The effect of probe density coverage on the detection of oenological tannins in Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) experiments

Running title: Probe Density Impact on Oenological Tannin Detection in QCM-D

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Abstract

Background: Polyphenols are a group of compounds found in grapes, musts, and wines. Their levels are crucial for grape ripening, proper must fermentation, and final wine characteristics. Standard chemical analysis is commonly used to detect these compounds, but it is costly, time-consuming, and requires specialized laboratories and operators. To address this, this study explores a functionalized acoustic sensor for detecting oenological polyphenols.

Results: The method involves utilizing a Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) to detect the target analyte by using a gelatin-based probe layer. The sensor is functionalized by optimizing the probe coverage density to maximize its performance. This is achieved by using 12-mercaptododecanoic acid (12-MCA) to immobilize the probe onto the gold sensor surface, and dithiothreitol (DTT) as a reducing and competitive binding agent. The concentration of 12-MCA and DTT in the solutions is varied to control the probe density. QCM-D measurements demonstrates that the probe density can be effectively adjusted using this approach, ranging from 0.2×10^{13} molecules cm^{-2} to 2×10^{13} molecules cm^{-2}.

This study also investigates the interaction between the probe and tannins, confirming the sensor ability to detect them. Interestingly, the lower probe coverage achieves higher detection signals when normalized to probe immobilization signals. Moreover, significant changes in mechanical properties of the functionalization layer are observed after the interaction with samples.

Conclusion: The combination of QCM-D with gelatin functionalization holds great promise for future applications in the wine industry. It offers real-time monitoring capabilities, requires minimal sample preparation, and provides high sensitivity for quality control purposes.

Keywords: QCM-D, sensor functionalization, gelatin, tannins, polyphenols

1. Introduction

Acoustic sensors are devices that can detect several kinds of analytes in various applications by exploiting acoustic waves [1]. These sensors are widely used due to their sensitivity and versatility [2-4]. There are various types of acoustic sensors: the main classes are Surface Acoustic Wave (SAW) sensors and Bulk Acoustic Wave (BAW) sensors. The working principle of SAW sensors is based on the generation and detection of acoustic waves propagating along the surface of a piezoelectric material [5], while BAW sensors utilize acoustic waves propagating through the entire thickness of the piezoelectric material [6]. Mechanical waves are then converted into electrical signals by the transducer composed of piezoelectric materials [7], such as the quartz. Piezoelectric materials are highly sensitive to mass variations, thus piezo transducers are highly responsive to detect mass changes at a molecular level. Due to their ability to measure mass changes on the sensor surface, these sensors are categorized as gravimetric sensors.

A widely used commercial apparatus based on BAWs is the Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) [8]. The QCM-D provides real-time measurements of mass changes and viscoelastic properties on the sensor surface. The main characteristics of the QCM-D include its ability to monitor mass deposition and the viscoelastic behavior of thin films, as well as providing valuable information about molecular interactions [9].

Acoustic sensors can find interesting applications in the agrifood sector. The ability to detect different analytes, like pollutants, pathogens, and chemical compounds, is crucial in ensuring the safety and quality of food products. Acoustic sensors offer rapid and reliable detection of

contaminants and allow monitoring food quality parameters [10]. In this field, the monitoring of oenological polyphenols plays a crucial role in the winemaking process [11-13].

Oenological polyphenols are a class of chemical compounds found in grapes, musts and wine. Such molecules have a profound impact on wine quality and aging potential, also contributing to color, taste, and mouthfeel [14]. Monitoring the levels of polyphenols can be useful in different stages of wine production, such as grape ripening, must maceration and wine aging. Polyphenol content can guide winemakers in optimizing their processes to achieve desired flavor profiles and enhance the overall wine quality. Gallic tannins, belonging to the family of polyphenols, play a crucial role in wine by contributing to its structure, color, and stability, providing a balanced flavor and a good tasting experience [15].

The advantages of using acoustic sensors for monitoring gallic tannins in the oenological industry are numerous and significant. Traditional analytical techniques for gallic tannins analysis (e.g. spectroscopy and chromatography) often involve time-consuming sample preparation, complex instrumentation and specialized operators. In contrast, acoustic sensors, such as the QCM-D, offer fast and cheap monitoring capabilities with minimal requirements.

The literature on acoustic sensor applications for monitoring polyphenols in oenology provides substantial evidence of its effectiveness. The QCM-D analysis, combined with tribology, was applied to evaluate the mouthfeel and astringency of red wines, demonstrating the effect of the shear forces on wine astringency while tannins interacted with salivary proteins [16]. A similar approach was used to deepen the role of tartaric acid in the interactions between salivary proteins and tannins [17], by studying the formation of complexes at a macromolecular level. Furthermore, the QCM-D was used to discriminate wines rich in tannins from those rich in proanthocyanidins [18]. A QCM-D approach was also proved to be useful to detect toxic compounds, like Ochratoxin, in red wines [19] in less than one hour and in low concentrations (ng mL^{-1}).

In a performed QCM-D analysis, it is critical to find a suitable probe for the analyte of interest. Gelatin finds extensive usage in winemaking for various purposes, like the purification process, where gelatin acts as a fining agent [20]. The amine groups in gelatin interact with tannins, forming crosslinks that precipitate out of the wine [21,22], resulting in a smoother and more palatable final product.

In the present work we have exploited the high affinity of gelatin toward tannins to develop the chemical functionalization of QCM-D sensors. We have analyzed the effect of gelatin

surface density with the aim to identify the best probe density coverage. The gelatin density was tuned by using an appropriate adlayer, composed of 12-mercaptododecanoic acid and dithiothreitol. Samples composed of commercial oenological tannins in water were measured, and the sensor response was analyzed and related to the probe density. Results indicates that the effect of probe coverage can be significant to maximize the sensor response.

2. Materials and Methods

2.1. Reagents

All the reagents utilized in this study were procured from Sigma Aldrich, unless otherwise specified. To enhance thiol-gold chemistry and saturate the sensor surface, 1,4-Dithiothreitol (DTT, Mw 154.2 Da), a reducing agent, was employed. The adlayer was obtained using 12-mercaptododecanoic acid (12-MCA, Mw 232.4 Da, purity degree 96%). For sensor functionalization, gelatin from porcine skin type A (Gel-A, High bloom Mw 75.0 kDa) was used as the probe. To form reactive esters essential for the probe immobilization (purity degree > 98%), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCl) and N-hydroxysuccinimide (NHS) were employed. Ethylene diamine (EDA) was used as blocking agent to deactivate residual active esters before the probe immobilization. Additionally, the oenological tannin commercial blend TANIN GALALCOOL (TG, polyphenol content > 95%) was utilized for sample preparation. All reagents were used in their as-received form.

2.2. QCM-D Measurements

The QCM-D (E4 model, Q-Sense AB, Sweden) measurements were conducted using polished AT-cut quartz crystals with gold electrodes. The crystals had a fundamental resonance frequency (F1) of 5 MHz, an overall diameter of 14 mm, a gold sensor diameter of 10 mm, and a quartz thickness of 300 µm (Biolin Scientific, Västra Frölunda, Sweden). The measurements were performed in static mode (stop flow) within fluidic cells that were thermostatted at 25 °C.

The apparatus simultaneously recorded the resonance frequency shift (Δf) and energy dissipation (ΔD) for up to n = 7 overtones by exciting the fundamental resonance frequency of the crystal. Throughout this study, all the overtones were monitored, and subsequently, the 7th resonance was selected as it exhibited the most stable performance across all acquisitions. By convention, the values of $\Delta F_n = \Delta f_n/n$ are reported below.

Overall, the QCM-D system effectively allowed for precise and simultaneous measurement of the resonance frequency and energy dissipation, providing valuable data for the study.

2.2.1. Preliminary analysis

To evaluate the ΔF solely due to DTT, a preliminary analysis was conducted. Five DTT solutions in water/ethanol (1:1 v/v) were prepared, with concentrations of 0.1 mg mL^{-1}, 1.2 mg mL^{-1}, 2.5 mg mL^{-1}, 4.3 mg mL^{-1}, and 6.2 mg mL^{-1}. Sensors mounted in microfluidic chambers were pre-rinsed with a mixture of water and ethanol (1:1 v/v), and data were acquired for 5 minutes. Subsequently, the DTT solution dissolved in a water/ethanol mixture (1:1 v/v) was injected into the microfluidic chamber and data were acquired for 30 minutes. Finally, the sensors were rinsed with water/ethanol (5 minutes) and water (5 minutes).

2.2.2. Sensor Functionalization

Before usage, the quartz crystals underwent a series of treatments. They were first rinsed with 2% sodium dodecyl sulfate, dried, and exposed to plasma oxygen (Femto Diener) for 2 minutes at a power of 100 W. Next, they were immersed in a 5:1:1 solution of water, ammonia (32% v/v), and hydrogen peroxide (25% v/v) at 75°C for 15 minutes. Afterward, the crystals were rinsed with water and isopropanol, and plasma oxygen treatment was applied again for 2 minutes at 100 W.

To modify the gold surface of the quartz crystal, an adlayer based on 12-mercaptododecanoic acid (12-MCA) and dithiothreitol (DTT) was formed using thiol-gold chemistry. To achieve this, sensors mounted in microfluidic chambers were pre-rinsed with a mixture of water and ethanol (1:1 v/v), and data were acquired for 5 minutes. Subsequently, a solution of 12-MCA dissolved in a water/ethanol mixture (1:1 v/v) containing DTT as a reducing agent (adlayer solution) was injected into the microfluidic chamber for 30 minutes. The 12-MCA concentration ranged from 0.1 mg mL^{-1} to 4 mg mL^{-1}, while the amount of DTT moles added to the adlayer solution was 0.1× or 1.0× in respect to those of 12-MCA. DTT concentrations were comprised in the range from 0.01 mg mL^{-1} to 2.65 mg mL^{-1}. Finally, the sensors were rinsed with water/ethanol (5 minutes) and water (5 minutes). The resulting adlayer exposed the carboxylic functionalities of 12-MCA towards the water phase, allowing subsequent Gel-A immobilization.

To immobilize the probe molecules, the carboxylic functionalities were activated by injecting a water solution containing EDCl/NHS (10 mM each, activation solution) into the QCM-D chambers for 20 minutes, followed by rinsing with water for 5 minutes. The next step involved the conjugation of the probe at a controlled pH of 9 using a phosphate buffer. Sensors were pre-rinsed with the buffer (5 minutes), and then the protein solution (probe solution) was injected (1 mg mL^{-1}) for 30 minutes. After the conjugation, sensors were rinsed with buffer (5 minutes) and water (5 minutes). The final step was the deactivation of residual NHS-esters, achieved by rinsing the sensors with a 0.1 M EDA solution in water (blocking solution) for 5 minutes, followed by water rinsing (5 minutes).

A schematic of the functionalization process is depicted in Figure1a, as well as the traces of a typical QCM-D run is reported in Figure 1b.

< Figure 1 >

2.3. Sample Detection

After the functionalization, sample solutions containing 0.1 mg mL^{-1} of TG in water were injected (15 min). Finally, the sensors were rinsed with water (5 min).

2.4. Data Analysis

The results for ΔF and ΔD were obtained by calculating the difference between the baseline (water) and the signals acquired after water rinsing. Four experiments were conducted for each condition, and ΔF and ΔD were continuously recorded throughout the experiment. The reported data include ΔF (Hz), ΔD (dimensionless), normalized ΔF (dimensionless), and molecular density (molecules cm^{-2}). The values presented are the means of replicates (N), and error bars represent the standard errors. The fundamental frequency F_1 is strongly affected by environmental noise [23], thus we have decided to neglect it and consider only harmonics with n > 1.

The mass adhered to the sensor surface was evaluated at each functionalization step by using the Sauerbrey model [24]. The Sauerbrey equation reads:

$$\Delta m = -C \cdot \Delta F_n \quad \text{(Eq. 1)}$$

In this equation, Δm represents the variation in sensor mass (ng cm^{-2}), C is the mass sensitivity constant specific to the physical properties of the quartz crystal (17.7 ng cm^{-2} Hz^{-}

[1] for quartz crystals with F_1 = 5 MHz), n is the overtone number, and ΔF_n denotes the frequency shift at a given time and overtone.

The validity of the Sauerbrey model is limited to thin and rigid layers. Its limitations have been extensively discussed, and qualitative data analysis can estimate its applicability. Three widely accepted criteria are as follows: 1) a small dependence of ΔF_n on n, 2) ΔD_n values below 2×10^{-6}, and 3) $\Delta D_n/(-\Delta F_n) << 4\times10^{-7}$ Hz. When any of these criteria are met, the adlayer can be considered as rigid, and the Sauerbrey model remains valid [25,26].

To calculate the number of molecules and evaluate molecular density, the areal mass (ng cm^{-2}) was utilized as follows:

$$molecular\ density\ = \frac{areal\ mass}{M_w} \cdot N_A \qquad \text{(Eq. 2)}$$

In this equation, M_w represents the molecular weight of the considered compound, and N_A is the Avogadro number (6.022×10^{23} mol^{-1}). For the experiments with DTT 0.1×, an average molecular weight of 225.3 Da was employed, while for the experiments with DTT 1×, the average molecular weight used was 193.3 Da.

We have analyzed data from adlayer formation, probe immobilization, blocking and sample detection. We have neglected the analysis of carboxylic acid activation because signals were very low, due to the very limited mass loading.

3. Results and Discussion

3.1. Preliminary analysis

The formation of the adlayer caused a decrease in the resonance frequency of the sensors, resulting in a negative shift relative to the baseline. This behavior is typical for gravimetric acoustic sensors, where an increase in sensor mass (mass loading) leads to a decrease in vibrational frequency. The ΔF_7 values obtained in the preliminary analysis ranged from –17 Hz ± 1.8 Hz to –32 Hz ± 2.5 Hz, with no monotonic dependence on DTT concentration (Figure2a). Gravimetric acoustic sensors commonly exhibit positive dissipation shifts, indicating mechanical energy dissipative effects. The measured ΔD values in these experiments were consistently positive and ranged from 4×10^{-6} to 10×10^{-6} (Figure2b).

These data were used to determine the mass of the adhered DTT (Figure2c). As the ΔF_n showed no significant dependence on n, we calculated the DTT mass adhered to the sensor, considering the molecular layer as rigid. The number of molecules attached to the sensor

surface was then determined using the Sauerbrey Equation (Eq. 1), ranging from 1.17×10^{15} molecules cm^{-2} ± 1.22×10^{14} molecules cm^{-2} to 2.24×10^{15} molecules cm^{-2} ± 1.71×10^{14} molecules cm^{-2}.

The nonmonotonic of signal trends indicated better sensor coverages at low (0.10× and 0.25×) and high (2.00×) DTT concentrations, but smaller coverages for intermediate (0.50× and 1.00×) DTT concentrations. We can hypothesize that the solution concentration can affect the adlayer packing but also that the overall –SH concentration used for subsequent experiments was sufficient to cover the entire sensor surface. The proposed hypothesis suggests that exists a saturation limit for the thiol-gold binding sites, reached even at the lowest concentrations of DTT. At intermediate concentrations, interactions between DTT molecules in solution may limit the availability of thiol groups for binding to the gold surface. This could be attributed to intermolecular interactions hindering effective anchoring. However, at higher concentrations, a shift similar to those measured at lower concentrations is observed, implying a safe DTT concentration where, although molecules interact in the liquid phase, yet a significant number remains available for immobilization on the sensor surface.

Furthermore, the parabolic nature of the dissipation curve lends support to our hypothesis. The observed minimum in dissipation at intermediate concentrations aligns with the notion that interactions among DTT molecules affect the viscoelastic properties of the formed film. This suggests a delicate balance between the film formation and internal interactions of the molecules, highlighting a potential optimization or equilibrium at intermediate concentrations.

Basing on this preliminary analysis, the modulation of probe density in experiments involving adlayers with different 12-MCA/DTT ratios should rely solely on the –COOH groups concentration/availability, rather than any poor adlayer configuration due to a non-uniform sensor coverage.

< Figure 2 >

3.2. Sensor functionalization

3.2.1. Adlayer formation

The formation of the adlayer led to a decrease in the resonance frequency of the sensors, resulting in a negative shift relative to the baseline due to mass loading. During the adlayer formation, the measured ΔF_7 values did not exhibit a noticeable trend with varying the 12-MCA concentration (Figure 3a). This lack of concentration effect can be explained by considering that the overall concentrations of the adlayer solutions were very close to or higher than the –SH concentration calculated in the preliminary analysis (1.6 µmol mL^{-1}). Consequently, complete sensor coverage was achieved in all cases.

The mass loading after the adlayer formation led to positive dissipation shifts (Figure 3b). The Sauerbrey model can still be considered valid as values of ΔF_n did not significantly vary with n (data not shown).

The calculated values of molecular density (Figure 3c) exhibited a trend that was not observed in the ΔF signals analysis. We noticed a slight increase in molecular density with increasing 12-MCA concentration in the adlayer solutions, which tended to plateau for both DTT concentrations. This observation could suggest that in the competitive reactions between 12-MCA-Au and DTT-Au, the 12-MCA reactant is favored, and the final adlayer composition differs from the solution concentration, as initially assumed. This aspect was further supported by the subsequent analysis on probe immobilization.

3.2.2. Probe immobilization

The effect of mass loading was observed during probe immobilization, resulting in negative frequency shifts in all cases (Figure 3d). The ΔF7 values measured during probe immobilization demonstrated a clear dependency on the 12-MCA concentration in the adlayer solution. Absolute values of ΔF_7 increased with higher 12-MCA concentrations, except for the experiment involving the highest 12-MCA concentration with DTT 1×. In this particular case, it is likely that the DTT concentration was too high, leading to an unfavorable binding of 12-MCA. As Gel-A was the only molecule involved in this step, the signals were solely attributed to protein immobilization. The trend of increased immobilized protein molecules over the adlayer can be attributed to higher amounts of activated esters bearing 12-MCA in comparison to non-active sites bearing DTT towards Gel-A. This observation supports our previous hypothesis of a favored 12-MCA-Au reaction at higher 12-MCA concentrations in the adlayer solution, compared to the DTT-Au reaction. Similar trends were detected in ΔD_7 values (Figure

3e), suggesting that the overall behavior of the functionalization layer becomes more viscoelastic at higher 12-MCA concentrations in the adlayer solutions. This also aligns with our hypothesis, considering that proteins generally exhibit a viscoelastic behavior when conjugated to a solid substrate.

Although the Gel-A layer had a viscoelastic behavior, the Gel-A molecular density (Figure 3f) was calculated using the Sauerbrey model for coherence. This assumption only slightly underestimated the Gel-A molecular density. The detected trend indicated that for 12-MCA concentrations < 2 mg mL^{-1} in the adlayer solutions, the probe density increased with increasing 12-MCA concentrations, independently of the DTT concentration. However, for higher concentrations, the DTT had an effect, limiting the probe immobilization.

3.2.3. Blocking

In the blocking step, EDA was used to deactivate residual NHS-esters that had not reacted with Gel-A and were still active. The ΔF_7 values (Figure 3g) were slightly negative, indicating a small mass loading, and no specific trend with the adlayer composition was highlighted. On the other hand, ΔD_7 values (Figure 3h) showed a trend only in experiments with the adlayer obtained with 1.0× DTT. Values increased, suggesting a more pronounced viscoelastic behavior of the functionalization layer. The increased viscoelasticity could be attributed to the larger hydrophilicity of the adlayer and enhanced hydration of the probe layer, as EDA is a small and hydrophilic molecule bearing a free amine after reacting with the residual activated NHS-esters. The molecular density of EDA (Figure 3i) did not exhibit a clear trend with the adlayer composition.

Obtained results confirmed that our approach to tune the probe density is valid.

< Figure 3 >

3.3. Sample detection

The literature suggests that the interactions between tannins and Gel-A involve electrostatic bonds between positively charged proline units and negatively charged gallic acid units, leading to mild crosslinking and the formation of a stable Gel-A physical network. This caused the probe layer to shrink, expelling water molecules that hydrate Gel-A and resulting in a positive frequency shift in ΔF traces.

The ΔF_7 values increased at lower 12-MCA concentrations in the adlayer solution and decreased at higher 12-MCA concentrations (Figure 4a), indicating the importance of controlling the probe molecular density for optimizing sensor functionalization. The maximum ΔF_7 values were observed at 1 mg mL^{-1} for experiments with 0.1× DTT and 0.25 mg mL^{-1} for experiments with 1.0× DTT. A similar parabolic trend, but with negative values, was observed for ΔD_7 (Figure 4b), indicating a more marked viscoelastic behavior of the functionalization layer over the sensor after the interaction with the analyte. Notably, negative values of dissipation were observed during the detection of gallic tannins, suggesting that the functionalization layer became more rigid in all cases. The stiffest behavior was observed at intermediate 12-MCA concentrations in the adlayer solution. Considering the trend measured for the molecular density of the probe (Figure 3f), we can suppose that the amount of crosslinked Gel-A sites increased with increasing the 12-MCA concentration in the adlayer solution. The presence of –OH groups in the adlayer, particularly in experiments with DTT 1.0×, contributed to the stiffening of the probe layer even at lower 12-MCA concentrations. However, at higher 12-MCA concentrations, the stiffness of the probe layer tended to decrease, possibly due to the large number of crosslinkable Gel-A sites and the negative charges of the tannins being insufficient to saturate them, leading to an increase in ΔD_7 values and a lower stiffening effect.

An interesting observation pertained to the absolute ratio between ΔF_7 obtained after sample detection and after probe immobilization (Figure 4c). The data indicated that this ratio decreased with an increase in probe density, suggesting that, at the same sample concentration, a maximized signal was achieved with a lower probe density, regardless of the amount of DTT used for the adlayer preparation. The observed behavior can be attributed to two aspects: i) the prolines were more available and freer to interact with polyphenols at lower probe densities and ii) the positive frequency shift due to water expulsion after the probe layer crosslinking was partially balanced by a negative shift due mass loading, which was more pronounced when the sample/probe mass ratio increased.

< Figure 4 >

4. Conclusion

Tuning the probe coverage over acoustic sensors plays a crucial role in optimizing sensor performance. Effective regulation of probe density can be achieved through the use of a suitable adlayer for probe immobilization. In this study, we utilized 12-mercaptododecanoic acid (12-MCA) and dithiothreitol (DTT) to create the adlayer, varying the concentration of both molecules in the sensor functionalization solution. Both 12-MCA and DTT molecules possess free –SH groups capable of binding to the gold surface of the sensor, resulting in a competitive functionalization process. Our findings indicate that modulation of adlayer properties is possible by adjusting the concentrations of both 12-MCA and DTT. Specifically, the adlayer exhibited a higher content of 12-MCA units when formed from more concentrated solutions. However, DTT concentration did not significantly impact adlayer formation or probe immobilization. Conversely, the influence of DTT was more noticeable during sample detection, where lower DTT concentrations resulted in more pronounced signals (absolute values) in both frequency and dissipation measurements. Nevertheless, the normalized ΔF was less affected by DTT concentration.

In the realm of acoustic sensor functionalization, providing additional details about the approach used to regulate the probe density holds significance, as highlighted in our findings. Through our investigation, the application of QCM-D has demonstrated efficacy and efficiency in detecting oenological gallic tannins. The real-time monitoring capabilities, minimal sample preparation, and high sensitivity of the proposed strategy suggest its potential for wine analysis.

The findings pave the way for further investigations aimed at gaining a deeper understanding of the interactions between tannins and Gel-A, potentially leading to the development of more efficient and tailored functionalization strategies for acoustic sensors. The successful implementation of QCM-D with Gel-A for wine-related analyses could extend its application in other aspects of the agrifood industry, such as the monitoring of polyphenols in olives and olive oils.

Integrating QCM-D technology with gelatin-based functionalization provides a tool to monitor the evolution of wine characteristics during fermentation, aging, and blending, enabling precise control over wine quality and flavor development.

In conclusion, the adaptability and potential of the proposed approach to tune probe density present promising prospects for various fields, creating opportunities for innovation and practical applications beyond wine analysis.

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