(III): 0.066					
Going	H_2SO_4	150	3.5	AA: 4.5	(10 min)	880	22,400	ε = 26400 (840 nm)	[81]
(1974)				Sb(III): 0.8				without Sb.	

Pai (1990)	H_2SO_4	200	5.4	AA: 4.8 Sb(III): 0.066	(30 min)	880	22,400		[82]
Harwood (1969)	H_2SO_4	200	2.7	AA: 23 Sb(III): 0.32	(10 min)	890	20,600		[83]
Edwards (1965)	H_2SO_4	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^{-1}	[84]
Murphy (1962)	H_2SO_4	200	5.4	AA: 4.8 Sb(III): 0.066	(10 min)	882	22,400		[85]
Drummond (1995)	H_2SO_4	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.

2.3.3. Nature of the reduced products

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

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

Table 4. Effect of protonation on IVCT absorption maxima and approximate molar absorptivities for β-$[X^{n+}Mo_{12}O_{40}]^{(12-n)-}$. Roman numerals denote the number of electrons the compound is reduced by.

Protonation and reduction state	**Absorption maxima (nm) in aqueous solution. Approximate molar absorptivities (L mol^{-1} cm^{-1}) and peak shapes are given in parentheses where possible; (br) = broad absorption peak, (sh) = absorption shoulder.**		
	$X = As^{5+}$	$X = Si^{4+}$	$X = P^{5+}$
IV	1110 (6600), 741(8400)		1050, 765
HIV	990 (9600),	1000 (br), 760 (br)	

	752 (9000)		
H_2IV	935 (16000), 719 (sh)	925, 720 (sh)	
H_3IV	885 (25000), 704 (sh)	880, 700 (sh)	880 (26000), 700-710 (sh)
H_4IV	840 (25000), 667 (sh)	820, 660-680 (sh)	820 (26000), 660 (sh)
II		1120 (br), 720	1030, 760
H_2II		1050, 680	~ 1000, 730 (7000, br)

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

As can be seen in Table 4, protonation has a profound effect on the absorptivities and positions of the PMB(4e$^-$) IVCT bands. It is now established that for four-electron reduced heteropoly acids, the first three protons are strongly acidic, whereas the remainder are weakly acidic [58, 61, 87]. For example, the weak pK_a values for SiMB(4e$^-$) are 2.8, 3.8, 7.1 and 9.5 [58], whilst those for AsMB(4e$^-$) are ~ 3, 4.5, 7.2 and 9.5 [61]. Given that orthophosphate MB methods are generally performed at pH < 1, it can be expected that PMB(4e$^-$) will almost always be present as $[H_4PMo_{12}O_{40}]^{3-}$, with the possibility of $[H_3PMo_{12}O_{40}]^{4-}$ existing at very low acid concentrations. This assignment is confirmed by the spectra of electrochemically formed PMB(4e$^-$) [42], characterisation of pure PMB(4e$^-$) at pH 1[86], and ion-pair HPLC [56]. These two highly protonated forms of PMB(4e$^-$) are amongst the most intensely absorbing of any PMB species, with virtually identical absorptivities at their respective wavelength maxima (Fig. 6).

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

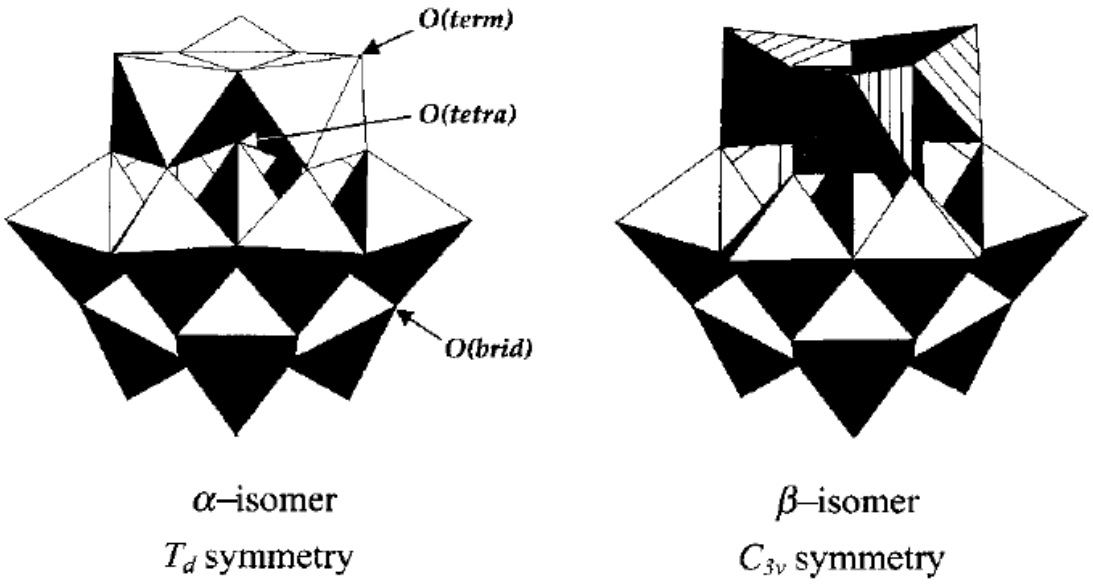

Figure 8. Polyhedral representations of the α- and β-isomers of the Keggin structure. Point group symmetries are indicated, and the labels in parentheses distinguish between the three types of O atoms (terminal, bridging, or part of the central tetrahedron). The β-isomer is obtained from the α-isomer by a 60^0 rotation of one Mo_3O_{13} subgroup. Reprinted with permission from [88]. Copyright 2015 American Chemical Society.

2.3.4. Isomerism of 12-MPA and its reduced forms

12-MPA is typically encountered as one of two isomers; the α-isomer is the nominal Keggin structure and is the stable form of unreduced 12-MPA, whereas the β-isomer, obtained by a 60^0 rotation of a Mo_3O_{13} group, is the more stable form of the reduced species (Fig. 8) [88, 89]. The optical and redox properties of the two isomers differ, in that the β-isomer is reduced at more positive potentials and exhibits more intense IVCT bands [34, 55, 89]. This phenomenon is well-known in silicomolybdate chemistry where the two isomers can be isolated in aqueous solution under distinctly different conditions [90-92], but has been overlooked in analytical methods based on phosphomolybdate. This is probably because β-MPA is less stable than its silicon counterpart [88], and whilst evidence suggests that the two isomers form simultaneously from different molybdate precursors in fresh 12-MPA solutions [89, 91, 93], β-MPA isomerises to α-MPA within minutes [89]. However, isomerisation from α → β appears to begin in earnest once the reduction process begins, and voltammetric data for aqueous 12-MPA indicate that nearly 10 min after the first reduction of pure α-MPA, the ratio of α- and β-isomers is nearly 1:1 [42]. Because the β-isomer is the thermodynamically favoured form of the reduced species, heating will accelerate the α → β transformation of PMB [94]. It should be noted, however, that the isomerism of PMB applies strictly only to $[H_nPMo_{12}O_{40}]^{(7-n)-}$, which is generally only obtained from the heating of organic reductants. Metallic reductants are capable of altering the composition of PMB and locking it into one particular isomer, a phenomenon discussed below.

2.3.5. Organic reductants

MB methods which use only organic reductants or hydrazine sulfate (the 'non-metallic' reductants) in combination with heating demonstrate a clear absorption maximum at 820 nm (Table 3), with a discernible shoulder at 660 nm. Electrochemical reduction of 12-MPA also demonstrates these features [42], which are attributed to the species β-$[H_4PMo_{12}O_{40}]^{3-}$ (Table 4). An exception to this is seen in the method of Salem [79] where the unusually low acid concentration encourages the predominance of β-$[H_3PMo_{12}O_{40}]^{4-}$ instead, with an absorption maximum at 880 nm instead of the usual 820 nm.

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

$$\text{Dehydroascorbic acid} + 2H^+ + 2e^- \leftrightarrows \text{Ascorbic acid} \qquad E_h = +0.33\ \text{V (pH 1)} \qquad (13)$$

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

Aside from methods using $SnCl_2$ as a reductant (discussed below), those which report an absorbance maximum at ca. 720 nm show vastly inferior molar absorptivities (Table 3). These methods do not use heating; as a result, the predominant reduction product is the two-electron reduced species $[H_2PMo_{12}O_{40}]^{3-}$, and the actual extent of 12-MPA → PMB($2e^-$) reduction is expected to be quite low as well. Furthermore, this species is only transient, and will exhibit an unstable UV-visible spectrum over time as it disproportionates to PMB($4e^-$) and 12-MPA.

2.3.6. Metallic reductants

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

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

$SnCl_2$ is very widely used due to its fast reduction kinetics which obviates the need for heating. However, it is quite clear that 12-MPA reduction with $SnCl_2$ yields a distinctly different visible spectrum to that of $[H_4PMo_{12}O_{40}]^{3-}$ (Fig. 9) which cannot be accounted for via (de)protonation. In fact, instead of the conventional four-electron reduction of 12-MPA, the presence of newly-formed Sn(IV) ions permits a different and much more favourable reaction in which the four-electron reduced product, still in the α-form, is immediately reacts with two Sn(IV) ions which substitute for Mo(VI) [94, 96]; whilst this behaviour has only been studied on 12-MSA, it appears analogous to that of 12-MPA. The vastly more positive reduction potential of 12-MPA in the presence of Sn(IV) has been discussed as the likely cause of the fast reduction kinetics observed with $SnCl_2$ [97]. The Sn(IV) substitution process is not observed with 12-MPA before reduction, nor does Sn(II) substitute for Mo after reduction.

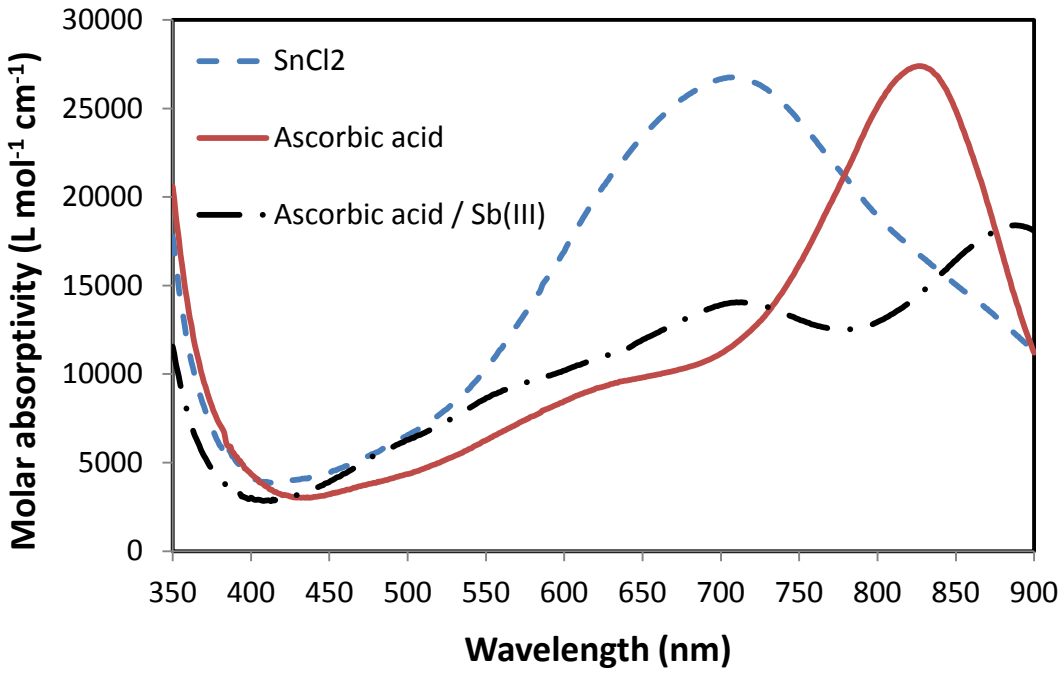

Figure 9. Comparison of product spectra for the three most commonly used reductants in MB methods. Ascorbic acid / Sb(III); prepared according to the procedure described in Standard

Methods for the Examination of Water and Wastewater [98]. Ascorbic acid; prepared as for ascorbic acid / Sb(III) procedure without the addition of Sb(III) and with heating at 100 ^{o}C for 10 min. $SnCl_2$; prepared using the working concentrations reported in [99]. All solutions contained 500 μg L^{-1} P as orthophosphate.

Thus, upon four-electron reduction of 12-MPA by $SnCl_2$, the immediate product appears to be α-$[PMo_{10}Sn_2O_{37}]^{5-}$ [94, 96], denoted as $Sn_2PMB(4e^-)$, with an absorption peak between 700 - 720 nm ($\varepsilon \approx 19{,}000$ L mol^{-1} cm^{-1}) and a pronounced shoulder at 620 nm.

$$\alpha\text{-}H_3PMo_{12}O_{40} + 4e^- + 2Sn^{4+} + 3H_2O \rightarrow \alpha\text{-}[PMo_{10}Sn_2O_{37}]^{5-} + 2MoO_3 + 9H^+ \quad E_h \approx +0.7\ V \qquad (14)$$

The rapid kinetics of this reduction process are attributed to a combined reduction/substitution step with a much more positive reduction potential than for the direct reduction of 12-MPA alone (Eq. (14)) [97]. Over time, this species spontaneously undergoes the loss of one Sn(IV) ion to form the incompletely characterised $SnPMB(4e^-)$ species, as has been observed with its silicate analogue [94]. $SnPMB(4e^-)$ absorbs more intensely at 720 nm than $Sn_2PMB(4e^-)$ whilst the absorptivity of the 620 nm band hardly changes, becoming a barely noticeable shoulder (Fig. 9) [96], and this mono-tin species is the main reduction product monitored in methods using $SnCl_2$ reduction. The spectral features of both products are reported in the analytical literature, i.e. $Sn_2PMB(4e^-)$ [85, 100] and $SnPMB(4e^-)$ [66, 99]. The nature of the transformation of $Sn_2PMB(4e^-)$ to the more intensely absorbing $SnPMB(4e^-)$ remains unclear, although it is not a redox process since $Sn_2PMB(4e^-)$ should be stable with respect to oxidation [96]. However, this process is spontaneous, and is likely the result of attack by acidified Mo(VI) species [97].

$SnPMB(4e^-)$ may ultimately be hydrolysed to $[H_4PMo_{12}O_{40}]^{3-}$ over time, although this process is accelerated with either heating [62, 94] or further acidification [40], indicated by the emergence of the 820 nm peak (Fig. 6). This process therefore appears to involve the displacement of Sn(IV) by molybdic acid, which seems to occur in the conversion of $Sn_2PMB(4e^-)$ to $SnPMB(4e^-)$ as well. These reactions are summarised in Fig. 6.

149 It should be pointed out that $SnCl_2$ only undergoes the chemistry discussed above when it reduces
150 α-isomers of heteropoly acids, and all of the stannomolybdate species discussed above are
151 themselves α-isomers [96]; the reduction of β-isomers yields PMB($4e^-$) directly without
152 incorporation of Sn(IV). Given the tendency for α- and β-MPA to form concurrently from
153 orthophosphate and acidified molybdate [89], and the equilibrium between SnPMB($4e^-$) and
154 PMB($4e^-$), it is to be expected that methods using $SnCl_2$ will form a mixture of PMB($4e^-$),
155 SnPMB($4e^-$) and Sn_2PMB($4e^-$) 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 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 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 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 $SnCl_2$, the presence of Sb(III) has a significant effect on the visible IVCT bands of the PMB
170 product, countering the early view that Sb(III) served only as a catalyst. 12-MPA reduced in the
171 presence of 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 $[H_3PMo_{12}O_{40}]^{4-}$ species (Table 4), except that the 710 nm band in the Sb(III) product is a
174 distinct peak.

It has long been known that antimony becomes part of the reduced complex and a central element in the stoichiometry of the reaction. However, the original 1:1 Sb:P ratio determined by Murphy and Riley [85] has been called into question by more recent and reliable results that show a 2:1 ratio of Sb:P in the reduced species [80, 81, 105] instead. This conclusion is in excellent agreement with observations about method linearity; a comparison of Sb(III) MB methods has shown that the linear range is limited by antimony concentration, and has a cut-off precisely where the Sb:P ratio becomes less than 2:1 [83]. This phenomenon has been exploited to extend the linear range to more than three times that of Murphy and Riley's method [83], although adding excessive amounts of Sb(III) results in solubility problems, presumably due to hydroxide salts [83, 85]. Of course, this linear range is only with respect to absorbance at 880 nm; the further addition of orthophosphate results in the additional formation of the familiar $[H_4PMo_{12}O_{40}]^{3-}$ species with IVCT bands at 820 and 680 nm, giving the false impression that the 880 nm band blue-shifts with added orthophosphate due to the rising 820 nm band [51, 81, 83].

The structure of the reduced phosphoantimonylmolybdate species $Sb_2PMB(4e^-)$, and the cause of its accelerating effect on the reduction kinetics, are not clearly established. The molar absorptivity of $Sb_2PMB(4e^-)$ at 880 nm compared with the conventional $[H_4PMo_{12}O_{40}]^{3-}$ product at 820 nm under the same conditions is actually lower by 20 - 30% [29, 81, 85]; this trend is clearly confirmed in Table 3 and in Fig. 9. Nevertheless, molar absorptivities in the vicinity of 20,000 L mol^{-1} cm^{-1} still suggest a four-electron reduced product. Aside from the Sb:P ratio of 2:1, the stoichiometry of the entire $Sb_2PMB(4e^-)$ complex has never been established with certainty. Whilst it has been held for some time that the complex is probably $[PSb_2Mo_{10}O_x]^{n-}$, wherein Sb(III) is substituted for Mo(VI), this conclusion appears to be based only on the findings of Going and Eisenreich in 1974 [81], which were derived from an average measured Mo:P ratio of 11.4:1 and the observed stoichiometry of other heteropoly species. A recent ESI-MS study addressing this question found no evidence of $[PSb_2Mo_{10}O_{40}]^{n-}$ or protonated derivatives under any conditions, but did observe peaks in aqueous solutions of pH ≈ 0.8 corresponding to $[PSb_2Mo_{12}O_{40}]^{n-}$ and species with one or both antimony

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

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

Sb(III) does not interact with acidified molybdate, nor does it alter the IVCT bands of pre-formed PMB [81]; it does not even appear to form an adduct with unreduced 12-MPA at ambient temperature [106], contrary to earlier assumptions [109]. However, it does redshift the characteristic UV charge transfer bands of 12-MPA, as do a number of other metals [81], suggesting at least weak coordination to the oxygen atoms of 12-MPA. The full $Sb_2PMB(4e^-)$ adduct seems to form only during 12-MPA reduction [106]. Heteropoly complexes which contain metals as counter-cations are known to exist as charge-transfer complexes, in which the metal acts as the electron donor and 12-MPA is the electron acceptor; isolation of antimony salts in the solid phase has shown that Sb(III) can indeed partially reduce 12-MPA when in this configuration, although again only after a heating step [107, 110]. However, the observation that Sb(III) does not bind to PMB after reduction would seem to discount it acting only as a countercation or loosely coordinating species. Taken together, all of the above evidence suggests that much like in the case of $SnCl_2$, Sb(III)-facilitated reduction is a reaction distinct from the normal four-electron reduction of 12-MPA. Interestingly, in the presence of both Sb(III) and Sn(II), only the $SnPMB(4e^-)$ product is observed, probably due to the much faster reduction process involving substitution of Sn(IV) into the product structure [111].

Despite these observations, the precise mechanism of $Sb_2PMB(4e^-)$ formation and the redox potential for the Sb(III)-facilitated reduction remain unknown.

Heating is sometimes applied in methods using Sb(III) to further enhance the rate of reduction [82, 112]. However, heating of this reaction can be problematic. Not only does it increase the impact of silicate interference [82, 109] as would be expected of any heated method (see Section 4.1.2), but higher reaction temperatures also appear to degrade the $Sb_2PMB(4e^-)$ product [109, 112]. If the product is already fully formed and is exposed to successively higher temperatures, the UV-visible spectrum of the product exhibits lower apparent molar absorptivities at 710 and 880 nm with an isosbestic point at 935 nm [112]. This evidence suggests that at high temperatures, the $Sb_2PMB(4e^-)$ product equilibrates with one or more species with an absorption maximum at wavelengths longer than 950 nm (see Section 2.3.2). However, this spectral change was found to be almost entirely reversible upon cooling the reaction mixture again. In contrast, if the reaction itself is performed at elevated temperatures, the concentration of $Sb_2PMB(4e^-)$ appears to decrease irreversibly, with considerably instability in absorbance at 880 nm during the reaction [112]. Pai et al. recommend that MB methods using Sb(III) should not exceed a reaction temperature of 35^0C.

3. MB method optimisation

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

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

However, the goals of flow-based techniques such as flow injection analysis (FIA), reverse flow injection analysis (rFIA) and sequential injection analysis (SIA) differ considerably from those of batch, automated discrete analyser (DA) and air-segmented continuous flow analyser (SFA) methodology [113]. Whilst the demands for sensitivity, low blank absorbance and a broad linear range remain, the temporal stability of the product is no longer of particular importance since sample detection occurs at a fixed point during the reaction, i.e. under non-equilibrium conditions. However, reaction kinetics are still important in flow systems; whilst the extent of reaction is inherently reproducible in these systems, it is obviously advantageous to maximise the extent of reaction in a short timeframe to enhance sensitivity. It is therefore unsurprising that the $SnCl_2$ and Sb(III) methods now dominate the literature on both flow-based and batch orthophosphate detection using MB, as the reduction reaction occurs almost instantaneously with the former reagent, and within a few minutes with the latter. Furthermore, whilst the low and zero dispersion of SFA and DA methods respectively allow for the exact replication of optimised solution conditions from batch methods, the much higher degree of dispersion encountered using FIA, rFIA or SIA makes the same task impossible using these methods. Rather than dealing with discrete volumes of reaction mixture, such systems inherently create concentration gradients between neighbouring liquid zones, which are further subjected to a parabolic flow profile arising from friction with the walls of the manifold tubing.

3.1. Reagent concentrations

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

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

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

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

Furthermore, prolonged heating appears to decompose both PMB($4e^-$) and Sb_2PMB($4e^-$) [60, 112], and any heating at all accelerates the spontaneous hydrolysis of the SnPMB($4e^-$) product [62, 94] obtained from methods using $SnCl_2$ as the reductant. Without heating, however, the effects of reagent concentrations on reaction kinetics and equilibrium positions become more important. At this point, it is important to highlight the trend shown in Fig. 7; a greater Mo concentration will always require a greater acidity to mitigate the reagent blank (discussed in Section 3.2), but the loss of sensitivity due to 12-MPA decomposition at these higher acidities is significant. Thus, whilst the acidity can easily be optimised for any given Mo concentration, method sensitivity will necessarily decrease as [Mo(VI)] is increased. Therefore, the concentration of Mo(VI) must be sufficiently high to stabilise 12-MPA at P concentrations appropriate for the analysis, but not so high as to warrant deleterious concentrations of acid. Going and Eisenreich originally recommended that Mo(VI) be used in at least a 40-fold molar excess over the maximum P concentration expected [81] although their experiments focused on the $[H^+]$/[Mo(VI)] ratio at different Mo(VI) concentrations. Nevertheless, this recommendation broadly agrees with other studies which have found that Mo(VI) concentrations as low as 3 – 5 mmol L^{-1} are sufficient for extensive 12-MPA formation from approximately 1 mg L^{-1} P (Table 3). The lowest possible acidity should be used, such that PMB decomposition is minimised but direct Mo(VI) reduction is still prevented or mitigated. El-Shamy and Iskander found that at 5 mmol L^{-1} Mo(VI), an acidity of only 0.20 mol L^{-1} HCl was effective in preventing isopoly MB formation [40]. However, whilst higher acidities at this Mo concentration drastically inhibited PMB formation, no acidities lower than this value were examined.

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

	Reagent concentrations at linear range maximum							
Author (Year)	**$[H^+]$ (mmol L^{-1})**	**[Mo(VI)] (mmol L^{-1})**	**[AA] (mmol L^{-1})**	**[Sb] (µmol L^{-1})**	**[P] (µmol L^{-1})**	**[Sb]/ [P]**	**[Mo(VI)]/ [P]**	**Ref.**
Harwood	200	2.7	23	329	> 97	Excess	< 28	[83]

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

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, $SnCl_2$
334 or Sb(III) methods require only low millimolar concentrations of reductant for effective use; most
335 $SnCl_2$ studies report reductant concentrations of 1.0 – 2.5 mmol L^{-1} $SnCl_2$, and Sb(III) / ascorbic
336 acid methods are effective with around 5 mmol L^{-1} ascorbic acid. Even a 1 mmol L^{-1} reductant
337 concentration represents an approximately 30-fold molar excess over 1 mg L^{-1} 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].

3.2. The reagent blank: isopoly molybdenum blue species

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

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

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

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

It is also interesting to note that in methods where a combined reagent solution containing acid, molybdate and reductant is prepared, such as those reported in Standard Methods for the Examination of Water and Wastewater [98], the reagent solution tends to exhibit a yellow colour [91] due to absorption bands at 490 and 385 nm. These bands can also be observed in some aged PMB products, manifesting as a green hue, and reflect the d-d transitions of dimeric Mo(V) present as $[Mo_2O_4(OH_2)_6]^{2+}$ [121, 122]. The presence of this species indicates the direct reduction of Mo(VI) under conditions where isopoly species cannot form, due to either the higher acidity of a combined reagent solution or the low Mo(VI) concentration used in some methods. Mo(V) halide complexes with a similar yellow colour have also been reported to form in marine sample matrices [123]. Whether or not the $[Mo_2O_4]^{2+}$ species itself acts as a reductant or is directly involved in forming PMB is currently unknown, but it does not interfere with the spectrophotometric determination of P. 12-MPA itself is also attributed a faint yellow colour, although this appears to arise from the tail of the 315 nm LMCT band extending into the visible region in concentrated 12-MPA solutions.

3.3. Product stability

The stability of the colour in MB chemistry is attributable to three factors; the extent of reduction at the time a measurement is taken, the stability of the product and the availability of excess reductant to protect PMB from re-oxidation by dissolved O_2. For example, it is frequently noted in studies which make use of organic reductants without heating that the absorbance of the product continually increases for periods up to several hours [49, 63, 70, 74]; given the slow reduction kinetics of these species, it is now clear that the reported 'instability' of the colour arises from the slow, ongoing disproportionation of PMB($2e^-$) to 12-MPA and PMB($4e^-$), which exhibits a different absorption spectrum. However, once formation of PMB($4e^-$) using organic reductants is complete, the product appears to be stable for at least several hours with respect to oxidation by dissolved O_2 [49, 60, 72, 73, 124]. Sb(III) methods behave in the same manner; once the reduction finishes after several minutes, the Sb_2PMB($4e^-$) adduct appears stable for many hours with respect to oxidation [51, 85]. By contrast, a recent study on the use of UV photo-reduction of 12-MPA found that PMB($4e^-$) immediately began to re-oxidise once irradiation ceased, as no reducing reagents other than PMB existed in the absence of UV light [125].

$SnCl_2$ methods are well known to yield comparatively short-lived products. However, this unusually short lifespan cannot be attributed solely to re-oxidation by O_2, since electrochemical measurements of Sn-substituted PMB complexes show that these products are in fact more stable toward oxidation than PMB($4e^-$) [96]. Instead, it has been found that the Sn-substituted products are hydrolytically unstable and are degraded in a stepwise fashion, presumably by acidified Mo(VI) [96], which may proceed to ultimately form PMB($4e^-$). However, in comparison to heated and Sb(III)-containing ascorbic acid methods, the reductant concentration in $SnCl_2$ methods is normally much lower, which presents a greater risk of untimely product re-oxidation. Therefore, $SnCl_2$ methods are often augmented with a sacrificial co-reductant such as hydrazine sulfate to extend the oxidative stability of the product to 30 – 60 min [49, 68, 126].

3.4. Method linearity

The linear range of a given MB method is the range of P concentrations over which 12-MPA formation and reduction occur to the same degree, and in which no deviation from the Bouguer-Beer-Lambert law is observed. MB method linear ranges, particularly those based on the Murphy and Riley method [85], typically extend up to around 1 mg L^{-1} P. In practice, modern methods typically achieve quantitative, or at least substantial reduction, even in the short timeframes of FIA measurements; $SnCl_2$ is well-known to reduce 12-MPA almost instantly, and Sb(III) methods can achieve full reduction within one minute if the chemical conditions are carefully chosen [29].

The actual extent of the linear range is governed by the presence of sufficient acidified Mo(VI) to stabilise 12-MPA, sufficient reductant, and in the case of antimony methods, sufficient Sb(III). Furthermore, linear ranges reported for FIA/SIA and batch/DA/SFA versions of the same method are not typically comparable, as they treat the sample volume in a fundamentally different way. Whereas sample aliquots are diluted to a fixed volume in batch procedures, they undergo dispersion in flow methods. Since the practical dispersion coefficient of a flow system describing the extent of sample dilution is unknown unless specifically characterised, it is easier to discuss method linearity in the context of batch methods.

Antimony-based methods are the simplest case. As discussed above, these form a complex in which the ratio of Sb:P is 2:1, and it is clear from a number of investigations [29, 83-85] that the upper end of the linear calibration range of each method occurs at P concentrations where the Sb:P ratio becomes less than 2, with the graph suddenly adopting a shallower gradient (Table 5). At this point, the $[H_4PMo_{12}O_{40}]^{3-}$ product forms which absorbs at 820 nm, contributing only slightly to the absorbance increase at 880 nm. By increasing the Sb(III) concentration used in the Murphy and Riley method fivefold, Harwood et al. were able to greatly extend the linear range of the former [83], whilst simultaneously halving the unnecessarily high Mo(VI) concentration. It has been found that even with a [Mo(VI)]/[P] ratio as low as 21, Sb(III) still acts as the limiting reagent (Table 5)

[84], suggesting that a 21-fold [Mo(VI)]/[P] ratio is sufficient for complete 12-MPA formation. Ascorbic acid concentrations of 5 – 100 mmol L^{-1} are reported (Table 3), with higher concentrations accelerating the reduction process somewhat [29, 81]; it has been suggested that ascorbic acid must be present in a 20-fold excess over 12-MPA to completely reduce it, with or without Sb(III) [81].

The addition of further Sb(III) does not accelerate the reduction process, nor does it appear to interfere with the reduction chemistry, but the extension of the linear range to arbitrarily high P concentrations by adding more Sb(III) is limited by solubility problems, which have been reported at working concentrations of ca. 10^{-4} mol L^{-1} Sb(III) [83, 85]. A similar phenomenon is well-known during the preparation of the mixed reagent solution in methods derived from that of Murphy and Riley [85], in which turbidity may become evident once both Sb(III) and Mo(VI) have been added to the solution [84, 98], and for which the general recommendation is to keep mixing the solution until the precipitate re-dissolves. This phenomenon is curious given much greater solubility of potassium antimonyl tartrate in pure water than in combined reagent solutions. In seems likely that the insoluble species being formed is in fact a salt of Sb(III) and molybdate [127] which forms at low [Mo(VI)] / [Sb(III)] ratios, rather than a basic salt as suggested by Murphy and Riley [85]. If a salt of Sb(III) and molybdate is responsible for the Sb(III) solubility issues, it is expected that pH manipulation will be ineffective in dissolving it [127]; dilution should be the preferred course of action.

Data on the linear ranges of MB methods other than those utilising Sb(III) have seldom been reported; many authors claim that their method demonstrates a linear response up to at least a given P concentration, rather than examining the maximum tolerable P concentration. As an approximation, non-Sb(III) methods appear to be limited by reductant concentration as long as [Mo(VI)]/[P] $\geq$ 21. For $SnCl_2$ methods, it appears that an approximately 8-fold $SnCl_2$:12-MPA excess is required for complete product formation; the standard $SnCl_2$ method deviates from linearity around this point, even though Mo(VI) is present in more than an 80-fold molar excess over orthophosphate [98]. Similarly, El-Shamy and Iskander found that the reduction of 12-MPA

continued up to a 5- to 6-fold molar excess of $SnCl_2$ over 12-MPA [40]. In methods using heating with organic reductants, 10-fold excesses of hydrazine sulfate and 1-amino-2-naphthol-4-sulfonic acid (ANS) have also been reported as sufficient for complete reduction of 12-MPA [65, 73], as the reduction inherently becomes much more favourable and rapid at higher temperatures.

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

3.5. Choice of acid

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

Neither nitrate nor perchlorate appear to coordinate to Mo(VI) species to any significant degree, even in 0.5 mol L^{-1} Mo(VI) solutions in 2.0 mol L^{-1} HNO_3 or $HClO_4$, as determined by ^{95}Mo NMR and Raman spectroscopic measurements [129]. However, the oxidising ability of nitric and perchloric acids should discourage their use in MB methods; even 0.1 mol L^{-1} HNO_3 interferes with $SnCl_2$ reduction of heteropoly acids [114] and $HClO_4$ has been reported to form a precipitate with $SnCl_2$ and partially oxidise hydrazine sulfate [74].

Literature reports of interactions of sulfate with heteropoly acid systems are varied [130, 131], including one 1915 report of a molybdosulfate species co-existing with 12-MPA, apparently precipitated as $[(NH_4)_2SO_4.5MoO_3]$ [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_2SO_4 is markedly more
496 dissociated into its precursor molybdates than in HNO_3[27] or $HClO_4$[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_4^- (pK_a = 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_2SO_4 (or
504 acidified sample matrices containing SO_4^{2-}) since this reaction must involve the release of HSO_4^-
505 from molybdosulfate species into a solution already containing a high concentration of HSO_4^-. 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_2SO_4 was seen to be far less than that observed with HNO_3 at a
508 range of pH values, and Linares et al. [134] have reported that even using HNO_3 instead of H_2SO_4
509 results in a significant enhancement in PMB($4e^-$) formation , despite the oxidising power of HNO_3.
510 A more detailed investigation of the impact of H_2SO_4 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_4^{3-} 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_4^{2-} and HSO_4^- are unavoidable in any case. Furthermore,
518 the ‘salt error’ encountered in $SnCl_2$ methods appears to have discouraged the use of hydrochloric

519 acid as an alternative to sulfuric acid, even in cases where the 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 $MoO_2Cl_2(H_2O)_2$ and $MoOCl_3(OH)(H_2O)$ 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 mol L^{-1}, and approximately 0.8 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 H_2SO_4 should be performed. However, if $SnCl_2$ 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 PO_4^{3-} forms a reducible heteropoly acid, so too does 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

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

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

arsenomolybdate complex, given the diverse range of As : Mo stoichiometries encountered in these compounds [35]. Whether or not the formation of combined As(V) / P species occurs in $SnCl_2$ reduction methods is unknown.

4.1.2. Silicate

SiO_4^{4-} has long been considered one of the main interferents in PMB methods as it also forms heteropoly acids (α- and β-12-MSA) [114] reducible to molybdenum blues. Silicate interference is well-known to become problematic when the reaction acidity is too low and when heating is employed [29, 109]. This is due to two separate phenomena; 12-MSA is only stable at lower Z values (higher pH) than 12-MPA [73], and the speciation of silicic acid itself is dependent on both temperature and acidity. At low pH, orthosilicic acid (H_4SiO_4) exists in equilibrium with polysilicic acids [141, 142] which form 12-MSA much more slowly than orthosilicic acid alone [142], presumably because only orthosilicate possesses the appropriate molecular geometry to form 12-MSA. Of note is a very recent ESI-MS study [20] suggesting that the use of H_2SO_4 actually decomposes polysilicic acids into a monosilicate complex with HSO_4^- which may still react to form 12-MSA, implying that silicate interference is therefore increased when H_2SO_4 is used. Unfortunately, no more data on this phenomenon are available at this time.

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

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

modern PMB methods (Table 3). The rate of 12-MSA formation decreases sharply when Z > 2 [90], which can be approximated as > 0.2 mol L^{-1} H^+ for low millimolar Mo(VI) concentrations and is within the optimal range for many PMB methods (Table 3). For example, in a previously reported flow injection method where working concentrations of 5 mmol L^{-1} Mo(VI) and 0.2 mol L^{-1} H_2SO_4 were used, 50 mg L^{-1} Si did not interfere at all and 100 mg L^{-1} Si generated only a + 2.6% error in the timeframe of the method [125]. Since acidity controls silicate interference by slowing the kinetics of 12-MSA formation, it therefore follows that the more rapidly a measurement is made, the more silicate can be tolerated in a sample without detectable interference [109].

An alternative approach to inhibiting 12-MSA formation is the use of organic acids. These species function as ligands, sequestering Mo(VI) and thus slowing the formation of 12-MSA, a phenomenon further discussed in Section 4.2.1. This approach exploits the observation that 12-MSA's formation kinetics can be greatly slowed by the presence of organic acids in comparison to 12-MPA which forms much more rapidly. However, this approach can be a double-edged sword since 12-MPA is decomposed by such species at higher concentrations, whereas 12-MSA is unaffected by organic acids if they are added after its formation is complete, a phenomenon often exploited in silicate determination [144]. For example, 12-MSA formation can be effectively suppressed with dilute oxalic acid whilst leaving 12-MPA mostly intact, whereas fully formed 12-MPA can be easily destroyed with more concentrated oxalic acid, which is ineffective in decomposing fully formed 12-MSA [145]. Tartaric acid ($C_4H_6O_6$) is also often used for inhibiting silicate interference in orthophosphate determinations [145, 146], yet it is reported to interfere with this determination quite substantially in its own right [145, 147], more so than oxalic acid [145]. Interestingly, since methods using Sb(III) almost invariably utilise potassium antimonyl tartrate ($K_2Sb_2(C_4H_2O_6)_2.3H_2O$) as the source of Sb(III), it may be expected that the tartaric acid acts to suppress silicate interference, even in micromolar quantitites. Zhang et al. have reported that the addition of this salt can reduce silicate interference by almost 50% when the method is heated to

619 70^0C [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

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

4.2.2. Fluoride

F^- is a strong negative interferent in the MB reaction [68, 147, 168, 169]. In a similar manner to that of organic acids, its mode of interference is binding to Mo(VI) to form discrete ions which cannot condense to form larger structures such as 12-MPA [170, 171]. This has the effect of slowing the MB reaction without necessarily changing the extent of the reaction, giving the appearance of a lower analytical signal if insufficient time is allowed for the inhibited reaction to reach completion [172]. H_3BO_3 is very effective at sequestering F^- as BF_4^- without negatively impacting the MB reaction [170, 173]. An interesting point is that silicate also complexes to F^- in solution, and can mitigate the inhibitory effect of F^- on the MB reaction in its own right [101]. Of course, this phenomenon interferes with the determination of silicate itself in waters containing sufficient F^-.

Interestingly, F^- can also alter the redox potentials of metallic reductants. The use of Fe(II) as a reductant combined with NaF has been reported, wherein the complexation of F^- to Fe(II) enhanced the latter's reducing power [174]. NaF has also been used to enhance the reducing power of $SnCl_2$

by converting its oxidation product to the more stable SnF_6^{2-} [175]. However, due to the formation of HF at the acidities used in the MB reaction, this approach cannot be recommended on practical grounds.

4.2.3. Chloride (salt error)

A major source of subtractive interference in MB methods is the 'salt error' [50, 85, 97, 105, 176], which manifests as a decrease in analytical signal when $SnCl_2$ is used as the reductant in assays of marine and estuarine waters or other matrices containing elevated chloride concentrations. Whilst a small (approx. 1 – 4%) decrease in analytical signal is generally expected at high ionic strengths due to the decrease in activity coefficients [50, 176], the decrease in signal is usually reported to be between 15 – 20% in marine samples [50, 64, 85, 176] and even higher in some cases [68, 97], implying a dependence on method conditions as well. The degree of analytical signal suppression varies with the Cl^- concentration in the matrix [68, 97] as well as the orthophosphate concentration [97], and method linear range is attenuated with increasing Cl^- concentration as well. As such, the salt error is troublesome not only because of the loss of method sensitivity, but because this loss varies in magnitude between sample matrices of different salinities and P concentrations.

The nature of the salt error is the decreased formation of SnPMB($4e^-$) due to the disruptive effect of Cl^- on the chemistry of Sn(IV). Chloro-complexes of Sn(II) and Sn(IV) readily form in the presence of Cl^- [105, 177-179], and their stability dramatically impedes the ability of Sn(IV) to substitute into PMB($4e^-$) to form Sn_2PMB($4e^-$) [97, 105]. As a result, the highly favourable combined reduction and substitution step responsible for producing Sn_2PMB($4e^-$) is impeded due to the depletion of uncomplexed Sn(IV). Thus, higher concentrations of Cl^- limit the extent to which SnPMB($4e^-$) can form, and force the system to reduce 12-MPA via the slower and much less favourable direct reduction process [97]. This is clearly shown in UV-visible spectra of products suffering from the salt error, in which the 710 nm SnPMB($4e^-$) peak is diminished and a distinct shoulder at 820 nm emerges due to the increased presence of PMB($4e^-$). Even though the two reduction products

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

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

4.3. Multifunctional interferents

4.3.1. Sulfide

Sulfide, present as H_2S under acidic conditions, is capable of both additive and subtractive interference in the MB reaction [181]. H_2S can act as a reductant for 12-MPA in its own right [182, 183] and causes distinct additive interference at low P concentrations as a result [181], particularly in terms of direct Mo(VI) reduction. At larger P concentrations, H_2S causes subtractive interference instead, apparently by complexing with Mo(VI) [181]. In Sb(III) methods, H_2S will also sequester Sb(III) to form insoluble antimony sulfides; not only is the precipitate problematic for spectrophotometry, but the loss of dissolved Sb(III) also forces the formation of the PMB($4e^-$) species, thus altering the spectral properties of the product [184]. H_2S can be conveniently oxidised by $KMnO_4$ 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 $KMnO_4$ concentration of 5.3 mmol L^{-1} can be reduced by a working ascorbic acid
720 concentration of 26.9 mmol L^{-1} with no impact on method sensitivity [183].

721 **4.3.2. Iron**

722 Fe(II), much like H_2S, can reduce 12-MPA. In fact, 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. 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 Fe(III) might precipitate orthophosphate from
728 solution [191]. Fe(III) interference is effectively controlled with a sufficient excess of reductant or
729 the presence of an organic acid to complex with 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 / Sb(III) or $SnCl_2$ reduction [193]. In this study, cationic surfactants such as
733 tetraalkylammonium chlorides were shown to cause severe negative interference even at low mg L^{-1}
734 concentrations; 5 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 / Sb(III) reduction was variable, whilst the $SnCl_2$ 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 g L^{-1} for ascorbic acid / Sb(III) reduction), and branched sulfonates such as
741 sodium dioctylsulfosuccinate only interfered above 25 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^{-1}.

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 $SnCl_2$ 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].

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

In terms of actual reaction chemistry, the Murphy and Riley method [85] using ascorbic acid / Sb(III) is by far the most popular of any MB method in flow analysis owing to its relatively fast reaction kinetics at room temperature, insensitivity to Cl^- interference and low susceptibility to silicate interference, since heating is not required [2]. $SnCl_2$ methods also have considerable utility in a flow analysis setting; their very rapid reduction kinetics and more intensely absorbing product [97] allow for greater sensitivity and sample throughput than ascorbic acid / Sb(III) methods, since their limited stability after several minutes is typically of little concern. However, the salt error has led to the diminished popularity of such methods, particularly as many flow analysis applications are aimed at ultra-trace P determination in marine or estuarine waters.

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

The coating phenomenon is dependent on the pH of the reaction mixture and the concentration of PMB product formed. The general observation that coating causes a smooth, elongated tail on FIA peaks suggests that the coating species exhibits a similar or identical spectral profile to the PMB product being measured. Combined with the observation that higher concentrations of PMB product increase sample carryover [109], it is apparent that the coating compound, which appears blue [109, 126], is the main reduction product and not the isopoly MB species formed in the reagent blank. The effect of acidity on sample carryover is interesting; for PMB($4e^-$), the amount of sample carryover appears to reach a minimum at pH 0.5 and varies little at higher pH, but is greatly increased below pH 0.5 [109, 195]. By contrast, Sb_2PMB($4e^-$) exhibits increased coating at pH values both above and below 0.5 [195], with precipitation of Sb(III) salts above pH 1.50 [109]. The coating phenomenon has previously been attributed to the colloidal behaviour of PMB species [109, 195] caused by their limited water solubility. This explanation is reasonable given the clear decrease in coating incidence at higher temperatures, and suggests that the solubilities of both PMB($4e^-$) and Sb_2PMB($4e^-$) decrease dramatically below pH 0.5, whilst Sb_2PMB($4e^-$) also experiences solubility problems at higher pH values. The actual reductant used, so long as the reduced product is the same, has no inherent effect on coating; assertions to the contrary are very likely due to differing extents of reduction between methods [195].

The lower solubility of the Sb_2PMB($4e^-$) complex than that of PMB($4e^-$) has been investigated by Zhang et al. in two studies [109, 195]. These authors showed that between pH 0.5 – 1.5, Sb_2PMB($4e^-$) gave rise to a carryover percentage of between 1 - 2% at room temperature, roughly double that of PMB($4e^-$) [109]. Curiously, these results differ greatly from those obtained in a subsequent study by the same authors in which carryover coefficients were significantly larger [195]. However, this later study also employed a commercial anionic surfactant formulation with unknown additives; the potential precipitation problems associated with such formulations have 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$^-$) 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$^-$) 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 $[H^+]$/[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. $[H_4PMo_{12}O_{40}]^{3-}$ is the reduction product in methods which use
841 organic reductants and/or heating, which may coexist with $[H_3PMo_{12}O_{40}]^{4-}$ in methods using very
842 low acidities. In contrast, the use of Sn(II) or Sb(III) in the reduction step yields MB species

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

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

6.1. Recommended reductants

In general, ascorbic acid and Sb(III) should be used since the $Sb_2PMB(4e^-)$ reduction product forms within minutes, is stable for hours and is insensitive to chloride interference. This is particularly the case in batch, DA and SFA methods, where the temporal stability of the product is of greater importance. However, when maximum sensitivity is desirable and sample matrices contain negligible chloride concentrations, $SnCl_2$ in combination with a sacrificial co-reductant (typically $N_2H_6SO_4$) should be used in preference to ascorbic acid and Sb(III) due to the faster reduction kinetics and significantly (~30%) higher molar absorptivity of $SnPMB(4e^-)$.

6.2. Recommended acids

Since both HNO_3 and $HClO_4$ are oxidising acids and interfere to a considerable extent with the reduction process, HCl and H_2SO_4 are recommended as the strong acids of choice for MB procedures. However, there is some evidence to suggest that H_2SO_4 inhibits the reaction, and a comparative study of H_2SO_4 and HCl is warranted for MB procedures. If $SnCl_2$ is used as the reductant, H_2SO_4 should be used due to salt error interference from the Cl^- ion unless it is desirable to use HCl as a means of buffering the Cl^- concentration. If converting acid concentrations between H_2SO_4 and HCl, or when consulting older literature, care must be taken to accurately calculate acidity, since the older normality scale of acidity is an inaccurate measure of $[H^+]$ when a diprotic acid such as H_2SO_4 is used.

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^{-1} Mo(VI)
873 is more than sufficient (and potentially excessive) for complete 12-MPA formation from 1 mg L^{-1}
874 (32 μmol L^{-1}) P, and that an acid concentration of around 0.20 mol L^{-1} H^+ is an effective match for
875 this Mo(VI) concentration range. Whether Na_2MoO_4 or $(NH_4)_6Mo_7O_{24}.4H_2O$ 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^{-5} mol L^{-1}, 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^+ 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^{-1}
886 $SnCl_2$ or 5 - 20 mmol L^{-1} 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 $[H^+]$/[Mo(VI)] ratio in method
892 optimisation as it is a chemically unjustifiable and entirely empirical variable which is no good

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

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

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

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

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

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