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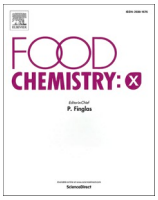

Semi-quantitative analysis of formaldehyde in food using calibration chart based on number of colored wells of microwell plate titration

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ABSTRACT

Microplate titration quantifies sodium hydroxide generated from formaldehyde reacting with excess sulfite in a 96-microwell plate. Phenolphthalein indicators change from red to colorless when all hydroxide ions react. Methodology optimized reagent concentrations, and reaction time and created a Calibration Chart for semi-quantitative determination. The chart shows formaldehyde concentration ranges corresponding to red well counts from 0 to 200 mM in 20 mM increments. Inter-operator repeatability demonstrates precision (3 replicates), correlating red wells with standard formaldehyde concentrations. This instrument-free technique uses readily available commercial plates, eliminating the need for specialized equipment and calibration. The methodology offers simplicity with its reliance on readily available commercial plates and minimal specialized equipment, hence it is cost-effective and easily transportable 96-microwell plates enhancing the methodology's portability, and efficient semi-quantitative analysis of formaldehyde. The analysis of twelve solutions from food samples agrees with the quantitative values using titration.

1. Introduction

Formaldehyde (FA), known as formalin when dissolved in water, has been classified as a human carcinogen by the World Health Organization (WHO) (Liteplo, Beauchamp, Chénier, & Meek, 2002). The improper use of FA as a preservative to extend the shelf life of food (Bianchi, Careri, Musci, & Mangia, 2007; Simeonidou, Govaris, & Vareltzis, 1997) raises concerns due to its potential health risks, causing both chronic and acute effects on the neurological system (Quackenboss, Lebowitz, Michaud, & Bronnimann, 1989; Songur, Ozen, & Sarsilmaz, 2010). Recognizing the importance of having stringent regulations, the Centre for Food Safety (CFS) has established acceptable FA levels, ranging from 100 to 406 mg per kg for fruits and vegetables, and lower than 140 mg per kg for seafood (Saiboh et al., 2023).

Formaldehyde, a chemical compound with potential health risks, can inadvertently infiltrate food products at various stages of the supply chain. Detecting and mitigating this contaminant swiftly is essential for safeguarding public health and ensuring the integrity of the food industry (Esteki, Regueiro, & Simal-Gándara, 2019; Gelbke, Buist, Eisert, Leibold, & Sherman, 2019; Rahman et al., 2023). A quick and efficient screening method plays a crucial role in addressing this issue. Early detection is paramount to preventing tainted products from entering the market and reaching consumers (Li et al., 2023). Contaminated products can have far-reaching consequences, affecting consumers across regions and potentially causing widespread health issues. Moreover, the proactive utilization of a screening method for formaldehyde enhances regulatory compliance efforts (Bhowmik, Begum, & Alam, 2016).

Table 1 presents various methods for the semi-quantitative analysis of FA in food products, with a summary employing devices, detection methods, reaction chemistry, analytical characteristics, performances, analysis time, and comparison of consumption of reagent and sample volumes (Kaewnu et al., 2023; Leblanc, Leblanc, & Ervin, 1988; Mostafapour, Mohamadi Gharaghani, & Hemmateenejad, 2021; Rungroadsri, Limsakul, Wongniramaikul, & Choodum, 2017; Taprab & Sameenoi, 2019; Tasangtong, Henry, & Sameenoi, 2023; Wang, Cui, & Fang, 2007). Employing paper-based devices (PADs) with color change detection leads to imprecisions in color change comparison with standards, in addition to variable spot sizes and uneven color distribution

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(Taprab & Sameenoi, 2019). The fabrication of these devices requires specialized equipment and a time-consuming process (Taprab & Sameenoi, 2019; Tasangtong et al., 2023). On the other hand, materials-assisted analysis, such as poly(vinylidene fluoride) (PVDF) indicator gel, activated carbon granules, and molybdenum disulfide quantum dots (MoS_2 QDs), involves material synthesis with limited accessibility, as shown in Table 1. (Kaewnu et al., 2023; Leblanc et al., 1988; Mostafapour et al., 2021; Rungroadsri et al., 2017; Wang et al., 2007). While the fabrication of these devices requires specialized equipment and a time-consuming process, there is a growing demand for instrument-free, simple, rapid, and cost-effective methods for semi-quantitative analysis of formaldehyde, aiming to achieve affordability through rapid and cost-effective means.

In this study, we adopt a visual counting approach to measure formaldehyde semi-quantitatively. The method involves microwell plate titration for quantifying the amount of sodium hydroxide produced from the reaction of formaldehyde contents with excess sulfite in wells of a 96-microwell plate for a rapid semi-quantitative method for detecting formaldehyde in food. This instrument-free technique involves observing color changes in sample solutions within a 96-microwell plate, facilitating convenient analysis. Formaldehyde concentration is assessed by counting red-colored wells, ranging from 0 to 200 mM in 20 mM intervals, without calibration. This approach also offers the advantage of portability for on-site analysis.

2. Materials and methods

2.1. Chemicals and standards

Chemicals: Sodium sulfite and potassium hydrogen phthalate (KHP) were obtained from Merck (Darmstadt, Germany). Analytical grade formaldehyde 37% *w/v* was acquired from QRëC™ (New Zealand) and phenolphthalein was obtained from CARLO ERBA Reagents (France).

Preparation of reagents: A 2 M sodium sulfite stock solution is prepared by dissolving 25 g of Na_2SO_3 in DI water in a 100 mL volumetric flask. A 1% w/v phenolphthalein solution is prepared by dissolving 5 g solid indicator with 75% *v*/v ethanol in a 500 mL volumetric flask and making to volume with ethanol.

Preparation of standard solutions: A 1000 mM stock solution of formaldehyde is prepared by diluting 81.30 mL of 37% w/v formaldehyde, in a 1000-mL volumetric flask with deionized (DI) water. This stock FA solution is standardized using the sulfite assay, which involves the formaldehyde and sodium sulfite mixture. The resulting NaOH product is titrated with standard potassium hydrogen phthalate (KHP) using phenolphthalein as an indicator. Working standard solutions of formaldehyde (10–190 mM) are prepared daily by diluting the stock solution. A 300 mM KHP standard solution is prepared by dissolving 30.63 g of dry pure KHP in distilled water and adjusting the volume to 500 mL using a volumetric flask.

2.2. Apparatus

The 96-microwell plate used were transparent polystyrene flat-bottom plates (SS244, 12 × 8 wells, Sero-Wel Sterilin®, UK). Each well measures 7 mm in diameter and 14 mm in depth, providing a total volume of 400 µL. An 8-channel micropipette (ONiLAB LLC Scientific Inc., USA) was utilized for the experiments. Mixing was performed using a ZX3 model Velp® vortex mixer from Scientifica (Italy). The pH values were measured with a STARTER 3100 model pH meter from OHAUS (USA). The ultrasonic bath employed was a 1510E-DTH model from

Table 1
List of semi-quantitative methods for formaldehyde detection in food products, with devices and detection methods, chemical reactions and detection products, analytical characteristics and performances, analysis time and reagent and sample volumes.

References	Device: Detection	Chemical reaction: Detection product	Device			Analytical characteristics and performances		Analysis time and reagent and sample volume consumption	
			Reusable device	No. of detection	Detection-assisted	Range of FA concentration, mM (mg/L)	LOD, mM (mg/L)	Estimated analysis time, min	Consumption volume, µL
Kaewnu et al., 2023	**Acrylic Sheet**: Indicator gel color change.	Oxidation by $Cr_2O_7^{2-}$: $Cr^{2+/3+}$	No	1	Material	0.03–200 (1–6000)	0.02 (0.47)	29	700
Wang et al., 2006	**Reference Card**: Color change with activated carbon in the injector.	Hantzsch reaction by $C_5H_8O_2$: 3,5-diacetyl-l-1,4-dihydrol-utidine	No	1	Material	0.03–0.7 (8×10^{-4}-0.02)	0.03 (8×10^{-4})	7	4000
Mostafapour et al., 2021	**PVDF*** **Film**: MoS_2 QDs** color change.	Converting electronic nose into opto-electronic nose: Not available	No	8	Material	0.03–0.8 (1–25)	Not reported	60	2000
Leblanc et al., 1988	**Test Strip**: Strip color change.	Oxidation by NAD^+: NADH	No	1	Fabrication device	0.8–150 mM (24–4500)	0.6 (18)	3	3500
Tasangtong et al., 2023	**PAD*** **Length**: Diameter length of color.	Reduction by sulfite: NaOH	No	1	Fabrication device	1.6–16 (50–500)	0.8 (25)	7	150
Taprab et al., 2019	**PAD Zones**: Number of colored zones.	Reduction by sulfite: NaOH	No	10	Fabrication device	3–30 (100−1000)	3 (100)	5	30
Rungroadsri et al., 2017	**Polymer Matrix in Vial**: Color change.	Hantzsch reaction by $C_5H_8O_2$: 3,5-diacetyl-l-1,4-dihydrol-utidine	No****	1	Device-free	0.03-1.7 (1–50)	5.3×10^{-4} (0.016)	7	2500
This work	**Colored Wells**: Counting for calibration chart.	Reduction by sulfite: NaOH	Yes	96	Device-free (96-microwell plate)	20–200 (600–6000)	10 (300)	6	155

* PVDF: poly(vinylidene fluoride).
** MoS_2 QDs: molybdenum disulfide quantum dots.
*** PAD: paper-based device.
**** Colorimetric reagents within the polymer matrix were entrapped at the bottom of the vial.

BRANSONIC (USA). Additionally, an Epson flatbed scanner with 24-bit color and 1200 dpi resolution (Perfection V39, EPSON, Thailand) was used to record images of the plates.

2.3. Procedure for construction of calibration chart

Two 96-microwell plates were used, each comprising 8 rows (labeled A to H) and 11 columns (labeled 1 to 11). Only 3 rows of the second plate were utilized, designated as I to K. All wells were filled with 50 μL of 0.8 M Na_2SO_3 using an 8-channel micropipette. Aliquots of 50 μL from various standard FA solutions were added to 11 wells of a row of the microplate. Row A contained 0.0 mM FA, Row B contained 10 mM FA, and Row C contained 30 mM FA. Subsequently, the concentration of FA increased in increments of 20 mM from 50 to 190 mM for addition into wells of Rows D to K, respectively. Thus, all wells in a row contained the same concentration of FA. The plates were then vortexed at 1200 rpm for 5 s. After a 5-min reaction time, 5 μL of 0.1% *w/v* phenolphthalein indicator was added to all 121 wells, and the plates were vortexed for an additional 5 s. Following this, aliquots of 50 μL from various standard KHP solutions were added to 11 wells of a column of the microplate, with Column 1 containing 0.0 mM KHP, Column 2 containing 20 mM KHP, and Column 3 containing 40 mM KHP. Subsequently, the KHP concentration was increased in increments of 20 mM from 60 to 200 mM for addition into wells of Columns 4 to 11, respectively. Thus, all wells in a column contained the same concentration of KHP. The plates were vortexed for 5 s after each addition of the KHP solution. Fig. 1 illustrates the schematics of the procedure for constructing the Calibration Chart. Table S1 (Supplementary Information A) provides a detailed list of the operational steps.

2.4. Procedure for estimating the limit of detection (LOD)

Five wells of a row of a 96-microwell plate were filled with 50 μL of 0.8 M Na_2SO_3. Then, 50 μL aliquots of 0.0, 2.5, 5.0, 7.5, 10, and 20 mM FA standard were added to the five wells, and the plate was shaken on a vortex mixer at 1200 rpm for 5 s. Following a 5-min. Reaction period, 5 μL of 0.1% *w/v* phenolphthalein indicator solution was added to the wells, followed by mixing for 5 s. Next, 50 μL of water was dispensed into the 5 wells and the plate was vortexed for an additional 5 s.

2.5. Method for sample analysis

2.5.1. Preparation of food samples

All samples were purchased from a local market in Bangkok, Thailand comprising shrimp (S1), squid (S2), oyster (S3), jellyfish (S4), boiled dried squid (S5), beef tripe (S6), coagulated pig blood (S7), ginger (S8), galangal (S9), straw mushroom (S10), shiitake mushroom (S11), and bean sprouts (S12). The purchased samples were obtained in small sections, each weighing <50 g per piece. Multiple pieces of each food type were selected to create a sample with an accurate weight of approximately 50 g. Subsequently, each sample was placed in 100 mL of DI water, sonicated for 10 min, and then filtered using Whatman No. 1 filter paper. The pH of the filtrate was measured using a pH meter (refer to Table S3 of Supplementary Information D for the pH of sample solutions) and adjusted to pH 7 using either 0.1 M HCl or 0.1 M NaOH. A sample aliquot of 50 μL was employed for analysis using the microwell-plate process.

2.5.2. Procedure for semi-quantitative analysis of samples

An empty row of a 96-well plate was filled with 50 μL of 0.8 M Na_2SO_3 using the auto-pipette. Then, 50 μL of the sample solution was added to each well and the plate was shaken on the vortex mixer at 1200 rpm for 5 s. After a 5 min reaction period, 5 μL of 0.1% *w/v* phenolphthalein indicator solution was added to every well of the 96-microwell plate, followed by mixing for 5 s. Then, 50 μL of KHP at concentrations ranging from 0 to 200 mM, in increments of 20 mM, was dispensed into the 11 wells and the plate was vortexed again for 5 s. The number of red wells was counted, and the concentration of the formaldehyde in the sample solution was read off from the Calibration Chart. Each sample was analyzed in duplicate by utilizing two empty rows of the plate.

2.5.3. Procedure for titration analysis

The sulfite titration method was used as a reference method by the titration procedure following the NIOSH analytical method (Eller &

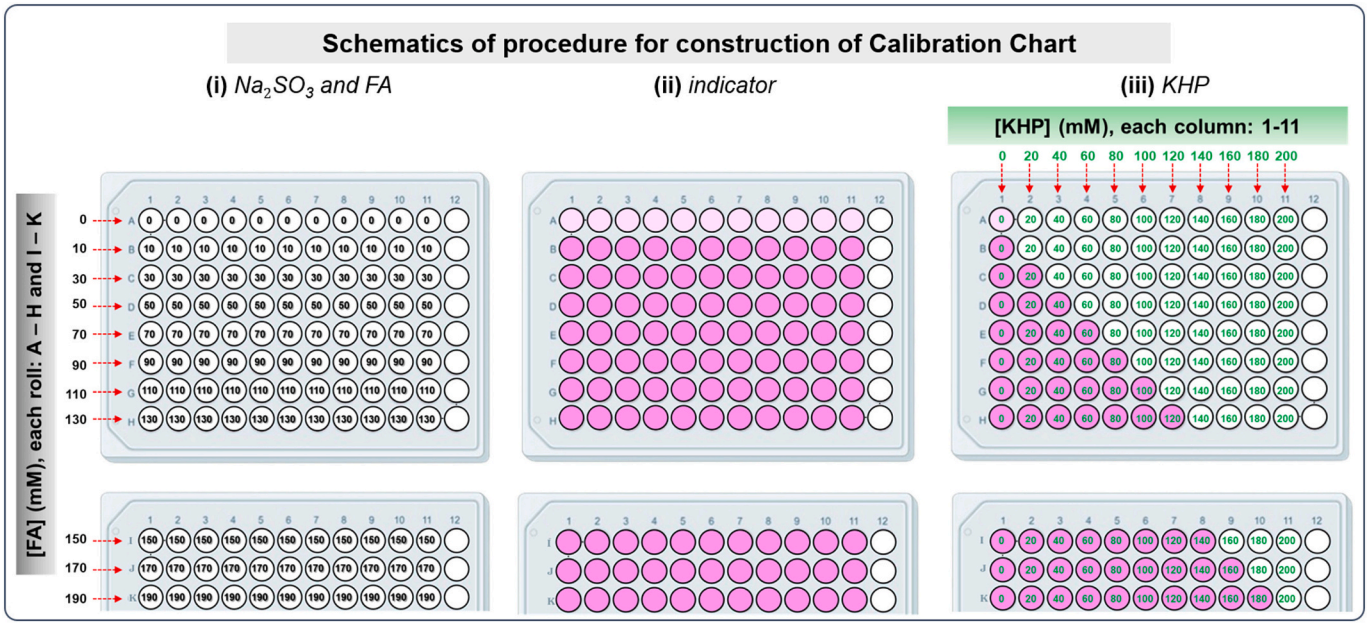


Fig. 1. Schematics of the procedure for the construction of Calibration Chart based on the counting of red wells for instrument-free analysis: (i) pipetting of Na_2SO_3 in 121 wells and FA standard solution (0, 10, 30, 50, 70, 90, 110, 130, 150, 170 and 190 mM) in rows A to H of the first plate and in rows I to K of the second plate, (ii) addition of indicator solution in 121 wells and (iii) addition of KHP solutions (0, 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 mM) in columns 1 to 11. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cassinelli, 1994) with some modifications. Titration was carried out by pipetting 5.0 mL of the sample solution (see Section 2.5.1 for sample preparation), followed by the addition of 5 mL of 0.8 M Na_2SO_3 and 20 μL of 0.1% w/v phenolphthalein indicator. The sample was titrated with 25.0 mM standard KHP, with the endpoint marked by the indicator changing from pink to colorless. The volume of blank solution of DI water was 5.0 mL.

The concentration of FA in the sample solution was then calculated from the following formula: S: sample, Bk: blank.

$$\text{FA concentration (mM)} = \frac{\left[\text{Volume of KHP}_{(S)}\text{, mL–Volume of KHP}_{(Bk)}\text{, mL}\right] \times 25\ \text{mM}}{5\ \text{mL.}}$$

3. Results and discussion

An instrument-free approach based on visualized colored well counting by the naked eye is developed for semi-quantitative analysis of formaldehyde. The following sections are the chemistry of the reaction, optimization studies, construction of the Calibration Chart, and its characteristics.

3.1. Semi-quantitative formaldehyde analysis: An approach of instrument-free analysis

3.1.1. Chemistry of the reaction

This approach relies on counting indicator spots (wells), with the number of colored spots reflecting the concentration range of formaldehyde (FA). It utilizes the formation of color in the well from the phenolphthalein indicator to quantify the production of NaOH resulting from the sulfite reduction of FA (Saiboh et al., 2023). The net reaction is as follows:

$$CH_2O(aq) + Na_2SO_3(aq) + H_2O(aq) \rightarrow NaOH(aq) + HOCH_2SO_3Na(aq) \quad (1)$$

The NaOH product is quantitated by titration with potassium hydrogen phthalate.

$$KHP(aq) + NaOH(aq) \rightarrow K^+(aq) + Na^+(aq) + P^{2-}(aq) + H_2O(l) \quad (2)$$

where P^{2-} is the phthalate dianion.

Initially, formaldehyde (CH_2O) reacts with sodium sulfite (Na_2SO_3) solution to produce sodium hydroxide (NaOH) and sodium bisulfite ($HOCH_2SO_3Na$), as represented by Eq. (1). The resulting NaOH is then quantified through the addition of potassium hydrogen phthalate (KHP), depicted in Eq. (2), where P^{2-} represents the phthalate dianion. The color of the added phenolphthalein indicator changes from red to colorless when the mole ratio of NaOH to KHP reaches equivalence, enabling semi-quantitative analysis of FA.

3.1.2. Microwell plate titration

In microwell plate titration, 96-microwell plates are employed for stepwise titration by adding increasing amounts of potassium hydrogen phthalate into one row of wells. Each well contains the same amount of formaldehyde (from the sample/standard), Na_2SO_3 (in excess), and phenolphthalein indicator. When a well has excess KHP the solution is neutral, and the indicator is colorless. A well containing NaOH that has not completely reacted with the added KHP will be alkaline, and the indicator will be red. The number of red wells corresponds to the amount of KHP within a defined interval at the equivalence point, providing a semi-quantitative analysis of formaldehyde present in the sample solution (See Section 2.3 for semi-quantitative analysis of FA).

3.2. Optimization studies

The proposed semi-quantitative analysis for formaldehyde (FA) is investigated by optimizing key parameters; the concentration of Na_2SO_3, phenolphthalein, and the reaction time between FA and Na_2SO_3 (see Fig. 2). The optimization study aimed to determine the optimal increment and maximum amount (in mols) of KHP suitable for the formaldehyde content that may be found in the selected types of food, such that all the wells are either red or colorless. The following concentrations of standard formaldehyde were chosen for the optimization work, viz., 0.0, 50, 90, 130, and 170 mM, based on literature studies (See Table 1), as representative concentrations of possible samples. The volumes of FA, sulfite and KHP solutions were set at 50 μL each and the phenolphthalein solution at 5 μL to ensure that the micro-well plate can be shaken on a vortex mixer without liquid spillage from the wells.

The optimization is performed by using aliquots of 50 μL of various standard FA solutions, which were added to 11 wells of a row of the microplate, with Row A containing 0.0 mM FA, Row B containing 50 mM FA, Row C containing 90 mM FA, Row D containing 130 mM, and Row E containing 170 mM, respectively. This procedure was repeated for 4 plates (see Figs. 2(A) – 2(C)).

3.2.1. Concentration of sodium sulfite

Sulfite is an important component of the analysis because its reaction with formaldehyde produces NaOH, which is used as the chemical marker for formaldehyde. The concentration of sulfite must be sufficiently high to ensure the complete conversion of formaldehyde, but an excessive amount can interfere with the phenolphthalein indicator and increase costs. Aliquots of 50 μL of 0.4 M Na_2SO_3 were added to all wells of the first plate. This addition was repeated with 0.8 M Na_2SO_3 for the second plate, 1.2 M Na_2SO_3 for the third plate, and 1.6 M Na_2SO_3 for the fourth plate (See Fig. 2(A)). The concentration of the sulfite added is denoted in the upper right corner of the scanned image of the plates in Fig. 2(A). The plates were vortexed at 1200 rpm for 5 s. Following a 5 min reaction time, 5 μL of 0.1% *w/v* phenolphthalein indicator was added to all wells of the four plates, and the plates were vortexed for 5 s. Then, 50 μL of pure DI water was dispensed into the five wells of the first column. The plates were vortexed for 5 s. This addition was repeated for solutions of standard KHP from 0 to 200 mM, in increasing increments of 20 mM, for the five wells in columns 2 to 11, respectively.

Fig. 2(A) presents the scanned images of the four plates. Fig. 2(A) shows that there are 3, 5, 7, and 9 red wells in the rows with added FA concentrations of 50, 90, 130, and 170 mM, respectively, for the plates with 0.4, 0.8, 1.2, or 1.6 M Na_2SO_3. The plate containing 0.4 M Na_2SO_3 has only 7 red wells for the last row containing 170 mM FA. This discrepancy is due to the insufficient amount of sulfite required to completely reduce the 170 mM FA solution. Therefore, 0.8 M Na_2SO_3 was selected for subsequent optimization studies since this concentration is sufficient.

It should be noted that the first well of the top row of all four plates exhibits a pink color. These wells contain only sulfite. The sulfite undergoes partial hydrolysis by water, leading to the formation of hydrogen-sulfite anion and hydroxide ion through the following reversible reaction:

$$SO_3^{2-}(aq) + H_2O(l) \rightleftarrows HSO_3^-(aq) + OH^-(aq) \quad (3)$$

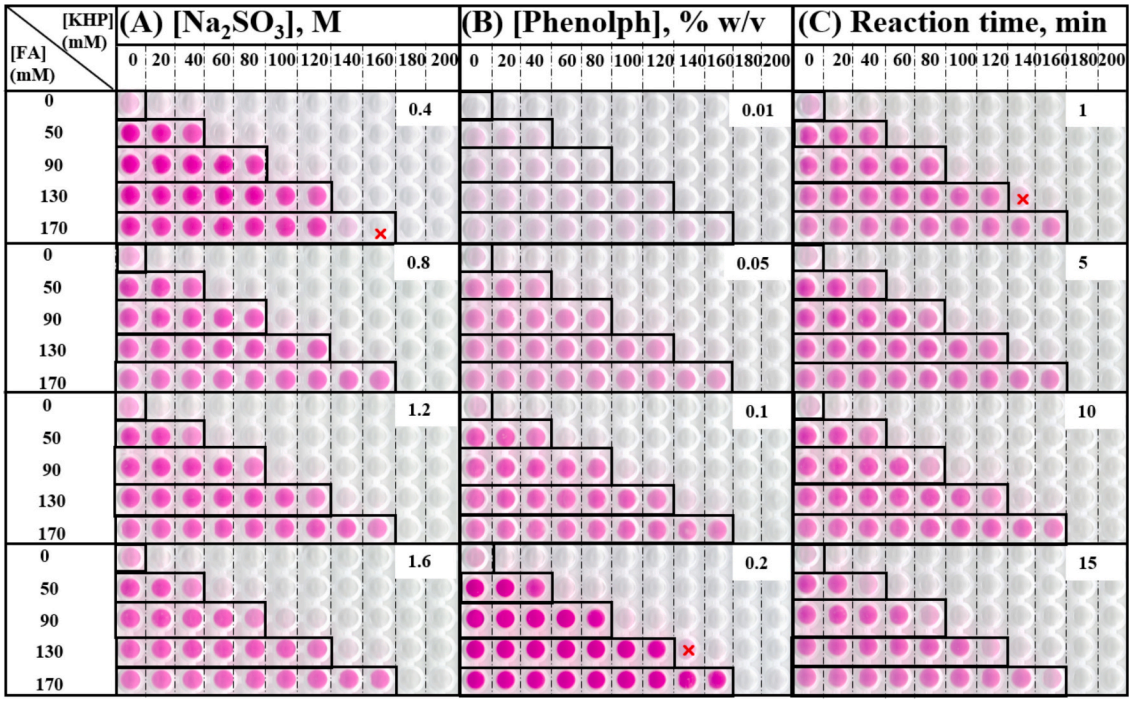
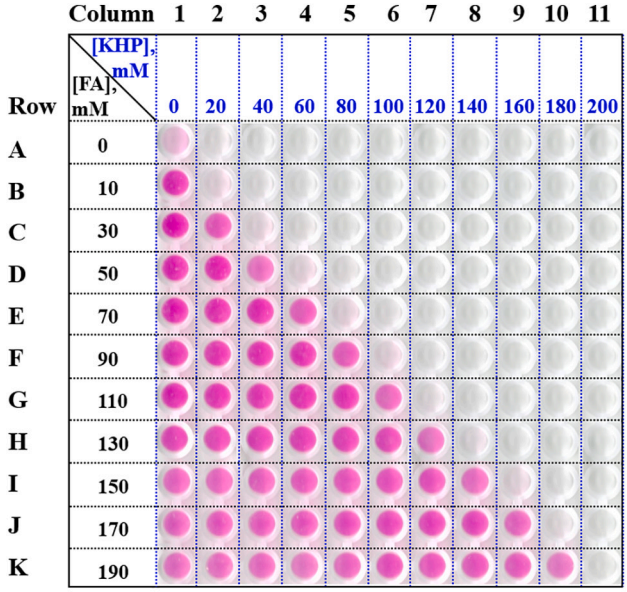


Fig. 2. Scanned images of plates used for optimization in the semi-quantitative method of FA analysis by counting the number of red wells in a 96-microwell plate. The variables investigated are: (A) Na_2SO_3 concentration: 0.4 M, 0.8 M, 1.2 M, 1.6 M; (B) phenolphthalein concentration: 0.01% *w/v*, 0.05% w/v, 0.1% w/v, 0.2% w/v; and (C) reaction time between FA and sodium sulfite: 1, 5, 10 and 15 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The solution is thus weakly basic and hence the indicator is pink in color.

3.2.2. *Concentration of phenolphthalein indicator*

The concentration of the phenolphthalein indicator is crucial for obtaining a well-defined color in the wells during the counting procedure. It is noted that Saiboh et al. (2023) also selected phenolphthalein concentration for optimization. The concentrations of the phenolphthalein indicator were set at 0.01, 0.05, 0.1, and 0.2% *w/v* for the four plates, respectively. Fig. 2(B) shows scanned pictures of the four plates. The same preparation and procedure of the four plates, as described in Section 3.2.1, were employed, but now using only 0.8 M Na_2SO_3 (See Table S2 of Supplementary Information B). As observed in Fig. 2(B), the higher the concentration of the indicator, the more intense the color of the wells. However, when a high concentration of 0.2% w/v is used, some wells exhibit a pink color, which may lead to difficulty in the counting of the right numbers of red wells. Thus 0.1% w/v concentration was considered suitable for a clear distinction between red



Number of red wells	Concentration interval (mM)
0	$0 \leq [FA] < 10$
1	$10 \leq [FA] < 20$
2	$20 \leq [FA] < 40$
3	$40 \leq [FA] < 60$
4	$60 \leq [FA] < 80$
5	$80 \leq [FA] < 100$
6	$100 \leq [FA] < 120$
7	$120 \leq [FA] < 140$
8	$140 \leq [FA] < 160$
9	$160 \leq [FA] < 180$
10	$180 \leq [FA] < 200$

Fig. 3. Scanned images of wells for constructing the Calibration Chart. The experimental conditions are 0.8 M Na_2SO_3, 0.1% w/v phenolphthalein (3 mM), and reaction time of 5 min. The number of red wells was counted, and the concentration of the formaldehyde in the solution read off from the Calibration Chart. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and pink wells.

3.2.3. Reaction time for complete reduction of formaldehyde

Investigating the reaction time is crucial to ensure the complete reduction of formaldehyde (FA) and achieve full conversion to NaOH. Reaction times of 1, 5, 10, and 15 min were tested using four plates. The preparation and procedure followed the method described in Section 3.2.1, with the addition of a 0.1% w/v phenolphthalein indicator. As shown in Fig. (C), a reaction time of 1 min was insufficient, indicated by the presence of pink wells. Inspection of the plates revealed that a reaction time of 5 min is optimal for the distinct formation of red wells, ensuring the accuracy and repeatability of the method.

3.2.4. Increment of KHP concentration between wells

The precision of the measurements relies on the step increment of the KHP concentrations. If the step increment is too small, the color distinction between wells may be difficult to evaluate, resulting in uncertainty in the number of observed pink wells. Conversely, if the increment is too large, it will provide a reproducible number of observed red wells but a wider interval of the concentration range, i.e., lower precision. Studies have shown that an increment of 20 mM of the concentration of KHP is fit for purpose (data not shown), providing the necessary precision and visual color differentiation.

3.3. Calibration chart and its performance evaluation

Calibration chart: The procedure for constructing the Calibration Chart is given in Section 2.3. Rows A and B show distinct red color patterns, leading to semi-measurement formaldehyde concentration ranges. The final Calibration Chart (Fig. 3) lists concentration intervals based on observed red wells.

Limit of detection (LOD): An LOD of 10 mM is obtained, see detailed in Section 2.4. Fig. S2 of Supplementary Information C depicts scanned images of six wells, with the fifth well distinctly appearing red. This suggests that concentrations equal to or >10 mM would produce a visible red color of the reaction, while concentrations lower than this might not be distinguishable.

Precision: Inter-operator repeatability is evaluated for precision study. Fig. S1 of Supplementary Information C shows three replicates of formaldehyde detection using 96-microwell plates performed as outlined in Section 2.3. The developed semi-quantitative method demonstrates precision, with color spot counts correlating with standard formaldehyde concentrations. This method provides consistent results among different users with inter-operator repeatability.

The Calibration Chart is a one-time task that produces a reusable reference for future analyses. This minimizes the necessity for repeated calibration, saving time and effort while enhancing efficiency and convenience.

3.4. Semi-quantitative analysis of real samples and its validation

The developed instrument-free approach is further evaluated for semi-quantitative FA analysis in various fresh foods (seafood, local food, and vegetables) (See Section 2.5.1 for sample preparation). These foods are frequently discovered to be contaminated with FA in Thailand (Suwanaruang, 2018; Taprab & Sameenoi, 2019; Tasangtong et al., 2023; Yodpach, Chantiwas, Wilairat, Choengchan, & Praditweangkum, 2023). Fig. S3 of Supplementary Information D depicts visual representations derived from duplicate analyses for semi-quantitative method of 12 sample solutions with the picture samples labeled as follows: S1: shrimp, S2: squid, S3: oyster, S4: jellyfish, S5: boiled dried squid, S6: beef tripe, S7: coagulated pig blood, S8: ginger, S9: galangal, S10: straw mushroom, S11: shiitake mushroom, and S12: bean sprouts. The semi-quantitative method results, depicted in Fig. 4 and Table 2, list FA

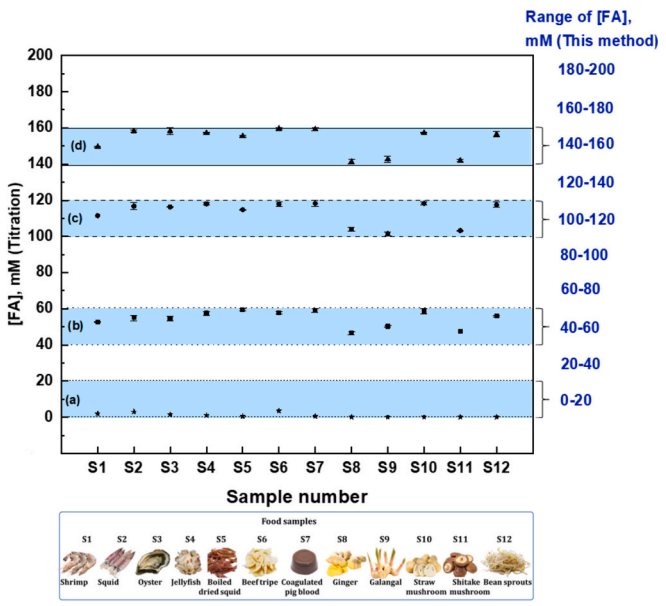


Fig. 4. Titration values presented as a scatter plot alongside concentration ranges from the Calibration Chart, represented as four bands: (a) formaldehyde concentrations in food samples, (b) formaldehyde concentration in spiked samples at 50 mM, (c) formaldehyde concentration in spiked samples at 110 mM, and (d) formaldehyde concentration in spiked samples at 150 mM ($n = 2$). The picture samples include S1: shrimp, S2: squid, S3: oyster, S4: jellyfish, S5: boiled dried squid, S6: beef tripe, S7: coagulated pig blood, S8: ginger, S9: galangal, S10: straw mushroom, S11: shitake mushroom, and S12: bean sprouts.

Table 2
FA content in fresh food samples (S1-S12) using the developed semi-quantitative method and the titration method.

Sample code: Type of sample type	FA content in the sample (mM)	
	Semi-quantitative method, counting of red wells	Titration
S1: Shrimp	$0 \leq [FA] < 10$, 0	2.0
S2: Squid	$0 \leq [FA] < 10$, 0	3.0
S3: Oyster	$0 \leq [FA] < 10$, 0	1.5
S4: Jellyfish	$0 \leq [FA] < 10$, 0	1.0
S5: Boiled dried squid	$0 \leq [FA] < 10$, 0	0.5
S6: Beef tripe	$0 \leq [FA] < 10$, 0	3.5
S7: Coagulated pig blood	$0 \leq [FA] < 10$, 0	0.5
S8: Ginger	$0 \leq [FA] < 10$, 0	ND *
S9: Galangal	$0 \leq [FA] < 10$, 0	ND *
S10: Straw mushroom	$0 \leq [FA] < 10$, 0	ND *
S11: Shitake mushroom	$0 \leq [FA] < 10$, 0	ND*
S12: Bean sprouts	$0 \leq [FA] < 10$, 0	ND*

* ND: Not Detected. The KHP volume used for titration in samples S8-S12 was not significantly different from that used for the reagent blank titration.

content in the fresh food samples (S1-S12) determined by the developed microwell plate titration. The semi-quantitative analysis method was performed through Calibration Chart analysis (Section 2.5.2) and titration method (Section 2.5.3). As shown in Table 2, the results of titration in sample S1-S12 agreed well to those obtained from semi-quantitative analysis, the values are within the range of measurement (0 mM $\leq$ [FA] $<$ 10 mM). It should be noted that FA was not detected by titration in samples S8-S12 because the KHP volume used for titration was not significantly different from that used for the reagent blank titration. Based on titration analysis, the FA contents for samples S1, S2, S3, S4, S5, S6, and S7 were 114, 174, 86, 58, 28, 204, and 30 mg/kg,

respectively. Fig. 4 depicts titration values presented in a scatter plot, accompanied by concentration ranges derived from the Calibration Chart. These ranges represent four levels of FA concentration: non-spiked samples, along with samples spiked at three different levels of FA concentrations (50 mM, 110 mM, and 150 mM, respectively). All 36 spiked solutions were analyzed for the content of FA utilizing both the developed method and titration methods. The results of the semi-quantitative analysis align well with titration. These analyses contribute to establishing validation results for the developed 96-micro-well plate method, indicating its efficiency and reliability in food applications.

4. Conclusion

This work presents an innovative, instrument-free approach for semi-quantitative FA analysis in various fresh foods, highlighting its suitability for rapid and convenient analysis using the Calibration Chart. The method involves visually counting red-colored wells, facilitating practical and efficient FA analysis. Optimization studies were conducted to construct a Calibration Chart, focusing on parameters such as sodium sulfite concentration, phenolphthalein indicator concentration, reaction time for complete formaldehyde reduction, and the increment of KHP concentration between wells. The method offers a rapid determination of FA, within only 6 min, requiring minimal sample and reagent volumes of 155 μL per measurement. The developed method was validated by analyzing real samples using conventional titration, yielding reliable results for determining FA content. Its cost-efficiency and portability make it suitable for practical on-site analysis in the semi-quantitative evaluation of food.

CRediT authorship contribution statement

Mintra Tongdee: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Prapin Wilairat:** Writing – review & editing, Methodology. **Wiboon Praditweangkum:** Writing – review & editing, Supervision. **Rattikan Chantiwas:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Grammarly and ChatGPT 3.5 to improve sentence fluidity and grammar. Subsequently, the authors thoroughly reviewed and edited the content as necessary, taking full responsibility for the content of the publication.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101617.

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