Development of low-cost methodologies for the determination of total phosphorus, total nitrogen and COD, as an alternative of commercially available kit tests

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Research Article

Keywords: Wastewater assessment, total phosphorus, total nitrogen, COD, method validation, comparison study, method greenness evaluation

Posted Date: November 12th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-5360999/v1

Additional Declarations: No competing interests reported.

Abstract

Water quality assessment is essential for understanding and managing water resources and identifying potential health and environmental risks. A method for a parameter valuation should be characterized by high accuracy and precision. This study presents a comprehensive comparative analysis of three crucial water quality parameters (especially in wastewater) - total phosphorus (TP), chemical oxygen demand (COD), and total nitrogen (TN_b) - by employing two distinct analytical methodologies for each parameter. Widely adopted commercial kits and low-cost in-house developed photometric procedures were rigorously developed, validated, and compared across various analytical performance metrics. Notably, the TN_b method represents an approach where nitrogenous compounds are oxidized and measured photometrically as nitrate without the conventional reduction step. The two methods for each parameter were statistically compared and the results demonstrate that the in-house methods consistently achieved lower detection limits, with precision and accuracy comparable to those of the commercial kits.

Highlights

• The present research applies an extensive approach on how to validate a method and utilizes statistical tools and criteria to compare two methods for the determination of a common parameter.

• COD and total phosphorus and total nitrogen determination methods were validated with excellent analytical characteristics and LOQs of 67.45, 0.03 and 0.09 mg/L, respectively.

• In-house methodologies demonstrate similar efficiency with commercially available cuvette tests. In-house method cuvette test accuracy (%) were 101.29 115.50, 107.38 119.20 and 88.42 112.95, for TP, COD and TN, respectively.

1. Introduction

Water is essential for the survival of most living organisms, including humans. Aquatic ecosystems are of paramount importance in maintaining the equilibrium between biodiversity and ecological stability. However, recent changes and human activities in these ecosystems pose a significant threat to their sustainability and raise concerns about the quality of environmental waters and biodiversity(Wang and Yang, 2016; Jin et al., 2022; Pawan Kumar et al., 2023). Pollution in aquatic environments can cause acute and long-term effects on fish species, such as immune suppression, reduced metabolism, and damage to gills and epithelia(Bukola et al., 2015). Recent study indicates that aquatic pollution significantly alters the transcriptome of fish gill tissues, affecting energy metabolism, protein folding, and degradation pathways, potentially impacting their health and survival(Mitra et al., 2020).

Over the past century, rapid industrial growth and increased human activities have significantly transformed our environment. It is estimated that over 80% of wastewater worldwide is discharged into the environment without proper treatment(Geetha, 2024). Water pollution is a significant problem with far-reaching impacts on human existence (complications in the gastrointestinal tract, hepatobiliary system, brain, etc.), influencing not just the quality of drinking water but also the individuals who partake in its consumption(Khalid and Shahid, 2017; Rodríguez-Tapia and Morales-Novelo, 2017; Kumar Gupta, Shah and Mishra, 2020; Pagano et al., 2023). Chemical pollutants often persist in the environment for extended periods, can bioaccumulate through the food chain affecting human health(Kalaboka et al., 2020). Pharmaceuticals, pesticides, industrial chemicals, surfactants, sweeteners, and personal care products are the most common emerging pollutants, typically not regulated under current environmental laws(Sui et al., 2015; Kapsi et al., 2019; Tran et al., 2019; Kalaboka et al., 2020).

In recent years, emerging organic contaminants have been increasingly recognized as in various aquatic environments, including surface water and groundwater(Gros, Petrović and Barceló, 2006; Sui et al., 2015). The persistence of these substances in the aquatic ecosystem can be attributed to insufficient removal during the wastewater treatment process, leading to their continued distribution throughout the water cycle(Kalaboka et al., 2020). WWTP monitoring is important for

sustainable development as it assesses the impact of effluent on ecosystem-level functions and toxicity levels, which can enhance existing water management strategies(Pereao, Akharame and Opeolu, 2021).

Water quality encompasses the overall composition of water, including its physical, chemical, and biological characteristics, while the analysis of these parameters is referred to as water quality assessment(Farrell-Poe, Payne and Emanuel, 2005). Typical monitoring of detrimental compounds and wastewater effluent quality has relied on the utilization of biochemical and physicochemical parameters, including but not limited to chemical oxygen demand (COD) and biological oxygen demand (BOD)(Aydin et al., 2015; Pereao, Akharame and Opeolu, 2021). In addition, the presence of total bounded nitrogen (TN_b) (gaseous dissolved elemental nitrogen N_2 is not included) and total phosphorus (TP) in both sediment and water is of paramount significance in the context of surface water quality maintenance and ecosystem sustenance(Wang et al., 2012). In the management of heavily polluted urban waters in China, the concentrations of these elements serve as critical indicators, providing valuable information to decision-makers regarding changes in water quality(Liu et al., 2015; Li et al., 2021). All these parameters provide valuable insights into the overall health of water bodies, reflecting the levels of organic pollutants and essential nutrients.

The effects of water pollution pose potential threats as the pollutants introduce novel risks that may not be fully understood, highlighting the importance of ongoing research and stringent environmental management. Traditionally, assessing water quality involves labor-intensive and costly processes, including sampling, transportation to laboratories, and extensive analyses, consuming considerable time and resources(Gazzaz et al., 2012). As a result, there is an urgent need for reliable and affordable techniques to evaluate water quality for scientific research and environmental management purposes. To address this challenge, there has been a paradigm shift in water quality assessment methodologies, which has been facilitated by the integration of cost-effective detection methods for physicochemical properties in environmental waters(Yao et al., 2009; Borzoei et al., 2019).

"Standard Methods for the Examination of Water and Wastewater"(Lipps, Braun-Howland and Baxter, 2023) (published by American Public Health Association, American Water Works Association and Water Environment Federation) being the definitive guide for water analysis, since 1905, encompasses an enormous amount of widely accepted procedures for testing water, wastewater, and similar matrices. The ascorbic acid method is the most frequently used technique for the colorimetric analysis of total phosphorus(Gross and Boyd, 1998; Yang et al., 2007; Li et al., 2022). In this procedure, ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) and antimony potassium tartrate ($K(SbO)C_4H_4O_6 \cdot ½H_2O$) react with orthophosphate ions in an acidic medium to create a vividly colored antimony-phospho-molybdate complex. This complex is then reduced by ascorbic acid to form a highly blue-colored complex, whose absorbance is measured at 880 nm(Murphy and Riley, 1962; Lipps, Braun-Howland and Baxter, 2023).

Chemical Oxygen Demand (COD) is a measure of the level of organic pollutants in water, indicating the amount of oxygen needed to oxidize these pollutants. A higher COD value signifies greater organic pollution in the sample. The principle of COD determination relies on the oxidation of nearly all organic compounds to carbon dioxide using a strong oxidizing agent under acidic conditions(Lipps, Braun-Howland and Baxter, 2023). The standard method involves using potassium dichromate ($Cr_2O_7^{2-}$) as the oxidizing agent, which is reduced to chromium (III) (Cr^{3+}) during the process. This reaction occurs under acidic conditions typically provided by sulfuric acid. The dichromate ion absorbs light strongly around 400 nm, while the chromic ion absorbs in the 600 nm region. The reduction of potassium dichromate results in a color change from orange to green, corresponding to the transition from hexavalent chromium (Cr_2) to trivalent chromium (Cr^{3+})(Lipps, Braun-Howland and Baxter, 2023). The absorbance of these chromium species is measured to determine COD. These measurements allow for an accurate assessment of the organic content in the water sample. This method is widely used(Ma, 2017; Wu, Hu and Liu, 2022; Li et al., 2024) because it provides a reliable estimate of the organic pollutants present, regardless of their biodegradability, making it a crucial parameter in water quality monitoring and wastewater treatment.

The alkaline persulfate digestion method is employed to fully oxidize nitrogenous compounds to nitrate for total nitrogen determination(Ameel, Axler and Owen, 1993). This method replaces the older, more cumbersome, and hazardous Kjeldahl

method(Kjeldahl, 1883; Patton and Kryskalla, 2003). The persulfate digestion typically begins under alkaline conditions, using equimolar concentrations of persulfate and hydroxide ions to yield samples with a pH > 12 after a 1:2 dilution(Patton and Kryskalla, 2003; Studt et al., 2020). During the digestion and thermal decomposition of persulfate, the pH of the solution becomes highly acidic (pH 1–2) due to the formation of bisulfate ions (Eq. 1).

$$S_2O_8^{2-} + H_2O \xrightarrow{\Delta} 2\,HSO_4^- + ½\,O_2 \quad (1)$$

These circumstances effectively convert all organic nitrogen and ammonia in the sample into nitrate. This step is well-established and widely accepted for determining total nitrogen content(Patton and Kryskalla, 2003; De Borba et al., 2014; Studt et al., 2020). Reduction of nitrate to nitrite is general the next step prior total nitrogen determination(Chen and Ding, 2008; Studt et al., 2020; LI et al., 2023). Although direct UV measurement of nitrate has been proposed by ISO 11905-1:1997('ISO 11905-1:1997 - Water quality — Determination of nitrogen — Part 1: Method using oxidative digestion with peroxodisulfate', no date), this method is not preferred by the scientific community.

This study undertakes a comprehensive comparative analysis of three key water quality parameters (TP, COD, TN_b), utilizing two distinct measurement methodologies. The first approach involves the use of a commercially available kit, a widely adopted method known for its convenience and efficiency. The second approach, in contrast, entails an in-house developed photometric procedure, demonstrating the potential for low-cost methodologies. Our primary objective is to rigorously develop and validate both methodologies and compare them in terms of analytical performance, practicality, analysis time, greenness and costs, in order to determine which method would be preferable for implementation in a routine laboratory.

2. Materials and methods

2.1 Total phosphorus methods

For the determination of total phosphorus an in-house method was developed based on the "4500-P E. Ascorbic Acid Method" as it is described in the 24th Edition of "Standard Methods for the Examination of Water and Wastewater", 2023(Lipps, Braun-Howland and Baxter, 2023).

Detailed description of the used reagents is presented in the following lines. Sulfuric acid 5N was prepared by diluting 70 mL concentrated H_2SO_4 to 500 mL distilled water. Antimony potassium tartrate solution was prepared by dissolving 1.3715 gr $K(SbO)C_4H_4O_6 \cdot ½H_2O$ in 500 mL distilled water. Ammonium molybdate solution was prepared by dissolving 20 gr $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 500 mL distilled water. Ascorbic acid 0.1M was prepared by dissolving 1.76 gr ascorbic acid in 100 mL distilled water. A mixture of the above four solutions, named combined reagent, was prepared following the ratio 5 mL 5N H_2SO_4, 0.5 mL antimony potassium tartrate solution, 1.5 mL ammonium molybdate solution, and 3 mL ascorbic acid solution. The combined reagent was prepared daily, as it is stable for 4 h only(Lipps, Braun-Howland and Baxter, 2023). Phenolphthalein indicator was prepared by dissolving 0.5 gr phenolphthalein in 50 mL ethanol.

In order to develop the in-house method, standard phosphate solution was used for calibration curve construction and validation. Dissolving 219.5 mg anhydrous KH_2PO_4 in 1000 mL distilled water equals a stock solution of 50 mg/L PO_4^{3-}-P. The same standard solution was used for the reference method validation.

To treat the sample, 5 mL of standard solution was added into a 1 cm diameter Pyrex® screw cap culture tubes followed by the addition of 0.05 mL phenolphthalein indicator, and 0.8 mL combined reagent. The culture tube was screwed, the solution was mixed and after 20 min absorbance was measured at 880 nm directly from the culture tube. The same process was performed for blank, standard solutions and QC samples.

As reference method for the determination of total phosphorus LCK 349 cuvette test (Hach Lange GmbH, Düsseldorf, Germany) was utilized and the manufacturer's instructions were followed strictly.

In order to perform a comparison study between the two methods, in-house and LCK 349 kit, Quality Control (QC) samples were analyzed. A multi-parameter standard solution LCA 708 Addista Lot no. 23223 (Hach Lange GmbH, Düsseldorf, Germany) was used as QC for both methods, while a spectrophotometer DR 3900 (Hach Lange GmbH, Düsseldorf, Germany) has been utilized to determinate the TP. All tests were completed at room temperature.

2.2 COD methods

An in-house method was developed for determining COD, based on the "5220 D. Closed Reflux, Colorimetric Method" (high range) as described in the 24th Edition of "Standard Methods for the Examination of Water and Wastewater," 2023(Lipps, Braun-Howland and Baxter, 2023).

This method involves only two reagents. Digestion solution (for high working range) is a mixture of 10.216 gr $K_2Cr_2O_7$ previously dried at 150°C for 2 h, and 33.3 gr $HgSO_4$ dissolved in 1000 mL distilled water containing 167 mL concentrated H_2SO_4. Sulfuric acid reagent was prepared by dissolving 10 gr Ag_2SO_4 in 1000 mL conc H_2SO_4. The reagent was let to stand for 2 days to be dissolved completely.

Potassium hydrogen phthalate (KHP) solution was used for constructing the calibration curve and validation. Therefore, 425 mg $HOOCC_6H_4COOK$ (KHP), previously dried at 110°C, were dissolved in 1000 mL distilled water. KHP has a theoretical COD of 1.176 mg O_2/mg(Well, 1983), consequently this solution has a theoretical COD of 500 mg O_2/L. The same standard solution was used for reference method validation.

The digestion process was performed in Pyrex® screw cap culture tubes and diameter of 1 cm. An amount of 1.665 mL of samples was added in test tube followed by the addition of 1 mL digestion solution and 2.335 mL sulfuric acid reagent, concluding to a final volume of 5 mL. The samples were incubated in a thermo-reactor LT 200 (Hach Lange GmbH, Düsseldorf, Germany) at 150°C for 2 h. The samples were cooled at room temperature and the absorbance was measured at 420 nm directly from the culture tube. Any digested standard that has a COD value gave lower absorbance than the digested blank because of the decrease in dichromate ion(Lipps, Braun-Howland and Baxter, 2023), thus the absorbance data were collected in absolute values. The same process was performed for blank, standard solutions and QC samples.

As reference method for the determination of COD LCK 514 cuvette test (Hach Lange GmbH, Düsseldorf, Germany) was utilized and the manufacturer's instructions were followed strictly.

A comparison study was performed between in-house and LCK 514 kit, and a multi-parameter standard solution LCA 708 Addista Lot no. 23223 (Hach Lange GmbH, Düsseldorf, Germany) was used as QC for both methods. The analyses for COD determination were done with a spectrophotometer DR 3900 (Hach Lange GmbH, Düsseldorf, Germany). All tests were completed at room temperature.

2.3 Total nitrogen methods

Total nitrogen was determinate by alkaline persulfate digestion method according to a modified version of U.S. Geological Survey National Water Quality Laboratory(Patton and Kryskalla, 2003), followed by direct photometrical measurement of nitrate based on the "4500-NO_3^- B. Ultraviolet Spectrophotometric Screening Method" as described in the 24th Edition of "Standard Methods for the Examination of Water and Wastewater," 2023(Lipps, Braun-Howland and Baxter, 2023).

Sodium hydroxide 1.5M was prepared by dissolving 92 gr NaOH in 1000 mL distilled water. Alkaline persulfate digestion reagent was prepared by dissolving 18 gr sodium persulfate(Studt et al., 2020) $Na_2S_2O_8$ in 450 mL distilled water containing 45 mL NaOH 1.5M. Standard solutions were not previously acidified so NaOH 1.5M was sufficient to perform alkaline persulfate digestion with initially pH > 12.

The alkaline persulfate reaction oxidizes all forms of nitrogen in the sample to nitrate. Consequently, calibration curve of NO_3^--N was constructed using a standard nitrate solution prepared by dissolving 0.7218 gr KNO_3, previously dried at 105°C

for 24 h, in 1000 mL distilled water (nominal concentration 100 mg NO_3^--N /L). The validation of both methods (in-house and reference) was carried out using a TN_b standard solution LCA 700 Addista Lot no. 23208 (Hach Lange GmbH, Düsseldorf, Germany).

Calibration curve was constructed by treating 5 mL nitrate standard solutions at different concentration levels with the addition of 0.1 mL HCl 1N. On this step the samples were not treated with digestion reagent and not digested. The absorbance was then measured at 220 nm to obtain NO_3^--N value and at 275 nm to determine interference due to dissolved organic matter, subtracting finally, two times the absorbance reading at 275 nm from the reading at 220 nm. Moving on to the validation process, to perform the alkaline persulfate digestion, 2 mL of samples were mixed with 1 mL of persulfate digestion reagent inside of Pyrex® screw cap culture tubes. Tubes were screwed and incubated in a thermo-reactor LT 200 (Hach Lange GmbH, Düsseldorf, Germany) at 121°C for 1 h. Once the samples had cooled to room temperature, the TN_b in the form of nitrate, was measured by absorbance, following the same procedure as for the nitrate standards. Specifically, cooled samples' absorbance was measured at 220 nm and 275 nm but there was not acidification with HCl 1N due to the fact that the finally pH was already < 2 (Borba, Jack and Rohrer, 2014). As the TN_b standards were diluted with digestion solution in a ratio of 2:1, and the calibration curve was constructed without dilution of nitrate standards, the concentration of the digested standards was then corrected according to the dilution factor. Blank, TN_b standard solutions and QC samples were treated accordingly.

As reference method for the determination of TN_b LCK 138 cuvette test (Hach Lange GmbH, Düsseldorf, Germany) was utilized and the manufacturer's instructions were followed strictly.

A comparison study was performed between in-house and LCK 138 kit, and a multi-parameter standard solution LCA 708 Addista Lot no. 23223 (Hach Lange GmbH, Düsseldorf, Germany) was used as QC for both methods. Total nitrogen determination in in-house method was measured with a 6405 UV/Vis spectrophotometer (Jenway, [a Cole Parmer company], Stone, Staffordshire, UK). Reference method measurements were collected by a spectrophotometer DR 3900 (Hach Lange GmbH, Düsseldorf, Germany). All tests were done at room temperature.

2.4 Statistical analysis

The three calibration curves of PO_4^{3-}-P, COD and NO_3^--N, were constructed in the form of $y = b (\pm S_b) x + \alpha (\pm S_\alpha)$, where slope is indicated as "b" (S_b: random error of slope) and intercept indicated as "α" (S_α: random error of intercept). Calibration curves examined for outliers and homoscedasticity assumptions studying the residuals behavior. Linearity assumption was evaluated by LOF (lack-of-fit) *F*-test(Stalikas and Sakkas, 2024). All the methods were further validated evaluating LOD and LOQ, accuracy, precision in terms of intra-day repeatability and inter-day repeatability (reproducibility), and uncertainty (20 replications were measured for each concentration level). Comparing the two methods' outcomes (reference and in-house methods) across various levels of analyte concentrations, least squares regression was utilized which further was statistically evaluated(Dimitrova et al., 2013; Stalikas and Sakkas, 2024). As a second way to compare the methods, Bland-Altman plots were constructed identifying any potential outliers within the data(Giavarina, 2015; Stalikas and Sakkas, 2024). The regression data results are acquired by utilizing the *Excel* spreadsheet of *Microsoft*.

The experiments were performed by one analyst to minimize potential variation from external influences. All instruments, including pipettes and other laboratory equipment used in this study, were calibrated by an accredited laboratory according to the ISO/IEC 17025 requirements. These ensure the accuracy and reliability of the measurements and results obtained.

2.5 Greenness determination

All methods were evaluated for their green character. The Analytical GREEnness (AGREE) metric approach can precisely forecast the greenness profile utilizing all 12 principles of green analytical chemistry (GAC)(Pena-Pereira, Wojnowski and Tobiszewski, 2020). The AGREE: The Analytical Greenness Calculator (version 0.5 beta, Gdansk University of Technology, Gdansk, Poland, 2020) estimated the AGREE score (method greenness) for each method.

3. Results and discussion

3.1 Total phosphorus in-house method evaluation

The calibration curve for total phosphorus was generated using standard solutions ranging from 0.01 to 1.00 mg PO_4^{3-}-P /L (seven concentration levels, five replications each), with confidence and prediction intervals shown in Fig. 1a. In the residuals plot (Fig. 1b) it is clear that there is no trumpet-shaped opening toward higher concentration levels; therefore, homoscedasticity test was passed successfully. In addition, the horizontal lines (± t(0.05, [conc. levels]-2) × $S_{y/x}$) in the residuals plot indicate the deviation limits for each individual point, concluding that there are no outliers among the measurements.

The linearity assumption was examined via *LOF F*-test as previously described(Stalikas and Sakkas, 2024). Residual error, SS_{RES}, is the combination of the "sum of squares for lack of fit (SS_{LOF})" and the "sum of squares for pure error (SS_{PE})," (Eq. 2). The F_{LOF} value was calculated by dividing the mean square for lack of fit (MS_{LOF}) to mean square for pure error (MS_{PE}), as it is described in Eq. 3. Sum of squares for pure error SS_{PE} was computed by summarizing the squares of the differences of the responses from the average response, while SS_{LOF} was computed by subtracting SS_{PE} from SS_{RES} (Eq. 2), which is output from the regression analysis (Table 1).

$$SS_{RES} = SS_{LOF} + SS_{PE}$$

2

$$F_{LOF} = \frac{MS_{LOF}}{MS_{PE}} = \frac{\frac{SS_{LOF}}{d.f.(p-2)}}{\frac{SS_{PE}}{d.f.\ ((p\times\ n)-p)}}$$

3

Table 1
Regression output of PO_4^{3-}-P calibration curve.

Regression Statistics					
Multiple R	0.999890479				
R Square	0.999780970				
Adjusted R Square	0.999774333				
Standard Error	0.003895547	[a]			
Observations	35				
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2.285877101	2.285877101	150631.539	5.72448E-62
Residual	33	0.000500785	1.51753E-05		
Total	34	2.286377886			
	Coefficients	*Standard Error*	*t Stat*	*P-value*	
Intercept	0.003016915	0.000888160 [b]	3.396813385	0.001793437	
X Variable 1	0.707109868	0.001821918 [c]	388.1127916	5.72448E-62	
	Lower 95%	*Upper 95%*			
Intercept	0.001209939	0.004823890			
X Variable 1	0.703403147	0.710816589			

[a] $S_{y/x}$ = Residual standard deviation, [b] S_a = Intercept standard deviation, [c] S_b = Slope standard deviation

In Table 2 is clear that the F_{LOF} value is greater that the critical *F*, indicating that the regression model is acceptable and the variations in the response can be explained linearly by the model.

Table 2
Lack of fit *F*-test outcomes of TP, COD and TN_b in-house methods.

	SS_{RES}	SS_{LOF}	SS_{PE}	MS_{LOF}	MS_{PE}	F_{LOF}	F_{crit}
TP	0.000500785	0.000031185	0.000469600	0.000006237	0.000016771	0.371876838	2.558127499
COD	0.014509060	0.003079460	0.011429600	0.000513243	0.000357175	1.436951796	2.399079631
TN	0.003957288	0.001134888	0.002822400	0.000283722	0.000117600	2.412601353	2.776289289

The developed method was further validated. Limits of detection (LOD) and quantification (LOQ) were calculated from the calibration curve as LOD = 3 × σ / b and LOQ = 10 × σ / b, respectively, where b is the slope of the calibration curve and σ is the standard deviation of the response. This standard deviation was determined in three different ways, using the calibration curve (residuals standard deviation $S_{y/x}$ and standard deviation of intercept S_a) and standard deviation of blank measurements. Finally, LODs and LOQs were the lowest theoretical values that could be confirmed experimentally. The analytical performance of the developed method was examined and the results are presented in Table 3, against the also validated reference method.

Table 3
Analytical parameters of TP in-house method (n = 20).

	in-house method		LCK 349	
LOD (mg/L)	0.01		0.02	
LOQ (mg/L)	0.03		0.06	
Working range (mg/L)	0.03–1.00		0.06–1.50	
Uncertainty (%)	5.18		3.05	
Accuracy (%)	LOQ	101.29	LOQ	115.50
	0.5 mg/L	100.69	1 mg/L	98.79
	1 mg/L	97.84	1.5 mg/L	101.30
Intra-day repeatability (% RSD)	LOQ	8.39	LOQ	5.02
	0.5 mg/L	2.20	1 mg/L	0.93
	1 mg/L	1.48	1.5 mg/L	0.87
Inter-day repeatability / reproducibility (% RSD)	LOQ	7.16	LOQ	5.16
	0.5 mg/L	2.33	1 mg/L	0.94
	1 mg/L	1.39	1.5 mg/L	0.88

Results indicate that the in-house method offers slightly better sensitivity, as reflected by its lower LOQ (0.03 mg/L) compared to the LCK method (LOQ: 0.06 mg/L). However, the LCK method demonstrated superior precision, although both methods maintained good accuracy across tested concentrations. Despite the slightly higher variability observed in the in-house method, particularly at lower concentrations, it is a robust alternative mainly because it requires much less analysis time.

3.2 COD in-house method evaluation

A calibration curve was established for COD by preparing standards with concentrations ranging from 60 to 500 mg O_2 /L (eight concentration levels, five replications each). Figure 2a illustrates the curve, along with its confidence and prediction intervals. Examination of the residuals plot (Fig. 2b) confirmed homoscedasticity, as no funneling pattern was observed at higher concentrations. The residuals analysis further demonstrated the absence of outliers, as all data points fell within the expected deviation boundaries.

The regression output is summarized in Table 4, while linearity was assessed via the lack-of-fit *F*-test, in alignment with previous descriptions and the positive results are presented in Table 2. According to the results, the method demonstrates great fitting ability with 0.9968 determination of coefficient (R^2) and linear relationship between concentration levels and the absorbance. The method was validated and the experimentally verified LODs and LOQs represent the minimum detectable concentrations, with the method's analytical parameters compared against the validated reference method in Table 5.

Table 4
Regression output of COD calibration curve.

Regression Statistics					
Multiple R	0.998397865				
R Square	0.996798297				
Adjusted R Square	0.996714041				
Standard Error	0.019540147	[a]			
Observations	40				
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	4.517159715	4.517159715	11830.68197	5.15340E-49
Residual	38	0.014509059	0.000381817		
Total	39	4.531668775			
	Coefficients	*Standard Error*	*t Stat*	*P-value*	
Intercept	0.019204083	0.005407553 [b]	3.551344165	0.001041666	
X Variable 1	0.002194186	2.01729E-05 [c]	108.7689384	5.15340E-49	
	Lower 95%	*Upper 95%*			
Intercept	0.008257064	0.030151103			
X Variable 1	0.002153348	0.002235024			

[a] $S_{y/x}$ = Residual standard deviation, [b] S_a = Intercept standard deviation, [c] S_b = Slope standard deviation

Table 5
Analytical parameters of COD in-house method (n = 20).

	in-house method		LCK 514	
LOD (mg/L)	20.26		35.80	
LOQ (mg/L)	67.45		119.20	
Working range (mg/L)	67.45–500.00		119.20–1000.00	
Uncertainty (%)	10.51		3.21	
Accuracy (%)	LOQ	107.38	LOQ	119.20
	125 mg/L	99.67	500 mg/L	107.34
	500 mg/L	98.01	1000 mg/L	100.98
Intra-day repeatability (% RSD)	LOQ	7.47	LOQ	0.95
	125 mg/L	10.64	500 mg/L	0.18
	500 mg/L	3.97	1000 mg/L	0.15
Inter-day repeatability / reproducibility (% RSD)	LOQ	11.35	LOQ	0.96
	125 mg/L	10.91	500 mg/L	0.17
	500 mg/L	7.33	1000 mg/L	0.16

For COD analysis, the in-house method exhibited a way lower LOQ at 67.45 mg/L compared to 119.20 mg/L for the LCK method, indicating better sensitivity at lower concentrations. However, the LCK method provided a broader working range (119.20–1000.00 mg/L) and significantly lower uncertainty (3.21%). The accuracy of both methods was comparable. The LCK method outperformed the in-house method in terms of repeatability, with notably lower %RSD values across all concentration levels in both intra-day and inter-day measurements, suggesting superior precision. Despite the higher variability observed in the in-house method, particularly at lower concentrations, method's precision still lays in an acceptable level (RSD < 15%), being the most appropriate choice especially when lower LOQ is a priority.

3.3 Total nitrogen in-house method evaluation

Calibration curve was constructed from nitrate standards at concentration levels 0.05 up to 7.00 mg NO_3^--N /L (six concentration levels, five replications each), and it is depicted with the presence of confidence and prediction intervals in Fig. 3a. The residuals analysis revealed a uniform spread across all concentration levels, indicating the absence of heteroscedasticity (Fig. 3b). Additionally, the residuals plot did not exhibit any outliers, as confirmed by the horizontal deviation limits for each data point.

The regression output is presented in Table 6. Lack-of-fit F-test confirms the linearity assumption in the working range, with the F_{LOF} value being slightly smaller than critical F (Table 2). Further validation of the method provided limits of detection (LOD) and quantification (LOQ), calculated using the calibration slope (b) and the response's standard deviation (σ). The method's analytical performance is presented in Table 7, alongside the reference method results.

Table 6

Regression output of NO_3^--N calibration curve.

Regression Statistics					
Multiple R	0.999872094				
R Square	0.999744205				
Adjusted R Square	0.999735069				
Standard Error	0.011888301	[a]			
Observations	30				
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	15.46658168	15.46658168	109434.6235	7.67408E-52
Residual	28	0.003957288	0.000141332		
Total	29	15.47053897			
	Coefficients	*Standard Error*	*t Stat*	*P-value*	
Intercept	0.004309677	0.002879029 [b]	1.496919965	0.14560271	
X Variable 1	0.26721295	0.000807756 [c]	330.8090439	7.67408E-52	
	Lower 95%	*Upper 95%*			
Intercept	-0.001587748	0.010207101			
X Variable 1	0.265558337	0.268867562			

[a] $S_{y/x}$ = Residual standard deviation, [b] S_a = Intercept standard deviation, [c] S_b = Slope standard deviation

Table 7
Analytical parameters of TN_b in-house method (n = 20).

	in-house method		LCK 138	
LOD (mg/L)	0.03		0.67	
LOQ (mg/L)	0.09		2.22	
Working range (mg/L)	0.09–7.00		2.22–16.00	
Uncertainty (%)	12.29		2.00	
Accuracy (%)	LOQ	88.42	LOQ	112.95
	1.4 mg/L	108.44	6 mg/L	101.48
	5 mg/L	102.81	16 mg/L	98.22
Intra-day repeatability	LOQ	12.77	LOQ	2.54
(% RSD)	1.4 mg/L	0.86	6 mg/L	1.93
	5 mg/L	0.83	16 mg/L	1.07
Inter-day repeatability / reproducibility	LOQ	15.89	LOQ	3.64
(% RSD)	1.4 mg/L	1.25	6 mg/L	2.02
	5 mg/L	1.13	16 mg/L	1.13

The TN_b in-house method exhibited a lower LOQ at 0.09 mg/L compared to 2.22 mg/L for the LCK method, indicating superior sensitivity. However, the LCK method demonstrated a broader working range (2.22–16.00 mg/L) and significantly lower uncertainty (2.00%). In terms of accuracy, the in-house method showed a slightly lower performance at the LOQ level (88.42%) but was comparable at higher concentrations. The in-house method also outperformed the LCK method in terms of repeatability, with lower %RSD values, indicating superior precision. Despite these differences, the in-house method offers a viable alternative.

Notably, the TN_b in-house method represents a pioneering approach, where total nitrogenous compounds are directly oxidized and measured as nitrate without requiring a prior reduction step to nitrite. This technique not only simplifies the TN_b determination process by minimizing the total analysis time, but also enhances accuracy and efficiency, potentially setting a new standard for nitrogen analysis in water quality assessments.

3.4 Comparison of analytical methods

3.4.1 Statistical evaluation

The comparison of methods experiment is critical for assessing the systematic errors. There's a lot of debate and discussion about the right way to analyze data from a comparison of methods experiment(Petersen et al., 1997). The most fundamental data analysis technique is to graph the comparison results and visually inspect the data. Measurements obtained for TP, COD and TN_b by in-house methods were plotted against the corresponding values of the standard methods across various levels of analyte concentrations (Fig. 4). The equations of the best-fit lines through the data were determined using the method of least squares. It was found that the relationships between the measurements, by both methods, were approximated accurately.

The obtained regression lines for each one of measured indicators were statistically evaluated by their comparison with a hypothetic ideal line the slope of which is unit and the intercept is zero. Intercept and slope were examined if they

significantly deviate from zero and one, respectively. Confidence limits for the values of both are calculated, usually at a significance level of 95%.

To compare the in-house and cuvette test method for the determination of TP, Fig. 4a was plotted and regression line was computed with the intercept be determined to be -0.0262005918838936 and the slope 1.08880285401549. Confidence limits of both intercept and slope (Table 8) establishing the range within which the slope and intercept values are likely to fall, indicating excellent agreement between the methods. The data from the reference technique and the COD in-house approach were compared, and the measurements of both methods were fitted linearly with 0.9908 coefficient of determination (R^2) (Fig. 4c). In Table 8 are depicted the confidence limits of the slope and intercept, confirming the assumption of a regression line with slope of 1 and intercept of 0. Similarly, both TN_b methods were compared in Fig. 4e, with the 95% confidence intervals of the slope (from 0.75988511832115 to 1.33374645590445) and intercept (from − 1.00737171646407 to 1.50844057626652) encompassing the desired value of one and zero, respectively (Table 8).

Table 8
Regression summary of comparison study with 95% confidence limits.

	TP	COD	TN_b
Slope	1.08880285401549	0.955955560842498	1.0468157871128
Slope lower 95%	0.895691640644734	0.786886535377449	0.75988511832115
Slope upper 95%	1.28191406738624	1.12502458630755	1.33374645590445
Intercept	-0.0262005918838936	6.09161611027611	0.250534429901223
Intercept lower 95%	-0.197532795700492	-52.8817829908609	-1.00737171646407
Intercept upper 95%	0.145131611932705	65.0650152114132	1.50844057626652

Bland-Altman plots serve as a valuable graphical tool to compare and evaluate the agreement between two sets of data obtained from different measurement techniques. Illustrating the difference between two measurements on the y-axis, a horizontal line is incorporated to represent the mean difference between the two measurements and the limits of agreement indicate the standard deviation, typically ± 1.96 × (standard deviation of the differences) from the mean difference. Figure 4 depicts the Band-Altman plots of all three comparison studies without identifying any potential outliers within the data and good agreement between methods.

3.4.2 Greenness evaluation

The environmental impact of the in-house methods for COD, TN, and TP determination was assessed using the AGREE software. This tool evaluates the greenness of analytical methods based on 12 principles of green chemistry. According to the AGREE methodology, the AGREE score for each GAC principle is assigned from 0 to 1. The AGREE analysis showed that the in-house methods demonstrated a similar overall greenness score compared to the commercially available kits (Fig. 5). In the case of COD determination both in-house and reference methods demonstrated very low greenness index, particularly due to the large amount of hazardous sulfuric acid. While the commercial kits were slightly more environmentally friendly, the TP in-house method scored better in terms of shorter analysis time and energy consumption. Overall, no significant differences were observed making in-house methods equivalent alternatives for laboratories focused on sustainable practices.

3.4.3 Practicality, analysis time and cost

To evaluate the applicability of the in-house methods a comparative analysis was conducted with commercially available kits, focused on two critical metrics: analysis time and cost per measurement. The analysis time was defined as the total duration from sample preparation to obtaining the final result, while the cost per measurement included the expenses for

reagents and consumables. The results are presented in Table 9 declaring that the financial analysis was done in the 2nd semester of 2024. The in-house methods demonstrated extremely low costs per measurement for all three parameters compared to the commercially available kits, primarily due to lower reagent expenses. In addition, the analysis time for the in-house methods was generally the same with the reference methods, except for TP determination, where the in-house method showed a slight advantage in speed. These findings suggest that the in-house methods are cost-effective, being ideal for routine analysis.

Table 9
Comparison of analysis time and cost per measurement between in-house methods and commercially available kits.

Parameter	In-house method Approx. time (min)	Commercial kit Approx. time (min)	In-house method/ Commercial kit cost ratio
TP	25	45	1/450
COD	125	125	1/11
TN_b	65	50	1/1300

4. Conclusions

Methods for the determination of three key water quality parameters - total phosphorus (TP), COD and total nitrogen (TN_b) - were developed and validated. Specifically, the determination of TN_b was performed utilizing the widely known persulfate oxidation method combined with the direct UV measurement of nitrate ions. The need of a prior reduction step before TN_b determination was eliminated optimizing the total analysis time. Analytical characteristics were compared between the low-cost in-house developed method and the corresponding reference one. In all cases the in-house methods had lower detection limits, while precision and accuracy where comparable and all six methods demonstrated excellent analytical performance. The comparison study of in-house *vs.* reference method showed great agreement among measurements at different concentration levels. In terms of analytical performance in-house methods are presented as an excellent, robust and reliable alternative approach, lacking nothing from widespread commercially available kits, ideal for implementation in routine laboratories.

Declarations

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Declaration

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

All the authors have consented to participate and to publish the results of this work.

Ethical Approval

Not applicable for this work

Author Contributions

Conceptualization: Christoforos Chrimatopoulos; Methodology: Christoforos Chrimatopoulos, Christos Tsiostas; Formal analysis and investigation: Christoforos Chrimatopoulos, Vasilios Sakkas; Writing - original draft preparation: Christoforos Chrimatopoulos; Writing - review and editing: Christoforos Chrimatopoulos, Christina Diamanti, Vasilios Sakkas, Lampros Nousis; Resources: Konstantinos K. Tsilidis; Supervision: Lampros Nousis, Evangelia Ntzani, Konstantinos K. Tsilidis, Vasilios Sakkas.

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Figures

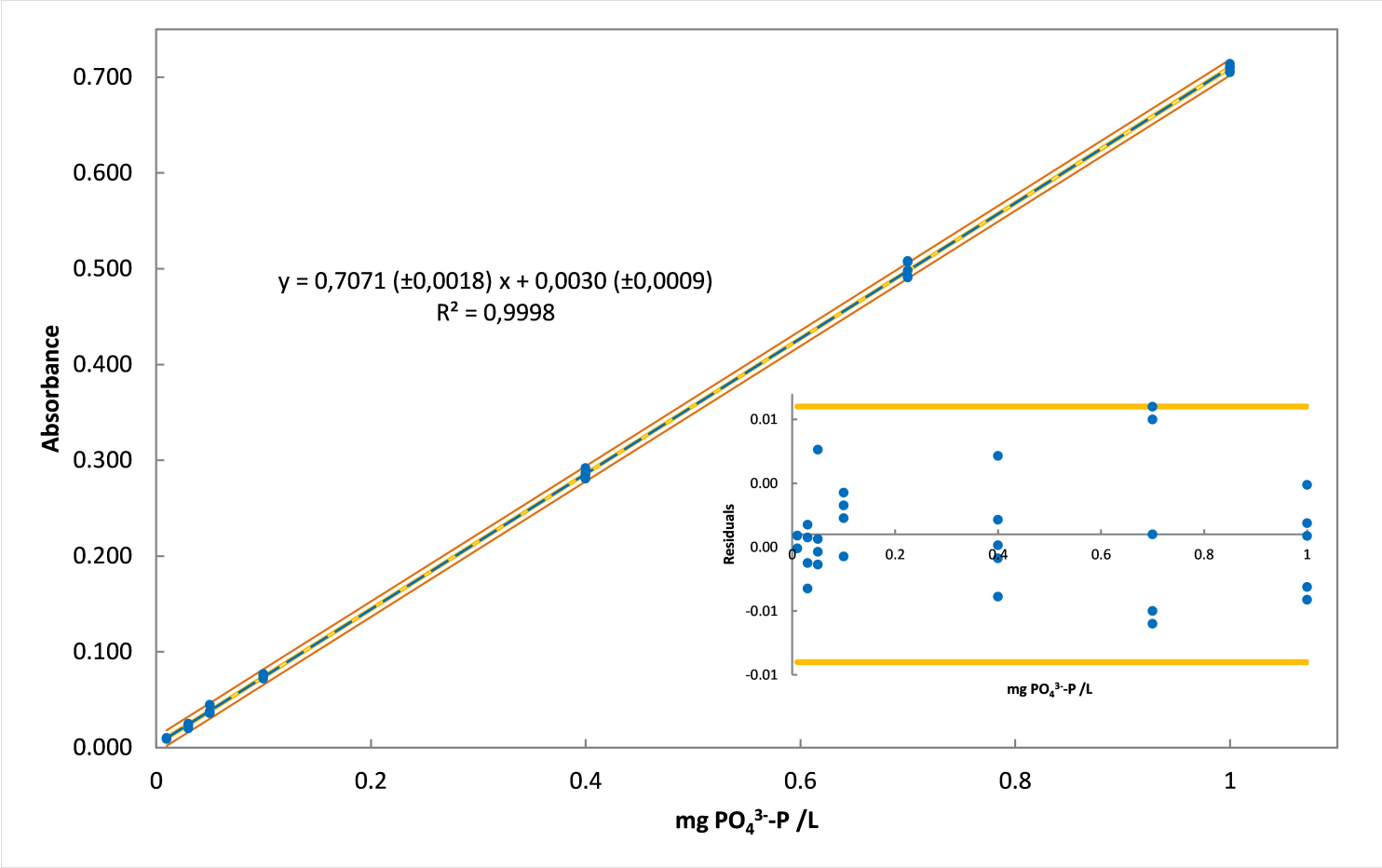

Figure 1

(a) Calibration curve of TP with confidence and prediction 95% intervals, (b) regression residuals plot with deviation limits.

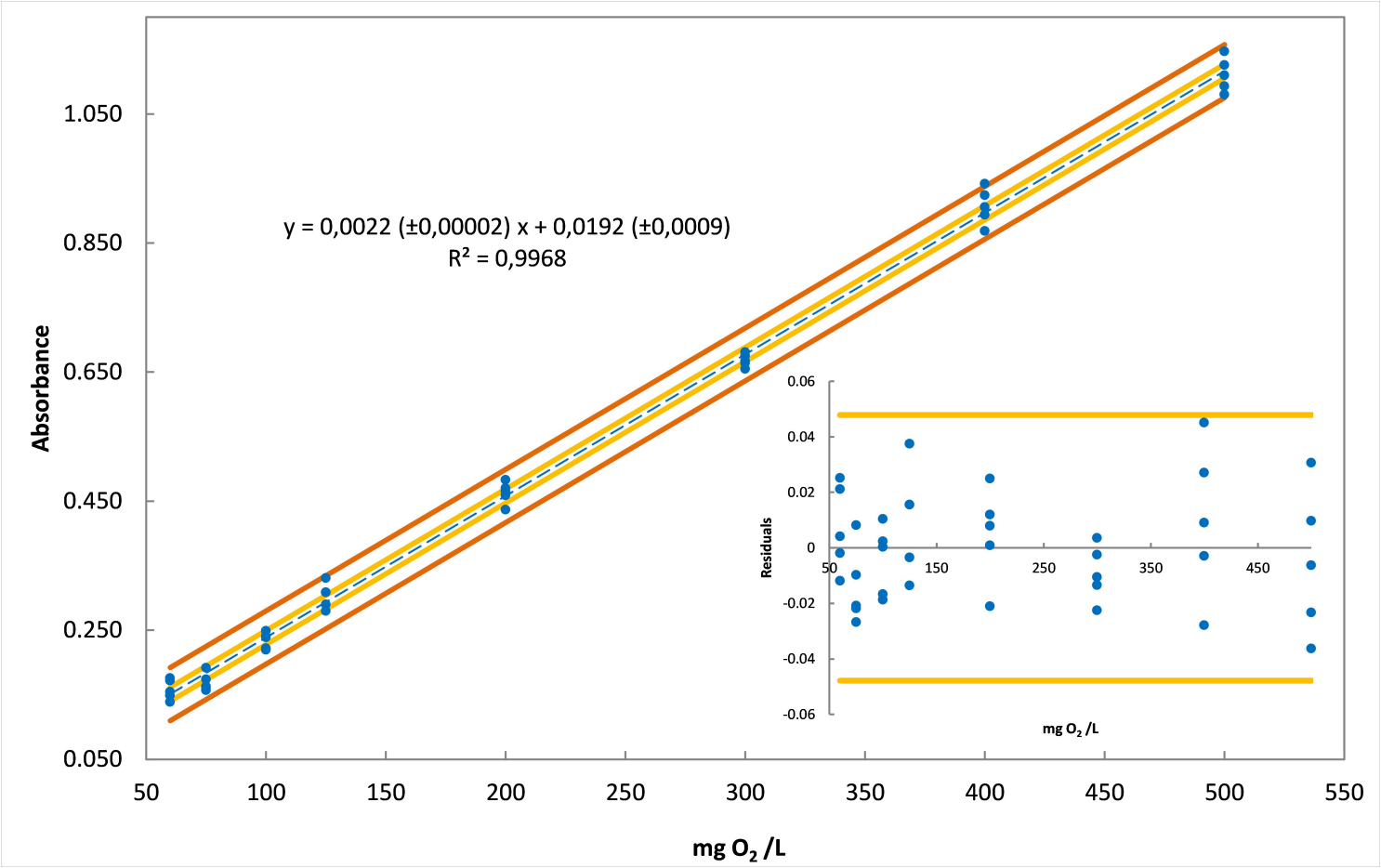

Figure 2

(a) Calibration curve of COD with confidence and prediction 95% intervals, (b) regression residuals plot with deviation limits.

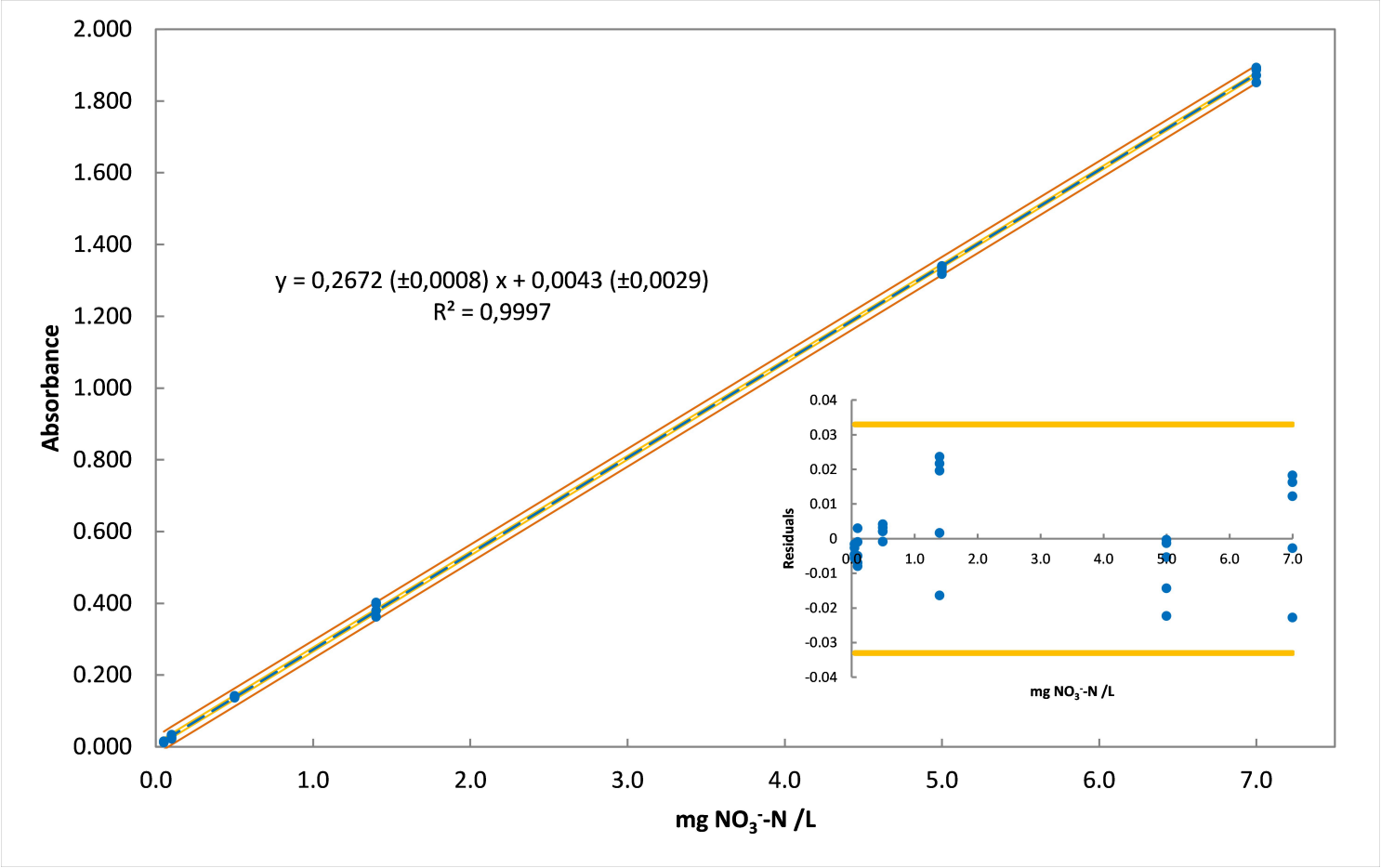

Figure 3

(a) Calibration curve of TN_b with confidence and prediction 95% intervals, (b) regression residuals plot with deviation limits.

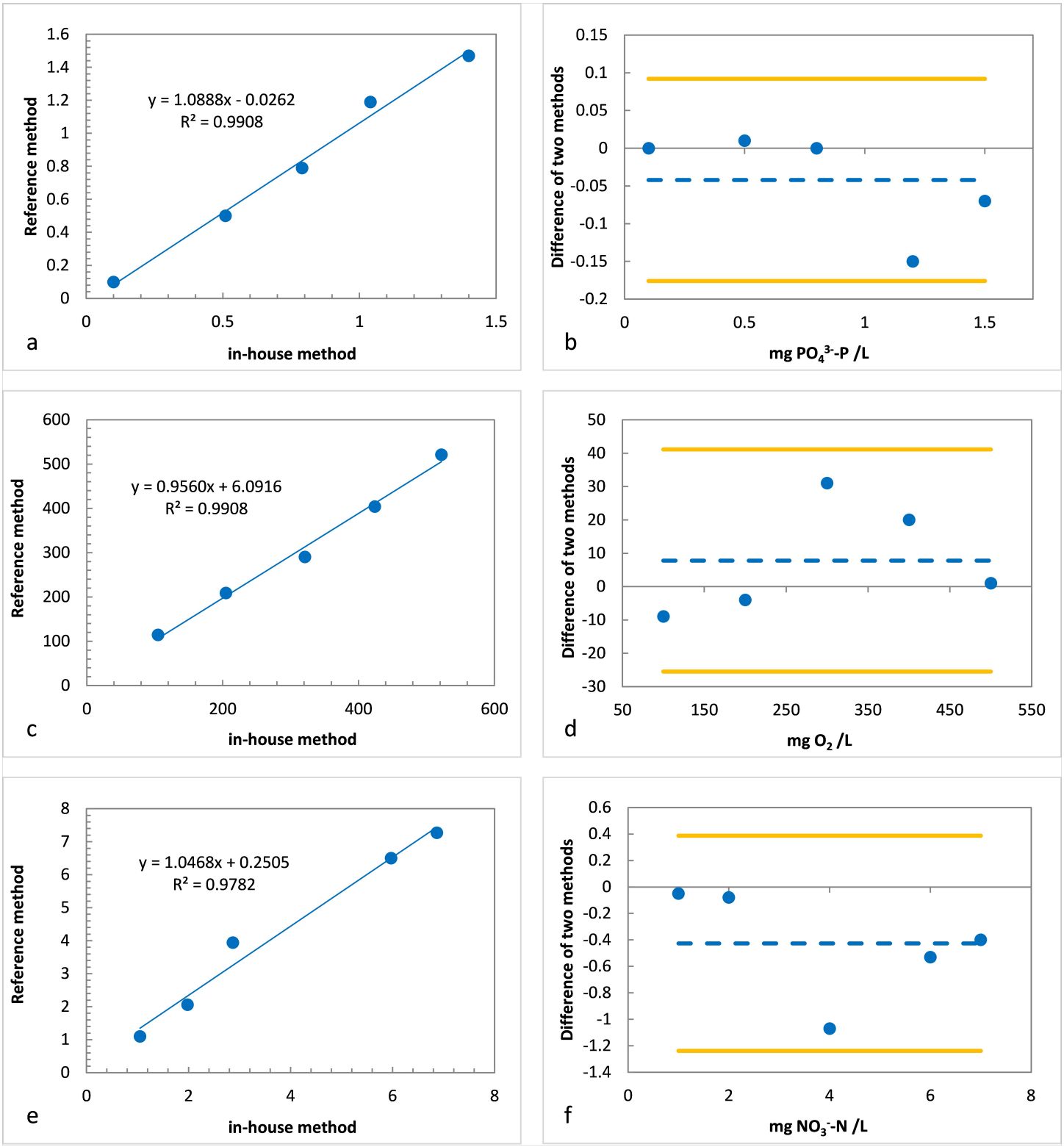

Figure 4

Comparison of in-house *vs.* reference methods of (a) TP, (c) COD and (e) TN_b with regression analysis. Bland and Altman plots for (b) TP, (d) COD and (f) TN_b, with the representation of the mean difference and limits of agreement.

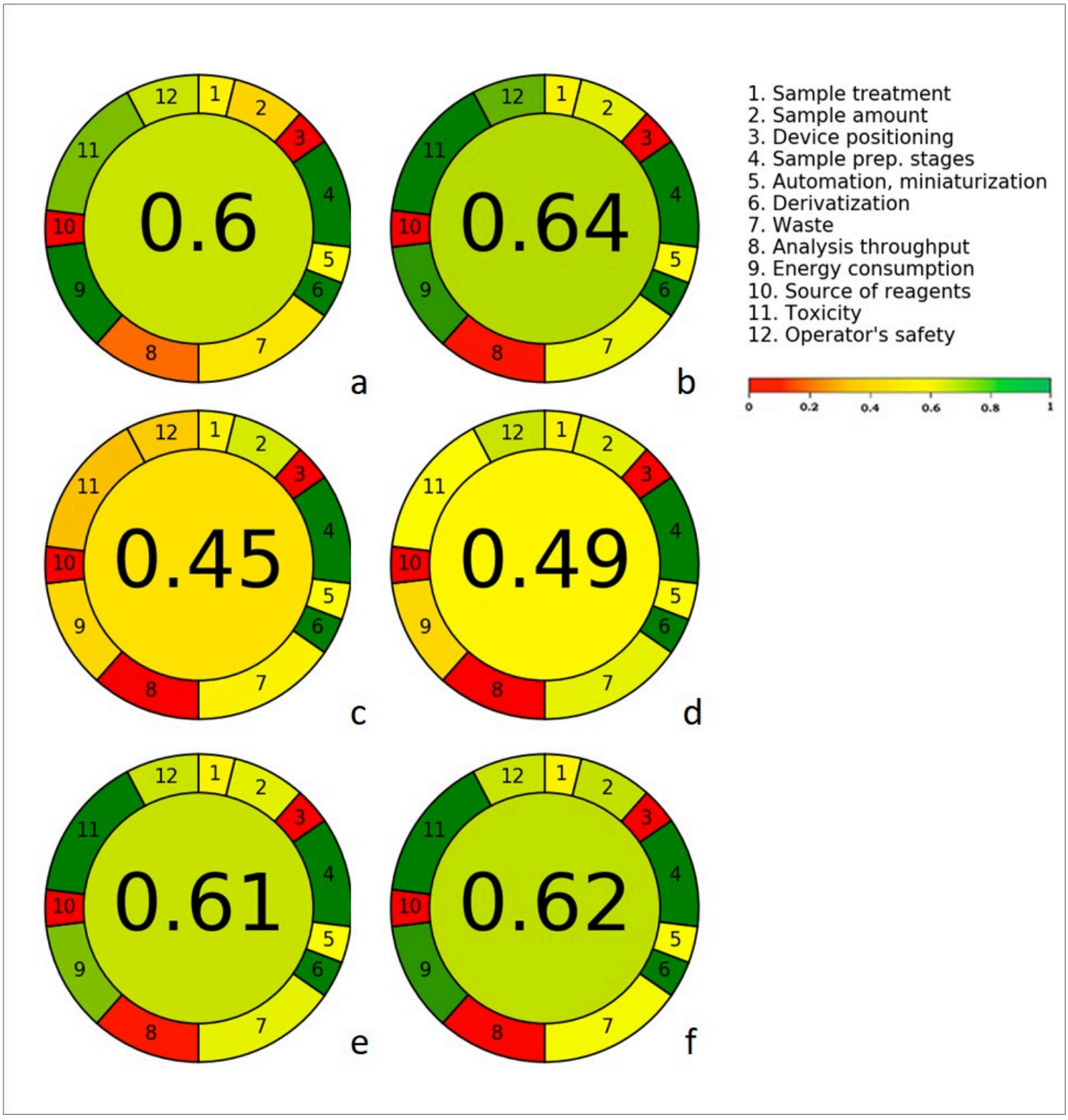

Figure 5

AGREE scores obtained using the AGREE methodology for the a) in-house TP method, b) TP reference method, c) in-house COD method, d) COD reference method, e) in-house TN method and f) TN reference method.