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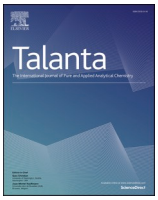

Automated calibration by a single standard solution prepared in deionized water by flow programming eliminates the schlieren and salinity effects and is applied to the determination of phosphate in sea water of different salinities

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ABSTRACT

A novel approach to calibration, based on automated dilution of a single standard solution, is combined with monitoring of the sample and reagent mixture under batch conditions, that is at equilibrium in a homogenous solution that has been arrested in a flow cell. This procedure eliminates the schlieren effect and demonstrates that there are no differences between reaction rates in deionized water and sea water. The method, based on flow programming, is applied to the determination of phosphate in sea water and is validated by analysis of certified reference materials.

1. Introduction

Due to changes in temperature, reagent composition, flow rates and other factors, the response of flow-based analyzers to target analytes can change with time. Therefore, these instruments must be periodically calibrated by standard solutions, which are usually prepared manually by serial dilution - a laborious technique, that is prone to error.

Yet another well-known issue in oceanography, is that calibration data obtained by analyzing standards prepared in deionized (DI) water and sea water maybe different due to two types of interference. In flow systems, spectrophotometric measurements are compromised by the schlieren effect [1], which is caused by the moving boundary between solutions of different ionic strengths and refractive indexes as they pass through the flow cell. Next, any difference in reaction rates, of an analyte with chromogenic reagents in sea water and DI water, can result in a difference between the calibration lines when they are obtained under nonequilibrium conditions. Obviously, these interferences cannot be eliminated from flow-based systems when the sample and reagent mixture moves through a flow cell under nonequilibrium conditions. Therefore, attempts to eliminate the interference of salinity on measurements obtained by flow-based methods has so far had limited success [1–5].

As shown in our previous work [6], flow programming eliminates these interferences by reproducing conditions of a manual batch type determination in that the final spectrophotometric measurement is performed when the reacting sample is held stationary in the flow cell until equilibria are reached. In this communication, we combine auto calibration, which is implemented in the first step of the flow protocol, with batch type monitoring used in the last step of the protocol, to develop a novel approach to the analysis of a nutrient, phosphate, in sea water. Therefore, this communication comprises two parts: introduction and validation of an automated Single Standard Calibration method (Sections 3.1. and 3.2.) and validation of the feasibility of DI based calibration by analyzing certified reference materials of open ocean sea water (Sections 3.3. and 3.4.).

2. Experimental

2.1. Principles

Programmable Flow Injection (pFI) [7], is performed in a lab-on-valve (LOV) manifold (Fig. 1) run by two milliGAT pumps via

Abbreviations: pFI, programmable Flow Injection; SSC, Single Standard Calibration; psu, practical salinity units; DI, deionized water.

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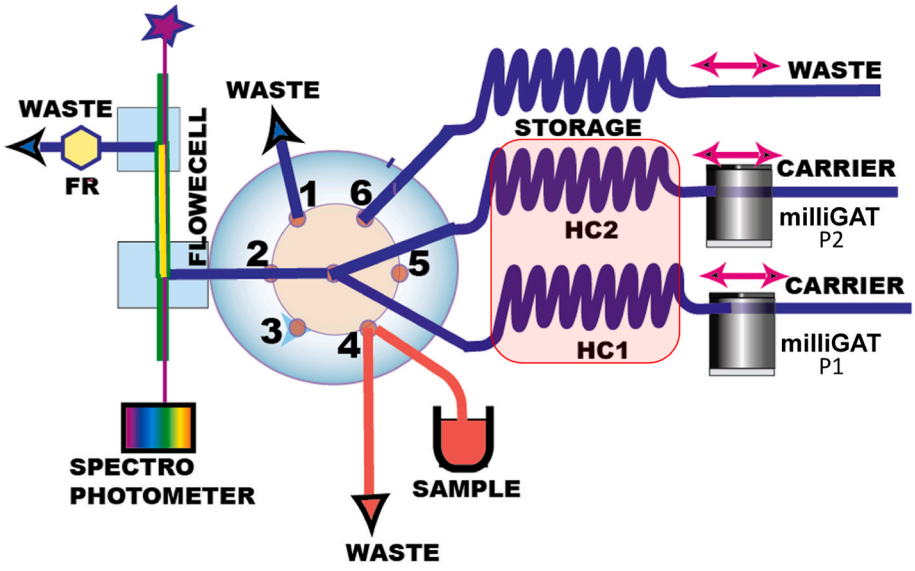


Fig. 1. Lab-on-valve instrument comprises two bidirectional milliGAT pumps connected with a six position valve through two holding coils (HC1 and HC2). Reagents are added via ports 3 and 5, sample via port 4, a 20 cm flow cell is attached to port 2, storage coil to port 6, waste to port 1. FR is a flow restrictor maintaining internal pressure above 40 psi to prevent formation of microbubbles.

two temperature-controlled holding coils (HC 1 and HC 2). A long light path flow cell (20 cm length), mounted on port 2 within the LOV is connected by optical fibers to a light source and a spectrophotometer. The remaining ports are used to introduce the sample (port 4), reagents (ports 3 and 5) and to accommodate a waste line (port 1) and a temperature controlled storage coil (port 6). All of the temperature-controlled coils were set to 40 °C.

The two-reagent phosphate determination pFI flow protocol (Fig. 2), used to validate the automated Single Standard Calibration (SSC) method is comprised of three parts:

- sample metering and dilution by the carrier stream
- mixing of sample with reagents

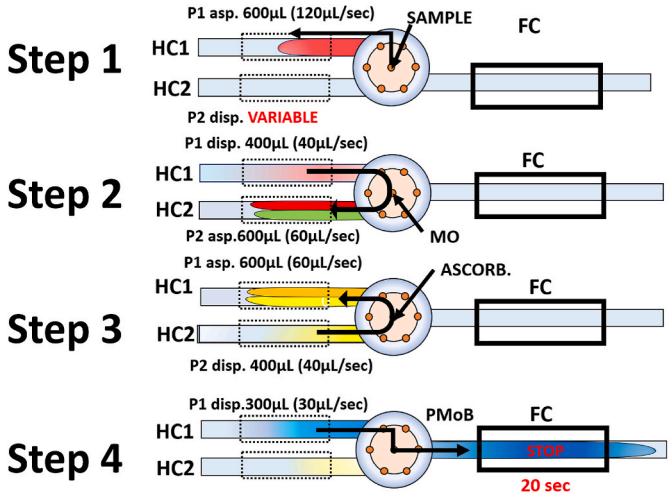


Fig. 2. Flow scheme for the two reagent determination of phosphate. MO molybdenum reagent, ASCORB ascorbic acid with SDS, PMoB phosphomolybdenum blue, FC flow cell. P1, P2 milliGAT pumps (see text for details). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

- monitoring of the reaction products

This is accomplished by means of the following sequence:

Step 1. A sample is aspirated upstream into a holding coil (HC 1) through a confluence point where it is diluted by carrier solution.

Step 2. Molybdate reagent (MO) is added to the sample as it travels from HC 1 to HC 2.

Step 3. Ascorbic acid (ASCORB) is added to the sample/molybdate mixture as it travels from HC 2 to HC 1.

Step 4. Reaction mixture (PMoB) is transferred from HC 1 into the flow cell, where it is arrested for 20 s for monitoring of the absorbance.

Auto-diluting a single, concentrated primary standard solution prior to its metering into an analyzer, is an obvious approach to auto calibration, and it can be achieved using a standalone unit comprising a valve and a syringe. But integrating auto calibration into any assay protocol of a flow analyzer has not yet been accomplished because the continuous flow format is not suitable for metering variable discrete volumes, and also because flow rates generated by peristaltic pumps are not reliable as they pulse and their flow rates decrease with time as the tubing ages. The miniSIA instrument [6,7], on the other hand, delivers precisely metered volumes and flowrates because the pump "milliGAT" dispenses volumes with a reproducibility of ±0.5% over a one-year period based on our experience.

To be useful, an auto calibration procedure must reproduce the results obtained by manual serial dilution of the most concentrated (primary) standard solution and therefore it must be accomplished within the first metering step of the pFI protocol (Fig. 2). A variety of calibration modes can be designed by aspirating the same total volume (Tv) of the primary standard solution into the holding coil (HC 1) by a pump (pump 1) while the other pump (pump 2) delivers variable volumes of the carrier solution as a diluent (D). For this work, we chose to construct a five-point descending *linear* calibration mode by programming using Tv = 600 & D = 0, Tv = 600 & D = 150, Tv = 600 & D = 300, Tv = 600 & D = 450 and Tv = 600 & D = 600 (all volumes in microliters, µL), while using DI water as the carrier solution that dilutes the primary standard on the way to HC 1. Further details are given in the software protocol (Fig. 3) and the accompanying explanation in Section 3.

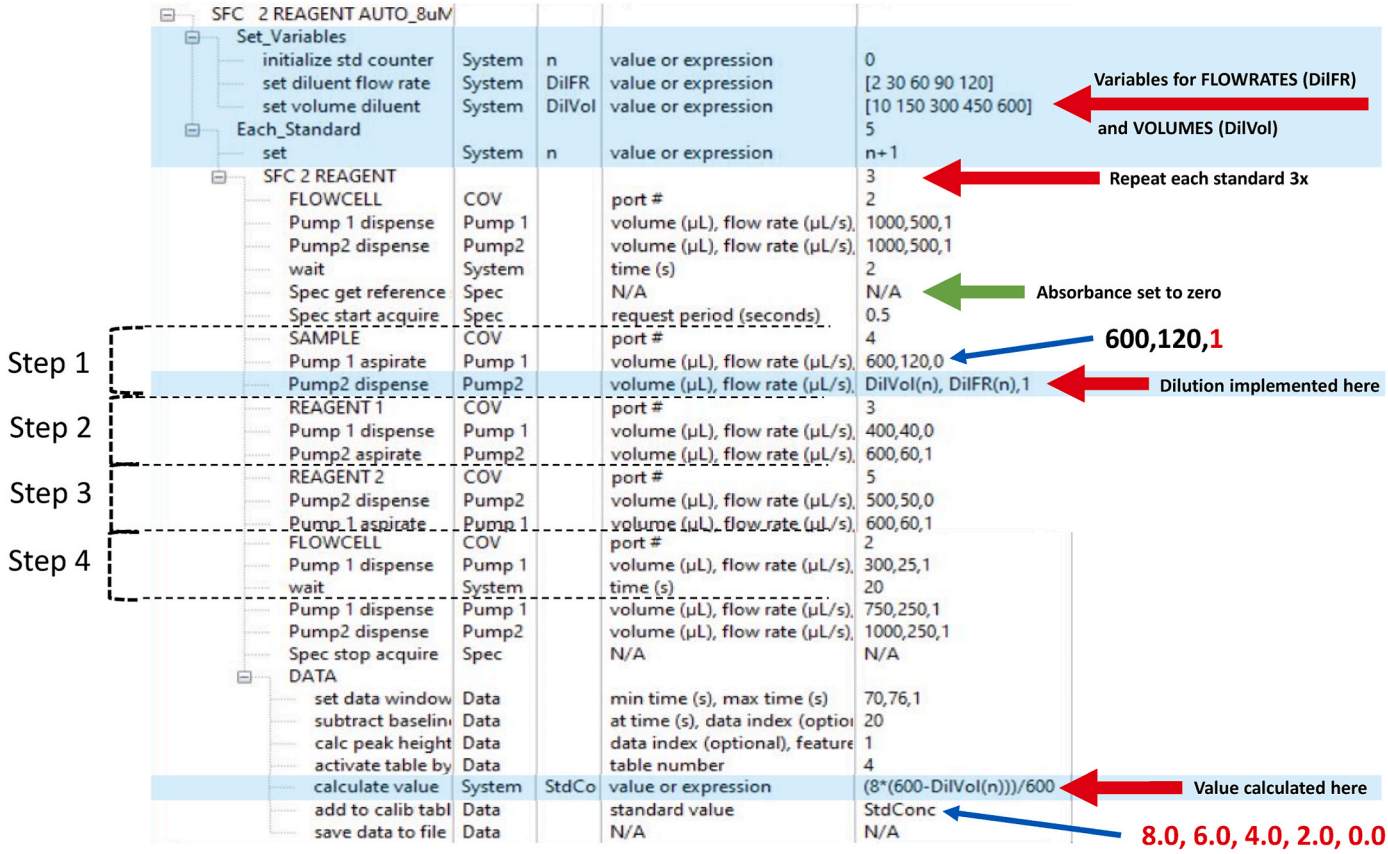


Fig. 3. Software protocol for the two reagent determination of phosphate (For details see text).

In this way, the primary standard solution, prepared in DI water is stepwise diluted by the carrier solution made of DI water.

However, in order to be applicable to the analysis of sea water (SW) samples, the flow protocol must be designed to eliminate the difference between DI and SW calibrations, caused by the schlieren effect and also by any difference between the reaction rates within DI and SW solutions. This is accomplished by programing the final step of the protocol (Fig. 2) in a batch, stop-flow mode [6] whereby the reaction mixture is arrested in the flow cell for 20 s while being monitored.

2.2. Instrumentation

The miniSIA-2 instrument (Global FIA, Fox Island, WA, USA), comprises two high precision, synchronously refilling milliGAT pumps, two thermostated holding coils, a 6-port LOV (model COV-MANI-6, constructed from polymethyl methacrylate, Perspex®) furnished with a module for an external flow cell. All tubing connections, downstream from the milliGAT pumps including the holding coils (volume 1000 µL), were made with 0.8 mm I.D. polytetrafluoroethylene (PTFE). The holding coils were thermostated at 40 °C for all experiments unless stated otherwise. The tubing between the carrier stream reservoirs and the milliGAT pump was made from 1.6 mm I.D. PTFE tubing to minimize degassing under reduced pressure at higher aspiration flow rates. A spectrophotometer (USB 4000 or Flame, Ocean Insight, Orlando, FL, USA) and a light source were connected to the flow cells by using optical fibers with 500-µm silica cores encased in 0.8 mm I.D. green PEEK tubing. The end of each fiber exposed to the liquid was cemented with epoxy, cut square, and polished. The Linear Light Path (LLP) flow cell (Fig. 1) can be purchased from Global FIA, ours was constructed in house [6]. The outlet of the LLP flow cell, was fitted with a 40-psi flow restrictor (GlobalFIA, Fox Island, WA, USA), which, by elevating the pressure within the flow path, efficiently prevented the formation of microbubbles from spontaneous outgassing. An Ocean Optics Tungsten Halogen (HL-2000, Ocean Insight, Orlando, FL, USA) light source was used. All assay steps were computer-controlled using commercially available software (FloZF, version 5.2, GlobalFIA, Fox island, WA, USA).

2.3. Water and reference materials

DI water was prepared using an Elix Type 2 Pure Water Systems, and then passed through a Milli-Q Integral Water Purification Systems (Merck Millipore, Massachusetts USA.).

Surface sea water was collected at the Hawaii Ocean Time-series station ALOHA (22° 45' N, 158 °W), in the North Pacific Ocean, and was filtered through a 0.45 µm filter (Supor 450 Membrane filter, Pall), and was stored in the dark.

Sodium chloride solutions were prepared by dissolving analytical grade NaCl in DI water.

The certified reference material (CRM), Reference Material Nutrient Seawater (RMNS) was purchased from KANSO TECHNOS CO., LTD. (KANSO) (http://www.kanso.co.jp/eng/production/).

2.4. Phosphate reagents

Phosphate stock standards were prepared by diluting a commercial PO_4 standard containing 1000 ppm phosphate (P) standard solution (LC185701, LabChem) in DI water. The 100 µM P stock solution was prepared weekly, and further diluted daily to obtain working standards (1 µM–8 µM P) in DI water or sea water.

Potassium antimony tartrate stock solution (15 mM) was prepared by dissolving 0.45 g of potassium antimony (III) oxide tartrate trihydrate (383376-100G, CAS28300-74-5, Sigma-Aldrich) in 45 mL of DI water. The mixed molybdate reagent (MO, Fig. 2) was prepared by dissolving 1.8 g of ammonium molybdate tetrahydrate crystalline (A674-500, CAS

12054-85-2, certified ACS, Fisher Scientific) in 100 mL of DI water. Next, 1.6 mL of 15 mM potassium antimony tartrate stock solution was added, followed by 15 mL of conc. H_2SO_4 (A300-500, CAS7664-93-9, certified ACS, Fisher Scientific). Finally, DI water was added to make a final volume of 200 mL. This solution is stable for one month.

The mixed solution of ascorbic acid and sodium dodecyl sulfate (SDS) solution (ASCORB, Fig. 2) was prepared by dissolving 1.0 g of L-ascorbic acid (A15613, CAS50-81-7, Alfa Aesar) in 50 mL of DI water, followed by the addition of 1.0 g of ultrapure sodium dodecyl sulfate (J75819-22, CAS151-21-3, Thermo Scientific) into the prepared ascorbic acid solution. Note that it is recommended this solution to be prepared one day or at least 30 min before use, in order to stabilize its reducing strength, as otherwise, the slope of the calibration line with freshly prepared reagent will be up to 15% steeper than those obtained later.

3. Results and discussion

3.1. Auto calibration by single standard solution is validated by spectrophotometric determination of phosphate

Determination of phosphate, based on spectrophotometry of the phosphomolybdenum blue (PMoB), has been widely used for the determination of this analyte in sea water [8–10]. The PMoB is produced in two steps; first yellow phosphomolybdate (PMo) is rapidly formed, followed by the gradual formation of the PMoB compound upon addition of a reducing agent. Both reactions are performed in a strongly acid solution (pH 0–1) to prevent formation of colloidal molybdenum blue (MoB) that coats the flow cell and increases the non-specific absorbance over the entire wavelength range.

The assay protocol can be designed in two ways; as a two-reagent assay (Figs. 2 and 3), by mixing of the sample with molybdate (MO), followed by addition of ascorbic acid (ASCORB.), or as a single reagent assay where the sample is added to a previously mixed solution of molybdate and ascorbic acid (described in Section 3.2). To validate the SSC method, we use the two-reagent assay, and for validation of the DI based calibration we use the single reagent assay because it is more sensitive (discussed in Section 3.2).

To verify that auto calibration with a single standard solution yields a calibration identical to that obtained by conventionally manually diluted standards, first, the automated SSC method was performed by using assay protocol (Fig. 3) using the DI based 8.0 μM P primary standard solution. Absorbance values obtained within WIN stop flow periods (Fig. 4, left panel) were used to construct the calibration graph (Fig. 4, right panel).

Next, a series of standards prepared by *manual* serial dilution with DI water from the primary 8.0 μM P standard solution (Fig. 5) was analyzed by the same assay protocol, from which the automated function was eliminated. This was accomplished by using the software script (Fig. 3) modified by removing the lines in the blue shaded areas, by modifying the sample aspiration script (blue arrow) and by typing the standard values prior to manually changing each diluted standard (blue arrow at bottom). Results, summarized in Table 1 reveal that the automated SSC and the manual serially diluted standard solution methods yield identical results.

3.2. Phosphate, single reagent protocol

In our previous work, we investigated the PMoB method [11] and optimized the conditions for the assay of traces of phosphate and used the method for analysis of sea water [9,10]. To minimize sample dilution in order to maximize the sensitivity of the determination, we used a mixture of molybdate with ascorbic acid rather than using separate reagents. However, since this pre-mixed reagent mixture decomposes during storage, it must be prepared immediately before use, during the first part of the assay protocol. Therefore, the flow protocol (Fig. 6) designed for this purpose is composed of three stages: reagent preparation, sample dilution, and reaction monitoring, which are executed in the following five steps:

- Mixing of reagents and rinsing of manifold (Steps 1 and 2)
- Dilution of the standard (red) with carrier solution. (Note no dilution of a sample) (Step 3)
- Mixing of reagents (green) with sample (red) (Step 4)
- Transfer of reaction mixture (blue) into flow cell monitoring of absorbance (Step 5)

Further details are given in the software protocol (Fig. 7) designed for auto calibration using a primary 4.0 μM phosphate standard solution prepared in DI water, to be diluted to five different concentrations with each diluted sample determined in triplicate.

This single reagent assay was used in the next section to examine the reproducibility of the automated SSC method.

3.3. Reproducibility of the automated Single Standard Calibration method

The advantages of automated calibration, saving of materials, time and labor etc., can only be realized if the reproducibility of the method will be equal to, or better than, that of the present way of preparing a series of standards by manual serial dilution. Therefore, the performance of the SSC was verified by investigating its reproducibility by repeating the single reagent phosphate protocol six times, while using the same

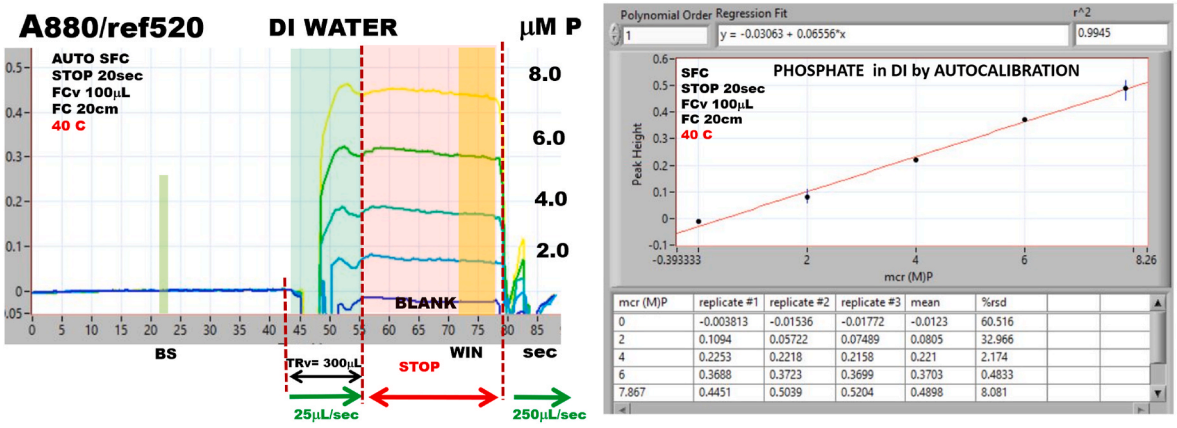


Fig. 4. Two reagent determination of phosphate obtained by automated SSC. Left: time/absorbance responses recorded for an automated SSC with standards in DI water (range 0–8 μM phosphate). The dip between 45 and 50 s is the schlieren effect as the refractive boundary passes through the flow cell. Right: Graph using absorbance values recorded within stop flow period (WIN). (For details see text).

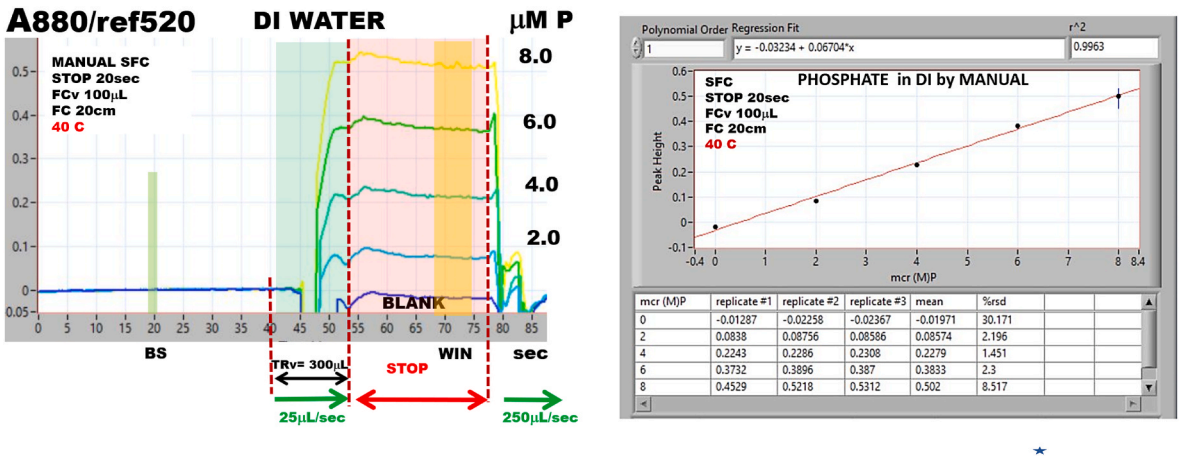


Fig. 5. Two reagent determination of phosphate obtained by manually diluted standard solutions. Left: time/absorbance responses recorded for manually prepared calibration standards in DI water (range 0–8 µM phosphate). The dip between 45 and 50 s is the schlieren effect as the refractive boundary passes through the flow cell. Right: Calibration graph obtained from absorbance values recorded within stop flow period (WIN). (For details see text).

Table 1
Comparison of auto calibration with manual calibration. SSC is the single standard calibration; CR is the ratio of the slopes.

Method	Calibration equation	r^2	Calibration slope ratio (CR)
Two reagent assay with SSC[*1]	$y = -0.03063 + 0.06556\ x$	0.9945	0.98
Two reagent assay with manual dilution[*2]	$y = -0.03234 + 0.06704\ x$	0.9963	–

[*1] Data from Fig. 4.
[*2] Data from Fig. 5.

4.0 µM phosphate primary standard solution prepared in DI water. The absorbance values, collected within the WIN stop flow interval, were used to construct a calibration graph in the same way as described in the previous section (Fig. 8). The results of all calibration slopes (Table 2), show a relative standard deviation of 1.6% around the average slope value of 0.1159 ± 0.0018. Therefore, we conclude that the automated SSC is reproducible to within 1.6%. The intercept of the standard curves ranges from −0.00265 to +0.00203 (run #1 and #6 in Table 2) equivalent to ~40 nM P.

In summary, the Single Standard auto calibration and calibrations obtained by manual serial dilution yield data precisions that are better than, or equal to, the recognized precision of spectrophotometry which is estimated to be 3% [12]. This finding confirms that auto and manual calibrations yield identical results when applied to spectrophotometry. It also suggests that SSC will become useful with optical and other methods of instrumental analysis.

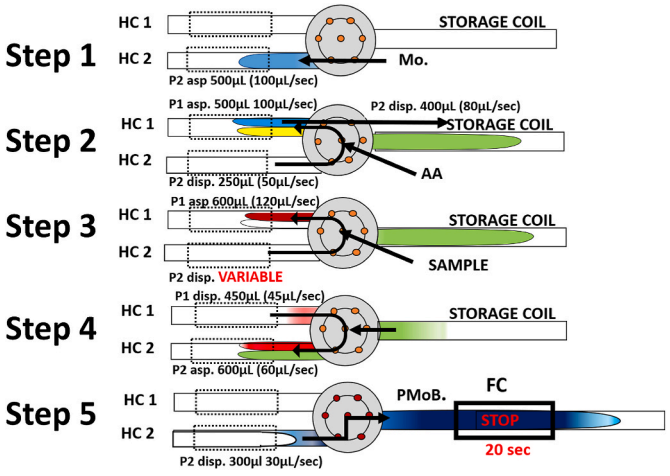


Fig. 6. Flow scheme using the single reagent scheme for the determination of phosphate. MO molybdenum reagent, ASCORB ascorbic acid mixed with SDS, PMoB phosphomolybdenum blue, FC flow cell. P1, P2 miliGAT pumps. The 4th step (not shown) is a wash of HC1 and HC2 with carrier (see Fig. 7). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. *Analysis of certified sea water materials by auto calibration with a single DI water based standard solution*

To become applicable, the assay protocol for analysis of sea water by means of DI based calibration, must resolve two issues: elimination of interference by the schlieren effect and identification and resolution of any reaction rate or other factors between the two matrices, that might lead to differences in calculated results between the DI and SW based standards. The second issue was addressed by analyzing certified reference seawater materials that are within 0–4 µM phosphate concentration range, the single reagent protocol (Fig. 7) was used because it accommodates this range of concentrations and has higher calibration slope than the two-reagent protocol.

The schlieren effect distorts the propagation of light through the flow cell in a random fashion thus degrading the reproducibility of the spectrophotometric measurement. It manifests itself by the dramatic dip in absorbance value as the boundary layer between DI water carrier and sample + reagent passes through the flow cell (green shaded area left panel of Fig. 5). However, reproducibility of absorbance measurements obtained during the following stop flow period (red shaded area) confirms that the schlieren effect was eliminated.

To validate the feasibility of using a DI based calibration for analysis of sea water certified materials, the calibration equation must be amended to:

$$y = [(a + sc) + b\ x] * sr \quad (1)$$

where "*y*" is the observed sample absorbance, "x" is the phosphate

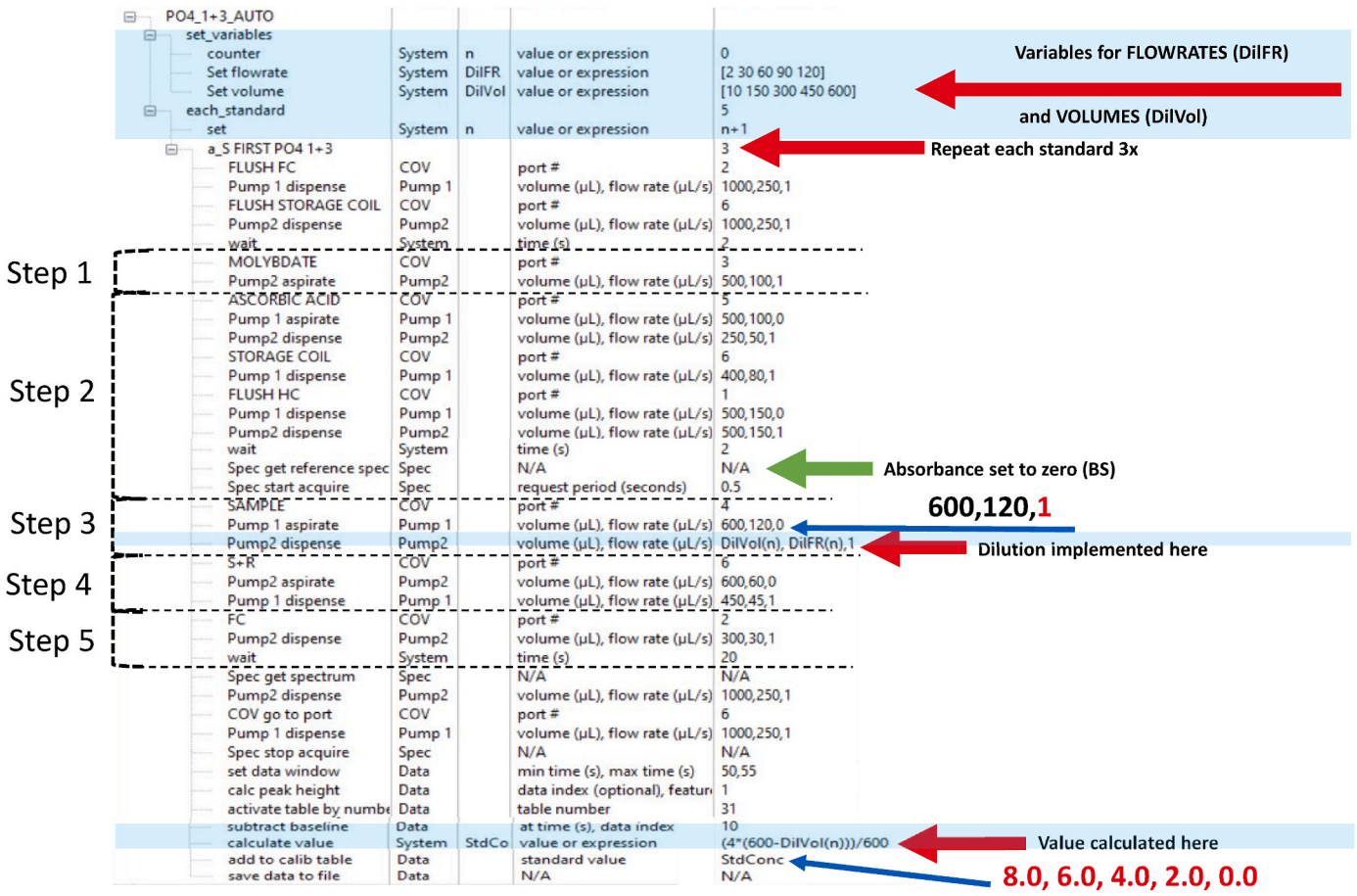


Fig. 7. Software protocol for the single reagent determination of phosphate. (For details see text).

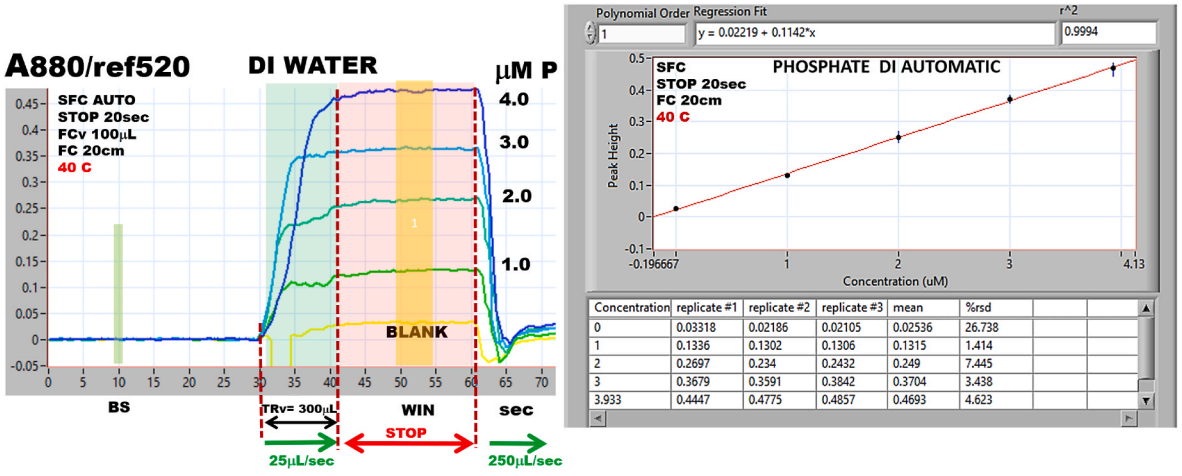


Fig. 8. Single reagent determination of phosphate by automated SSC. Left: time/absorbance responses recorded for automated SSC with standards in DI water (range 0–4 µM phosphate) Right: Auto calibration graph obtained from absorbance values recorded within stop flow period (WIN). (For details see text).

concentration (µM), "a" is the absorbance at the DI calibration curve intercept, "b" is the slope of the DI calibration curve, "sr" is the ratio of the DI/SW calibration slopes, and "sc" is the salinity correction in absorbance units – the difference in absorbance between the DI and SW calibration lines at the zero intercept.

In our previous work [6] we found that if PMoB spectrophotometry is performed in batch-flow mode, and the chemical reactions in DI and SW reach equilibria we found sr = 1.026, which is within the methods analytical error and is thus indistinguishable from 1. This means that the DI and SW calibration lines are parallel and the sr value in eq.1 can be neglected.

To determine the effect of salt on the absorbance at the calibration curve intercept (sc), a series of solutions (4, 3, 2 and 1%, of NaCl w/w) in DI were prepared from a 4% NaCl solution using the same automated

Table 2
Reproducibility of six auto calibration runs with standard in DI water.

Run#	Obtained auto standard curve	Slope/Average slope
1	$y = -0.00265 + 0.1194\ x$	1.03
2	$y = 0.00057 + 0.1154\ x$	1.00
3	$y = 0.00137 + 0.1159\ x$	1.00
4	$y = 0.00167 + 0.1154\ x$	1.00
5	$y = 0.00065 + 0.1146\ x$	0.99
6	$y = 0.00203 + 0.1145\ x$	0.99
ave.	$y = 0.0010 + 0.1159\ x$	

Note: Averaged slope = 0.1159 ± 0.0018 (1.6%); Averaged offset = 0.0010.

protocol, as described for the automated single reagent phosphate calibration (Fig. 7). The results (Fig. 9), show a *linear* dependance of the effect of salt on the difference in the absorbance at the zero intercept between the DI and seawater calibration lines (sc). The value of sc is derived from Fig. 9 using the salinity of the sample.

Therefore, when we use the DI based calibration equation:

$$y = 0.0010 + 0.1159\ x \quad (2)$$

The average value of calibrations in Table 2. The universal amended equation will be:

$$y = 0.0010 + (0.0013 * \%\ \mathrm{SAL}) + 0.1159\ x \quad (3)$$

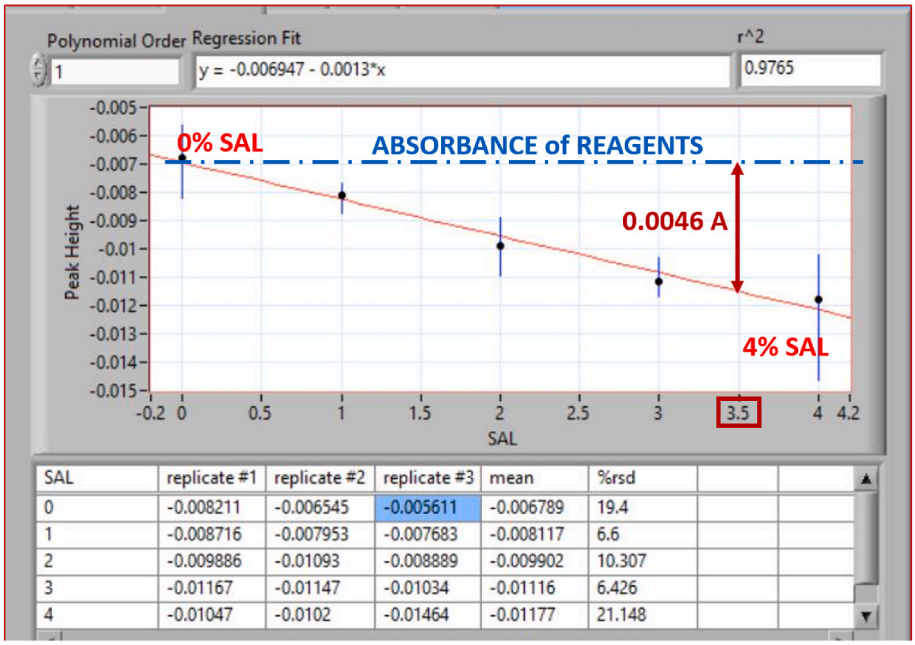
And for open ocean SW water using the values of 35 psμ ~3.5% salt shown in Fig. 9:

$$y = (0.0010 + 0.0046) + 0.1159\ x \quad (4)$$

It should be noted that the 0.0046 correction is equivalent to approximately 40 nM P.

To analyze the certified sea water samples, the software protocol (Fig. 7) was modified by removing the automated dilution function. This was done by deleting lines within the blue shaded areas and the line of "add to calib table". All absorbance measurements were performed in triplicate and the averaged absorbance values are listed in the second column in Table 3.

To illustrate the influence of salinity on phosphate determination, Table 3 shows the concentration and % of certified value obtained with an uncorrected DI calibration/eq. 2./(columns 5 and 6) and the same values using a corrected DI calibration/eq.4./(columns 7 and 8). These results show, not surprisingly, that the ~40 nM correction has most



SAL	replicate #1	replicate #2	replicate #3	mean	%rsd
0	-0.008211	-0.006545	-0.005611	-0.006789	19.4
1	-0.008716	-0.007953	-0.007683	-0.008117	6.6
2	-0.009886	-0.01093	-0.008889	-0.009902	10.307
3	-0.01167	-0.01147	-0.01034	-0.01116	6.426
4	-0.01047	-0.0102	-0.01464	-0.01177	21.148

Fig. 9. Influence of salt content on the absorbance at the intercept obtained by SSC (for details see text).

Table 3
Determination of phosphate in certified SW samples by auto calibration with a single standard prepared in DI water. F (%) is the ratio between the obtained concentration against the reported CRMs value [13].

Reference material/ Certified value (in μmol/kg)	Averaged absorbance	Standard deviation	% standard deviation	P concentration using DI standard curve *1	F % of certified value	P concentration using adjusted standard curve *2	F % of certified value
CL 0.425 ± 0.019	0.0423	0.0040	9.495	0.38	87	0.41	97
CO 1.177 ± 0.014	0.1330	0.0034	2.566	1.16	98	1.195	102
CP 1.753 ± 0.018	0.2050	0.0031	1.517	1.77	101	1.72	98
CN 2.94 ± 0.03	0.3463	0.0088	2.551	2.99	102	3.04	103

Note: *1 y = 0.0010 + 0.1159 × (eq. 2); *2 y = 0.0056 + 0.1159 × (eq. 4).

effect on the recovery of the lowest concentration certified material. The corrected values also all now fall within ~3% of their certified values, within the expected error of the method.

Overall, these results confirm that the batch-flow mode, on which the SSC assay is based, yields DI water calibration lines that are well reproduced (Table 2) and yields results that are in excellent agreement with the certified data.

Also, since the (sc) value in absorbance units (AU) is linearly dependent on salinity (Fig. 9), the DI based SSC method can be applied to the analysis of a wide range of water samples collected in rivers, in brackish areas/eq. 3./as well as in the open ocean/eq. 4./. Note that implementation of DI based SSC method for routine analysis of sea water is simple, because all that is needed is to amend the daily DI calibration by the (sc) value based on salinity (Fig. 9). Therefore, Low Nutrient Sea Water is no longer needed, which is not only convenient, but also positions the calibration process on well-defined materials – DI water and NaCl.

During routine use on samples at sea, day to day variations in the DI calibration slope should not affect the calculations, since sr is equivalent to 1. However, in regions of strong salinity gradients such as coastal areas the slope of the salinity effect (Fig. 9) should be evaluated to ensure that this small correction is applied appropriately.

To achieve the salinity correction value (as shown in Fig. 9), the SSC method must be run using a 0 μM phosphate standard in a 4% w/w NaCl solution, along with molybdate and ascorbic acid reagents. As the instrument automatically dilutes the 0 μM phosphate standard, the salinity of the sample reagent mixture in the flow cell will be incrementally diluted with DI carrier, allowing the user to calculate the salt correction.

Finally, results obtained in our previous work [6] indicate that DI based calibration has the potential to be successfully used for the determination of nitrite, nitrate and silicate in the same way as documented here for determination of phosphate.

4. Conclusion

Although the Single Standard Calibration method is introduced and documented here in a lab setting as the means to assist automated serial assays of sea water, its automated version can facilitate work in other fields. For example, continuous monitoring of environmental and of industrial processes by automated analyzers will be facilitated by Single Standard Calibration as this requires only a single (primary) standard solution. In addition, the optimization of reagent-based assays can be facilitated by using the SSC to produce automated calibration data obtained under changing reaction conditions (pH, reagents, incubation time, temperature etc.) in a systematic manner. In this way we had investigated the complex chemistry [8] on which phosphomolybdenum blue spectrophotometry of phosphate determination is based. Since each data point on the H^+ diagrams in Ref. [11] was obtained by plotting the slope of the calibration line obtained manually at many different acidities of molybdate reagents, the use of the Single Standard Calibration method, to generate each of these slopes would have much facilitated our work, which, at that time, had to be prepared from a large number of standard solutions prepared by manual serial dilution.

Determination of nutrients in sea water is based on a matrix matching technique that is designed to eliminate the schlieren effect and the influence of salinity on data. While widely used [3,5] it is well recognized that the matrix matching method has several flaws.

In the ocean, salinity can vary from 0 psμ in estuarine waters up to 35 psμ in the open ocean (the latter roughly equivalent to 3.5% by weight dissolved solids), which makes sample/standard matrix matching of samples collected from coastal areas with variable salinity impractical. The influence of day to day variations of DI calibration on the value of the coefficients sc and sr will probably be small, but understanding the stability of those values along with analysis of samples of different salinities will need to be carried out by the users.

Application of the matrix matching for analysis of samples collected in the open ocean, where the salinity is relatively constant, faces another challenge: sea water to be used for the preparation of matrix matched standards has to be obtained from regions with extremely low existing nutrients in order to reduce blank values (typically these are surface waters from oceanic gyres). This, Low Nutrient Sea Water (LNSW) that must be prepared for making these standards, must be filtered to remove microorganisms and be stored in darkness to prevent the (further) growth of organisms. Therefore, LNSW collected at different locations and prepared by different methods may differ in composition. This issue led to yet another approach, by some: the use of artificial sea water currently available in five different formulations including a commercial, one-(Sigma) [14].

Automation of nutrient assays is an important issue and is therefore being continuously developed. An alternative approach to pFI, is the "lab in syringe" approach which may be of interest to the reader [15].

Hopefully the advantages of DI based calibration, combined with the automated Single Standard Calibration technique will be recognized and more widely used.

Author contribution

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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