



Research article

Rapid analysis to distinguish porcine and bovine gelatin using PANI/NiO nanoparticles modified Quartz Crystal Microbalance (QCM) sensor

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ABSTRACT

Rapid analysis to distinguish porcine and bovine gelatin using a modified Quartz Crystal Microbalance (QCM) sensor has been studied. The PANI was deposited on the sensor surface using electropolymerization, and then nickel nanoparticles were deposited by layer by layer (LbL) technique. The modified QCM sensor's performance was compared to an unmodified sensor in porcine and bovine gelatin at neutral, acidic, and alkaline conditions. The result shows that the unmodified sensor cannot distinguish between porcine and bovine gelatin, whereas the modified QCM sensor produces a different response. Porcine gelatin shows an increasing frequency response, but in contrast, bovine gelatin decreases frequency response at the alkaline condition. The time response was 2 min with a detection limit of 51.2 ppm and 8.7 ppm for porcine and bovine gelatin, respectively. Further investigation shows that the modified sensor can analyze porcine gelatin contamination in the a mixed gelatin sample.

1. Introduction

Gelatin is widely used in the pharmaceutical and food industry [1, 2, 3, 4, 5], whereas it is used as capsule shells and tablets, particularly in the pharmaceutical industry. It is also commonly used as a stabilizer, a thickener, and gelling agent for the food industry [6]. Gelatin as foodstuff is easily found in a sweet, a jelly, ice cream, and a marshmallow [7]. Some of these products have limited information on their original source of gelatin. Ingredient of pig derivative, i.e., 45.8%, is the largest source of gelatin [1, 2, 3, 5]. The gelatin source should be clearly and truthfully declared on the food package [4, 8]. This information is important for legal, decent, and honesty. In addition, for a host of people, porcine and bovine products are unacceptable. Therefore, it is necessary to develop a method that can distinguish the species origin of gelatin.

Today, a gelatin source can be determined using the protein test method, which can be observed based on its structure. This method was reported by Hofmann (1985), which used an isoelectric method. The method focuses on a polyacrylamide gel to identify muscles derived from a pig [9]. Aristoy and Toldra developed a peptide test as a qualitative measurement for porcine gelatin identification [9]. The gelatin sources also were determined using the enzyme-linked immunosorbent assay

(ELISA) method [10]. Another protein test reported is using the chromatography technique [11, 12, 13]. However, all methods mentioned above encountered a problem when these were used to analyze processing products as the samples' proteins degraded during the food processing. This problem was overcome by Polymerase Chain Reaction (PCR) method, which has good selectivity for analyzing fresh and processing products by recognizing its DNA [14, 15]. Nevertheless, one of the reasons which often caused failures in the determination of gelatin using PCR is the low quality and quantity of extracted DNA. The PCR method's general disadvantages are time-consuming and high operating costs [16].

PANI/Ni(OH)₂ nanoparticles were used to modify the QCM sensor [6, 17]. The QCM sensor is suitable for fast, simple, and real-time analysis [18, 19, 20, 21]. Gelatin analysis using the QCM sensor is based on a change in the sensor frequency due to the target compound attached to the QCM sensor surface. However, this work used PANI/NiO nanoparticles modified QCM sensor to distinguish porcine and bovine gelatin. The sensor works based on the different reactivity between both gelatins with the active materials on the sensor. The performance of the QCM sensor before and after modification was compared. This modified QCM sensor works well at room temperature in alkaline conditions with a

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response time and detection limit of 2 min and 51.2 ppm, respectively, for porcine gelatin. This sensor has a better performance than the other methods that have already exist. It also shows a good performance in the real sample (marshmallow) due to no interference was observed during the measurement.

2. Experimental

2.1. Chemicals and materials

Bovine and porcine gelatin were bought from a local market in Surabaya, East Java, Indonesia. Aniline (Merck, Darmstadt, Germany) was purified prior to use. Hydrochloric acid (37% HCl, Merck, Darmstadt, Germany), sodium hydroxide (NaOH, Merck, Darmstadt, Germany), and demineralized water were used without purification. Nickel hydroxide nanoparticles were synthesized by electrochemical method from our previous work [22, 23]. The nanoparticles were used as a starting material in the fabrication of NiO nanoparticles. Four marshmallows from the local market containing gelatin based on the ingredients list (two contain porcine gelatin and the others contain bovine gelatin) were used as a real sample. A commercial Quartz Crystal Microbalance (QCM) with 5 MHz, AT-cut quartz crystal, and 25.4 mm diameter were purchased from Renlux Crystal (Shenzhen, China).

2.2. Fabrication of PANI/NiO nanoparticles modified QCM sensor

The QCM sensor was cleaned ultrasonically in demineralized water for 10 min, immersed into piranha solution (concentrated H_2SO_4 : 30% $\text{H}_2\text{O}_2 = 1:3$ v/v) for 5 min, and rinsed with demineralized water consecutively. Subsequently, the clean QCM sensor's surface was coated by a PANI layer using polymerization of 0.1 M aniline at pH 1.5 [24]. The electropolymerization process was performed using a cyclic voltammetry method. A conventional three-electrode cell system with a QCM sensor acted as a working electrode (WE), Ag/AgCl (KCl 3 M) acted as the reference electrode (RE), and platinum as a counter electrode (CE) was used. It was done at a scan rate of 50 mV s^{-1} (vs. Ag/AgCl (KCl 3 M)) over the potential of -0.5 V to $+1.0 \text{ V}$ for 40 cycles. After that, nickel hydroxide nanoparticles [23] were deposited on the surface of the PANI modified QCM sensor using the Layer by Layer (LbL) technique for 5 min and were dried at room temperature ($30 \text{ }^\circ\text{C}$). This step was repeated three times before being dried for 24 h at room temperature ($30 \text{ }^\circ\text{C}$). Next, the sensor was calcined at $400 \text{ }^\circ\text{C}$ to form the NiO nanoparticles [23]. All the modification steps were observed using optical microscopy (Olympus BX60, California, USA) and confirmed by scanning electron microscopy (SEM, TESCAN MIRA, Kohoutovice, Czech Republic).

2.3. Measurement of standard gelatin by QCM sensor

Every 3 g of porcine and bovine gelatins were diluted in 100 mL demineralized water to prepare 30,000 ppm gelatin stock solutions. The gelatin stock solution was diluted in demineralized water to obtain various standard gelatin solutions with 100, 200, 300, and 400 ppm concentrations. All these standard gelatin concentrations were determined using QCM sensor, PANI modified QCM sensor, and PANI/NiO nanoparticles modified QCM sensor consecutively under acidic (pH 4), neutral (pH 7), and alkaline (pH 9) conditions at room temperature ($30 \text{ }^\circ\text{C}$). A schematic picture of the QCM system is shown in Figure 1.

2.4. Determination of limit of detection (LOD) and repeatability of the PANI/NiO nanoparticles modified QCM sensor

The various porcine and bovine gelatin concentrations used to obtain the calibration curves were 0, 100, 200, 300, and 400 ppm. It was performed by emptying the QCM cell each time when changing to the next concentration of gelatin. The measurement was done at room temperature ($30 \text{ }^\circ\text{C}$). The calibration curve was plotted based on various concentrations versus the ΔF at the constant measurement. The repeatability was investigated using three different PANI/NiO nanoparticles modified QCM sensors. Five replications of each sensor were conducted in 100 ppm for both bovine and porcine gelatin solution under alkaline conditions. All the measurements were conducted at room temperature ($30 \text{ }^\circ\text{C}$) under the stirring condition of 200 rpm for 10 min.

2.5. Interference study of PANI/NiO nanoparticles modified QCM sensor

Common interferences for gelatin testing in food are glucose, milk, and starch. A 100 ppm of glucose, milk, or starch solutions were measured by the PANI/NiO nanoparticles modified QCM sensor. Further interferences study was performed by measuring a 100 ppm mixture of porcine and bovine gelatin with several compositions. The various compositions are porcine gelatin:bovine gelatin = 1:9; 2:8; 3:7; 4:6 and 5:5. All measurements were conducted at room temperature ($30 \text{ }^\circ\text{C}$) under the stirring condition of 200 rpm.

2.6. Measurement of the real sample using PANI/NiO nanoparticles modified QCM sensor

The real four samples described in the previous chemicals and materials section were prepared with the same treatment. Each of them was cut into small pieces before being dissolved in demineralized water.

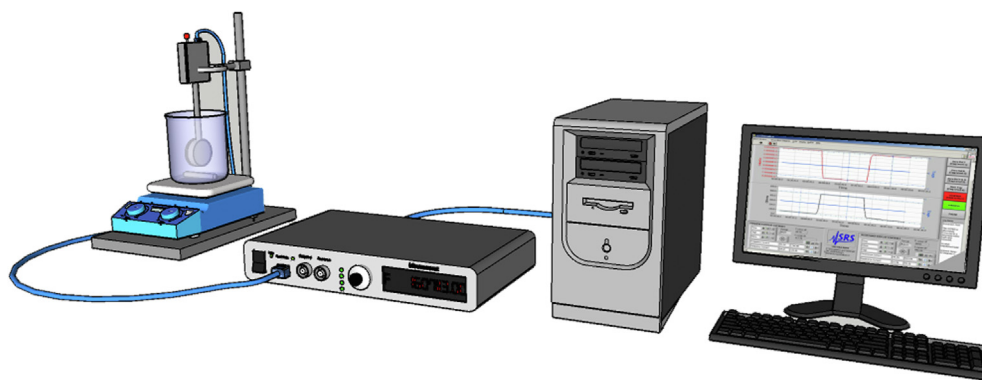


Figure 1. Schematic picture of the QCM system.

Latterly, 1 g of each marshmallow was diluted in 300 mL alkaline solution (pH 9) under a stirring condition until a homogenous mixture was obtained. The real samples were measured at 30 °C under the stirring condition of 200 rpm for 10 min. The amount of gelatin in the real sample was calculated using a linear regression equation from the calibration curve.

3. Results and discussion

3.1. Fabrication of PANI/NiO nanoparticles modified QCM sensor

PANI/NiO nanoparticles modified QCM sensor was successfully fabricated. The modification steps are shown in Figure 2a-d. The surface optical images of the QCM sensor before and after modification can be seen in Figures 2e-h. The QCM sensor shows a bright gold color (Figure 2e). The black spots and cavities are visible with PANI modification on the QCM sensor's surface (Figure 2f). In this state, the PANI layer serves as an adhesive between the QCM sensor surface and nickel hydroxide nanoparticles. Nickel hydroxide nanoparticles are shown as blue spots on the QCM sensor (Figure 2g). The blue spots came from the nickel hydroxide nanoparticles, which were deposited between PANI cavities.

Moreover, the blue spots became brighter than the previous ones after the calcination process, as seen in Figure 1h. The brightening of the color gives a strong indication that NiO nanoparticles have been formed on the PANI-modified QCM sensor's surface. Furthermore, the QCM sensor's morphology with and without modification was also confirmed by SEM images (Figure 2i-l). Both optical microscopy and SEM indicate that PANI/NiO nanoparticles modified QCM sensor has been successfully fabricated.

3.2. Performance of QCM sensor for gelatin identification

Detection of bovine and porcine gelatin in neutral conditions (pH 7) show a similar response as displayed in supplementary material at Figure S1a and S1d, respectively. Both bovine and porcine gelatin frequencies are decreased until -425 Hz and -20 Hz, respectively, for all the concentrations. The measurement continued in acidic conditions (pH 4). The results also indicated a similar sensor frequency response for bovine (supplementary material, Figure S1b) and porcine gelatin (supplementary material, Figure S1e). Each frequency was also decreased to less than -320 Hz for bovine gelatin and -40 Hz for porcine gelatin. A similar condition was also shown to detect bovine and porcine gelatin (supplementary material, Figures S1c and S1f, respectively) at alkaline conditions using the QCM sensor. The frequency was decreased until -240 Hz for bovine gelatin and -15 Hz for porcine gelatin. All the results show that the QCM sensor produced the same response for both bovine and porcine gelatin in all conditions. The decreasing frequency response is caused by the increase of mass on the QCM sensor surface. The increase in mass is due to the similar interactions for both gelatins with the QCM sensor. This same response proves that the QCM sensor cannot be used to distinguish between bovine and porcine gelatin.

3.3. Performance of PANI modified QCM sensor for gelatin identification

The PANI modified QCM sensor was also used to measure the porcine and bovine gelatin using the same previous procedure. All the measurements at neutral (supplementary material, Figures S2a and S2d), acidic (supplementary material, Figures S2b and S2e), and alkaline conditions (supplementary material, Figures S2c and S2f) for both bovine and porcine gelatins, respectively, also do not demonstrate a different

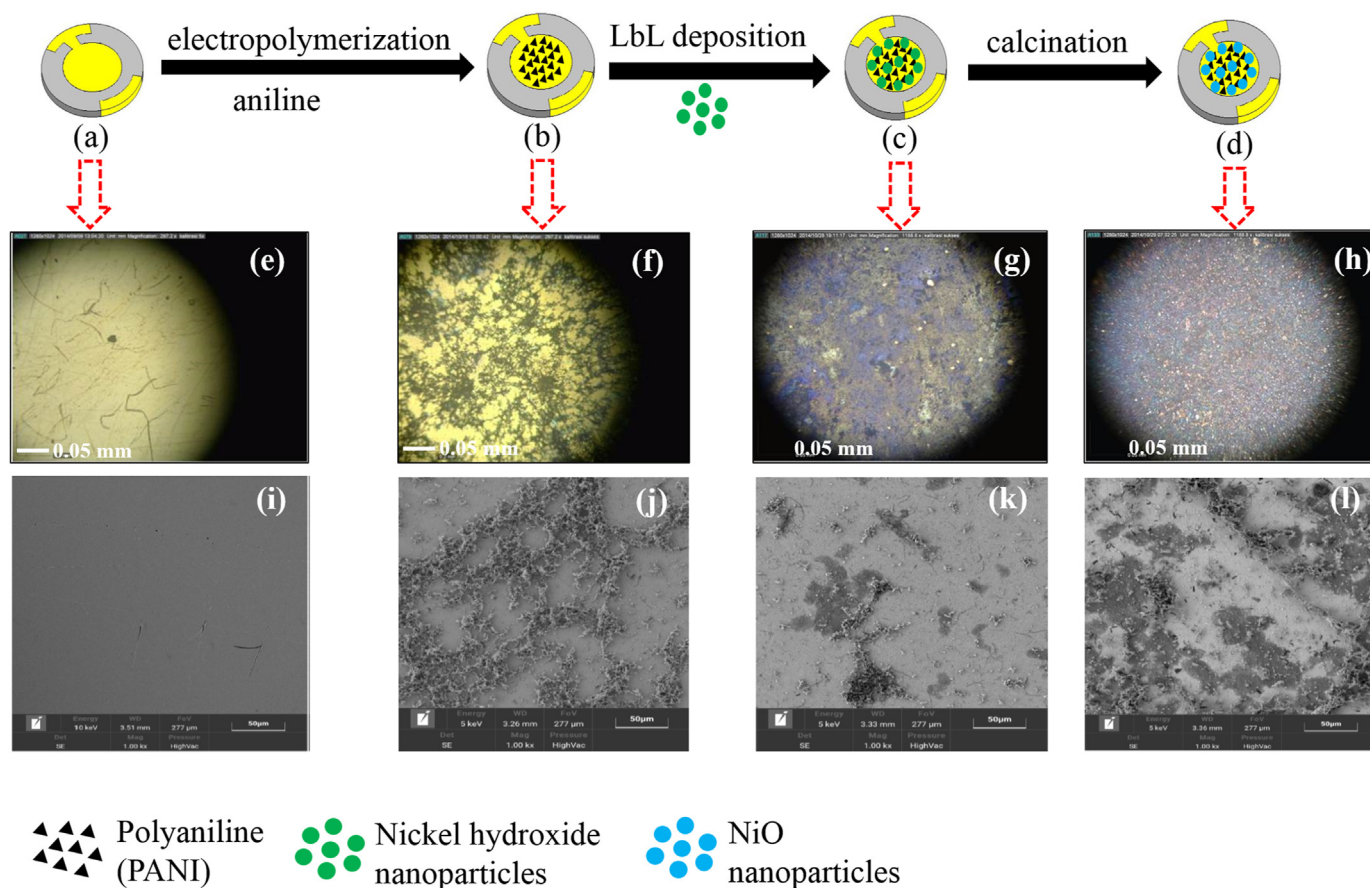


Figure 2. The fabrication steps of PANI/NiO nanoparticles modified QCM sensor (a-d) and its surface image (by optical microscope (e-h) and SEM (i-l)).

pattern of the sensor frequency response between both gelatins. This result signifies that the presence of PANI on the QCM sensor is not enough to distinguish the porcine from bovine gelatin.

3.4. Performance of PANI/NiO nanoparticles modified QCM sensor for gelatin identification

PANI/NiO nanoparticles' response modified QCM sensor in a gelatin solution was also studied at neutral, acidic, and alkaline conditions. A similar response to bovine (Figures 3a and 3d) and porcine (Figures 3b and 3e) gelatins were observed at neutral and acidic conditions. It implies that PANI/NiO nanoparticles do not work well at neutral and acidic conditions, similar to the QCM and PANI-modified QCM sensors. A significant difference in the results was observed for the real-time frequency measurement of both porcine and bovine at alkaline conditions as shown in Figures 3c and 3f, respectively. The real-time frequency response for bovine gelatin is decreasing, but it is increasing for porcine gelatin. According to the Sauerbrey equation, shown in Eq. (1),

$$\Delta F = -C_f \times \Delta m, \quad (1)$$

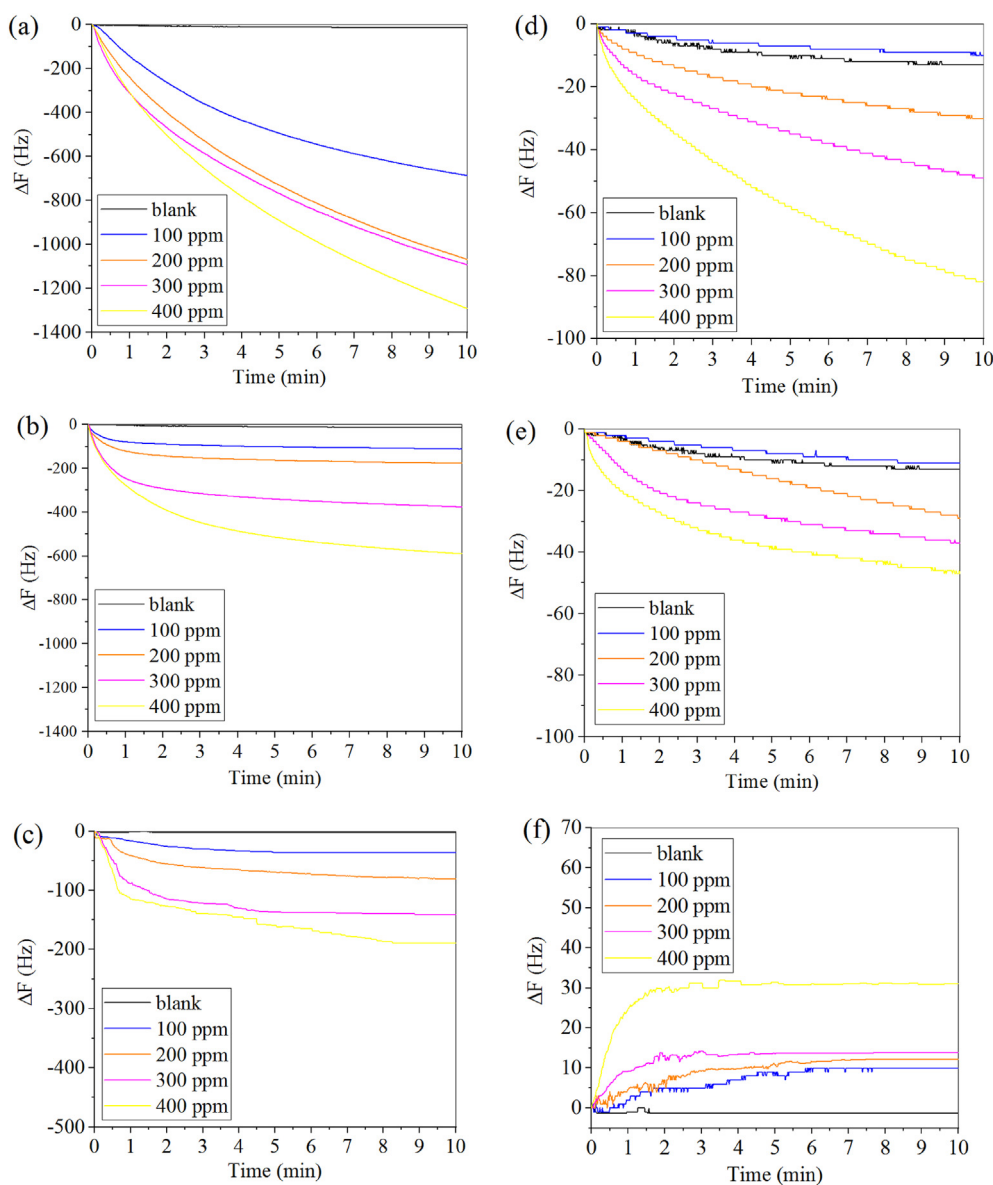


Figure 3. Real-time response of the PANI/NiO nanoparticles modified QCM sensor in the standard gelatin solution of bovine (a–c) and porcine (d–f). The measurement was carried out at neutral (a and d), acidic (b and e), and alkaline (c and f) conditions. All measurement was conducted at room temperature for 10 min.

Decreasing frequency response implies that some gelatin molecules attach to the QCM sensor, but in contrast, increasing frequency response implies that some of the active materials were removed from the surface of the QCM sensor by the gelatin molecule. The different porcine and bovine gelatin interaction mechanisms on the sensor surface cause the opposite direction of the calibration curves (supplementary material, Figure S3). The porcine gelatin shows a positive slope (supplementary material, Figure S3a), while bovine gelatin shows a negative slope (supplementary material, Figure S3b).

3.5. Possible interaction of PANI/NiO nanoparticles modified QCM sensor in presence of gelatin

The results obtained prove that PANI/NiO nanoparticles modified QCM sensor can successfully distinguish between bovine and porcine gelatin qualitatively at alkaline conditions. The response time of this sensor is only 2 min. The calibration curve for calculating the Limit of Detection (LOD) of the sensor towards porcine gelatin and bovine gelatin is shown in Figures S3a and S3b (supplementary material), respectively, in addition to using Eq. (2):

Table 1. Comparison of the PANI/NiO nanoparticles modified QCM sensor with several methods.

Method	Limit of detection for porcine gelatin	Analysis time	Disadvantage	Reference
Conventional PCR	0.1% w/w	~120 min	Require long step preparation	[15]
Real-time PCR	1% w/w	~120 min	Require long step preparation	[14]
ELISA	8% w/v	~15–20 min	Less sensitive to a specific species, only specific to bovine gelatin, and produce false-positive for gelatinized heated meat	[2]
FTIR	4% w/v	~10–15 min	Require high purity sample, unable to differentiate a mixture of raw gelatins	[29]
MALDI-TOF-MS	1%	N/A	Complicated preparation step	[30]
LC-MS/MS	0.4% w/w	~40–50 min	Complicated preparation step	[5]
PANI/NiO nanoparticles modified QCM sensor	0.0051%	2–5 min	-	This work

$$LOD = \frac{3\sigma}{S}, \quad (2)$$

In Eq. (2), σ is the pure deviation of the response (0.4627), and S is the calibration slope. It was found that this method has a better detection limit of 51.2 ppm for porcine gelatin when compared to the other method (Table 1). In comparison, the LOD of the sensor for bovine gelatin is 8.7 ppm. The LOD of porcine gelatin is higher than bovine gelatin because it has higher interaction with the sensor's surface. The interaction between porcine or bovine gelatin and the sensor depends on the amino acid constituents.

Hafidz et al. (2011) have reported the amino acid constituents of bovine and porcine gelatins, summarized in supplementary material at Table S1 [25]. These different constituents give different gelatin structures, which give different in the unique property of gelatin. Porcine gelatin has a higher degree of cross-linking [26]. It also has higher gel strength than bovine gelatin in almost all pH [25]. According to Aewsiri et al., the highest gel strength of porcine gelatin was achieved at pH 9 [27]. This result agrees with this work which the best measurement was conducted at alkaline conditions (pH = 9) to distinguish porcine and bovine gelatin. The results obtained show a different interaction of bovine and porcine gelatin on the PANI/NiO nanoparticle QCM sensor's surface, as shown in Figures 4a and 4b.

The different frequency response shifting is probably due to these different amino acid constituents in the bovine and porcine gelatins. Bovine gelatin gives a decreasing frequency response, while porcine gelatin gives an increasing frequency response. This phenomenon suggests that an active material of the QCM sensor tied up bovine gelatin. In contrast, the porcine gelatin pulls the active material off from the QCM sensor. The comparison of the PANI/NiO nanoparticles modified QCM sensor to other methods is shown in Table 1.

3.6. Reproducibility and interference study of PANI/NiO nanoparticles modified QCM sensor

The reproducibility of PANI/NiO nanoparticles modified QCM sensor in identifying gelatin can be seen from repetition study. This study was conducted by fabricating three PANI/NiO nanoparticles modified QCM sensors under the same conditions. Figure S4a, S4b, and S4c in supplementary material display the real-time response of sensors 1, 2, and 3, respectively. It was found that there were no significant differences in the responses of all PANI/NiO nanoparticles modified QCM sensors. Decreasing frequency response is always observed for bovine gelatin measurement, whereas an increasing frequency response was consistently observed for porcine gelatin measurement. This suggests that PANI/NiO nanoparticles modified QCM sensor has an excellent reproducibility to distinguish bovine from porcine gelatin.

The PANI/NiO nanoparticles modified QCM sensor response was studied in glucose, milk, and starch solutions which can be seen in supplementary material (Figure S5). They all give a decrease response. Moreover, the presence of porcine gelatin at all variations in the bovine gelatin mixture resulted in an increased response (supplementary material, Figure S6), indicating that porcine gelatin is more dominant and possible to identify contaminant porcine gelatin in the food sample. These results show that the sensor is selective to porcine gelatin, and it is because the response of the sensor consistently decreases in the absence of porcine gelatin.

3.7. Performance of PANI/NiO nanoparticles modified QCM sensor in the real sample

The performance of PANI/NiO nanoparticles' modified QCM sensor in the real sample matrices, a marshmallow, was also investigated. The main ingredients of marshmallows are sucrose, glucose syrup, water, and gelatin. About 1.5% of gelatin is usually used in marshmallow production to avoid sugar crystallization, resulting in a soft and easily formed product [28]. Figure 5 shows the frequency response obtained from the measurements using PANI/NiO nanoparticles modified QCM sensor in marshmallow samples. The marshmallow A and marshmallow B samples known as commercial marshmallows containing bovine gelatin. Both marshmallows show a decreased frequency response, similar to the standard bovine gelatin detection. The marshmallow gelatin analysis in C and D (both well-known contain porcine gelatin) show an increased frequency response, as shown in Figure 5. The results obtained are in good agreement with the information on the label. The amount of gelatin in real samples was calculated as shown in Table 2.

This measurement shows no interference response from the matrices in marshmallow samples, signifying that the PANI/NiO nanoparticles modified QCM sensor has good sensitivity and selectivity to porcine gelatin. The matrices in marshmallows are common matrices that can be found in many other foods. Thus, this sensor presents further probabilities that it can also be applied for different various food samples.

4. Conclusion

Porcine and bovine gelatin can be distinguished using PANI/NiO nanoparticles modified QCM sensor. The sensor works well only in the alkaline condition, which can be seen from the different frequency responses obtained. A decreased frequency response was observed for the bovine gelatin source, whereas an increased frequency response was observed for a porcine gelatine source. The PANI/NiO nanoparticles modified QCM sensor was also proven to be used for marshmallows without interferences from the matrices sample. The limit detection of

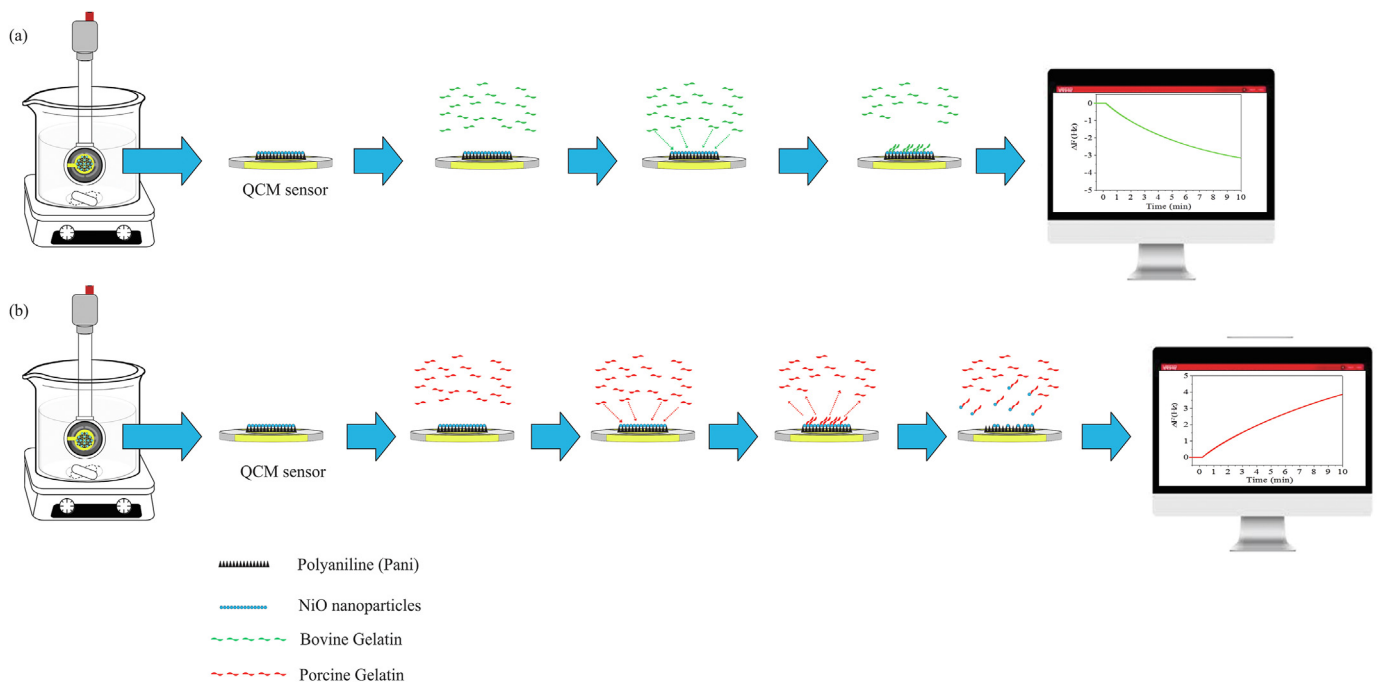


Figure 4. Possible interaction of bovine (a) and porcine gelatin (b) on the PANI/NiO nanoparticles modified QCM sensor at alkaline condition.

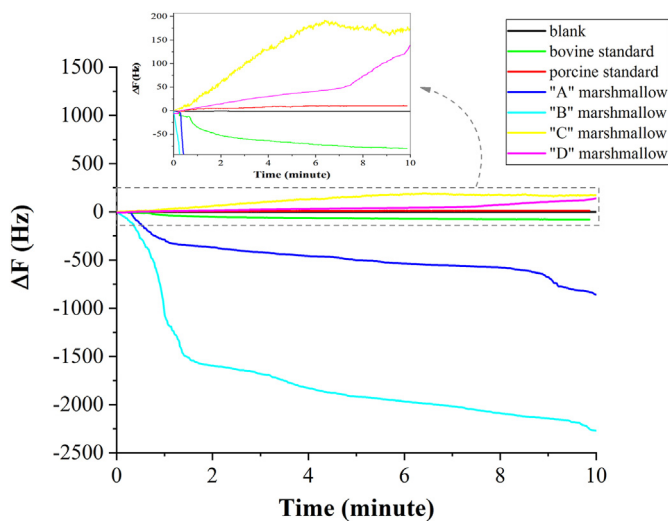


Figure 5. Real-time response of the PANI/NiO nanoparticles modified QCM sensor for marshmallow sample in variant types. All measurement was conducted at room temperature for 10 min.

Table 2. Calculation of the amount of gelatin in real samples.

Real sample	The amount of gelatin (%)
Marshmallow A	0.35
Marshmallow B	1.52
Marshmallow C	0.32
Marshmallow D	0.08

the sensor is relatively small with a fast response time compared to other methods.

Declarations

Author contribution statement

Fredy Kurniawan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ari Nugroho, Rangga Aji Baskara, Lourentia Candle, Diwasasri Pradini: Performed the experiments; Analyzed and interpreted the data.

Kartika A. Madurani: Analyzed and interpreted the data; Wrote the paper.

Raden Djarot Sugiarso, Hendro Juwono: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2022.e09401>.

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