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The Relationship of Modified Bismuth and Ninhydrin Methods with Periodontal Pathogens in Saliva

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Abstract

Periodontal diseases are prevalent and chronic oral conditions. This study was conducted to determine the performance, validity, and applicability of the modified bismuth and ninhydrin methods to detect bacterial degradation for the development of periodontal screening tools. The experiments were conducted to test the performance and validity of both methods using standard solutions and evaluate their applicability using human saliva. Spearman's correlation was used to find the correlation between the quantity of periodontal pathogens, salivary H_2S, and total amines. The experiments showed good performance. The bismuth solution had a limit of detection (LOD) of 1-2 ppm NaHS. The stability test revealed that the 23-week bismuth solution produced the same quantity of bismuth sulfide precipitate as fresh preparations under optimal conditions. The validity test showed acceptable results. The color intensities of the modified ninhydrin method were quantified. The intensity of ΔG was chosen for the quantitative detection of total amines due to its strong correlation with putrescine concentration. The calibration curve exhibited linearity over putrescine concentrations ranging from 0.5 to 4.0 mM ($R^2 = 0.929$). The precision test showed that %RSD ranged from 2.40 to 22.50. The applicability tests demonstrated that both methods detected and scored salivary H_2S and total amines in relation to the quantity of analytes. Three periodontal pathogens—*F. nucleatum*, *P. gingivalis*, and *P. intermedia*—were significantly correlated to salivary H_2S. Only *F. nucleatum* was significantly correlated to salivary total amines. The results of this study indicate that the modified bismuth and ninhydrin methods can effectively identify periodontal degradation with acceptable performance and validity.

Keywords: Periodontal pathogens, Modified bismuth method, Modified ninhydrin method, Bismuth scores, Ninhydrin scores, Saliva

Introduction

Periodontal diseases are common and ongoing conditions that cause damage to both the hard and soft tissues of the periodontium through a combination of microbial dysbiosis and human immunological responses [1-3]. According to the 2017 Thai National Oral Health Survey, 81.00% of working-age adults aged 35 - 44 years had gingivitis, and 32.60% had periodontitis with periodontal pocket. Additionally, 83.90% had experience in tooth loss, which is the major oral health problem among this age group [4]. Measuring radiographic and clinical periodontal parameters constitutes a thorough evaluation of

periodontal disorders. Conducting a comprehensive periodontal examination on a large population necessitates the presence of highly qualified practitioners and accurate diagnostic devices, both of which consume significant time and resources.

Human saliva contains several biomarkers that may have diagnostic significance in identifying the person with periodontal diseases resulting in a useful screening tool. The advantages of saliva are that the collection process is minimally invasive, it offers many sample possibilities, and it provides simple access, enabling collection by non-specialist dental personnel [5,6]. Several studies have explored possible biomolecules for the detection of periodontal diseases, including antibodies, periodontal bacteria, inflammatory cytokines, proteins, and other degradation products.

It is widely recognized that gram-negative bacteria produce sulfur-related compounds as metabolic byproducts. One of the most common sulfur compounds is H_2S, which is made when bacteria break down sulfur-containing amino acids like cysteine and homocysteine. The periodontal bacteria that can produce H_2S are *Fusobacterium nucleatum*, *Treponema denticola*, *Prevotella tannerae*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Aggregatibacter actinomycetemcomitans* [7,8]. Each bacterium exhibits a different capacity for H_2S production. Several clinical studies have reported that sulfide byproducts are associated with periodontal diseases [9-13]. Patients with a high sulfide level are at a higher risk for further periodontal diseases progression [14,15]. Oral H_2S can be found in supra- and subgingival plaque, gingival crevicular fluids (GCF), and saliva [16,17].

Total amines or polyamines, such as cadaverine, putrescine, spermine, and spermidine, are associated with inflammatory processes, cellular proliferation, tissue regeneration, and bone formation, all of which are influenced by periodontal diseases. Bacteria include *Fusobacterium* spp., *Prevotella* spp., and *Porphyromonas* spp., which degrade host amino acids and produce polyamines that enhance metabolism, communication, virulence, and antibiotic resistance [18-21]. Previous research has shown that salivary total amines are associated with periodontal inflammatory status and can serve as a crucial biomarker for the extent of periodontal inflammation [22,23].

Oral biomarkers, H_2S and total amines, can be identified through various methods, including organoleptic test, gas chromatography (GC), sulfide level monitoring devices, high-performance liquid chromatography (HPLC), and several chemical methods [21,24,25]. Researchers develop the possibility of simple and rapid detection of periodontal diseases. Previous studies have shown that smartphone digital image colorimetry provides a complementary, modified analytical method for the determination of biomolecules [26-28].

The bismuth method is a semi-quantitative colorimetric method used for sulfide detection. The bismuth ion was combined with the sulfide ion and produced bismuth sulfide, a brown to black precipitation. The previous study revealed that the bismuth method can visually detect subgingival H_2S produced by periodontopathogens [8,29]. The ninhydrin method is one of the colorimetric methods for amine and amino acid quantitative analysis. An amino acid reacts with ninhydrin to produce Ruhemann's purple, a dark purple compound. Ninhydrin was utilized to detect salivary amine compounds as compared to other halitosis detection methods. The study revealed a significant correlation between salivary amine levels and VSC levels and organoleptic scores [30]. However, no studies have been performed on both methods for salivary H_2S and total amines in screening analysis, the rapid collection of semi-quantitative information about all the components of a sample [31]. Therefore, this study developed these methods for detecting salivary H_2S and total amines utilizing smartphone digital image colorimetry for practical application in saliva analysis. This study aims to evaluate the performance and validity of the modified bismuth and ninhydrin methods, and their applicability was also tested using human saliva.

Materials and methods

The recent study was approved by the Human Research Ethics Committee of the Faculty of Dentistry, Prince of Songkla University, Thailand (EC6411 - 074).

Reagents and chemicals

The sulfide standard (NaHS) and amine standard (putrescine) were purchased from Sigma Aldrich (Bornem, Belgium). The bismuth chloride ($BiCl_3$) was purchased from Himedia Laboratories (Himedia, India).

All other chemical reagents were prepared with analytical-reagent-grade chemicals and deionized water.

Modified bismuth method

The bismuth method established by Basic *et al.* [7,8] has been modified for visual detection. The freshly prepared bismuth solution consisted of 0.4 M Triethanolamine HCl (pH 8.0), 10 mM Bismuth (III) chloride, 20 µM Pyridoxal 5 - phosphate monohydrate, 20 mM EDTA and 40 mM L-cysteine. The different concentrations of NaHS were mixed with the freshly prepared bismuth solution. The black precipitation of bismuth sulfide at the bottom of the microcentrifuge tube was visually observed within 30 min after sample addition [29].

The performance of the modified bismuth method was determined using the visual detection limit (LOD) and the detection box was used to control the light and position of the photograph of each reaction tube (**Figure 1**). A stability test was conducted to determine the optimal storage time for the bismuth solution. A ready-to-use bismuth solution would be prepared and stored at 4 °C, and it would be analyzed using standard 20 ppm NaHS (daily prepared) for 7 days. The long-term stability would be evaluated every week for 12 weeks to determine the optimal maximum storage time.

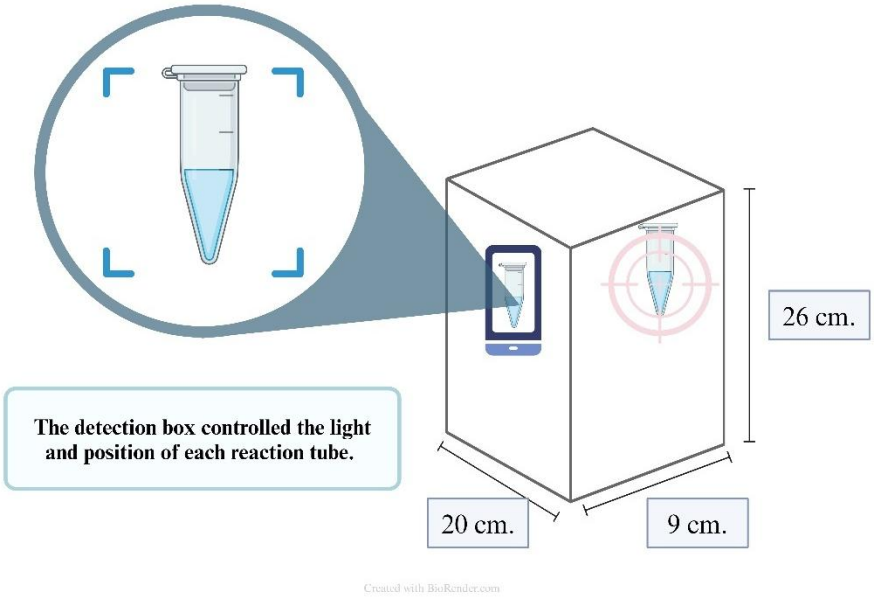

Figure 1 The detection box.

The validity of the modified bismuth method was designed to assess the method's ability to produce reliable outcomes over repetition. Using serial dilutions of NaHS, the repeatability of the bismuth method was validated 3 times on the same day, and the inter-day precision of the bismuth method was validated on three separate days, with triplicates on each day.

This study also utilized artificial saliva as a solvent to ensure that matrix variability did not affect the performance of the modified bismuth methods [32]. Artificial saliva was prepared by the pharmaceutical unit, Faculty of Dentistry, Prince of Songkla University. The standard sulfide solution was dissolved in artificial saliva, and the performance study was performed as described above.

Modified ninhydrin method

The reaction between ninhydrin and an amino acid produces a dark purple compound. Based on the findings of the previous study, which demonstrated no significant difference in absorbance between putrescine, cadaverine, and their combination, putrescine was chosen as the amine standard for this study [33]. After conducting a pilot study (data not shown), the researcher determined that the optimal conditions for the ninhydrin method were as follows: 300 µL of standard putrescine solution was placed in a microcentrifuge tube with 0.2% ninhydrin dissolved in deionized water and phosphate buffer (pH 8.0), then vortexed and refluxed at 85 °C for 7 min. After completing the reflux time, each reaction

tube was placed at room temperature to cool for 15 min. The color change of the solution was captured by a smartphone's camera using the detection box, and the RGB values of the digital image were analyzed by the ColorPicker application. The color intensities would be shown as ΔR, ΔG, and ΔB, respectively. The calibration curves were plotted against the concentration of putrescine versus the color intensities. The standard calibration curve for measuring the amount of total amines in the next experiment would be the one with the steepest slope on the calibration curve [27,28].

The performance of the modified ninhydrin method was determined using linearity test of different concentrations of putrescine. In addition, using the standard method, light absorbance readings at 570 nm were determined using Multiskan™ FC Microplate Photometer (Thermo Scientific™).

The validity of the modified ninhydrin method was determined by intra-day and inter-day precision. The percentage relative standard recovery (%RSD) was studied to obtain the validity of the developed method. In order to ensure precision repeatability, the %RSD should be within the range of 0.5 to 2.0 of a theoretical value as determined by the Hortwitz function [34]. The following equation's formula provided the percentage relative standard recovery (%RSD) calculation: [35]

$$\%RSD = \frac{\text{(Standard deviation of peak area)}}{\text{(Average peak area)}} \times 100 \quad (1)$$

The performance testing was repeated after the various putrescine concentrations were dissolved in artificial saliva to demonstrate that matrix variability had no effect on the method's effectiveness [32].

Measurement of salivary H_2S and total amines

Subject selection

The number of subjects in this study was calculated using sample size calculators for designing clinical research [36]. Unstimulated saliva was obtained from 105 adult volunteers with the following criteria: Not receiving any periodontal treatment within 6 months, not receiving antibiotic treatments within the last 3 months, and not receiving medication that may induce xerostomia. Before the appointment, the sample collection protocol was explained and informed consent was obtained verbally and legally document, and the participants were instructed to maintain their standard dietary and oral hygiene practices. They also received instructions to abstain from using commercial mouth rinse for 24 h before this appointment. The sample collection process was conducted between 9:00 a.m. and 12:00 a.m.

Sample collection and clinical examination

Unstimulated saliva was collected by spitting into a disposable cup with a plastic lid over a period of 5 min and using parafilm to prevent sample contamination and the release of H_2S into the atmosphere [37]. Saliva samples were immediately tested with the modified bismuth and ninhydrin methods following the completion of each day's oral examination. The remaining samples were then placed in microcentrifuge tubes and encapsulated with parafilm before being frozen and stored at −80 °C for further real-time PCR analyses.

Each saliva sample was mixed with the freshly prepared bismuth solution. After the reaction time, the production of H_2S was observed visually and photographed using the detection box. The technical personnel would evaluate the bismuth sulfide precipitation using a computer with a fixed screen brightness and image size. Three technical staff members scored the precipitation at the bottom of the microcentrifuge tube using a visual scale, from no color production (score 0), small color change to black (score 1), medium color change (score 2), to maximum color change production (score 3). Three technical personnel underwent training and calibration before assigning a score for bismuth sulfide. After training, Fleiss Kappa values indicated almost perfect agreement among examiners regarding consistency among stage raters (K value: 0.85; 95% confidence interval [CI]: 0.74 to 0.91). Cohen's kappa values for intra-examiners ranged from 0.81 to 0.85, indicating near-perfect agreement [38].

To determine the quantities of salivary total amines, the mixture of ninhydrin reagent, phosphate buffer, and saliva samples was mixed, refluxed in a water bath, and cooled. The color change of the solution was then captured by a smartphone's camera, and the RGB values of the digital image were analyzed using the ColorPicker application, and salivary total amines were found by converting the values into mM units using a

standard calibration curve. Both salivary H_2S and total amines were tested in triplicate.

DNA extraction and real-time PCR analyses

Saliva samples were centrifuged for 5 min at 10,000 rpm. The resultant pellet was resuspended in 20 mg/mL freshly prepared lysozyme (Amresco Inc., Solon, OH, USA) and was incubated at 37 °C for 60 min to lyze the bacterial cell wall. Then, bacterial DNA was extracted, purified, and eluted using the PureDirex® Genomic DNA Isolation kit (Bio-Helix Co., LTD., Keelung City, Taiwan) according to the manufacturer's instructions.

Periodontal bacteria, *F.nucleatum*, *P.gingivalis*, *P.intermedia*, and *A.actinomycetemcomitans* were identified using real-time PCR analysis. The primer sequences for detecting each bacterial species are presented in **Table 1**. An aliquot of qPCR master mix, comprising 5 µL of purified DNA template from a saliva sample, 2 µL of a specific primer pair, 3.4 µL of nuclease-free water, and 10 µL of 2× SensiFAST SYBR® No-ROX mix (Bioline Reagent Ltd., Foster City, CA, USA), was amplified using the CFX96 Touch™ Real-Time PCR detection system (Bio-Rad Laboratories, Inc). Duplicate samples were routinely used for determination.

Table 1 Specific primer sequences for real-time PCR analyses.

Primer pairs		**Ref.**
1. Total bacteria		Nadkarni *et al.* [39]
Forward primer	5'- TCCTACGGGAGGCAGCAGT-3'	
Reverse primer	5'- GGACTACCAGGGTATCTAATCCTGTT-3'	
2. *Fusobacterium nucleatum* ATCC 25568		Saygun *et al.* [40]
Forward primer	5'- GCGGAACTACAAGTGTAGAGGTG-3'	
Reverse primer	5'- GTTCGACCCCCAACACCTAGTA-3'	
3. *Porphyromonas gingivalis* ATCC 33327		Morillo *et al.* [41]
Forward primer	5'- CCTACGTGTACGGACAGAGCTATA-3'	
Reverse primer	5'- AGGATCGCTCAGCGTAGCATT-3'	
4. *Prevotella intermedia* ATCC 25611		Saygun *et al*. [40]
Forward primer	5'-CGTGGACCAAAGATTCATCG-3'	
Reverse primer	5'-CCGCTTTACTCCCCAACAAA-3'	
5. *Aggregatibacter actinomycetemcomitans* ATCC 33384		Morillo *et al.* [41]
Forward primer	5'-ACGCAGACGATTGACTGAATTTAA-3'	
Reverse primer	5'-GATCTTCACAGCTATATGGCAGCTA-3'	

The cycle threshold values (Ct values) from a real-time PCR detection system and the standard curves of serial dilution of each periodontal pathogen from the Oral Microbiology Laboratory, Faculty of Dentistry, Prince of Songkla University were utilized to determine the quantity of bacteria in each sample, which was expressed as $\log_{10}$ CFU/mL.

Statistics

Statistical analyses were conducted using the IBM SPSS Version 29.0 software package (SPSS®, IBM, USA). Descriptive statistics were used to describe the main features of modified bismuth and ninhydrin methods and characteristics of the study population. Correlations were assessed using the Spearman correlation coefficient (r_s) determination to find out if

there was a correlation between the quantity of periodontal pathogens in saliva, salivary H_2S, and salivary total amines. A *p*-value less than 0.05 was considered statistically significant.

Results and discussion

This study evaluated the performance and validity of the modified bismuth and ninhydrin methods using standard solutions, in addition to their applicability in biological samples.

Modified bismuth method

Based on visual analysis, the bismuth solution's limit of detection (LOD) was approximately 1 - 2 ppm NaHS dissolved in deionized water. A stability test was also conducted to determine the optimal storage time for the bismuth solution during the manufacture of a sulfide test kit for early detection of salivary H_2S. The same amount of bismuth sulfide precipitate was produced by maintaining the bismuth solution at 4 °C for 23 weeks as when the bismuth solution was freshly prepared. (**Figure S1**) This study conducted both intra- and inter-day experiments, and the results indicated that the modified bismuth method's validity was acceptable.

The standard sulfide solution was spiked into artificial saliva, and sensitivity testing was performed. According to **Figure 2**, after 30 min of reaction time, the bismuth sulfide precipitation in artificial saliva exhibits a similar appearance to that obtained with deionized water.

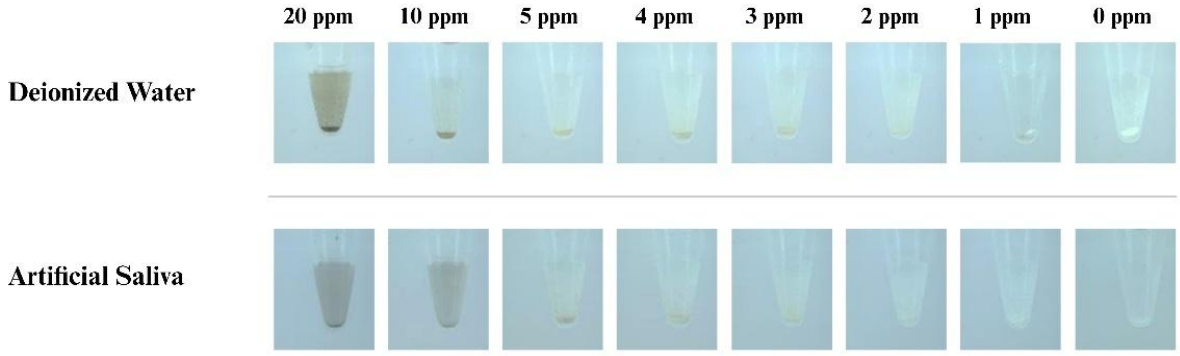

Figure 2 Visual detection limit of the bismuth solution dissolved in deionized water and artificial saliva.

The results revealed that the performance of the modified bismuth method was close to previous studies, with the visual detection limit (LOD) of 1 - 2 ppm NaHS [7]. Consistent with the previous study, it showed that H_2S from standard sulfide and periodontal bacteria degradation can be detected and graded using the bismuth method [7,8]. This finding suggests that the modification of the bismuth method might be used as a screening for periodontal diseases. The study's results on the stability of bismuth solutions are interesting. The same amount of bismuth sulfide precipitate was produced by maintaining the bismuth solution at 4 °C for 23 weeks as when the bismuth solution was freshly prepared. This result suggests that the bismuth solution could be suitable for long-term storage when producing a sulfide test kit for early detection of salivary H_2S, which would be advantageous for future studies. The precision test demonstrated that the validity of the modified bismuth method was acceptable. In addition, using artificial saliva instead of deionized water revealed that 30 min of reaction time was enough to ensure that the viscosity of the matrix did not affect the bismuth sulfide precipitation.

Modified ninhydrin method

The calibration curves were plotted against the concentration of putrescine versus the change in slope for the R, G, and B values. The delta-G (ΔG) values were chosen for measuring putrescine because they had the steepest slope (slope = 30.06) in the calibration plot with a linear range of 0.5 to 4.0 mM (**Figure 3**).

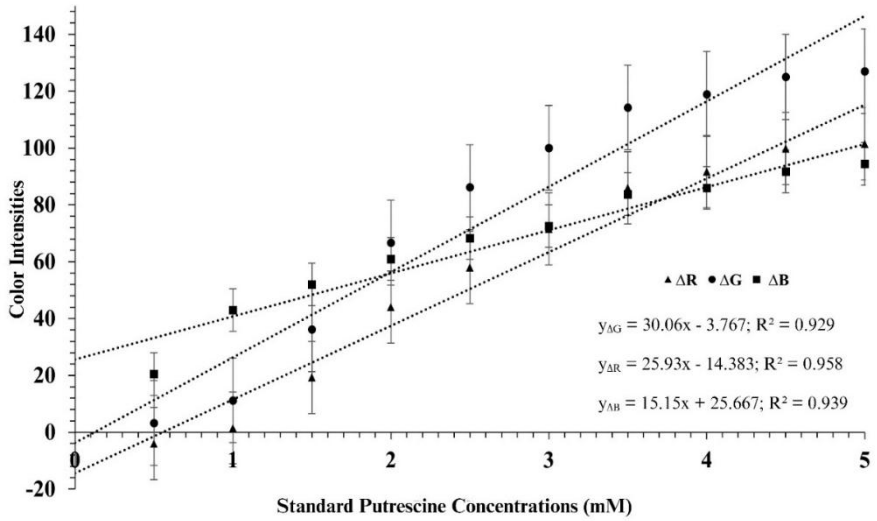

Figure 3 The relationship between standard putrescine concentrations and color intensities (ΔR, ΔG, and ΔB).

The linearity test showed that the pale-yellow solution turned into purple solution at 1.5 to 2.0 mM putrescine. The similar trend of the standard calibration curve and the linearity of Abs_{570nm}, ΔG, and putrescine concentrations suggested that the modified ninhydrin method's results could also be analyzed using ΔG values.

The scoring system of ninhydrin method was developed using the standard calibration curve of ΔG and putrescine concentrations by determining the slope between each adjacent pair of dots on the standard calibration curve. The changing slope was related to the color change in the reaction tube. After calculating the average slope, the total amine detection scoring system could range from score 1 to score 3. (**Table S1**)

The intra- and inter-day precision of the modified ninhydrin method was evaluated using %RSD. **Table 2** showed that %RSD of modified ninhydrin method ranged from 2.40 to 22.50

Table 2 The intra- and inter-day precision of the ninhydrin method.

Putrescine concentrations	Intra-day precision		Inter-day precision	
	SD	%RSD	SD	%RSD
1.0 mM	0.07	13.54	0.06	12.96
2.0 mM	0.02	2.40	0.25	20.33
3.0 mM	0.29	22.50	0.45	19.73
4.0 mM	0.26	13.35	0.34	12.29
5.0 mM	0.28	11.61	0.25	7.69

The researcher spiked the putrescine solution into artificial saliva and conducted linearity testing. **Figure 4** showed a trend that was similar to the standard calibration curve between ΔG and putrescine concentrations. The following equation could explain the relationship between G and various putrescine concentrations spiked in artificial saliva:

$$y = 33.08x + 0.606; R^2 = 0.948 \quad (2)$$

The amount of salivary total amines in the next experiment would be calculated using this equation.

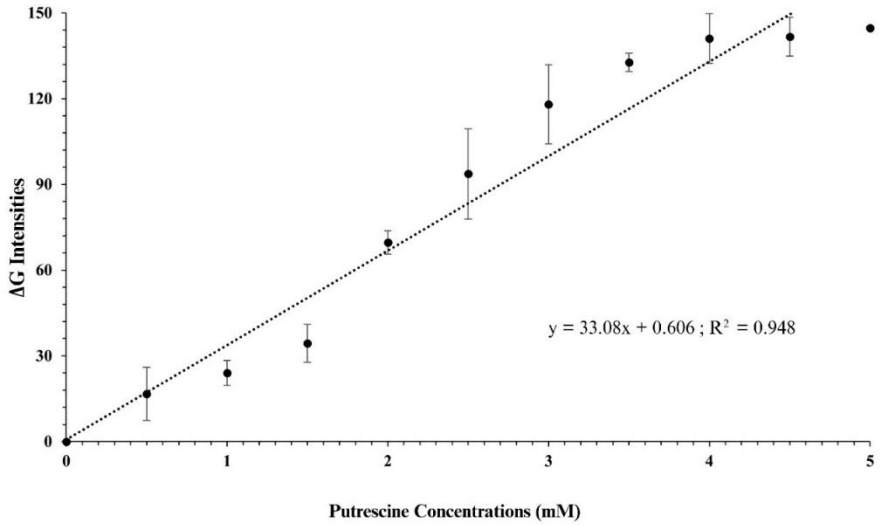

Figure 4 The relationship between ΔG and different putrescine concentrations spiked in artificial saliva.

The ninhydrin scoring system can also grade salivary total amines in mM units and categorize them into 3 groups. **(Table S1)**

The modified ninhydrin method showed that the pale-yellow solution changed to a purple solution when there was 1.5 to 2.0 mM putrescine, and the Abs_{570nm} at that point was similar to Iwanicka's study, which suggested that the turning point can distinguish healthy subjects from halitosis patients resulting from the overactivity of bacterial degradation of amino acids [30]. The process of amino acid degradation also happens in the inflammatory process of periodontal diseases. This suggests that the modified ninhydrin colorimetric reaction might be useful in the alternative method of screening periodontal diseases. The validity test showed that, under optimal conditions, the ninhydrin technique has a higher %RSD than the Horwitz RSD values; [34] however, for use as a screening test, the precision was acceptable. Furthermore, the precision study used only putrescine as an analyte, but in human saliva, other amines and amine compounds can be detected via ninhydrin.

Measurement of salivary H_2S and total amines

Demographic data of the study populations and salivary analysis

One hundred and five adult participants were recruited from the Dental Hospital of the Faculty of Dentistry, Prince of Songkla University. The findings demonstrated that nearly all participants exhibited a positive response to H_2S and total amines. **Table 3** presented the demographic data of the study's population, the results of both methods, and the number of participants in each score category.

Table 3 Demographic data of the study population and results for saliva analysis.

Variables	**Total (n = 105)**
Gender	
Female	74 (70.48)
Male	31 (29.52)
Age (years [mean ± SD])	43.7 ± 15.1
Age (years [range])	77 – 20
Smokers	
Current smokers	5 (4.76)

Variables	Total (n = 105)
Non-smokers	100 (95.24)
Systemic diseases	
Systemic diseases	30 (28.57)
Non-systemic diseases	75 (71.43)
Modified bismuth methods	
Score 0	46 (43.81)
Score 1	34 (32.38)
Score 2	9 (8.57)
Score 3	16 (15.24)
Modified ninhydrin methods	
Score 1	20 (19.05)
Score 2	22 (20.95)
Score 3	63 (60.00)
Total amines concentration (mM)	
Mean ± SD	3.51 ± 1.75
Range	5.98 – 0.01

DNA extraction and real-time PCR analyses

Crude genomic DNA samples were extracted from saliva samples. Periodontal pathogens and total bacteria were determined using real-time PCR. However, this study population did not detect *A. actinomycetemcomitans*. The quantities of 3 periodontal pathogens in each saliva sample varied in relation to H_2S and total amines. In **Table 4**, the median and range of periodontal bacteria amounts were shown for bismuth and ninhydrin scores.

Table 4 The median and range of periodontal bacteria quantities in bismuth and ninhydrin scores.

Bacteria	Bismuth score				Ninhydrin score		
	0	**1**	**2**	**3**	**1**	**2**	**3**
Total bacteria (logCFU/mL)							
Median	9.32	10.44	11.04	11.90	9.75	10.76	10.41
Range	12.20 - 2.34	12.31 - 8.22	15.29 - 10.18	12.99 - 9.52	12.03 - 6.47	12.36 - 7.87	15.29 - 2.34
F. nucleatum (logCFU/mL)							
Median	5.21	5.42	6.00	6.10	5.07	5.44	5.46
Range	6.11 - 4.28	6.27 - 4.45	6.36 - 5.09	6.60 - 5.28	5.84 - 4.28	6.27 - 4.57	6.60 - 4.45
P. gingivalis (logCFU/mL)							

Bacteria	Bismuth score				Ninhydrin score		
	0	**1**	**2**	**3**	**1**	**2**	**3**
Median	1.01	1.51	1.84	2.66	1.07	1.40	5.30
Range	3.27 - 0.00	3.42 - 0.00	3.50 - 0.63	4.04 - 0.71	2.97 - 0.01	2.99 - 0.00	4.04 - 0.00
P. intermedia (logCFU/mL)							
Median	4.89	5.20	6.01	6.15	5.07	5.30	5.29
Range	6.72 - 0.00	6.87 - 0.00	7.01 - 3.96	7.42 - 5.06	6.59 - 3.65	6.87 - 2.91	7.42 - 0.00

Correlation analyses

Correlation analysis of the whole study population showed a significant correlation between the levels of 3 periodontal pathogens and bismuth score. Using the ninhydrin score, the quantities of *F. nucleatum* were significantly correlated with salivary total amines. (**Table 5**).

Table 5 The correlation between the quantity of periodontal pathogens, bismuth, and ninhydrin scoring system.

Periodontal pathogens	Bismuth score		Ninhydrin score	
	r_s	***p*-value**	r_s	***p*-value**
Total Bacteria	0.641	<0.001*	0.169	0.084
F. nucleatum	0.599	<0.001*	0.215	0.028*
P. gingivalis	0.356	<0.001*	0.154	0.118
P. intermedia	0.385	<0.001*	0.06	0.546

* Analysis was carried out using Spearman's correlation coefficient (r_s), *p-value* <0.05 was considered statistically significant.

The results indicated that it might be possible to find the aqueous form of H_2S and total amines in saliva in a simpler way. The higher bismuth score indicates high H_2S levels in saliva and is related to the quantity of periodontopathic bacteria. It was discovered that the amounts of the periodontal pathogens *F. nucleatum*, *P. gingivalis*, and *P. intermedia* were correlated to the H_2S level in saliva, consistent with a previous study finding that *F. nucleatum* had the most rapid H_2S production. High H_2S production was also seen for *P. gingivalis*, which was associated with periodontal diseases [7,8]. The result could be explained by the presence of periodontopathic bacteria in saliva and subgingival plaque, and there were significant positive correlations between the bacterial counts of *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, and *P. intermedia* in the supragingival plaque, subgingival plaque, and saliva samples [42,43].

This study indicated that we could measure total amines in saliva using the ninhydrin method and a simpler way with a smartphone application to determine RGB values, both of which can give clearer and easier-to-understand results. Previous studies try to use smartphone applications for monitoring the RGB values of colorimetric reactions, which have been used in many studies; the results show no significant difference between traditional and alternative methods [28]. However, the results and accuracy in the modified ninhydrin method might be different from those of other studies. This study only used putrescine to create the standard calibration curve; however, when researchers use the ninhydrin method on human saliva, the ninhydrin reagent can identify other amine compounds. Earlier study showed that measuring polyamines in human saliva can be done easily with a simple sample preparation method and HPLC, which is reliable and

accurate, but the process still needs HPLC, which is costly and needs a skilled technician [44].

The ninhydrin score was related to the quantity of total amines; however, only the quantity of *F. nucleatum* and salivary total amines showed a significant correlation. However, several studies indicated that amines are involved in the inflammatory process of periodontal diseases [5,22]. An increase in periodontal inflammation elevates salivary amine levels [22,45]. Oral bacteria, including periodontal pathogens, generate putrescine and other polyamines, which are released into the mouth cavity after the breakdown of bacterial cells in reaction to the host immune response [20]. The synthesis of H_2S and amines in the oral cavity is complicated and requires more investigation in future studies to strengthen our knowledge of the processes behind H_2S and total amines production, periodontal parameters, and its implications for periodontal diseases screening.

This research minimized sampling bias by employing a large, representative sample. Limitations of this research include the lack of periodontal risk factors, radiographic exams, criteria, and definitive diagnosis. The subsequent studies should evaluate and control confounding variables, including gender, age, and smoking behaviors, that might have influenced the study's results.

Conclusions

The study's results indicate that the modified bismuth and ninhydrin methods have acceptable validity and can be used to find periodontopathic bacteria and their byproducts; however, implications for periodontal screening require more investigation to clarify the mechanism and clinical consequences. Future research should focus on a more severe group of periodontal diseases and investigate other periodontal risk factors in order to obtain a greater comprehension of the mechanisms underlying bacterial H_2S and amines production and their influence on clinical parameters.

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No generative AI or AI-assisted technologies were used in this article.

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Supplementary Materials

This manuscript contains one supplement figure and one supplement table with the figure and table captions listed below:

Figure S1 The stability of the bismuth solution.

Table S1 Scoring system for total amines detection.

Total amines concentration (mM)	Slope	Average slope	Score
0.00 - 0.50	16.00	16.733	Score 1
0.50 - 1.00	19.33		
1.00 - 1.50	14.00		
1.50 - 2.00	36.67	37.133	Score 2
2.00 - 2.50	42.67		
2.50 - 3.00	32.67		
3.00 - 3.50	36.00		
3.50 - 4.00	20.00	14.733	Score 3
4.00 - 4.50	15.33		
4.50 - 5.00	8.67		