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Corresponding Author:	Eduard Rott University of Stuttgart Stuttgart, Baden-Württemberg GERMANY
Corresponding Author's Institution:	University of Stuttgart
Corresponding Author E-Mail:	eduard.rott@iswa.uni-stuttgart.de
First Author:	Eduard Rott
Other Authors:	Tobias Reinhardt
	Stephan Wasielewski
	Ellen Raith-Bausch
	Ralf Minke
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TITLE:

Optimized Procedure for Determining the Adsorption of Phosphonates onto Granular Ferric Hydroxide Using a Miniaturized Phosphorus Determination Method

AUTHORS & AFFILIATIONS:

Eduard Rott[1], Tobias Reinhardt[1], Stephan Wasielewski[1], Ellen Raith-Bausch[1], Ralf Minke[1]

[1]Institute for Sanitary Engineering, Water Quality and Solid Waste Management, University of Stuttgart, Stuttgart, Germany

eduard.rott@iswa.uni-stuttgart.de
tobias.reinhardt@iswa.uni-stuttgart.de
stephan.wasielewski@iswa.uni-stuttgart.de
ellen.raith-bausch@iswa.uni-stuttgart.de
ralf.minke@iswa.uni-stuttgart.de

CORRESPONDING AUTHOR:

Eduard Rott
eduard.rott@iswa.uni-stuttgart.de
Tel: (49)-711-68560497

KEYWORDS:

Phosphonates, NTMP, Adsorption, Granular ferric hydroxide, Total P determination, Good buffers, Modified molybdenum blue method.

SUMMARY:

This paper introduces a procedure to investigate the adsorption of phosphonates onto iron-containing filter materials, particularly granular ferric hydroxide, with little effort and high reliability. In a buffered solution, the phosphonate is brought into contact with the adsorbent using a rotator and then analyzed via a miniaturized phosphorus determination method.

ABSTRACT:

This paper introduces a procedure to investigate the adsorption of phosphonates onto iron-containing filter materials, particularly granular ferric hydroxide (GFH), with little effort and high reliability. The phosphonate, *e.g.*, nitrilotrimethylphosphonic acid (NTMP), is brought into contact with the GFH in a rotator in a solution buffered by an organic acid (*e.g.*, acetic acid) or Good buffer (*e.g.*, 2-(*N*-morpholino)ethanesulfonic acid) [MES] and *N*-cyclohexyl-2-hydroxyl-3-aminopropanesulfonic acid [CAPSO]) in a concentration of 10 mM for a specific time in 50 mL centrifuge tubes. Subsequently, after membrane filtration (0.45 µm pore size), the total phosphorus (total P) concentration is measured using a specifically developed determination method (ISO_{mini}). This method is a modification and simplification of the ISO 6878 method: a 4 mL sample is mixed with H_2SO_4 and $K_2S_2O_8$ in a screw cap vial, heated to 148–150 °C for 1 h and then mixed with NaOH, ascorbic acid and acidified molybdate with antimony(III) (final volume of 10 mL) to produce a blue complex. The color intensity, which is linearly proportional to the

phosphorus concentration, is measured spectrophotometrically (880 nm). It is demonstrated that the buffer concentration used has no significant effect on the adsorption of phosphonate between pH 4 and 12. The buffers, therefore, do not compete with the phosphonate for adsorption sites. Furthermore, the relatively high concentration of the buffer requires a higher dosage concentration of oxidizing agent ($K_2S_2O_8$) for digestion than that specified in ISO 6878, which, together with the NaOH dosage, is matched to each buffer. Despite the simplification, the ISO_{mini} method does not lose any of its accuracy compared to the standardized method.

INTRODUCTION:

The efforts to reduce nutrient inputs into surface waters, which are necessary, *inter alia*, in the context of the implementation of the European Water Framework Directive[1], require a more detailed examination of phosphorus emissions. The substance group of phosphonates (**Figure 1**), which are used as bleach stabilizers in textile and paper industries, as antiscalants in drinking water treatment, as hardness stabilizers of cooling water and in detergents and cleaning agents, is particularly relevant in terms of quantity and environmental relevance[2]. Phosphonates are suspected of contributing to long-term eutrophication of water bodies[2-4]. For example, due to UV radiation of sunlight or in the presence of Mn^{II} and dissolved oxygen, phosphonates can be degraded into microbiologically available phosphates[5-6]. The oversupply of phosphate is an essential characteristic of ecologically unbalanced water bodies, which makes phosphorus an important target substance for the sustainable improvement of the ecological status of water bodies.

Phosphonates can be removed from wastewater by precipitation/flocculation when using iron or aluminum salts[7-10]. In this process, metals are transformed into hardly soluble metal hydroxides. These polar flocks with a relatively large specific surface serve as adsorbents for the negatively charged phosphonates. However, the flocculation process can have two main disadvantages. Depending on the wastewater, sludge volumes of up to 30% of the sample volume can occur[11]. This sludge has to be separated, treated and disposed of in a further sedimentation or filter stage. Furthermore, phosphonates can complex the added flocculants and thus prevent the formation of flocks, especially in wastewater with low water hardness. This effect can be compensated by increased quantities of flocculant. However, this leads to increased β values (β = molar ratio of flocculant to phosphorus in wastewater)[11-12]. A complex wastewater matrix, therefore, can complicate the control of an optimum flocculant dosage.

[Place Figure 1 here]

A possible alternative that exploits the high adsorption affinity of phosphonates to metal-containing surfaces and that does not have the above-mentioned disadvantages are filter materials based on iron (hydr)oxides. For such filter materials, the literature mainly presents investigations into the elimination of phosphate[13-16]. This paper introduces a procedure which allows the investigation of the adsorption capacity of selective granulated filter materials, in this work in particular with granular ferric hydroxide (GFH), regarding phosphonates with little workload and significant cost saving. The study of the adsorption capacity can be divided into the following steps: preparation of the phosphonate solution, adsorption test (contact of the

phosphonate solution with the granulate) and phosphonate analysis. All steps must be perfectly coordinated.

Concept for adsorption test and the use of suitable buffers

For the study of adsorption capacity, batch or column tests can be carried out. In order to determine adsorption isotherms or pH-dependencies of the adsorbent, the batch approach is preferred since many results can be obtained within a short period of time by the possibility of varying several parameters. The pH value is one of the most important factors influencing adsorption. Compliance with or adjustment of the pH value is a great challenge for the laboratory technician, as the simple adjustment of the pH value in the sample solution previously to the contact with the adsorbent is usually not sufficient. Each adsorbent material is usually striving to approximate the pH around its point of zero charge (PZC). Accordingly, it is possible that an aqueous solution, *e.g.*, adjusted to pH 3, changes to a pH value of 8 when in immediate contact with the adsorbent. Wastewater mostly has a natural buffering capacity, which attenuates this effect. If, however, only the removal of a particular target substance is to be investigated with a particular adsorbent, synthetic wastewater must be used, *i.e.*, pure water, which is specifically spiked with the target substance or, *e.g.*, competitive anions. In contrast to powdered adsorbents, where the pH value can be easily maintained in the desired range by adding acids and bases in the open stirring vessel, no pH adjustment in this form can be done in a batch approach with granulates. In order to keep granules homogeneously suspended, very high stirring speeds are required, which would result in very rapid abrasion of the material. If such abrasion is unintended, the gentlest method is to rotate closed centrifuge tubes to keep the granules mixed continuously in the solution. The only way to keep the pH value constant in this case is to use buffers.

The following requirements for buffers must be met in order to be able to investigate the adsorption of phosphate and phosphonates on iron-containing filter materials: free of phosphorus; colorless; soluble;at best, no complexing agents; no competition with phosphonates regarding adsorption onto polar filter materials; similar structure of the different buffers used; and buffers or their degradation products must not have a negative effect on the spectral absorbance of the color complex after digestion for total P determination. For the biochemical research field, so-called Good buffers were developed[17-19], which have exactly these properties. Thus, for the investigations of this work, the buffers in **Table 1** were selected. The pK_a value of each buffer indicates the range that can be kept constant by the buffer. For the pH range < 5, however, organic acids such as citric acid (CitOH) and acetic acid (AcOH) must be used. Citric acid is a complexing agent, but it buffers in a pH range where most iron-containing filter materials become unstable anyway. Acetic acid and MOPS were already used by Nowack and Stone[7] to investigate the adsorption of NTMP on slurry goethite (α-FeOOH) at pH 4.6 and 7.2. However, their experiments on the pH-dependency of adsorption took place without buffering.

[Place Table 1 here]

Total P determination (ISO_{mini}) adapted to the buffer solution

Following each adsorption test, each solution must be analyzed for the residual phosphonate concentration. Only recently, a method for the determination of phosphonates in environmental samples with limits of quantification in the range of 0.1 µg/L was introduced. It is based on the IC-ICP-MS method and the use of cation exchangers (for the conversion of phosphonates into "free" phosphonic acids) and anion exchangers (for the pre-concentration of phosphonates)[21]. Furthermore, already in 1997 a method from Nowack[22] was introduced with higher limits of detection of 15–100 µg/L, which is based on the pre-complexation of phosphonates with Fe^{III}, retention using HPLC and the photometric detection of these complexes. However, these methods are very time-consuming and expensive. In studies with synthetic wastewater in which the only phosphorus-containing compound is a phosphonate, it is sufficient to determine the phosphonate concentration by determining the total P concentration. The determination of inorganic phosphate presents the experimenter with far fewer problems than the determination of total P, as the latter requires previous digestion. The amount of chemicals that have to be added priorly must be matched precisely to the compounds present in the sample.

The determination of phosphate is currently carried out mainly using the method introduced by Murphy and Riley[23]. This method is based on the spectrophotometric detection of an intensely colored phosphomolybdenum blue complex ($[PSb_2Mo_{12}O_{40}]^-$ with λ_{max} at 880 nm) which is formed in the presence of phosphate and acidified molybdate using ascorbic acid and antimony(III) as reducing agents[24]. In other studies, the optimal ratio of $[H^+]$:[Mo] was determined to be 60–80[25-26]. In order to determine total P, digestion, *i.e.*, the breaking of P–O–P, C–O–P and C–P bonds in phosphorus-containing compounds and the oxidation of phosphorus to phosphate must be carried out prior to the phosphomolybdenum blue formation[24]. Eisenreich *et al.*[27] presented a simplified method based on the use of the oxidizing agent peroxodisulfate ($K_2S_2O_8$) in the acidic milieu. Many of these findings have been incorporated into the development of ISO 6878[28], which systematically explains the procedure for the determination of phosphate-P and total P concentrations in water samples (wastewater and seawater).

The total P determination according to ISO 6878 (**Figure 2**) requires the sample to be digested in an Erlenmeyer flask by $K_2S_2O_8$ at an acidic pH (use of sulfuric acid) for at least 30 min. After digestion, the pH value is set to 3–10 using NaOH and the content of the Erlenmeyer flask is transferred to a 50 mL volumetric flask. In this flask, ascorbic acid and an acidic solution containing molybdate and antimony are added to the sample and then filled with water. After 10–30 minutes, the intensity of this blue coloration is measured at a wavelength of 880 nm. In the case of phosphate determination, the digestion is omitted. This means, the sample is mixed in a 50 mL volumetric flask with ascorbic acid and a solution containing molybdate as well as antimony, and the intensity of the blue coloration is measured in the photometer.

[Place Figure 2 here]

The procedure of total P determination is very complex since during digestion it must always be taken care of that the sample does not boil over and the adjustment of the sample to pH 3–10 takes a long time. In order to be able to analyze as many samples as possible in a very short time,

a miniaturized form of the total P and ortho-phosphate determination was developed based on this ISO method. **Figure 3** summarizes the individual steps of this method. In this miniaturized determination method (ISO_{mini}), the final volume of the color solution is 10 mL (in the ISO method, this is 50 mL). Accordingly, the ISO_{mini} method reduces the amount of the solutions to be used to one-fifth. In the ISO_{mini} method, the digestion is carried out in a thermostat (in contrast to the ISO method, where digestion is proposed in an Erlenmeyer flask on a hotplate) at 148–150 °C to obtain the highest possible oxidation. NaOH is added after digestion together with the ascorbic acid and acidic molybdate solution.

[Place Figure 3 here]

The organic buffers contained in the samples must be present in relatively high concentrations (10 mM) in comparison to the phosphonate (5–30 µM) in order to maintain the pH value effectively. These buffers must be digested for the analysis of the total P after the adsorption test. Accordingly, the dosed amount of oxidizing agent must be matched to each buffer, taking into account that too much oxidizing agent should not interfere with the formation of the color complex formed after digestion. In order to be able to estimate the $K_2S_2O_8$ quantity required for the digestion of each buffer in the total P determination based on the analyzed chemical oxygen demand (COD), a comparison of how many electrons can be converted during the reduction of O_2 and $K_2S_2O_8$ is necessary:

$$O_2 + 4\ H^+ + 4\ e^- \rightarrow 2\ H_2O$$

$$S_2O_8^{2-} + 2\ e^- \rightarrow 2\ SO_4^{2-}$$

Thus, the oxidation of a particular molecule requires twice as many peroxodisulfate molecules as O_2 molecules. Accordingly, in the case of a sample volume of 20 mL, the COD of the sample must not exceed 500 mg/L when using the ISO method. However, even in the case of MES, the Good buffer with the smallest molar mass from **Table 1**, already a COD of 2.4 g/L is present at a concentration of 10 mM. In addition to the step-by-step protocol of the adsorption test and ISO_{mini} method, this paper, therefore, investigates the required buffer concentration, the influence of the buffers on phosphonate adsorption and the $K_2S_2O_8$ quantity and NaOH dosage required for their digestion in the ISO_{mini} method.

Freundlich model of adsorption

Adsorption isotherms, *i.e.*, loading q (*e.g.*, in mg P/g adsorbent) applied over the dissolved concentration c (in mg/L P) of adsorptive after a specific contact time, can be modeled using the equation proposed by Freundlich[29]:

$$q = K_F c^{1/n}$$

If the experimentally obtained values q and c are plotted in the form of a function ln(q) over ln(c), the slope of this function determined by linear regression corresponds to 1/n and the y-axis intercept to the K_F value[30].

Overview of the procedure

The entire process for determining the adsorption capacity of granular ferric hydroxide with regard to phosphonates is divided into several steps and is described in the protocol section. For the analysis, it is necessary to prepare a sufficient amount of reagent solutions (Section 1 in the protocol). These are durable for several weeks. The phosphonate-containing solution is then prepared (Section 2), followed by the adsorption test (contact of the phosphonate solution with the granular material) (Section 3) and the analysis of the total P according to the miniaturized ISO method (Section 4).

PROTOCOL:

1. Preparation of All Required Solutions for the Total P Determination

Note: The preparation of some of the solutions described below is explained in ISO 6878[28]. These preparation methods have been slightly adapted to the method of this work. The required degree of purity of chemicals can be found in the attached material list.

1.1. Preparation of H_2SO_4 solutions (13.5, 9 and 0.9 M H_2SO_4)

Caution: Work under fume hood.

1.1.1. Preparation of 13.5 M H_2SO_4

1.1.1.1. Fill a 100 mL graduated cylinder with 25 mL of water and transfer it into a 100 mL glass bottle surrounded by ice cubes placed in a beaker.

1.1.1.2. Fill the same graduated cylinder with 75 mL of concentrated sulfuric acid and transfer it under stirring to the water in the bottle. **Caution:** Heat development.

1.1.1.3. Take the bottle carefully out of the beaker as soon as it is sufficiently cooled down (max. 40 °C).

1.1.2. Preparation of 9 M H_2SO_4 (required for the preparation of molybdate solution)

1.1.2.1. Fill a 1 L graduated cylinder with 700 mL of water and transfer it into a 3 L glass beaker surrounded by ice cubes placed in a bucket.

1.1.2.2. Fill the same 1 L graduated cylinder with 700 mL of concentrated sulfuric acid and transfer it under stirring to the water in the 3 L beaker. **Caution:** Heat development.

1.1.2.3. Take the 3 L beaker carefully out of the bucket as soon as it is sufficiently cooled down (max. 40 °C) and transfer its content into a 2 L glass bottle.

1.1.3. Preparation of 0.9 M H_2SO_4

1.1.3.1. Fill a 250 mL volumetric flask with about 100 mL of water.

1.1.3.2. Transfer 25 mL of 9 M H_2SO_4 (see 1.1.2) into the 250 mL volumetric flask using a 25 mL volumetric pipette. **Caution:** Heat development.

1.1.3.3. Fill the 250 mL volumetric flask with water up to the 250 mL ring mark.

1.1.3.4. Close the volumetric flask with a stopper, shake it several times for homogenization and transfer the content of the volumetric flask into a 250 mL glass bottle.

1.2. Preparation of HCl rinsing solution (approx. 2 M)

Caution: Work under fume hood.

1.2.1. Fill a 2 L graduated cylinder with 1 L of water.

1.2.2. Fill this graduated cylinder with 400 mL of 32% HCl (w/w) solution.

1.2.3. Now add 600 mL of water to gain a total volume of 2 L in the graduated cylinder.

1.2.4. Stir the content of the graduated cylinder with a rod (*e.g.*, graduated pipette) and transfer the content of the graduated cylinder into a 2.5 L glass bottle.

1.2.5. Close the bottle and shake it upside down several times for homogenization.

1.2.6. Reuse this solution only until a color change becomes apparent. Then discard the rinsing solution and prepare a new one.

1.3. Preparation of HCl solutions (10.2 and 2 M)

Caution: Work under fume hood.

1.3.1. Use 32% HCl (w/w) as 10.2 M HCl.

1.3.2. Preparation of 2 M HCl

1.3.2.1. Fill a 100 mL volumetric flask with 15 mL of 32% HCl (10.2 M) using a 15 mL volumetric pipette.

1.3.2.2. Add another 4.67 mL of 32% HCl (10.2 M) to the volumetric flask using a micropipette.

1.3.2.3. Fill the volumetric flask with water up to the 100 mL ring mark.

1.3.2.4. Close the volumetric flask with a stopper and shake it upside down several times for homogenization and transfer the content of the volumetric flask into a 100 mL glass bottle.

1.4. Preparation of NaOH solutions (10, 2, 1.5 M NaOH)

Caution: Work under fume hood.

1.4.1. Weigh 100.0 g (for 10 M), 20 g (for 2 M) or 15 g (for 1.5 M) of NaOH into a small beaker and transfer the content of the beaker into a 250 mL volumetric flask.

1.4.2. Fill the volumetric flask with water up to the 250 mL ring mark. Close the volumetric flask with a stopper and shake it upside down several times for homogenization (**Caution:** solution can become hot). If the height of the water level no longer corresponds to the ring mark, add more water (the total volume changes as a result of the dissolving process).

1.4.3. Transfer the content of the volumetric flask into a 250 mL plastic bottle (**Caution:** Do not use glass bottles for NaOH solutions).

1.5. Preparation of $K_2S_2O_8$ solution/suspension (8.33, 41.67, 50.00, 58.33, 66.66 g/L)

Note: Differently concentrated peroxodisulfate mixtures are required for phosphorus determination. Since some of them are above the saturation limit of $K_2S_2O_8$ of approx. 50 g/L at 20 °C, it is advisable to weigh the $K_2S_2O_8$ directly into a brown glass bottle and pour a corresponding volume of water over it (do not use volumetric flasks for the preparation).

1.5.1. Weigh 2.08 g (for 8.33 g/L), 10.42 g (41.67 g/L), 12.50 (50.00 g/L), 14.58 g (58.33 g/L) or 16.67 g (66.66 g/L) of solid $K_2S_2O_8$ directly into a brown 250 mL glass bottle.

1.5.2. Fill a graduated cylinder with 250 mL of water and pour this water over the $K_2S_2O_8$ in the bottle.

1.5.3. Stir the content of the bottle until all ingredients are dissolved or until there is only a slight turbidity.

1.5.4. Carry out the extraction of $K_2S_2O_8$ under high turbulence on the magnetic stirrer to ensure that the undissolved $K_2S_2O_8$ can also be extracted as homogeneously as possible.

1.6. Preparation of 100 g/L ascorbic acid solution

1.6.1. Weigh 50 g of ascorbic acid into a 500 mL volumetric flask.

1.6.2. Fill the volumetric flask with water up to the 500 mL ring mark.

1.6.3. Stir the content of the volumetric flask on the magnetic stirrer until the ascorbic acid is completely dissolved. It may be necessary to correct the level of the water surface to make it congruent with the ring mark by adding a little more water (be careful of the stirring bar giving volume as well). Then transfer the content of the volumetric flask into a brown 500 mL glass bottle.

1.7. Preparation of Molybdate I solution (required for phosphate determination)

1.7.1. Weigh 13.0 g of solid $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ directly into a 100 mL glass bottle. Fill a graduated cylinder with 100 mL of water and pour it into the bottle. Stir the content of the bottle on a magnetic stirrer until it is completely dissolved.

1.7.2. Weigh 0.35 g of solid $K(SbO)C_4H_4O_6\cdot \frac{1}{2}H_2O$ directly into a fresh 100 mL glass bottle. Fill a graduated cylinder with 100 mL of water and pour it into the bottle with $K(SbO)C_4H_4O_6\cdot \frac{1}{2}H_2O$. Stir the content of the bottle until it is completely dissolved.

1.7.3. Fill a graduated cylinder with 300 mL of 9 M H_2SO_4 (see 1.1.2) and pour it into a brown 500 mL glass bottle.

1.7.4. Add the $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ solution to the 300 mL of 9 M H_2SO_4. Then add the $K(SbO)C_4H_4O_6\cdot \frac{1}{2}H_2O$ solution to this mixture. Close the bottle and shake it several times upside down for homogenization.

1.8. Preparation of Molybdate II solution (required for total P determination)

1.8.1. Weigh 13.0 g of solid $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ directly into a 100 mL glass bottle. Fill a graduated cylinder with 100 mL of water and pour it into the bottle. Stir the content of the bottle on a magnetic stirrer until it is completely dissolved.

1.8.2. Weigh 0.35 g of solid $K(SbO)C_4H_4O_6\cdot \frac{1}{2}H_2O$ directly into a fresh 100 mL glass bottle. Fill a graduated cylinder with 100 mL of water and pour it into the bottle with $K(SbO)C_4H_4O_6\cdot \frac{1}{2}H_2O$. Stir the content of the bottle until it is completely dissolved.

1.8.3. Fill a graduated cylinder with 70 mL of water. Add 230 mL of 9 M H_2SO_4 (see 1.1.2) to the water in the graduated cylinder (*i.e.*, fill up to 300 mL). Carefully homogenize the content of the graduated cylinder with a rod (*e.g.*, graduated pipette). Transfer the content of the graduated cylinder into a brown 500 mL glass bottle (current content: 6.9 M H_2SO_4).

1.8.4. Add the $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ solution to the 300 mL of 6.9 M H_2SO_4. Then add the $K(SbO)C_4H_4O_6\cdot \frac{1}{2}H_2O$ solution to this mixture. Close the bottle and shake it several times upside down for homogenization.

1.9. Preparation of internal quality standard (IQS: 1 mg/L KH_2PO_4-P in 0.9 mM H_2SO_4)

1.9.1. Dry a few grams of KH_2PO_4 in a small glass dish at 105 °C in the drying oven until mass constancy is achieved and then cool the KH_2PO_4 down to room temperature in a desiccator.

1.9.2. Weigh 0.2197 g ± 0.0002 g of KH_2PO_4 directly from the desiccator into a 1 L volumetric flask and add approx. 800 mL of water into the volumetric flask.

1.9.3. Now add 5 mL of 9 M H_2SO_4 (see 1.1.2) to the flask using a 5 mL volumetric pipette and fill the flask with water up to the 1 L ring mark.

1.9.4. Stir the content of the volumetric flask on the magnetic stirrer and transfer the content of the volumetric flask into a 1 L glass bottle (current content: 50 mg/L KH_2PO_4-P in 45 mM H_2SO_4). This solution can henceforth be used as a stock solution for the preparation of IQS.

1.9.5. Transfer 10 mL of this solution into a 500 mL volumetric flask using a 10 mL volumetric pipette, fill the volumetric flask with water up to the 500 mL ring mark and stir the content of the volumetric flask on the magnetic stirrer.

1.9.6. Transfer the content of the volumetric flask into a 500 mL glass bottle (current content: 1 mg/L KH_2PO_4-P in 0.9 mM H_2SO_4). This solution is the IQS.

2. Preparation of Phosphonate-Containing Buffered Solutions

2.1. Weigh or pipette the desired buffer into a volumetric flask (at a target concentration of 0.01 M buffer in 1 L, *e.g.*: 572 µL of 100% AcOH, 2.1014 g of CitOH·H_2O, 1.9520 g of MES, 2.0926 g of MOPS, 2.3831 g of HEPES, 2.5233 g of EPPS, 2.3732 g of CAPSO, 2.2132 g of CAPS, 5 mL of 2 M NaOH).

2.2. Fill the volumetric flask to about three quarters with water and add a previously prepared 1 g/L phosphonate-P stock solution (for a target concentration of 1 mg/L P in 1 L, *e.g.*, 1 mL of 1 g/L phosphonate-P).

2.3. Fill the flask with water up to the ring mark, stir the content of the flask on the magnetic stirrer until all ingredients are dissolved and transfer it into a glass bottle.

2.4. While stirring, adjust the desired pH value in the buffer solution (*e.g.*, pH 6 at MES) with HCl (*e.g.*, 2 and 10.2 M) or NaOH (*e.g.*, 2 and 10 M) (the addition of both acidic and basic solution should be avoided to prevent an unnecessary increase of ionic strength).

2.5. To determine the phosphonate-P concentration, proceed according to step 4.

3. Procedure of the Adsorption Test

3.1. Wash the filter material thoroughly with distilled water (*e.g.*, over a sieve with a mesh size of 0.5 mm) and then dry it at 80 °C.

Note: The protocol can be paused here.

3.2. Weigh the filter material (*e.g.*, granular ferric hydroxide) into a 50 mL centrifuge tube.

Note: The protocol can be paused here.

3.3. Quickly fill the 50 mL centrifuge tube with the phosphonate-containing solution from step 2 up to the 50 mL mark.

3.4. Quickly close the tube and clamp it into the running rotator (the contact time starts from now on).

3.5. Rotate the tube at 20 revolutions per minute for a specific amount of time (*e.g.*, 1 h).

3.6. Filter approx. 10–20 mL of the supernatant with a syringe filter (0.45 µm pore size) into an empty glass bottle.

Note: The protocol can be paused here.

3.7. Determine the pH value of the filtrate and to determine the phosphonate-P concentration proceed with step 4. In the case of investigating phosphate adsorption, proceed with step 5.

4. Determination of Total P (Phosphonate-P) According to ISO_{mini}

Note: The following procedure is also shown in **Figure 3**.

4.1. Transfer an aliquot of the sample to be analyzed (V_{sample}, max. 4 mL) using a micropipette into a 10 mL screw cap vial (the vial including the cap should be pre-rinsed with HCl (see 1.2) and H_2O and dried at 80–100 °C).

Note: The protocol can be paused here.

4.2. Add water with a micropipette to obtain a total volume of 4 mL together with the sample previously added (V_{water} = 4 mL-V_{sample}).

Note: The protocol can be paused here.

4.3. Add 0.2 mL of 0.9 M H_2SO_4 solution (see 1.1.3) using a micropipette. If there is a concentration of 1 M NaOH in the sample, as is often the case with regeneration solutions, add 0.2 mL of 13.5 M H_2SO_4 solution (see 1.1.1) (**Caution:** This solution of sulfuric acid is highly concentrated).

Note: The protocol can be paused here.

4.4. Add 4.8 mL of a $K_2S_2O_8$ solution/suspension (see 1.5) the concentration of which depends on the buffer contained in the sample (corresponding to ISO at 0.01–1 M NaOH: 8.33 g/L $K_2S_2O_8$; 0.01 M CitOH, AcOH, MES: 41.67 g/L; 0.01 M MOPS: 50.00 g/L; 0.01 M HEPES: 58.33 g/L; 0.01 M EPPS, CAPSO, CAPS: 66.66 g/L).

4.5. Close the vial with the cap very tightly and shake it.

4.6. Heat the vial in a thermostat at 148–150 °C for 1 h.

4.7. Take the vial out of the thermostat and let it cool down to room temperature.

Note: The protocol can be paused here.

4.8. Open the vial and add 0.4 mL of 1.5 M NaOH solution (see 1.4).

Note: The protocol can be paused here.

4.9. Add 0.2 mL of 100 g/L ascorbic acid solution (see 1.6).

4.10. Subsequently, add 0.4 mL molybdate II solution (see 1.8).

4.11. Close the vial and turn it upside down for homogenization.

4.12. Wait minimum 15 minutes to a maximum of 4 h for color formation.

4.13. Measure spectral absorbance (A) at a wavelength of 880 nm using a photometer.

4.14. Carry out the steps 4.1–4.13 regularly for 4 mL of water (for the determination of A_{blind}) as well as for 4 mL of an IQS (see 1.9).

4.15. Calculate the total P or phosphonate-P concentration of the analysis sample on the basis of the specific absorbance of the analysis sample (A), the absorbance of the blind sample (A_{blind}) and the sample volume (V_{sample}) using the following equation (0.287 corresponds to the slope of the calibration line with 1 cm cuvettes and can deviate depending on the photometer):

$$P\left[\frac{mg}{L}\right] = \frac{(A - A_{blind})}{0.287} \cdot \frac{4\ mL}{V_{sample}[mL]}$$

5. Determination of o-PO_4^{3-}-P According to ISO_{mini}

Note: This determination method can be used when the adsorption of inorganic ortho-phosphate

on granulated filter materials is to be investigated. In this case, the sample to be tested does not have to be digested.

5.1. Transfer an aliquot of the sample to be analyzed (V_{sample}, max. 9.4 mL) by means of a micropipette into a 10 mL screw cap vial (the vial including the cap should be pre-rinsed with HCl (see 1.2) and H_2O and dried at 80–100 °C).

Note: The protocol can be paused here.

5.2. Add water with a micropipette to obtain a total volume of 9.4 mL together with the sample previously added (V_{water} = 9.4 mL-V_{sample}).

Note: The protocol can be paused here.

5.3. Add 0.2 mL of 100 g/L ascorbic acid solution (see 1.6).

5.4. Subsequently, add 0.4 mL of molybdate I solution (see 1.7).

5.5. Close the vial and turn it upside down for homogenization.

5.6. Wait minimum 15 minutes to a maximum of 4 h for color formation.

5.7. Measure spectral absorbance (A) at a wavelength of 880 nm using a photometer.

5.8. Carry out the steps 5.1–5.7 regularly for 9.4 mL of water (for the determination of A_{blind}) as well as for 4 mL of an IQS (see 1.9).

5.9. On the basis of the specific absorbance of the analysis sample (A), of the blind sample (A_{blind}) and the sample volume (V_{sample}), the ortho-phosphate-P concentration of the analysis sample can be calculated using the equation in 4.15.

REPRESENTATIVE RESULTS:

Example of isotherms gained with the proposed procedure

Figure 4 shows an example of the results gained when applying the protocol in the case of the investigation of the adsorption of NTMP by GFH at different pH values. NTMP was selected because, with three phosphonate groups, it is the most representative phosphonate for the broad spectrum of possible phosphonates of which the number of phosphonate groups vary between one (PBTC) and five (DTPMP). Furthermore, the molar mass of NTMP (299.05 g/mol) is also located in the middle range of phosphonates (HEDP: 206.03 g/mol, DTPMP: 573.20 g/mol). In **Figure 4**, the adsorption isotherms, *i.e.*, the loading of the phosphonate above the residual phosphonate concentration, are depicted at different buffers and pH values after a contact time of 1 h. Longer contact times could lead to undesirable abrasion of the material due to too long contact between the particles. For each isotherm, a solution with 1 mg/L NTMP-P and, depending on the desired pH range, buffer in the concentration of 0.01 M was prepared and adjusted to an

initial pH value by means of HCl or NaOH. This was 4.0 (AcOH), 6.0 (MES), 8.0 (EPPS), 10.0 (CAPS) and 12.0 (NaOH). Depending on the GFH concentration, as a result of the 1 h contact time, the pH value in the solution changed by a maximum of 2.0: 4.0–6.0 (AcOH), 6.0–7.3 (MES), 8.0–8.2 (EPPS), 9.4–10.0 (CAPS), 10.9–12.0 (NaOH). The PZC of GFH is approx. 8.6, so it is consequential that the pH value in the case of a set pH value > 8.6 decreased due to contact with GFH and increased at a pH value < 8.6. The further away this adjusted pH value was from 8.6, the stronger the pH change was.

[Place Fig. 4 here]

All isotherms in **Figure 4** were modeled using the Freundlich equation (R^2 values from left to right with increasing pH: 0.875, 0.905, 0.890, 0.986, 0.952; corresponding n values: 2.488, 3.067, 4.440, 2.824, 1.942; corresponding K_F values: 0.619, 0.384, 0.260, 0.245, 0.141). At pH values of 4–6, a loading of up to 0.55 mg NTMP-P/g was achieved, which corresponds to 1.8 mg NTMP/g. The higher the pH value, the lower the level of adsorption. Iron hydroxides have a large number of Fe-OH groups on their surface, which may be protonated or deprotonated depending on the pH value. With the depth of the pH value, the surface is predominantly protonated, *i.e.*, positively charged, which means that the multidentate phosphonates, which are negatively charged over the almost entire pH range, are attracted. A higher pH value shifts the charge of the iron hydroxide surface in the negative direction, which in turn leads to increased electrostatic repulsion[7]. Interestingly, even at pH 12, which corresponds to an OH^- concentration of 0.01 M, adsorption occurred. Therefore, for successful desorption, NaOH solutions with a much higher concentration must be used.

In comparison to the results of other researchers, the maximum loading of up to 0.55 mg NTMP-P/g of GFH in this work seems to be rather low. Boels *et al.*[14] found a maximum loading of 71 mg NTMP/g of GFH, which corresponds to 21.7 mg of NTMP-P/g GFH in their experiments with a synthetic reverse osmosis concentrate with 30 mg/L NTMP (9.3 mg/L NTMP-P) at pH 7.85. They used powdered GFH and stirred the synthetic solution, which contained HCO_3^- that also acts as a buffer, for 24 h. Therefore, their results cannot be directly compared to the findings of this work, as they used a much higher initial concentration and powdered GFH, which is likely to lead to a higher surface area and, therefore, results in a better adsorption performance. Additionally, the contact time was significantly longer as in this work. Nowack and Stone[7] conducted experiments with a 40 µM NTMP solution (3.72 mg of NTMP-P/L) in a 0.42 g/L goethite slurry at a pH of 7.2. The solution was stirred for 2 h leading to a maximum loading of approx. 30 µM NTMP/g goethite (2.79 mg of NTMP-P/g). 1 mM MOPS was used as a buffer. Again, the results cannot be compared directly to the results of this work due to the higher initial phosphonate concentration. In addition, the slurry, which consisted of goethite flocks, had a high surface area. However, the shapes of the isotherms from Boels *et al.*[14] and Nowack and Stone[7] agree with the ones of this work, and all of them could be fitted well by the Freundlich model.

Influence of buffer on phosphonate adsorption and required buffer concentration

Previous experiments to determine the adsorption kinetics had shown that also with the use of buffers, an equilibrium pH value is reached within a very short period of time. That pH can deviate

significantly from the pH value that was previously set in the phosphonate-containing solution (adjusted pH). This equilibrium pH tends to the PZC of the filter material, which was 8.6 for the granular ferric hydroxide discussed here (according to own investigations). Therefore, it can be assumed that the pH value after the contact time (final pH) is decisive for the extent to which the adsorption of the phosphonate occurs.

[Place Figure 5 here]

In the right-hand diagram in **Figure 5**, the pH values that were set in the NTMP-containing solution at different buffer concentrations are compared with the final pH values after the 1 h contact between 1 mg/L NTMP-P and 2.5 g/L GFH. It becomes evident that a specific correlation between the pH value previously set in the solution and the final pH value was only attainable and thus a relatively reliable pH adjustment was possible only when buffers in concentrations of 10 mM were used. This is reflected in the correlation function determined by means of polynomial regression and reproduced in the diagram on the right. The fact that in the case of buffer concentrations below 10 mM pH values of 2–4 had to be preset in order to obtain final pH values of 6–7 shows that the prediction of the final pH value, which is decisive for adsorption, and thus the safe execution of the adsorption tests for such buffer concentrations were challenging.

In the left-hand diagram in **Figure 5**, the extent of adsorption of 1 mg/L NTMP-P at 2.5 g/L GFH is depicted as a function of the final pH value for different buffer concentrations. Assuming a linear dependence of the loading on the pH value in the pH range 4–12 according to equation $y = ax + b$, the values calculated by linear regression for all buffer concentrations investigated were very similar (10 mM: a=−0.0673, b=1.0914, R^2=0.9837; 6.6 mM: a=−0.0689, b=1.1047, R^2=0.9512; 3.3 mM: a=−0.0672, b=-0.0672, R^2=0.9570; 0 mM: a=−0.0708, b=1.157, R^2=0.8933). The coefficient of determination, which was the highest for 10 mM buffer, showed very clearly that with this buffer concentration not only the final pH value was easier to adjust, but also the most reliable results with regard to adsorption were achieved. Only the course without buffer indicates possible deviations of the adsorption extent between pH 5 and 7. However, in order to achieve these final pH values without buffering, very low pH values had to be set in the stock solution, some of which were only slightly above 2. Due to the very strong difference between adjusted pH and final pH, it is, therefore, possible that the final pH value was not decisive for the extent of the adsorption in the case of no buffer. It can thus be assumed that the use of Good buffers mentioned in **Table 1** has no significant influence on the adsorption of phosphonates onto GFH, *i.e.*, there is no competition for adsorption sites between the phosphonate and the buffer. Such selectivity is only prevalent because the adsorption of NTMP onto GFH is mainly due to the formation of mono- and bidentate complexes[15]. Good buffers, on the other hand, have little tendency to form metal complexes[17, 19], which is why NTMP is preferably bound by GFH. In the case of adsorbents with a less polar surface, such as activated carbon, it can be assumed that Good buffers also occupy free adsorption sites and thus influence the adsorption of the phosphonate. The use of these buffers to study the adsorption of phosphonates on activated carbon is therefore not recommended.

Calibration of ISO_{mini} method and compliance with ISO

Figure 6 shows the calibration lines using the internal quality standard (IQS: 1 mg/L KH_2PO_4-P in 0.9 mM H_2SO_4) according to ISO 6878 as well as the modified ISO_{mini} method for total P and o-PO_4^{3-}-P determination. Based on a linear regression, the calibration function equivalent to ISO 6878 was y = 0.0033 + 0.2833x (R^2=0.99978). The linear regression applied to the miniaturized variant for phosphate determination resulted in the calibration function y = 0.0058 + 0.2864x (R^2=0.99999). With y = 0.0020 + 0.2890x (R^2=0.99985) the calibration function for total P determination according to the ISO_{mini} method was very similar and very precise as well. All variants had a very high coefficient of determination, which means that the ISO_{mini} method does not compromise accuracy by the reduction of the sample volume to one-fifth. The conversion equation determined by means of the calibration functions for determining the P concentration in the analysis sample from the measured spectral absorbances is given in the protocol in step 4.15. Experience has shown that the absorbance of the blind sample can usually be neglected since at 880 nm the signal emitted by the photometer can jump very strongly in the very small measuring range. Thus, a measured value of 0.287 at 4 mL sample volume (ISO_{mini}) corresponded to a phosphorus concentration of 1 mg/L P.

[Place Figure 6 here]

Plausibility and buffer-dependent dosage quantities of ISO_{mini} method

As already mentioned, a reliable pH adjustment in the adsorption test is only possible with a buffer concentration of 0.01 M. However, such a buffer concentration requires a higher $K_2S_2O_8$ dosage than specified in ISO 6878 for most buffers. In addition, the ISO stipulates that the pH value must be set to 3–10 using a pH probe after digestion. Since such a pH adjustment cannot be carried out in a small screw cap vial, the matching NaOH dosage quantity for different buffer solutions had to be determined. **Figure 7** shows the absorbance of different buffer-containing solutions with 1 mg/L NTMP-P when these were digested with different $K_2S_2O_8$ quantities according to ISO_{mini} and treated with varying amounts of NaOH after digestion. Accordingly, each matrix was based on the following procedure: 4 mL of a solution was mixed with 0.2 mL 0.9 M H_2SO_4, provided with different $K_2S_2O_8$ quantities and filled up with H_2O to the same total volume of 9 mL. This was now digested in accordance with the protocol (1 h at 148–150 °C). After cooling, different NaOH quantities were added and filled up to a total volume of 9.4 mL with H_2O. Subsequently, 0.2 mL of ascorbic acid solution and 0.4 mL of molybdate II solution were added. The determination of the absorbance (880 nm) was carried out 4 h after the addition of these color reagents. This time was chosen to ensure that the specific absorbance was stable. A solution with 1 mg/L NTMP-P and 1 M NaOH was also investigated. However, instead of the $K_2S_2O_8$ and NaOH amounts, the H_2SO_4 amounts were varied to ensure that the pH was low enough for digestion. The targeted absorbance value was 0.287 (see calibration line in **Figure 6**). Thus, in **Figure 7** those values are shown in light green that deviated from this target value by a maximum of 5%. One value in each matrix is highlighted with a dark green color. This marks the $K_2S_2O_8$ and NaOH dosage quantities recommended for the regular ISO_{mini} method for this type of buffer solution.

[Place Figure 7 here]

Although reductive conditions must prevail in the color formation process and excessive $K_2S_2O_8$ may interfere with this, the results for solutions a and b (**Figure 7**), for which no (IQS) or only a very small quantity of $K_2S_2O_8$ (only NTMP without buffer) is required, show that higher quantities of $K_2S_2O_8$ than required do not automatically lead to an abrupt reduction of the absorbance. It should also be mentioned here that other phosphonates in solutions analogous to solution b with 1 mg/L PBTC-P (absorbance: 0.3005), 1 mg/L HEDP-P (0.3035), 1 mg/L EDTMP-P (0.2952) or 1 mg/L DTPMP-P (0.2936) were digested entirely using the ISO_{mini} method according to the protocol with 0.04 g $K_2S_2O_8$ and 0.6 mmol NaOH. Thus, this method can also be used for phosphonates other than NTMP.

Table 1 shows the theoretical oxygen demand (ThOD) for the oxidation of each buffer and the chemical oxygen demand (COD) measured in a 0.01 M buffer solution by Hach LCK 514 cuvette rapid tests. It is known that potassium dichromate, the oxidant used for the COD determination, does not oxidize organically bound nitrogen[32]. For Good buffers, the measured COD was always between the theoretical amount for the oxidation of C and H and the oxidation of C, H and S. Only for buffers with a C–OH group (HEPES, EPPS, CAPSO) the measured value did correspond to the theoretical value for oxidation of C, H and S. In buffers that do not contain a C–OH group (MES, MOPS, CAPS), the sulfo group is obviously not degraded completely to sulfate.

For the solutions **7c** to **7j**, it can be seen very clearly that $K_2S_2O_8$ quantities significantly below the amount of oxidizing agent required according to the COD of the buffer, independently of the NaOH amount, did not contribute to the achievement of the target value. At 10 mM, the buffer in these solutions had a concentration of approx. 1000 times higher than that of NTMP. If the buffer is not digested, it cannot be guaranteed that the phosphonate can be completely oxidized. Only $K_2S_2O_8$ quantities beyond the COD contributed to the reliable attainment of the target value. Thus, it was not necessary for all buffers to apply the theoretical oxidant requirement for the complete oxidation of the buffer (ThOD) because the nitrogen and obviously also for some buffers, the sulfo groups were not completely decomposed. Any oxidizing agent beyond the COD did not react with the buffer, and, therefore, there was sufficient excess of $K_2S_2O_8$ to oxidize the phosphonate. NTMP also contains nitrogen. Although this may not be completely oxidized to nitrate, all phosphonate groups are obviously oxidized to phosphate. Otherwise, one would not find the absorbance that is present for 1 mg/L P. Abundant excess of $K_2S_2O_8$ did certainly also contribute to the complete oxidation of the phosphonate, but after the digestion some $K_2S_2O_8$ was still present and could react with ascorbic acid, which is necessary for the reduction of the blue molybdate-phosphate complex. The result was an absorbance lower than the target value.

In each row, the absorbance increased with the amount of NaOH starting from a certain amount of NaOH. Thus, it also occurred that below the amount of oxidizing agent required according to the COD of the buffer, the measured absorbance value could be in accordance with the target value, although NTMP was obviously not completely digested (see solutions **7c**, **7f**, and **7h**). In this case, the increase in absorbance was due to self-reduction of the molybdate ion due to a too low $[H^+]$:[Mo] ratio[26], and any correspondence is therefore only random. Accordingly, with higher $K_2S_2O_8$ quantities, more NaOH could be used after digestion, as $K_2S_2O_8$ reduces the pH value.

In most solutions, the absorbance was also in accordance with the target value even if no NaOH dosing was applied. Occasionally, however, deviations from this value occurred, which may be because the absence of NaOH resulted in the fact that the optimum $[H^+]$:[Mo] ratio was not maintained and thus the color complex became unstable. Therefore, irrespective of the analysis solution, a dosage of 0.6 mM NaOH is recommended, as, thereby, the color complexes proved to be the most stable. Regeneration solutions often have a concentration of 1 M NaOH. One such case is covered by matrix l. Here, it was shown that only a very narrow spectrum of H_2SO_4 dosage is permissible, proving that the use of a pH probe to adjust the pH after digestion may be a safer procedure here.

All dark green absorbance values in **Figure 7** (n=12), converted into the total P concentration according to the calibration line in **Figure 6**, give an average value of 1.013 mg/L. The standard deviation is 0.014 mg/L. The typical deviation from the target value (1.000 mg/L) is therefore only 0.11–2.67% ((1.013-0.014-1.000) / 1.000 × 100% = 0.11%; (1.013 + 0.014-1.000) / 1.000 × 100% = 2.67%). This shows a high accuracy of the ISO_{mini} method.

FIGURE AND TABLE LEGENDS:

Table 1: pK_a values[20], theoretical oxygen demand (ThOD) and analyzed actual chemical oxygen demand (COD) of buffers used in this study.

Figure 1: Structural formulae of important phosphonates[11].

Figure 2: Procedure of total P determination according to ISO 6878 applying digestion using sulfuric acid and potassium peroxodisulfate, a subsequent pH adjustment with NaOH and coloration using ascorbic acid and molybdate-containing solutions.

Figure 3: Procedure of total P determination according to a modified and miniaturized form of ISO 6878 (ISO_{mini}) using 10 mL screw cap vials, buffer-dependent potassium peroxodisulfate concentrations, heating in a thermostat and addition of color reagents directly to the digested sample without transferring it previously.

Figure 4: Loading of NTMP (initial concentration of 1 mg/L NTMP-P) onto granular ferric hydroxide dosed at concentrations of 0.7–14 g/L after 1 h contact time at room temperature. The following buffers at concentrations of 0.01 mol/L were used at the mentioned pH values in the graph: AcOH (pH 4.0–6.0), MES (pH 6.0–7.3), EPPS (pH 8.0–8.2), CAPS (9.4–10.0) and NaOH (pH 10.9–12.0). The curves plotted are Freundlich isotherms.

Figure 5: Left: loading of NTMP (initial concentration of 1 mg/L NTMP-P) onto 2.5 g/L granular ferric hydroxide as a function of the pH value at different buffer concentrations after a contact time of 1 h. Right: Comparison of the pH value after 1 h contact time with the pH value set in the stock solution before contact with the granular ferric hydroxide at different concentrations of the buffers AcOH, MES, MOPS, EPPS, CAPSO and CAPS.

Figure 6: Calibration lines for the determination of total P and ortho-phosphate-P according to ISO 6878 and ISO_{mini}. An IQS (1 mg/L KH_2PO_4-P in 0.9 mM H_2SO_4) was used in accordance with point 1.9 of the protocol. For the ISO method, the IQS was used in aliquots of 4, 8, 12, 16 and 20 mL and for the modified ISO_{mini} method in aliquots of 0.8, 1.6, 2.4, 3.2 and 4.0 mL.

Figure 7: Spectral absorbance (×1000) of different phosphonate- and buffer-containing solutions with different $K_2S_2O_8$ and NaOH dosage quantities at a wavelength of 880 nm in 1 cm cuvettes. Procedure: 4 mL solution (as shown in the figure and adjusted to the pK_a value of the buffer adapted from the thermodynamic pK_a values of Goldberg *et al.*[20] to a concentration of 0.01 M and 25 °C[31]) was placed in a 10 mL screw cap vial, mixed with 0.2 mL of 0.9 M H_2SO_4 and with different amounts of $K_2S_2O_8$ (as shown in the figure). Water was then added to obtain a total volume of 9 mL for all samples before digestion. Now the vials were heated in the thermostat at 148–150 °C for 1 h (digestion). After cooling to room temperature, different amounts of NaOH (as shown in the figure) were added and with the addition of water, it was ensured that a total volume of 9.4 mL was present in all vials. 4 h after addition of 0.2 mL of ascorbic acid solution and 0.4 mL of molybdate II solution, the absorbance at 880 nm was determined. In the case of solution l (1 mg/L NTMP-P in 1 M NaOH), the amount of H_2SO_4 was varied instead of $K_2S_2O_8$. Here, the dosed amount of NaOH in all samples corresponded to 0.4 mL of 1.5 M NaOH, *i.e.*, 0.60 mmol of NaOH. Light green: maximum 5% deviation from target value: 287. Dark green: the recommended setting for this buffer- and phosphonate-containing solution. Dashed line: COD, straight line: ThOD.

DISCUSSION:

The increasing significance of phosphonates requires research for reliable methods of removing these compounds from wastewater to protect wastewater treatment plants or receiving water bodies. At present, very few studies have been carried out on the removal of phosphonates from industrial wastewater[5, 11-14, 16]. The procedure presented here shows that investigations regarding the elimination of phosphonates by adsorption on polar iron oxide containing materials, in particular granular ferric hydroxide, can be carried out quickly and reliably when in accordance with the given protocol.

The decisive point in conducting adsorption studies is the maintenance of the pH value. This cannot be done in rotating centrifuge tubes without using a buffer. In this article, it was shown that Good buffers allow an acceptable pH adjustment only at a concentration of 0.01 M and even at this concentration have no significant influence on the adsorption of phosphonates onto GFH. The application of Good buffers is also the reason why the procedure presented here cannot be used for studies on adsorption of phosphonates onto rather non-polar materials such as activated carbon. Good buffers would compete with phosphonates for free adsorption sites.

Since the direct analysis of phosphonates by means of HPLC[22] or IC-ICP-MS[21] is very complex and expensive, the presented method suggests that the phosphonate after contact with the adsorbent should be measured indirectly via the determination of the total P. A standardized method (ISO 6878[28]) is generally used for the total P determination, in which a digestion is carried out by means of H_2SO_4 and $K_2S_2O_8$ on a hotplate, the pH value is then set to 3–10 by means of NaOH and a blue color complex (the color intensity of which is linearly proportional to the phosphate concentration) is formed with the aid of ascorbic acid and molybdate solution. This standardized method is very labor and time consuming, which is why a faster variant of the ISO method (ISO_{mini}) was developed. The ISO_{mini} method reduces the total volume to one-fifth. The digestion takes place comfortably in a thermostat and the NaOH dosage after digestion is fixed. This method enables a large number of phosphorus determinations to be carried out within a very short time and does not compromise accuracy in comparison to the ISO method.

Each buffer has a different COD. In addition, the relatively high necessary buffer concentration of 0.01 M means that, in order to ensure sufficient digestion of the sample constituents, considerably higher amounts of oxidizing agent have to be dosed than it is stipulated in the ISO method. If the $K_2S_2O_8$ dosage is too low or too high, incorrect measurement results do occur. In the ISO_{mini} method, this $K_2S_2O_8$ dosage is thus matched to each buffer individually. Another critical point is the dosage of NaOH. As a rule, regeneration solutions have NaOH concentrations of > 0.1 M. In order to avoid that the $[H^+]$:[Mo] ratio required for the formation of the color complex[25-26] is not adhered to, a proper adjustment of the H_2SO_4 quantity prior to digestion is therefore necessary. The problem arises when the regeneration solution is reused several times, thereby changing its pH value and COD. Since a reliable and simple pH measurement is not possible in screw cap vials and an appropriate pH adjustment is not provided, the ISO_{mini} method presented here, thus, reaches its limits for samples with very high pH values. For regeneration solutions it is therefore recommended to use the ISO method.

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No conflicts of interest declared.

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Fig 1 AllPhosphonates

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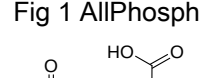
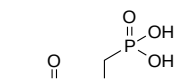
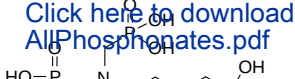


PBTC
2-Phosphono-butane-1,2,4-tricarboxylic acid

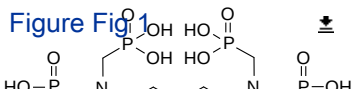
HEDP
1-Hydroxyethylidene-(1,1-diphosphonic acid)



NTMP
Nitrilotrimethyl-phosphonic acid



EDTMP
Ethylenediaminetetra-(methylene phosphonic acid)



DTPMP
Diethylenetriaminepenta-(methylene phosphonic acid)

Fig 2 ISO method

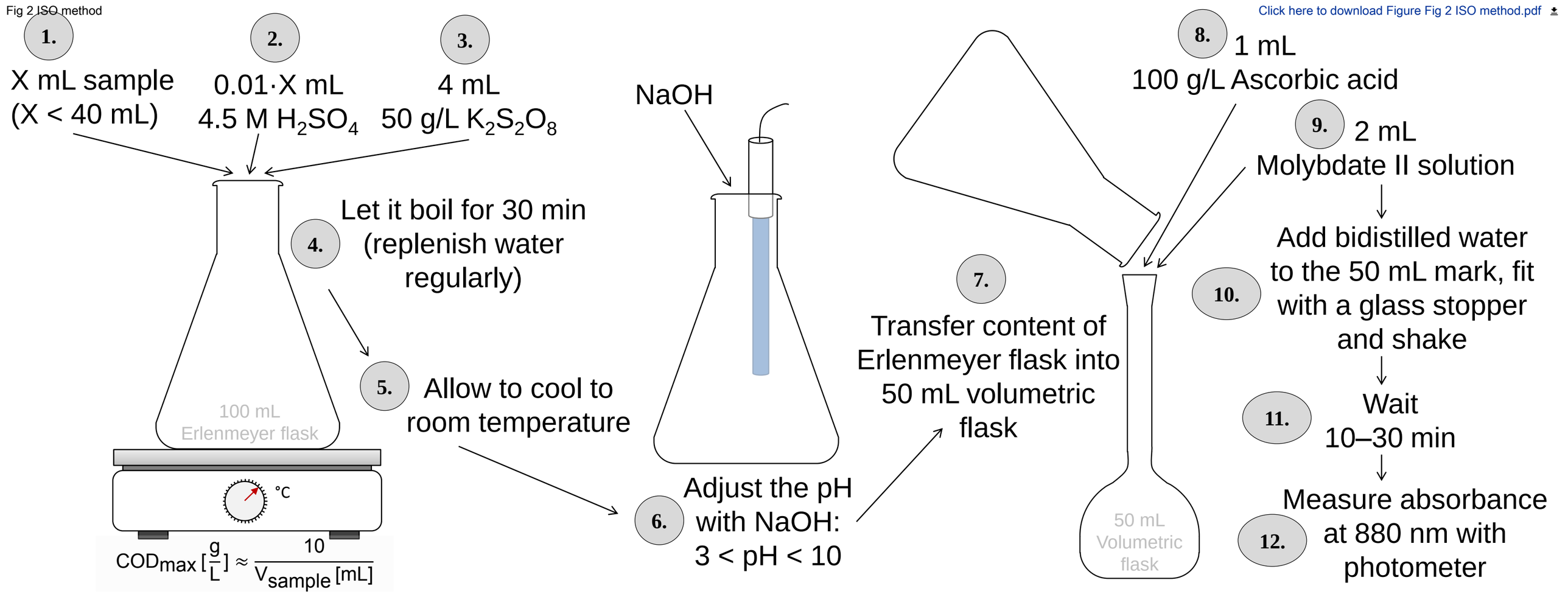
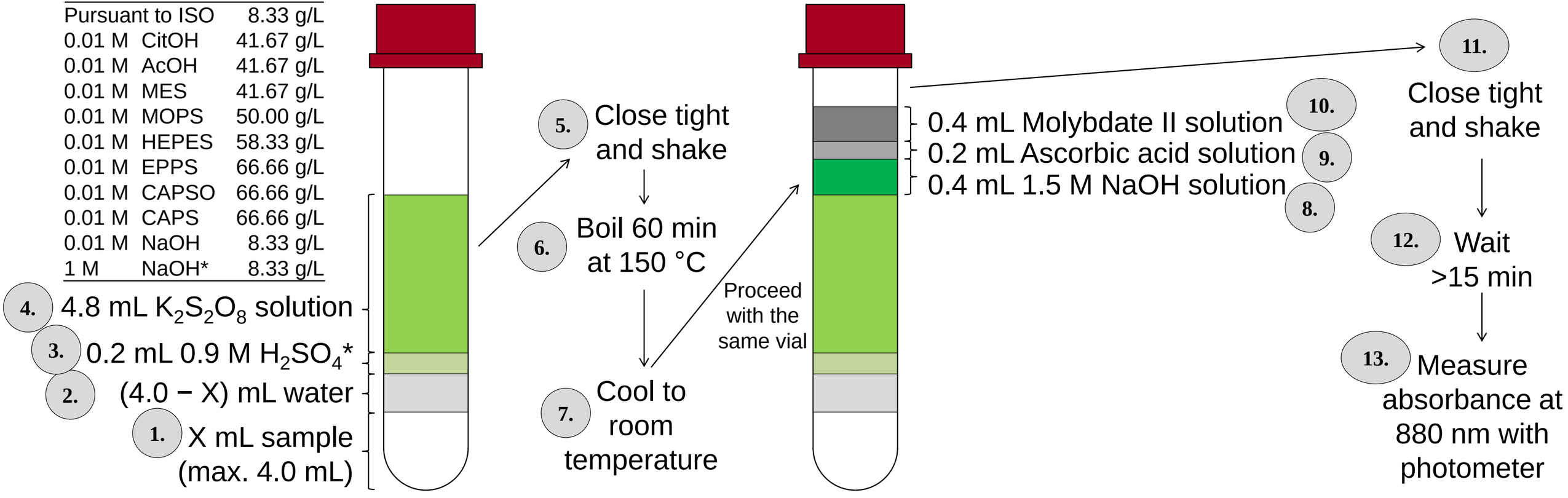


Fig 3 ISOmini method

Click here to download Figure Fig 3 ISOmini method.pdf

Buffer	$K_2S_2O_8$ solution
Pursuant to ISO	8.33 g/L
0.01 M CitOH	41.67 g/L
0.01 M AcOH	41.67 g/L
0.01 M MES	41.67 g/L
0.01 M MOPS	50.00 g/L
0.01 M HEPES	58.33 g/L
0.01 M EPPS	66.66 g/L
0.01 M CAPSO	66.66 g/L
0.01 M CAPS	66.66 g/L
0.01 M NaOH	8.33 g/L
1 M NaOH*	8.33 g/L



* At 1 M NaOH use: 0.2 mL 13.5 M H_2SO_4

Fig 4 RepresentativeResults

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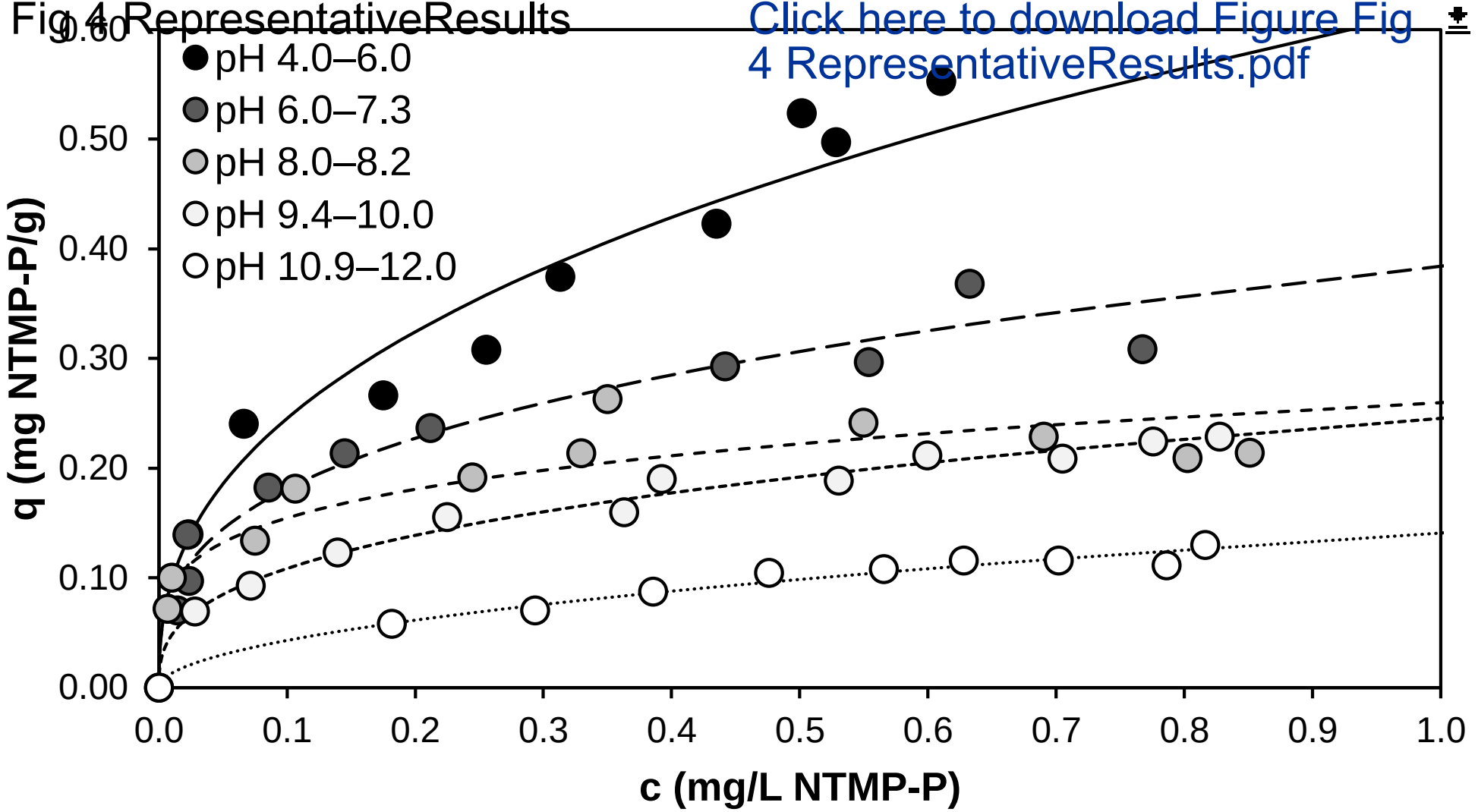

Fig. 5 Buffer-Influence

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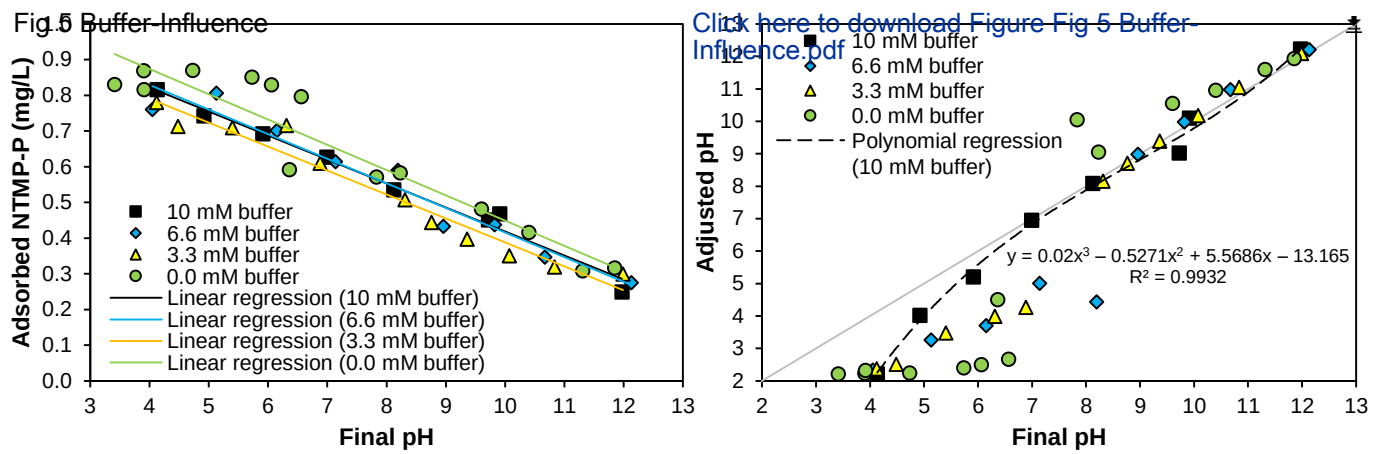

Fig. 6 Calibration

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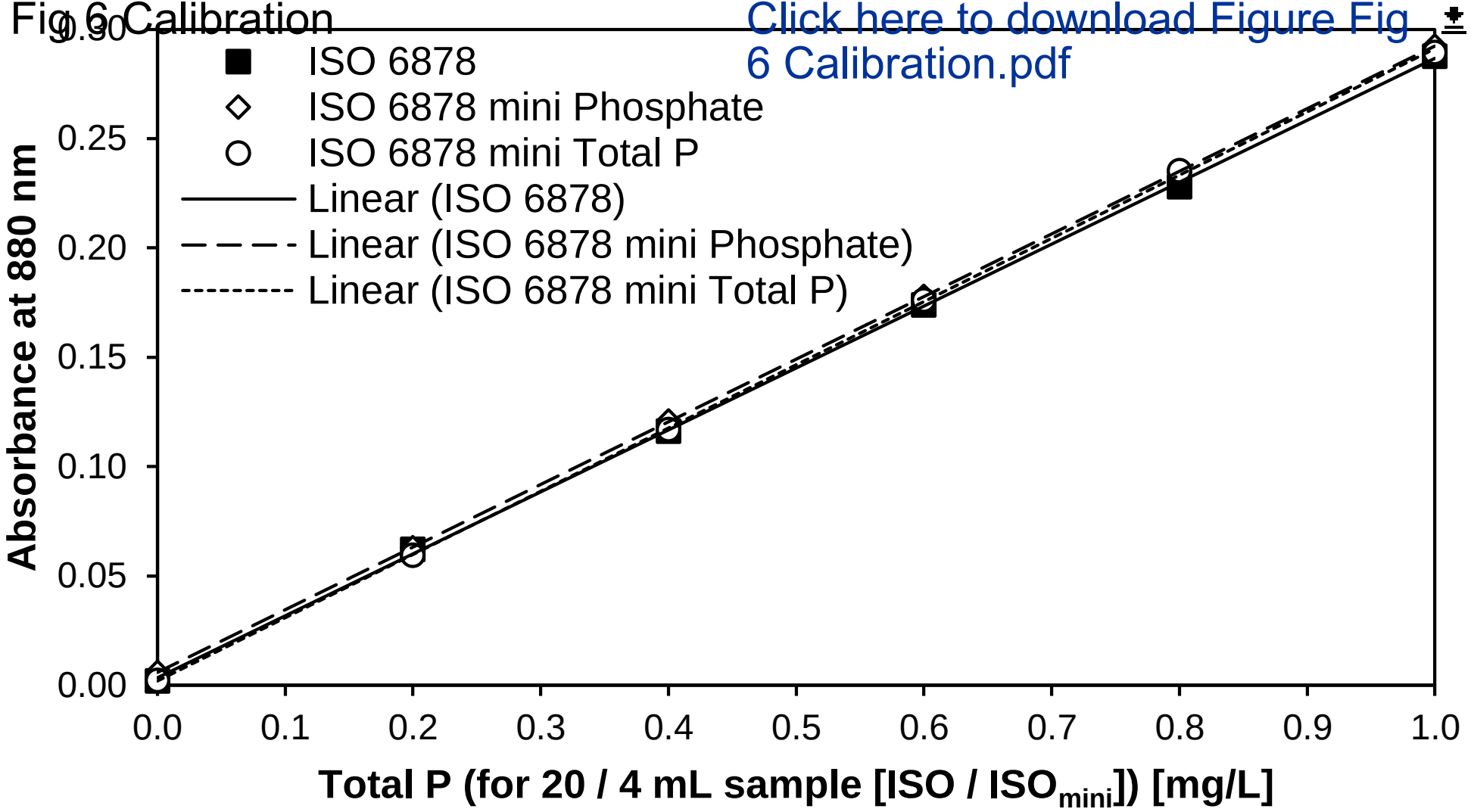

Fig 7 Plausibility

mmol NaOH

g $K_2S_2O_8$

	0.00	0.60	1.07	1.53	2.00	2.46
0.04	290	294	303	352	499	920
0.08	288	291	308	307	425	448
0.12	287	292	298	321	388	345
0.16	271	294	290	318	342	480
0.20						
0.24						
0.28						
0.32						
0.36						
0.40						

a) IQS: 1 mg/L KH_2PO_4-P + 0.9 mmol/L H_2SO_4 pH 2.63 (no pH adjustment)

	0.00	0.60	1.07	1.53	2.00	2.46
0.04	287	293	311	341	2226	2258
0.08	276	291	303	316	357	2229
0.12	293	293	300	307	354	460
0.16	287	289	294	304	312	322
0.20						
0.24						
0.28						
0.32						
0.36						
0.40						

b) 1 mg/L NTMP-P pH 3.63 (no pH adjustment)

	0.00	0.60	1.07	1.53	2.00	2.46
0.04	220	221	224	225	226	237
0.08	244	251	258	274	325	523
0.12	297	301	318	365	487	800
0.16	289	299	304	335	409	611
0.20	281	286	292	312	370	369
0.24	261	268	259	284	292	353
0.28						
0.32						
0.36						
0.40						

c) 1 mg/L NTMP-P 0.01 M CitOH pH 3.10

	0.00	0.60	1.07	1.53	2.00	2.46
0.04	293	303	347	426	716	1252
0.08	293	300	323	397	570	951
0.12	294	299	312	345	479	599
0.16	293	294	313	330	369	410
0.20	273	284	281	303	344	402
0.24						
0.28						
0.32						
0.36						
0.40						

d) 1 mg/L NTMP-P 0.01 M AcOH pH 4.72

	0.00	0.60	1.07	1.53	2.00	2.46
0.04						
0.08						
0.12	244	249	255	269	354	508
0.16	280	292	292	306	343	479
0.20	289	292	302	308	329	418
0.24	300	290	285	308	313	381
0.28	248	281	273	269	289	325
0.32	297	294	294	302	332	415
0.36						
0.40						

e) 1 mg/L NTMP-P 0.01 M MES, pH 6.17

	0.00	0.60	1.07	1.53	2.00	2.46
0.04						
0.08						
0.12						
0.16	236	242	244	251	289	370
0.20	283	284	287	298	325	394
0.24	280	285	283	292	305	356
0.28	272	280	265	274	289	318
0.32	150	263	259	263	264	273
0.36	197	246	203	211	233	242
0.40						

f) 1 mg/L NTMP-P 0.01 M MOPS, pH 7.15

	0.00	0.60	1.07	1.53	2.00	2.46
0.04						
0.08						
0.12						
0.16	225	231	233	238	265	350
0.20	268	267	277	277	295	357
0.24	287	291	295	294	315	370
0.28	284	293	289	294	304	331
0.32	281	289	284	288	294	314
0.36	273	282	267	276	275	286
0.40	192	248	191	210	235	254

g) 1 mg/L NTMP-P 0.01 M HEPES, pH 7.53

	0.00	0.60	1.07	1.53	2.00	2.46
0.04						
0.08						
0.12						
0.16						
0.20	226	228	231	233	248	288
0.24	295	293	295	300	308	352
0.28	233	295	293	297	304	330
0.32	286	292	289	292	304	323
0.36	274	290	284	288	289	300
0.40	265	256	249	268	254	281

h) 1 mg/L NTMP-P 0.01 M EPPS, pH 7.92

	0.00	0.60	1.07	1.53	2.00	2.46
0.04						
0.08						
0.12						
0.16	206	203	210	212	242	244
0.20	208	215	207	225	222	231
0.24	234	240	241	241	245	263
0.28	286	291	293	297	302	335
0.32	289	291	291	289	299	326
0.36	271	283	276	284	283	300
0.40	193	271	265	255	251	270

i) 1 mg/L NTMP-P 0.01 M CAPSO, pH 9.79

	0.00	0.60	1.07	1.53	2.00	2.46
0.04						
0.08						
0.12						
0.16	206	211	211	214	224	238
0.20	217	219	216	220	221	234
0.24	236	236	237	239	242	258
0.28	279	291	292	296	305	337
0.32	276	288	293	291	297	303
0.36	250	277	275	275	286	288
0.40	230	272	261	231	258	281

j) 1 mg/L NTMP-P 0.01 M CAPS, pH 10.47

	0.00	0.60	1.07	1.53	2.00	2.46
0.04	289	293	316	365	576	1032

k) 1 mg/L NTMP-P 0.01 M NaOH, pH 11.95

l) 1 mg/L NTMP-P 1 M NaOH, pH 13.90

mmol H_2SO_4 (prior to digestion)

0.00	1.38	2.76	4.14	5.52	6.90
585	2333	297	186	-1	-4

Table 1 MES

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Table 1 MOPS

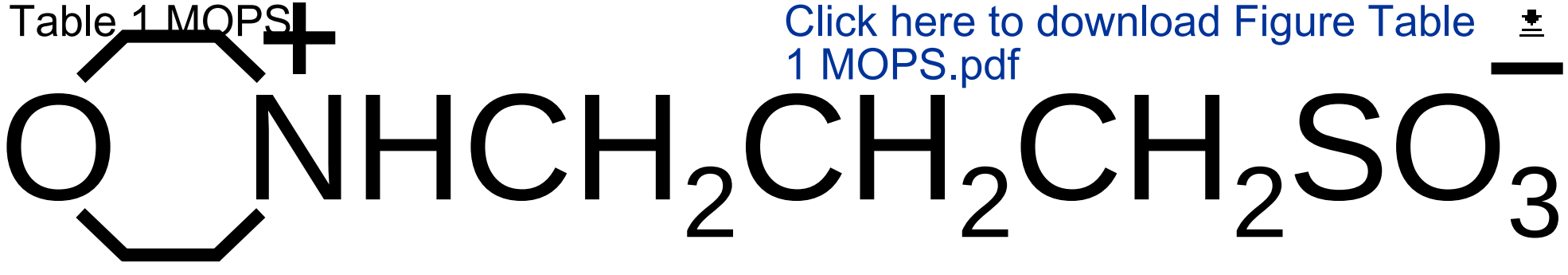

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Table 1 HEPES

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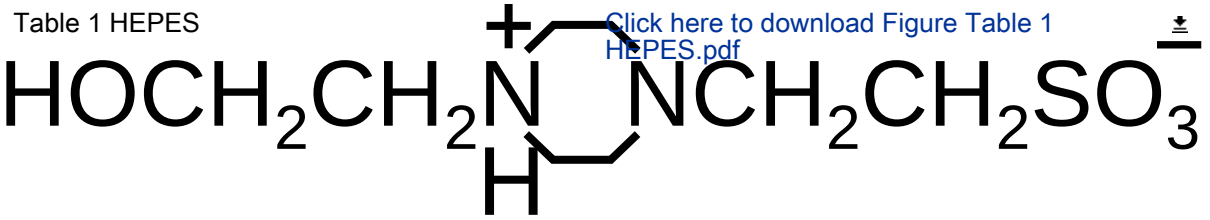

Table 1 EPPS

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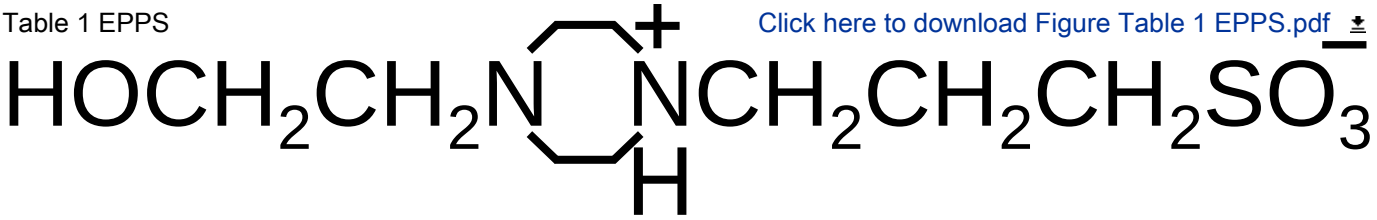

Table 1 CAPSO

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Table 1 CAPS

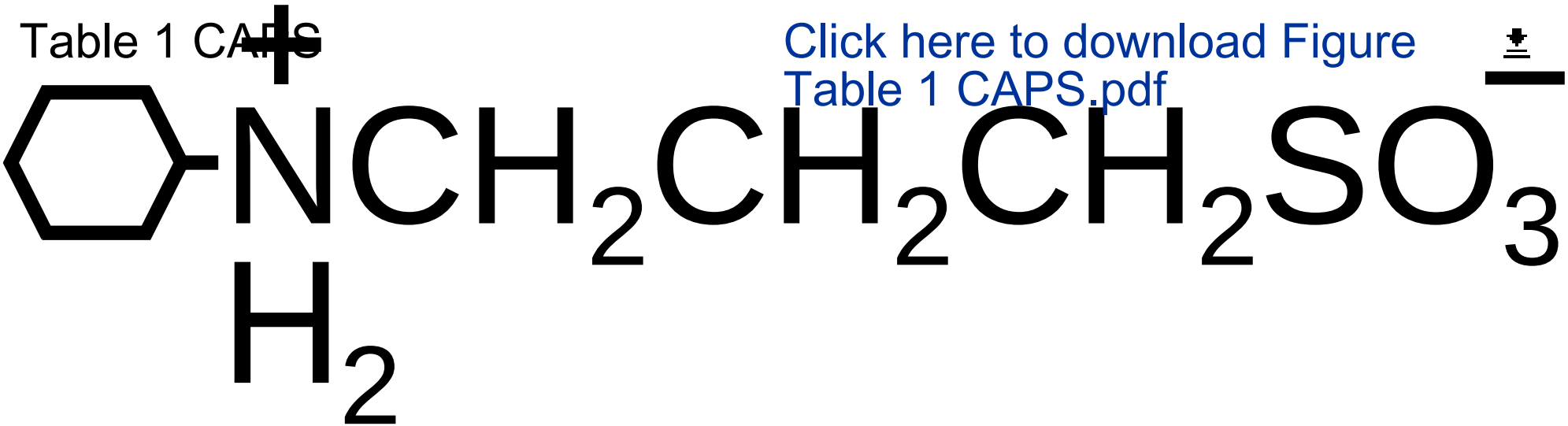

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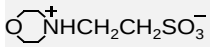
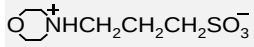
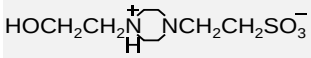
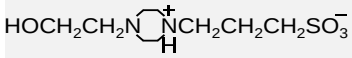
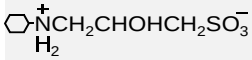
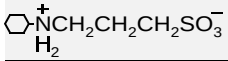
Table

Structure	Name	$pK_{a\ (25\ °C)}$	ThOD Oxidation of C, H $[O_2]/[mol]$	ThOD Oxidation of C, H, S $[O_2]/[mol]$	ThOD Oxidation of C, H, S, N $[O_2]/[mol]$	COD Analyzed oxidation [COD]/[mol]
$HOOCCH_2COH(COOH)CH_2COOH$	CitOH	3.128	4.5	4.5	4.5	4.58
$HOOCCH_3$	AcOH	4.756	2.0	2.0	2.0	1.93
	MES	6.270	6.5	8.0	10	7.38
	MOPS	7.184	8.0	9.5	11.5	9.04
	HEPES	7.564	9.0	10.5	14.5	10.54
	EPPS	7.957	10.5	12.0	16.0	11.84
	CAPSO	9.825	11.0	12.5	14.5	12.51
	CAPS	10.499	11.5	13.0	15.0	12.22

Materials Table

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Sulfuric acid (H_2SO_4)	Merck (Darmstadt, Germany)	1120802510	98% (p.a.)
Hydrochloric acid (HCl)	VWR Chemicals (Fontenay-sous-Bois, France)	20254.401	32% (AnalaR NORMAPUR, p.a.)
Sodium hydroxide (NaOH)	Merck (Darmstadt, Germany)	1064981000	≥99% (p.a.)
Citric acid monohydrate (CitOH·OH)	VWR Chemicals (Fontenay-sous-Bois, France)	20276.292	99.9% (AnalaR NORMAPUR, p.a.)
Acetic acid (AcOH)	VWR Chemicals (Fontenay-sous-Bois, France)	20104.334	100% (p.a.)
2-(*N* -morpholino)ethanesulfonic acid (MES)	SigmaAldrich (St. Louis, MO, USA)	M3671-250G	≥99%
3-(*N* -morpholino)propanesulfonic acid (MOPS)	SigmaAldrich (St. Louis, MO, USA)	M1254-250G	≥99.5%
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	SigmaAldrich (St. Louis, MO, USA)	H3375-250G	≥99.5%
4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS)	SigmaAldrich (St. Louis, MO, USA)	E9502-250G	≥99.5%
N -cyclohexyl-2-hydroxyl-3-aminopropanesulfonic acid (CAPSO)	SigmaAldrich (St. Louis, MO, USA)	C2278-100G	≥99%
N -cyclohexyl-3-aminopropanesulfonic acid (CAPS)	SigmaAldrich (St. Louis, MO, USA)	C2632-250G	≥98%
2-Phosphonobutane-1,2,4-tricarboxylic acid (PBTC)	Zschimmer & Schwarz (Mohsdorf, Germany)	CUBLEN P 50	50 % technical
1-Hydroxyethane 1,1-diphosphonic acid monohydrate (HEDP·H_2O)	SigmaAldrich (St. Louis, MO, USA)	54342-50G	≥95,0 %
Nitrilotris(methylene phosphonic acid) (NTMP)	SigmaAldrich (St. Louis, MO, USA)	72568-50G	≥97,0 %
Ethylenediamine tetra(methylene phosphonic acid) (EDTMP·1.4H_2O)	Zschimmer & Schwarz (Mohsdorf, Germany)	-	
Diethylenetriamine penta(methylene phosphonic acid) (DTPMP·6H_2O)	Zschimmer & Schwarz (Mohsdorf, Germany)	-	
Potassium dihydrogen phosphate (KH_2PO_4)	Merck (Darmstadt, Germany)	1048731000	≥99.5% (p.a.)
Potassium peroxodisulfate ($K_2S_2O_8$)	Merck (Darmstadt, Germany)	1050920250	≥99.0% (p.a.)
L(+)-Ascorbic acid ($C_6H_8O_6$)	Merck (Darmstadt, Germany)	1004680500	≥99.7% (p.a.)
Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}·4H_2O$)	Merck (Darmstadt, Germany)	1011800250	≥99.0% (p.a.)
Potassium antimony-(III) oxide tartrate hemihydrate ($K(SbO)C_4H_4O_6·½H_2O$)	Merck (Darmstadt, Germany)	1080920250	≥99.5% (p.a.)
Granular ferric hydroxide (GFH)	Hego BioTec (Berlin, Germany)	-	FerroSorp RW
Syringe membrane filters	Sartorius Stedim Biotech GmbH (Göttingen, Germany)	17765----------Q	Minisart RC Hydrophilic 25 mm 0.45 µm pore size
Single-use syringes for membrane filtration	Henke Sass Wolf (Tuttlingen, Germany)	5200.X00V0	3-part Soft-Ject Luer 20 mL
Rotator	LLG Labware (Meckenheim, Germany)	6.263 660	uniROTATOR2
Clamp for rotator	LLG Labware (Meckenheim, Germany)	6.263 664	Clamp for uniROTATOR2
Screw cap vial	Glasgerätebau Ochs (Bovenden, Germany)	135215	Präparatenglas Duran, 16x100 mm, thread GL18, cap with PTFE seal
Micropipette	Eppendorf (Hamburg, Germany)	3123000047	eppendorf Research plus 10–100 µL
Micropipette	Eppendorf (Hamburg, Germany)	3123000063	eppendorf Research plus 100–1000 µL
Micropipette	Eppendorf (Hamburg, Germany)	3123000071	eppendorf Research plus 0.5–5 mL
Precision balance	Precisa Gravimetrics (Dietikon, Switzerland)	-	Precisa LX 220 A SCS
Thermostat	Hach (Berlin, Germany)	LTV077	HT200S High Temperature Thermostat
Thermostat	Merck (Darmstadt, Germany)	1712000001	Spectroquant TR 320
Spectrophotometer	Jasco Labor- u. Datentechnik (Groß-Umstadt, Germany)	-	UV/VIS Spectrophotometer Jasco V-550
Centrifuge tube	Sarstedt (Nümbrecht, Germany)	62.559.001	Tube 50 mL, 115x28 mm, flat/conical base PP, assembled cap
pH probe	WTW (Weilheim, Germany)	103635	WTW pH-Electrode SenTix 41
pH device	WTW (Weilheim, Germany)	-	WTW Multi 350i
COD determination	Hach (Berlin, Germany)	LCK514	100–2000 mg/L O_2
Sieve	Retsch (Haan, Germany)	60.131.000500	Test sieve 0.5 mm mesh (ISO 3310/1) stainless steel
Drying cabinet	Memmert (Schwabach, Germany)	-	Modell 600

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Author(s): E. Rott, T. Reinhardt, S. Wasielewski, E. Reith-Bausch, R. Minke

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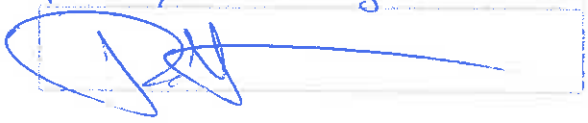
CORRESPONDING AUTHOR:

Name: Dr. Eduard Rott

Department: Institute for Sanitary Engineering, Water Quality and Solid Waste Management

Institution: University of Stuttgart

Article Title: Optimized procedure for determining the adsorption of phosphorates onto granular ferric hydroxide using a miniaturized phosphorus determination method

Signature: 

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Dr. Eduard Rott
INSTITUTE FOR SANITARY ENGINEERING, WATER QUALITY AND SOLID WASTE MANAGEMENT

UNIVERSITÄT STUTTGART

Institute for Sanitary Engineering, Water Quality and Solid Waste Management
● Bandtäle 2 ● 70569 Stuttgart

D-70569 Stuttgart (Büsnau)
Bandtäle 2

Phone +49 711 685 – 60497
Fax +49 711 685 – 63729

E-Mail:
eduard.rott@iswa.uni-stuttgart.de

Editorial Board
Journal of Visualized Experiments
Phillip Steindel, Ph.D.

Date
February 2nd 2018

Subject: Response to reviewers

On behalf of the authors, I like to thank the reviewers for their suggestions increasing the quality of the article. My co-authors and I have revised the manuscript according to the recommendations of the reviewers and the editors and hope that this was done to their full satisfaction. We also responded to some requests for change with a rebuttal in the case we did not agree with the recommendation.

Editorial comments:

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have proofread the manuscript (see trackings).

Protocol:
1. Please and align all text to the left margin to ensure the length of the protocol can be properly measured.
Done.

2. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed?
We do not think there is a more detailed way each step can be described.

Specific Protocol steps:
1. 1. This information should be moved to the Introduction.
The mentioned information was moved.

2. 2.1.2.3: What does 'sufficiently cooled down' mean?
We added the information (max. 40 °C):

“Take the 3 L beaker carefully out of the bucket as soon as it is sufficiently cooled down (max. 40 °C) and transfer its content into a 2 L glass bottle.” (lines 245 and 257).

3. 2.3.: Do you mean that the 32% HCl listed in the Table of Materials should be used as the 10.2 M HCl?
We changed the sentence to “Use 32% HCl (w/w) as 10.2 M HCl.”

Discussion:
1. Please move extensive discussion of the results to the Representative Results section.
We moved all paragraphs regarding „Influence of buffer on phosphonate adsorption and required buffer concentration”, “Calibration of ISO_{mini} method and compliance with ISO” and “Plausibility and buffer-dependent dosage quantities of ISO_{mini} method” to the Representative Results section.

2. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We changed the total Discussion section and added new texts:

“The increasing significance of phosphonates requires research for reliable methods of removing these compounds from wastewater to protect wastewater treatment plants or receiving water bodies. At present, very few studies have been carried out on the removal of phosphonates from industrial wastewater[5, 11-14, 16]. The procedure presented here shows that investigations regarding the elimination of phosphonates by adsorption on polar iron oxide containing materials, in particular granular ferric hydroxide, can be carried out quickly and reliably when in accordance with the given protocol.

The decisive point in conducting adsorption studies is the maintenance of the pH value. This cannot be done in rotating centrifuge tubes without using a buffer. In this article it was shown that Good buffers allow an acceptable pH adjustment only at a concentration of 0.01 M and even at this concentration have no significant influence on the adsorption of phosphonates onto GFH. The application of Good buffers is also the reason why the procedure presented here cannot be used for studies on adsorption of phosphonates onto rather non-polar materials such as activated carbon. Good buffers would compete with phosphonates for free adsorption sites.

Since the direct analysis of phosphonates by means of HPLC[22] or IC-ICP-MS[21] is very complex and expensive, the presented method suggests that the phosphonate after contact with the adsorbent should be measured indirectly via the determination of the total P. A standardized method (ISO 6878[28]) is generally used for the total P determination, in which a digestion is carried out by means of H_2SO_4 and $K_2S_2O_8$ on a hotplate, the pH value is then set to 3–10 by means of NaOH and a blue color complex (the color intensity of which is linearly proportional to the phosphate concentration) is formed with the aid of ascorbic acid and molybdate solution. This standardized method is very labor and time consuming, which is why a faster variant of the ISO method (ISO_{mini}) was developed. The ISO_{mini} method reduces the total

volume to one-fifth. The digestion takes place comfortably in a thermostat and the NaOH dosage after digestion is fixed. This method enables a large number of phosphorus determinations to be carried out within a very short time and does not compromise accuracy in comparison to the ISO method.

Each buffer has a different COD. In addition, the relatively high necessary buffer concentration of 0.01 M means that, in order to ensure sufficient digestion of the sample constituents, considerably higher amounts of oxidizing agent have to be dosed than it is stipulated in the ISO method. If the $K_2S_2O_8$ dosage is too low or too high, incorrect measurement results do occur. In the ISOmini method, this $K_2S_2O_8$ dosage is thus matched to each buffer individually. Another critical point is the dosage of NaOH. As a rule, regeneration solutions have NaOH concentrations of > 0.1 M. In order to avoid that the $[H^+]$:[Mo] ratio required for the formation of the color complex[25-26] is not adhered to, a proper adjustment of the H_2SO_4 quantity prior to digestion is therefore necessary. The problem arises when the regeneration solution is reused several times, thereby changing its pH value and COD. Since a reliable and simple pH measurement is not possible in screw cap vials and an appropriate pH adjustment is not provided, the ISO_{mini} method presented here, thus, reaches its limits for samples with very high pH values. For regeneration solutions it is therefore recommended to use the ISO method."

References:

1. Please ensure references have a consistent format.

The only thing we found that could have been not consistent with the template was we had added "doi:" in front of the doi link. However, the template is confusing here. In the example this "doi:" is missing while later it says "DOI (if available), preceded by a comma and listed as "doi:" then the number." However, we deleted all "doi:". Furthermore, the template only describes the citation style for scientific papers. Website links, laws and ISOs are not described, so we kept it the way we think it is ok.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We did not add glass materials such as flasks, beakers or cylinders in that list since it is common laboratory equipment. Materials that have to be bought especially for the protocol are contained in the materials table.

Reviewers' comments:

Reviewer #1: Manuscript Summary:

A very clearly written protocol on determining phosphonates and their adsorption in model and real system. The only additions I could think of are a specification of the quality of the chemicals to be used in the process, and an indication of the eventual accuracies and sensitivities to be expected for a few examples.

"Specification of the quality of the chemicals": The information about suppliers, chemicals and instruments is put in the materials excel table. Maybe the reviewers did not get the materials list? To ensure that the reader is aware of this aspect, we have inserted the following sentence at the beginning of the protocol: "The required degree of purity of chemicals can be found in the attached material list."

“indication of the eventual accuracies and sensitivities”: We added a new paragraph (lines 736–740) to address this aspect: “All dark green absorbance values in Figure 7 (n=12), converted into the total P concentration according to the calibration line in Figure 6, give an average value of 1.013 mg/L. The standard deviation is 0.014 mg/L. The typical deviation from the target value (1.000 mg/L) is therefore only 0.11–2.67% ((1.013 – 0.014 – 1.000) / 1.000 × 100% = 0.11%; (1.013 + 0.014 – 1.000) / 1.000 × 100% = 2.67%). This shows a high accuracy of the ISO_{mini} method.”

Reviewer #2: Manuscript Summary:
In this manuscript the authors reported the procedure to investigate the adsorption of phosphonates onto iron-containing GFH filter materials. Some good steps are obtained. The results seem to new support for constructing new self-assembly nanomaterials with good steps and details. I think it can be accepted after minor revisions.

Q1. Pages 1, Abstract part, the sentence of "the total P is measured using a determination method (ISOmini) that is a modification and simplification of the ISO 6878 method:….." should be modified as clear sentences; please definite P, etc. with the first time;
We separated the sentence into two sentences: “Subsequently, after membrane filtration (0.45 µm pore size), the total phosphorus (total P) concentration is measured using a specifically developed determination method (ISO_{mini}). This method is a modification and simplification of the ISO 6878 method: …”. Although we think that “P” is a common chemical element symbol for phosphorus, we defined “total P” with the first use.

Q2. Introduction part, some important and relative reports about self-assembled nanocomposites should be added to show clear background, such as the following literatures;
ACS Sustainable Chem. Eng., 2017, 5(6), 4948-4956.
ACS Sustainable Chem Eng, 2018, DOI: 10.1021/acssuschemeng.7b03635
Nanoscale Research Letters 2017, 12: 99
This is a very subjective opinion. In addition, all of the articles mentioned deal with materials that have not been examined in the context of our article. It is therefore not certain whether the process presented by us can be used with such materials. Own investigations with similar self-assembled nanocomposites showed that buffers in high concentrations contributed to strong influences on the adsorption behavior of phosphonates. The mention of self-assembled nanocomposites would thus possibly mislead the reader into thinking that the method we are proposing could be used with these materials. That is not the point of the article.

Q3. Materials part, some chemical and instrument suppliers should be listed;
The template does not include a materials part in the manuscript. The information about suppliers, chemicals and instruments is put in the materials excel table. Maybe the reviewers did not get the materials list? To ensure that the reader is aware of this aspect, we have inserted the following sentence at the beginning of the protocol: “The required degree of purity of chemicals can be found in the attached material list.”

Q4. Fig.6 the color plot line should be improved to show clear results;
In Figure 6, there is no colored line. Furthermore, the reviewer is not specific enough, so we cannot understand what is unclear in Figure 6. We think the figure is very clear and simple.

Q5. Some minor Language error should be modified;
We used the opportunity of revising the manuscript to thoroughly proofread the article.

Q6. Page 20-22, The references styles should be well corrected.
Without examples we do not know what the reviewer is referring to here. However, please see our comments above regarding the reference style.

With kind regards

Institut f. Siedlungswasserbau, Wassergüte
u. Abfallwirtschaft d. Universität Stuttgart
Bandtäle 2, 70569 Stuttgart (Büsnau)

Dr. Eduard Rott