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Molecular Structure Underlying the Allosteric Mechanism of Caffeine Detection in Taste Sensor

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Abstract: The use of taste sensors with lipid/polymer membranes is one of the methods to evaluate taste. As previously reported, taste sensors can detect non-charged substances such as caffeine by modifying the lipid/polymer membranes with hydroxybenzoic acids (HBAs). The mechanism of caffeine detection by taste sensors was identified to be an allosteric one. Generally, the allosteric mechanism, defined as "regulation at distant sites", is used to describe the regulation process for proteins. In this study, to improve the sensitivity of taste sensors to caffeine and its analogs using the allosteric mechanism, we used various modifiers of lipid/polymer membranes, and we detected caffeine using taste sensors with the modified membranes. The detection of the caffeine analogs theophylline and theobromine was also analyzed. The results of caffeine detection clarified that the molecular structure underlying the allosteric mechanism capable of effective caffeine detection involves both the carboxyl and hydroxyl groups, where the hydroxyl group can form intermolecular H bonds with caffeine. Furthermore, the taste sensors with a modifier, which has the molecular structure underlying the allosteric mechanism, showed high sensitivity to caffeine and caffeine analogs. The use of an allosteric mechanism may help improve the sensitivity of taste sensors to other non-charged pharmaceutical substances, such as dexamethasone and prednisolone, in the future.

Keywords: taste sensor; lipid/polymer membrane; allosteric mechanism; caffeine detection; surface modification

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1. Introduction

The technology to quantify the taste felt by humans has been developed worldwide. Up to now, one of the methods to evaluate taste is to use taste sensors or electronic tongues; many reports on measurements of plural kinds of foods and medicines have been made [1–8] and many review papers have been published [9–23]. Electronic noses are used to measure gas, and electronic tongues are used to measure liquid [18,20]. The working principle of these instruments is based on the employment of an array of a few different nonselective chemical sensors [24]. They have the following commonalities: nonselective sensing components [24], sensor arrays [18], data analysis with multivariate analysis or artificial neural networks [15,25]. Taste information of sample solutions can be evaluated systematically using electronic tongues and taste sensors. Electronic tongues measure the ions of sample solutions using ion-specific electrodes [25] or pulse voltammetry [15] and then provide taste information of sample solutions using multivariate analysis or artificial neural networks. Taste sensors with lipid/polymer membranes are intended to distinguish and quantify each taste in a manner similar to the human sense of taste. An example of taste sensors is TS-5000Z (Intelligent Sensor Technology, Inc., Kanagawa, Japan), which can quantify taste by detecting the potential difference between the reference and sensor electrodes [1,9]. The sensor electrode was attached

with membranes consisting of lipids, plasticizers, and supporting materials [2,10]. By changing the composition of the lipid/polymer membrane, one can obtain a taste sensor that can quantify the five basic tastes. Moreover, the study [26] confirmed the durability and repeatability of the astringency sensor by using 100 times repeated measurements of commercial black tea. Nevertheless, such a taste sensor is not sensitive to non-charged substances because the functionality of taste sensors depends on their capability to measure the change in membrane potential caused by charged substances.

Caffeine, one of the non-charged substances, can be detected by using high-performance liquid chromatography (HPLC) [27,28]. In a real solution, the caffeine content of beverages is roughly 2 mM [29]. In our previous study [30], we used taste sensors with lipid/polymer membranes modified with hydroxybenzoic acids (HBAs) to detect non-charged bitter substances, such as caffeine and caffeine analogs. Another previous study by our group [31] revealed the effect of HBAs on detection, and we indicated that the log P and pKa of HBAs affect the response of taste sensors to caffeine. Moreover, we confirmed by NMR measurement the formation of H bonds between caffeine and HBAs and identified the mechanism of caffeine detection to be an allosteric one. The formation of H bonds between HBAs and caffeine causes the intramolecular H bonds, which were formed between the carboxyl and hydroxyl groups of the HBAs, to be broken. Then, H^+ in the caffeine solution returns to the carboxyl group, resulting in increasing the membrane potential positively [32].

The allosteric mechanism, defined as "regulation at distant sites", is the most direct, rapid, and effective regulatory mechanism for sensing changes in the concentration of small-molecule compounds and the cellular responses [33,34]. Generally, the allosteric mechanism is used to describe the regulation process for proteins [33–36]. A previous biochemical study [37] revealed that an allosteric mechanism contains four elements: the active site, allosteric site, ligand, and substrate, and its hallmark feature was discussed as follows: When a ligand binds to an allosteric site of a protein, it will lead to a higher affinity for the second ligand or substrate at the active site. While our previous study [32] indicated that the detection of non-charged substances by taste sensors relies on the allosteric mechanism, we still need to clarify the molecular structure of modifiers underlying the allosteric mechanism capable of effective caffeine detection. On this basis, we use the allosteric mechanism to improve the sensitivity of taste sensors to caffeine and its analogs.

In this study, we used a three-step process to clarify the structure of the allosteric mechanism of detecting caffeine and improve the sensitivity of taste sensors to caffeine. Step 1: We detected caffeine using taste sensors modified with 2,6-dihydroxybenzoic acid (2,6-DHBA) and three other modifiers, such as aniline, resorcinol, and benzoic acid (BA). By comparing the results in Step 1, we determined the molecular structure underlying the allosteric mechanism of detecting caffeine. Step 2: To improve the sensitivity of the taste sensors to caffeine, we measured the electric response of caffeine using the taste sensors treated with another three modifiers with the same substructure as 2,6-DHBA, namely, 2,6-dihydroxyterephthalic acid (2,6-DHTA), 1,3-dihydroxy-2-naphthoic acid (1,3-DHNA), and 3-Bromo-2,6-dihydroxybenzoic acid (3-Br-2,6-DHBA). Step 3: to improve the sensitivity of the taste sensors to caffeine analogs, we detected theophylline and theobromine using taste sensors treated with 2,6-DHTA and 3-Br-2,6-DHBA. The results showed that the taste sensors treated with 2,6-DHTA and 3-Br-2,6-DHBA had high sensitivity to caffeine analogs.

2. Materials and Methods

2.1. Reagents

We used tetrahydrofuran (THF) as an organic solvent and the tetra(decyl)ammonium bromide (TDAB) as a lipid, both of which were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Dioctyl phenyl-phosphonate (DOPP) was used as a plasticizer, and polyvinyl chloride (PVC) was used as the supporting material. DOPP was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). PVC was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Caffeine, theophylline, and theobromine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Aniline, 1,3-DHNA, and 3-Br-2,6-DHBA were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Resorcinol and BA were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). 2,6-DHBA, tannic acid, and potassium chloride (KCl) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 2,6-DHTA was purchased from Ambeed, Inc. (Arlington Heights, IL, USA). Figure 1 shows the structural formula of theophylline, theobromine, aniline, resorcinol, BA, 2,6-DHBA, 1,3-DHNA, 3-Br-2,6-DHBA, and 2,6-DHTA.

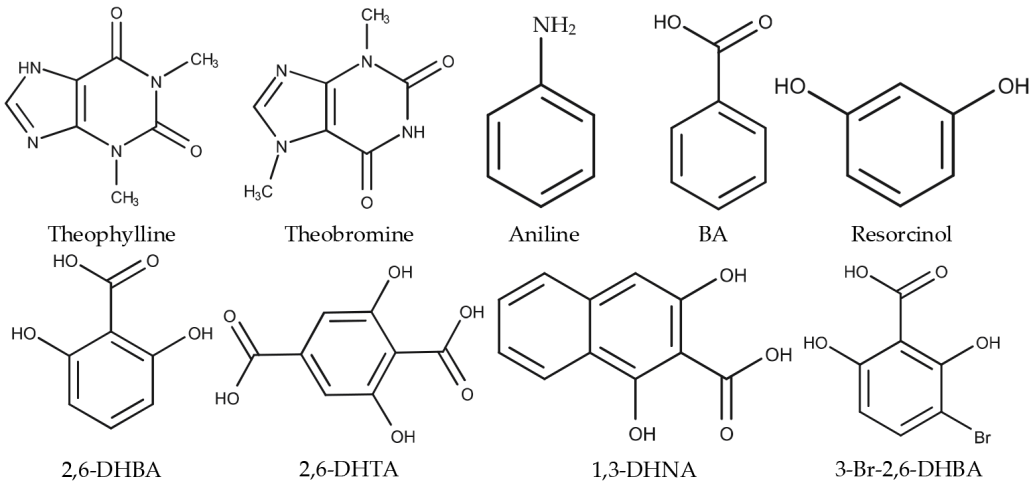

Figure 1. Structural formula of theophylline, theobromine, aniline, resorcinol, BA, 2,6-DHBA, 2,6-DHTA, 1,3-DHNA, and 3-Br-2,6-DHBA.

2.2. Lipid/Polymer Membrane and Surface Modification

To compare our previous research [30,31] on the same membrane composition, we used the membrane with lipid TDAB. The fabrication of the TDAB membrane included three steps. Firstly, 10 mL of 0.3 mM TDAB with THF, 1.5 mL DOPP, and 800 mg PVC were stirred in a screw tube. In the second place, by volatilizing THF of the resulting mixture solution on a Petri dish (90 mm ϕ), the 0.3 mM TDAB membrane was formed. Finally, a piece of the 0.3 mM TDAB was attached to a probe. The lipid membranes with 1 mM and 3 mM TDAB were also prepared similarly.

For the modifiers to be adsorbed onto the lipid/polymer membrane, the sensor electrodes were soaked in the modification solution for 72 h. A previous study [30] revealed that the sensitivity of a taste sensor to non-charged bitter substances was improved by soaking the lipid/polymer membrane in a modification solution. As is also shown in another study [38], liquid-membrane electrodes based on charged sites generally show permselectivity for opposite-charge ions, which indicates that TDAB membranes have a strong electrostatic interaction with negatively charged ions. While the Br^- from TDAB and the ionized modifiers have negative charges, modifiers such as 2,6-DHBA, BA, 2,6-DHTA, 1,3-DHNA, and 3-Br-2,6-DHBA have very high hydrophobicity, and ionized modifiers can be adsorbed onto the membrane surface through hydrophobic interactions. Thus, ionized aniline with a positive charge and resorcinol without charge can also be adsorbed onto the membrane surface through hydrophobic interactions.

2.3. Procedure of Taste Sensor Measurement

TS-5000Z taste sensing system (Intelligent Sensor Technology, Inc., Kanagawa, Japan) was used for all measurements. The taste sensor detection unit is outfitted with a reference electrode and four sensor electrodes. An Ag/AgCl (3.33 M KCl) reference electrode was used as the reference electrode. Figure 2 shows the structure of sensor and reference electrodes.

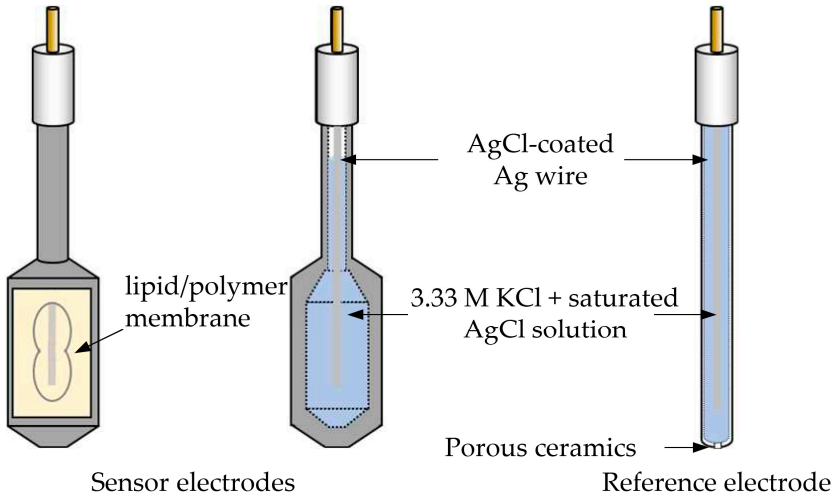

Figure 2. Structure of sensor and reference electrodes.

Figure 3 shows the measurement procedure for the taste sensor. Firstly, the detection unit was immersed in a reference solution containing 30 mM KCL and 0.3 mM tartaric acid for 30 s. The membrane potential change between the reference and sensor electrodes was recorded for 30 s. The membrane potential change at the 30th second is used as the sensor output. The potential difference between the reference and sensor electrodes was designated as Vr. Secondly, the detection unit was immersed in a sample solution for 30 s, and the potential difference between the reference and sensor electrode was designated as Vs. The difference between Vs and Vr was considered as the response value. Finally, the membrane surface was refreshed with a water-based cleaning solution of 10 mM KOH, 100 mM KCl, and 30 vol% EtOH. The entire process was performed four times. The mean values and standard deviations (SDs) were calculated from $n = 4$ (electrode) $\times$ 4 (rotation) = 16 electrical response values.

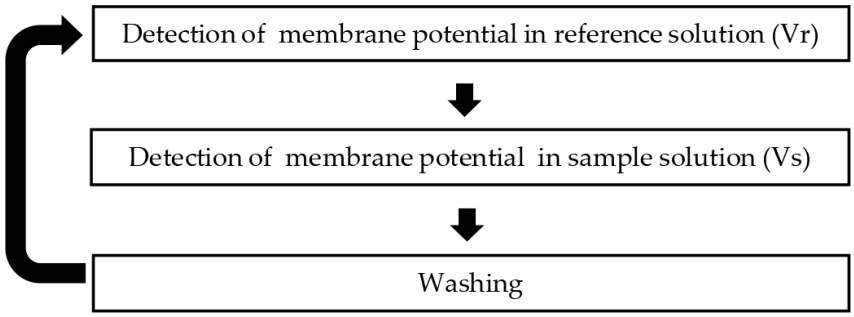

Figure 3. Measurement procedure for the taste sensor.

For the preparation of the measuring caffeine sample solution, caffeine was dissolved into the reference solution. Our previous studies [30–32] prepared the caffeine sample solution based on the reference solution. To compare the results with those of the previous studies, the preparation of caffeine sample solutions in this study used the same method. Since the solubility of theophylline and theobromine is lower than that of caffeine, the concentration of the theophylline and theobromine sample solution was prepared up to 30 mM.

2.4. Step 1: Measurement of Caffeine Using Taste Sensors with Aniline, BA, Resorcinol, and 2,6-DHBA

100 mM caffeine in reference solutions were detected by the taste sensors with the lipid/polymer membrane modified with 0.3 wt% 2,6-DHBA, BA, resorcinol, and aniline. To compare our previous research [30,32] on the same experimental conditions, we used the same measurement conditions: The pH of measuring conditions is 3.5, the measuring

temperature is 25 °C, and the measurement machine is TS-5000Z taste sensing system (Intelligent 122 Sensor Technology, Inc., Kanagawa, Japan). To confirm the reliability of the sensors during the measurement, we used four of the same type of sensor electrodes for each detection unit. Next, the taste sensor with the detection unit was repeatedly run four times according to the procedure in Section 2.3. The mean values and standard deviations (SDs) of measurement data were calculated, and the calculation results were used to make Figure 4. From the SDs, we can confirm the stability of the detection unit when measuring the sample.

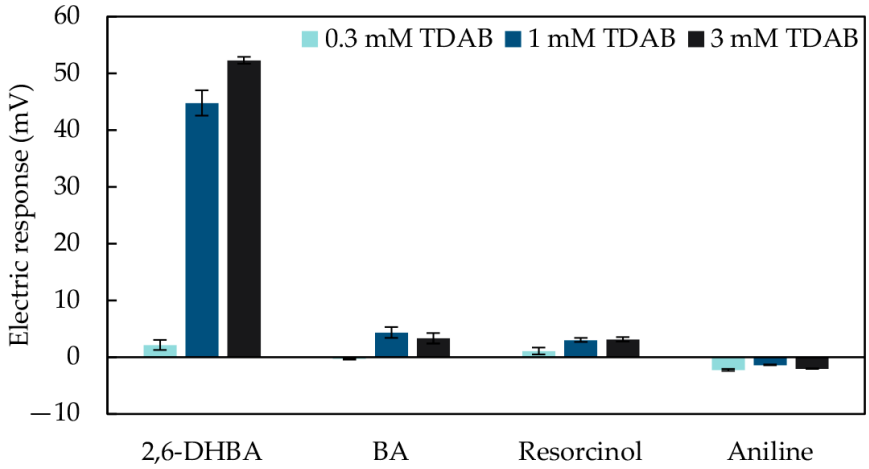

Figure 4. Response to 100 mM caffeine measured using taste sensors with lipid/polymer membrane modified with 2,6-DHBA, BA, resorcinol, and aniline. The error bar expresses the SD of the data, n = 4 (electrode) × 4 (rotation) = 16 values.

2.5. Step 2: Measurement of Caffeine Using Taste Sensors with 2,6-DHTA, 1,3-DHNA, and 3-Br-2,6-DHBA

We measured the electric response of caffeine (0.1, 0.3, 1, 3, 30, 100 mM in reference solution) using the taste sensors with the lipid/polymer membrane modified with 2,6-DHTA, 1,3-DHNA, and 3-Br-2,6-DHBA. The compositions and types of modification solutions used in Step 2 are shown in Table 1. The mean values and standard deviations (SDs) were calculated from n = 4 (electrode) × 4 (rotation) = 16 electrical response values.

Table 1. Compositions of and types of modification solutions used in Step 2.

Composition	Concentration
2,6-DHTA	0.001, 0.003, 0.01, 0.03 wt%
1,3-DHNA	0.001, 0.003, 0.01, 0.03, 0.06, 0.1 wt%
3-Br-2,6-DHBA	0.001, 0.003, 0.01, 0.03, 0.1, 0.3 wt%

2.6. Step 3: Measurement of Caffeine Analogs Using Taste Sensors with 2,6-DHTA and 3-Br-2,6-DHBA

We detected theophylline and theobromine under the experimental conditions that increased the sensitivity to caffeine in Step 2. The experimental conditions and compositions of sample solutions used in Step 3 are shown in Table 2. The mean values and standard deviations (SDs) were calculated from n = 8 (electrode) × 1 (rotation) = 8 electrical response values. To confirm the selectivity of the sensors treated with 3-Br-2,6-DHBA, we measured samples of five basic tastes and astringency. The compositions and concentrations of the measured samples were the same as those used in our previous study [30].

Table 2. Experimental conditions and compositions of sample solutions used in Step 3.

Experimental Conditions for Maximal Response to Caffeine	Sample
1 mM TDAB membrane modified with 0.03 wt% 2,6-DHTA 3 mM TDAB membrane modified with 0.03 wt% 3-Br-2,6-DHBA	0.1, 0.3, 1, 3, 30 mM theophylline and theobromine in reference solution

3. Results and Discussion

3.1. Step 1: Measurement of Caffeine with 2,6-DHBA, BA, Resorcinol, and Aniline

In our previous study [32], the interaction between caffeine and 2,6-DHBA was proved by NMR measurements as being due to H bond formation, and the mechanism of caffeine detection was proposed to be an allosteric one. However, our systematic understanding of the type of molecular structure underlying the allosteric mechanism capable of effective caffeine detection still needs to be improved. Thus, we detected caffeine using taste sensors with a lipid/polymer membrane modified with four modifiers (2,6-DHBA, aniline, BA, and resorcinol).

The results, shown in Figure 4, showed that the sensor with the membranes modified with 2,6-DHBA responds effectively to 100 mM caffeine, whereas the others are not responsive to caffeine. These results are consistent with the results reported previously [30–32]. This indicates that the structure of 2,6-DHBA in taste sensors has the allosteric mechanism capable of caffeine detection.

Comparing the structures of 2,6-DHBA and aniline shown in Figure 4, we found that the lipid/polymer membrane modified with aniline cannot effectively bind with caffeine through the H bonds because aniline cannot form H bonds with caffeine via the hydroxyl group, resulting in being nonresponse to caffeine by the taste sensor with this modified membrane. While BA can form H bonds with caffeine via the carboxyl group [39], taste sensors treated with BA cannot respond effectively to caffeine. This is because the H bonds formed by the carboxyl group and caffeine cannot induce a significant change in membrane potential by themselves. Similarly, resorcinol can form H bonds with caffeine, but the caffeine response obtained from resorcinol was insignificant. Thus, these findings revealed that the allosteric mechanism of detecting caffeine involves both carboxyl and hydroxyl groups, where the hydroxyl group can form intermolecular H bonds with caffeine, as shown in Figure 5.

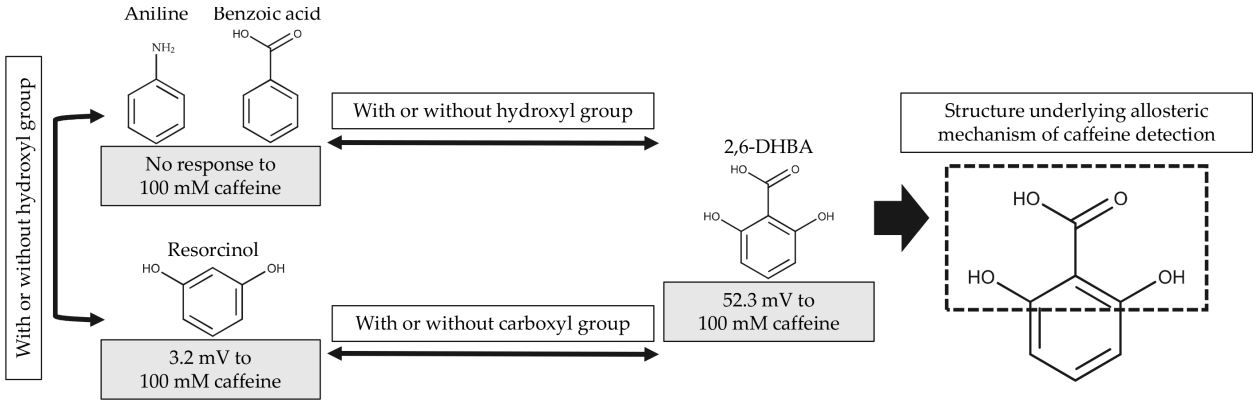

Figure 5. Flow diagram for the derivation of the allosteric mechanism of detecting caffeine. The grey box expresses the maximum response to 100 mM caffeine for the taste sensor modified with each modifier. The black dashed-line box expresses the structure of the allosteric molecule capable of detecting caffeine for taste sensors.

Moreover, the elements of a model of allosteric mechanism and caffeine detection are listed in Table 3. On the basis of Table 3 and Figure 5, we propose that the allosteric site is the hydroxyl group that can form hydrogen bonds with caffeine; the active site is the carboxyl group; the ligand is caffeine, and the substrate is H^+ ionized by the carboxyl group.

Thus, aniline did not have the allosteric mechanism of detecting; BA only has a part of the allosteric mechanism of detecting caffeine (the active site); aniline only has the allosteric site of the allosteric mechanism. As BA, resorcinol, and aniline do not have a complete allosteric mechanism of detecting caffeine, the sensors treated with them cannot respond to caffeine effectively. The taste sensor with lipid/polymer membrane modified with 2,6-DHBA showed good sensitivity to caffeine owing to 2,6-DHBA taking the completed allosteric mechanism of detecting caffeine.

Table 3. Elements of the allosteric mechanism and caffeine detection.

Model of Allosteric Mechanism	Elements of Caffeine Detection
Allosteric site	Hydroxyl group that can form H bonds with caffeine
Active site	Carboxyl group
Ligand	Caffeine
Substrate	H^+ ionized by carboxyl group

3.2. Step 2: Measurement of Caffeine with 2,6-DHTA, 1,3-DHNA, and 3-Br-2,6-DHBA

In Figure 5, we show the allosteric mechanism of detecting caffeine for taste sensors. Nevertheless, this mechanism was only derived from the modifiers (2,6-DHBA, BA, aniline, and resorcinol) that we investigated in our previous studies [30–32]. To further confirm the allosteric mechanism of detecting caffeine with taste sensors and improve the sensitivity of taste sensors to caffeine, we measured the electric response to caffeine using the taste sensors with the lipid/polymer membrane modified with three other modifiers whose substructure is the same as that of 2,6-DHBA. We chose three modifiers, 2,6-DHTA, 1,3-DHNA, and 3-Br-2,6-DHBA, for this confirmation. The results are summarized in Figures 6–8.

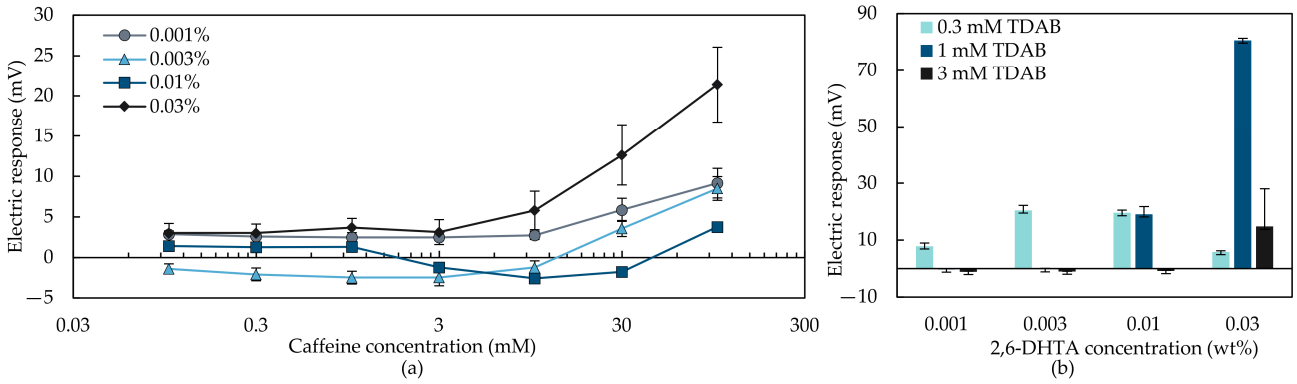

Figure 6. Response to caffeine in sample solutions measured using taste sensors with the membrane modified with 2,6-DHTA: (**a**) Concentration of caffeine determined using the taste sensors with 0.3 mM TDAB membrane modified with different concentrations of 2,6-DHTA as a function of the response value; (**b**) electric response to 100 mM caffeine when using lipid/polymer membrane modified with different concentrations of 2,6-DHTA. The error bar indicates the SD of data, n = 4 (electrode) × 4 (rotation) = 16 values.

3.2.1. 2,6-DHTA

Figure 6a shows the relationship between the caffeine concentrations and the response values obtained from the taste sensors treated with 2,6-DHTA. It indicated that the response to caffeine increased with the caffeine sample concentration. This phenomenon demonstrated that the taste sensors treated with 2,6-DHTA were able to detect caffeine. Figure 6b shows the response to 100 mM caffeine measured using taste sensors with the lipid/membrane modified with 2,6-DHTA. The sensor with the 1 mM TDAB membrane modified with 0.03 wt% 2,6-DHTA showed the highest response (80.1 mV) to caffeine, which is higher than that obtained with 2,6-DHBA (52.3 mV). Thus, the use of 2,6-DHTA improved the sensitivity of the taste sensor to caffeine.

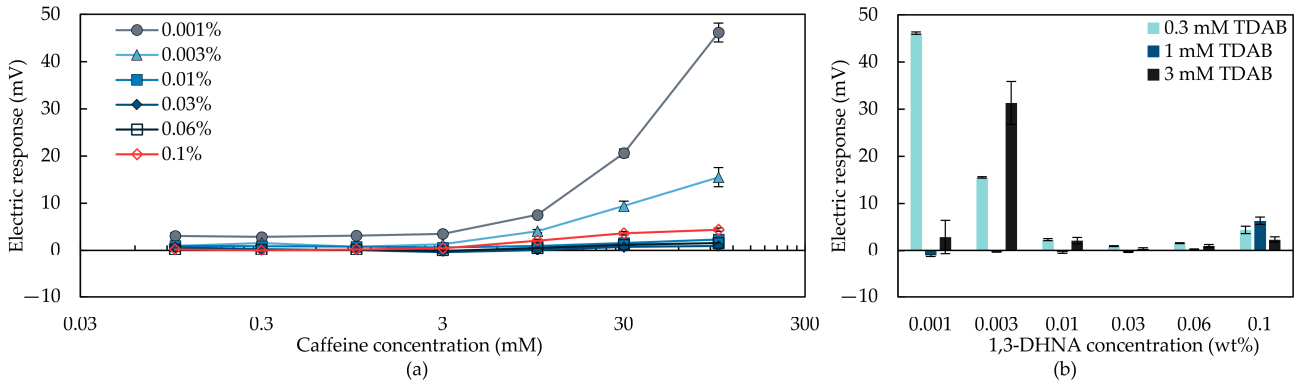

Figure 7. Response to caffeine in sample solutions measured using taste sensors with membrane modified with 1,3-DHNA: (**a**) Concentration of caffeine determined using the taste sensors with 0.3 mM TDAB membrane modified with different concentrations of 1,3-DHNA as a function of the response value; (**b**) electric response to 100 mM caffeine when using lipid/polymer membrane modified with different concentrations of 1,3-DHNA. The error bar indicates the SD of data, n = 4 (electrode) × 4 (rotation) = 16 values.

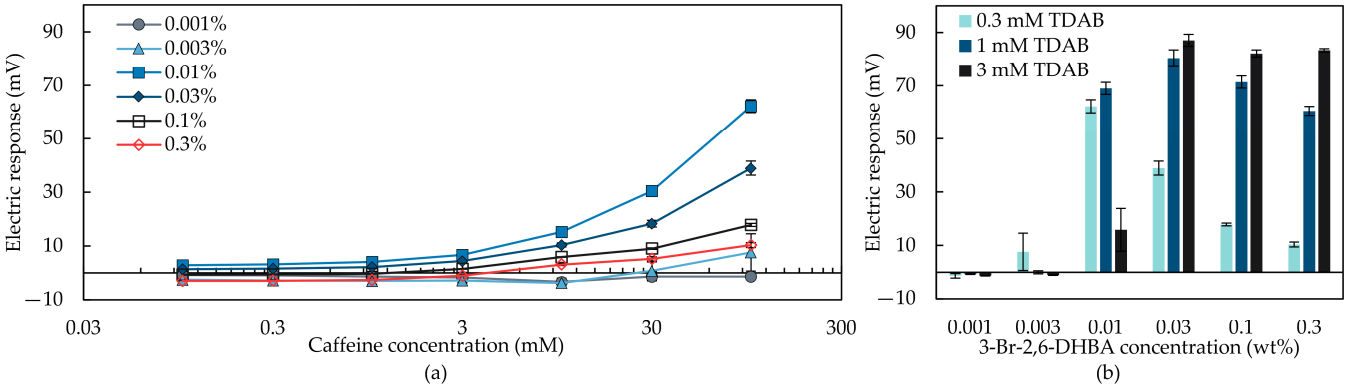

Figure 8. Response to caffeine in sample solutions measured using taste sensors with membrane modified with 3-Br-2,6-DHBA: (**a**) Concentration of caffeine determined using the taste sensors with 0.3 mM TDAB membrane modified with different concentrations of 3-Br-2,6-DHBA as a function of the response value; (**b**) electric response to 100 mM caffeine when using the lipid/polymer membrane modified with different concentrations of 3-Br-2,6-DHBA. The error bar indicates the SD of data, n = 4 (electrode) × 4 (rotation) = 16 values.

3.2.2. 1,3-DHNA

Figure 7a shows the relationship between caffeine concentration and response values determined using the taste sensors with the 0.3 mM TDAB membrane modified with 1,3-DHNA. As shown in Figure 7a, increasing the caffeine concentration resulted in an increase in the electric response to caffeine. This indicates that the taste sensors with the TDAB membrane modified with 1,3-DHNA are sensitive to caffeine. Figure 7b shows the response to 100 mM caffeine measured using taste sensors with the lipid/membrane modified with 1,3-DHNA. The sensor with the 0.3 mM TDAB membrane modified with 0.001 wt% 1,3-DHNA responded to caffeine sensitivity (46.2 mV).

3.2.3. 3-Br-2,6-DHBA

Figure 8a shows the concentration of caffeine measured using taste sensors with the TDAB membrane modified with 3-Br-2,6-DHBA. The results show that the response increased with the caffeine concentration in solution samples. This indicates that the sensors modified with 3-Br-2,6-DHBA are responsive to caffeine. Figure 8b shows the electric potential response of the sensors with TDAB membranes modified with six different

concentrations of the 3-Br-2,6-DHBA solution. The highest response to caffeine (87.1 mV) was obtained with the 3 mM TDAB membrane modified with 0.03 wt% 3-Br-2,6-DHBA; it was higher than that obtained with 2,6-DHBA. This implies that the taste sensors with the membrane modified with 3-Br-2,6-DHBA have good sensitivity to caffeine.

3.2.4. Comparison of Responses to Caffeine among 2,6-DHBA, 2,6-DHTA, 3-Br-2,6-DHBA, and 1,3-DHNA

To compare the sensitivity of the taste sensors to caffeine when using different modifiers, such as 2,6-DHTA, 1,3-DHNA, and 3-Br-2,6-DHBA, we chose the maximum value of caffeine response as the comparison standard. The maximum responses to caffeine obtained with Step 2 are summarized in Table 4, which shows that the sensors treated with the three other modifiers respond to caffeine sensitively (all the responses to 100 mM caffeine were above 45 mV). These findings indicate that the modifier with the allosteric mechanism structure can confer taste sensors the capability to detect caffeine sensitively.

Table 4. Log P and pKa of 2,6-DHTA, 1,3-DHNA, and 3-Br-2,6-DHBA; maximum response to caffeine obtained from each modifier.

Modifiers	Log P	pKa	Maximum Response to Caffeine (mV)	Experimental Conditions for Maximal Caffeine Response
2,6-DHBA	2.29	1.64	52.3	3 mM TDAB membrane modified with 0.03 wt% 2,6-DHBA
2,6-DHTA	1.81	1.19	80.1	1 mM TDAB membrane modified with 0.03 wt% 2,6-DHTA
1,3-DHNA	3.30	1.54	46.2	0.3 mM TDAB membrane modified with 0.001 wt% 1,3-DHNA
3-Br-2,6-DHBA	3.09	1.48	87.1	3 mM TDAB membrane modified with 0.03 wt% 3-Br-2,6-DHBA

In particular, the responses obtained from 2,6-DHTA and 3-Br-2,6-DHBA were higher than that obtained from 2,6-DHBA. These findings can be interpreted in terms of the log P and pKa of modifiers. According to our previous study [31], the response to caffeine was influenced by the log P and pKa of the modifier: membranes modified with modifiers with lower pKa responded more effectively to caffeine, and modifiers with higher log P were more easily adsorbed to the membrane. Thus, owing to the log P of 3-Br-2,6-DHBA (=3.09) being higher than that of 2,6-DHBA (=2.29), the more easily can 3-Br-2,6-DHBA be adsorbed to the lipid/polymer membrane through the hydrophobic interactions. Moreover, owing to the pKa of 3-Br-2,6-DHBA (=1.49) being smaller than that of 2,6-DHBA (=1.64), through the formation of intermolecular H bonds between caffeine and 3-Br-2,6-DHBA, the more ionized H^+ can be transferred from the caffeine solution to the carboxyl group of 3-Br-2,6-DHBA. This results in the 3-Br-2,6-DHBA-modified membrane being more sensitive to caffeine.

Similarly, since the pKa of 2,6-DHTA (=1.19) is smaller than that of 2,6-DHBA, the taste sensor treated with 2,6-DHTA shows greater sensitivity to caffeine than that treated with 2,6-DHBA. For 1,3-DHNA, because its log P (=3.30) is greater than that of 2,6-DHBA, it adsorbs more readily to the membrane than 2,6-DHBA, resulting in taste sensors with the lipid/polymer membrane modified with 0.001 wt% 1,3-DHNA being sensitive to caffeine. These findings indicate that the sensitivity of the taste sensor to caffeine can be improved by using a modifier with the allosteric mechanism structure and more favorable pKa and log P. All the log P and pKa values given in Table 4 were calculated using Marvin (Marvin 21.12.0, ChemAxon, Budapest, Hungary).

However, some limitations are worth noting. While we successfully found three new modifiers with the allosteric mechanism of detecting and confirmed that using them can make the taste sensors sensitive to caffeine, we found that, for these modifiers, appropriate membrane conditions (e.g., modifier concentration and lipid concentration) are required

for the modified taste sensors to respond effectively to caffeine. For example, at a modifier concentration of 0.03 wt%, the sensor treated with 1,3-DHNA did not respond to caffeine, while the sensors treated with 3-Br-2,6-DHBA responded effectively. Future work should therefore include follow-up work designed to investigate the relationship between modifiers and their appropriate membrane conditions.

3.3. Step 3: Measurement of Caffeine Analogs

In our previous study [30], the caffeine analogs theophylline and theobromine were measured using taste sensors with the lipid/polymer membranes modified with 2,6-DHBA; the responses to caffeine analogs were weaker than that to caffeine, and the low solubility of theobromine resulted in a small response value (about 3 mV). Therefore, to improve the sensitivity of taste sensors to caffeine analogs, we detected theophylline and theobromine under experimental conditions that increased the sensitivity to caffeine in Step 2. The results are summarized in Figures 9 and 10. Moreover, we confirmed the selectivity of the sensor treated with 0.03 wt% 2,6-DHBA and 3-Br-2,6-DHBA to caffeine.

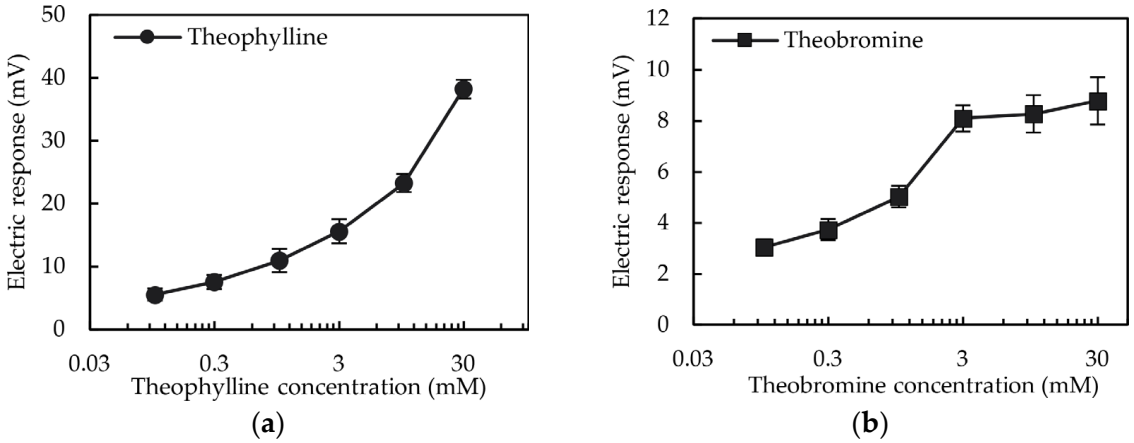

Figure 9. Concentration characteristics of each caffeine analog obtained from taste sensors with the 1 mM TDAB membrane modified with 0.03 wt% 2,6-DHTA: (**a**) Theophylline; (**b**) theobromine. The error bar indicates the SD of data, n = 8 (electrode) × 1 (rotation) = 8 values.

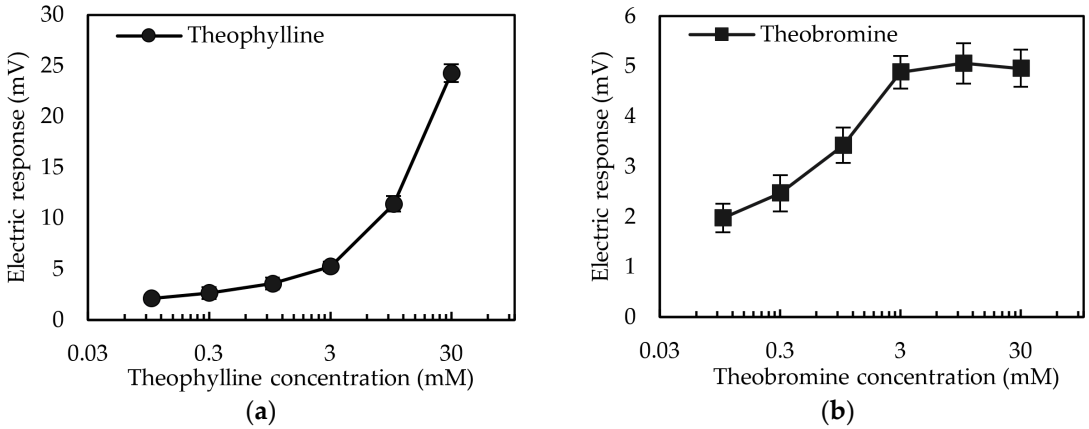

Figure 10. Concentration characteristics of each caffeine analog obtained with 3 mM TDAB membrane modified with 0.03 wt% 3-Br-2,6-DHBA: (**a**) Theophylline; (**b**) theobromine. The error bar indicates the SD of data, n = 8 (electrode) × 1 (rotation) = 8 values.

3.3.1. Detection of Theophylline and Theobromine Using Taste Sensors with Lipid/Polymer Membrane Modified with 2,6-DHTA

Figure 9 shows the results of theophylline and theobromine measurement using the sensors with the 1 mM TDAB membrane modified with 0.03 wt% 2,6-DHTA. The error

bar expresses the SD of data (n = 8 (electrode) × 4 (rotation) = 8 values). In Figure 9a, the response to theophylline in the sample solutions increased to around 38 mV when the theophylline concentration was increased to 30 mM. In Figure 9b, as the theobromine concentration increased, the response to theobromine increased and then plateaued at 8 mV because of its low solubility in water (saturates at ~3 mM).

3.3.2. Detection of Theophylline and Theobromine Using Taste Sensors with Lipid/Polymer Membrane Modified with 3-Br-2,6-DHBA

Figure 10 shows the results of detecting theophylline and theobromine with the 3 mM TDAB membrane modified with 0.03 wt% 3-Br-2,6-DHBA. The error bar expresses the SD of the data of n = 8 (electrode) × 1 (rotation) = 8 values. In Figure 10a, the response to theophylline increased significantly with increasing theophylline concentration, and the highest response to theophylline in the sample solution was 24 mV. In Figure 10b, the response to theobromine increased from 2 mV to 5 mV and plateaued at 5 mV, owing to its saturation at 3 mM.

3.3.3. Comparison of Responses to Caffeine Analogs among 2,6-DHBA, 2,6-DHTA, and 3-Br-2,6-DHBA

In Step 2, surface modification with 0.03 wt% 2,6-DHTA or 3-Br-2,6-DHBA could improve the sensitivity of the taste sensors to caffeine. Using the sensor whose sensitivity was improved by the modification with 2,6-DHTA or 3-Br-2,6-DHBA, we detected the caffeine analogs theophylline and theobromine and summarized their maximum response in Table 5. The results obtained with 2,6-DHBA was also listed in Table 5. The responses to theophylline (=38.2 mV) and theobromine (=8.1 mV) obtained from 2,6-DHTA were higher than those obtained from 2,6-DHBA (response to 30 mM theophylline = 12.8 mV; response to 3 mM theobromine = 3.3 mV). The results indicate that the modification with 2,6-DHTA improved the response sensitivity of the taste sensor to theophylline and theobromine. Similarly, the response to theophylline (=24.3 mV) or theobromine (=4.9 mV) obtained from 3-Br-2,6-DHBA was higher than that obtained from 2,6-DHBA; these results demonstrate that the sensitivity of taste sensors to caffeine analogs can be improved by modification with 3-Br-2,6-DHBA. These findings indicate that using a modifier with the allosteric mechanism structure and smaller pKa than that of 2,6-DHBA improves the sensitivity of taste sensors to caffeine analogs.

Table 5. Response to 30 mM theophylline and 3 mM theobromine obtained from 2,6-DHBA, 2,6-DHTA and 3-Br-2,6-DHBA.

Modifiers	Response to 30 mM Theophylline (mV)	Response to 3 mM Theobromine (mV)	Experimental Conditions
2,6-DHBA	12.8	3.3	3 mM TDAB membrane modified with 0.03 wt% 2,6-DHBA
2,6-DHTA	38.2	8.1	1 mM TDAB membrane modified with 0.03 wt% 2,6-DHTA
3-Br-2,6-DHBA	24.3	4.9	3 mM TDAB membrane modified with 0.03 wt% 3-Br-2,6-DHBA

3.3.4. Confirmation of Sensor Selectivity

Figure 11 shows the measurement results for the five basic tastes and astringency samples using the lipid/polymer membrane modified with 0.03 wt% 2,6-DHBA and 3-Br-2,6-DHBA. These sensors showed the highest response (all the responses to 100 mM caffeine were above 45 mV) for caffeine, while they showed negligible responses to astringency, sweetness, saltness, and two other bitterness. Concerning the response to sourness, we could eliminate it in the sample containing caffeine and sour substances by concurrently using the commercialized sourness sensor electrode, similar to the case of coexisting sweetness and bitterness [40] or coexisting sweetness and saltiness [41]. Thus, the sensor

treated with 0.03 wt% 2,6-DHBA and 3-Br-2,6-DHBA has good sensitivity and selectivity to caffeine. Moreover, the selectivity of the sensor treated with 3-Br-2,6-DHBA to caffeine is higher than that treated with 2,6-DHBA.

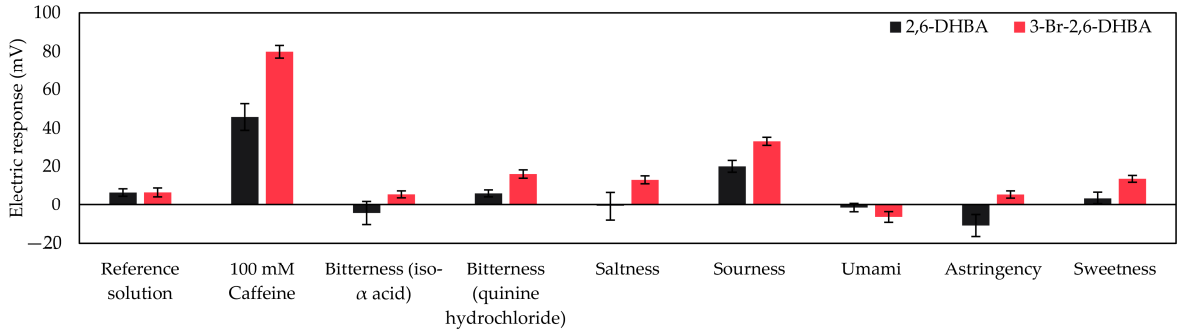

Figure 11. Response to five basic tastes and astringency in sample solutions measured using taste sensors with lipid/polymer membrane modified with 0.03 wt% 2,6-DHBA and 3-Br-2,6-DHBA. The error bar indicates the SD of data, n = 8 (electrode) × 1 (rotation) = 8 values.

3.3.5. Linearity and Repeatability of the Sensor Treated with 3-Br-2,6-DHBA to Caffeine and Its Analogs

As reported by the study in [42], the linearity and precision of the taste sensor were investigated. It indicated that taste sensors showed an adequate linearity range with a good coefficient of determination (R^2) at the same time, e.g., the R^2 for the astringency sensor is 0.999. For astringency taste sensors, repeatability was found with values of relative standard deviations (RSD) less than 4%, which were set to be the acceptable values for relative standard. In our study, the 3-Br-2,6-DHBA-treated sensors were modelled using partial least square regression, including the log-linear range, the slope of the regression line, Y-intercept, and coefficient of determination. The intra-day precision for caffeine and its analogs detection using the sensor treated with 3-Br-2,6-DHBA was also calculated. Table 6 shows the calculated results. As can be seen, the slope for caffeine and its analogs detection was positive, which is consistent with the results obtained in Step 2 and Step 3 measurements.

Table 6. Linearity and repeatability (intra-day) RSD of the taste sensor with 3 mM TDAB membrane modified with 0.03 wt% 3-Br-2,6-DHBA to caffeine and its analogs.

	Caffeine	**Theophylline**	**Theobromine**
Concentration range of linearity [mM]	0.1–100	0.1–30	0.1–30
Slope of the regression line	22.90	7.89	1.36
y-intercept	10.63	5.90	3.30
Coefficient of determination	R^2 = 0.77	R^2 = 0.75	R^2 = 0.89
Repeatability (intra-day) RSD [%]	1 mM 2.24 3 mM 1.27 10 mM 1.29 30 mM 1.16 100 mM 0.69	0.3 mM 7.09 1 mM 6.21 3 mM 4.56 10 mM 2.69 30 mM 1.69	1 mM 5.93 3 mM 3.29 10 mM 3.44 30 mM 2.86

For the linearity of caffeine detection, the sensor treated with 0.03 wt% 3-Br-2,6-DHBA, 3 mM TDAB in Table 5, had an extensive linearity range (0.1–100 mM) with a R^2 of 0.77. When detecting theophylline and theobromine, the same sensor showed relatively extensive concentration ranges (0.1–30 mM) with R^2 = 0.75 (theophylline detection) and R^2 = 0.89 (theobromine detection). Conclusively, the sensor treated with 3-Br-2,6-DHBA shows an adequate linearity range with a good R^2 for caffeine and its analogs.

Table 6 shows that the values of RSD are less than 3% for the repeatability of caffeine detection. RSD for the concentration is higher than the threshold reported by the study [43] (caffeine is 0.70 mM; theophylline is 0.20 mM; theobromine is 0.75 mM). These RSD results indicated that the sensor treated with 3-Br-2,6-DHBA showed good repeatability for

caffeine detection. Moreover, the values of RSD were less than 8% for the repeatability of theophylline detection, and the values of RSD were less than 6% for the repeatability of theobromine detection. Their repeatability was smaller than that of the caffeine detection. Further assumptions will be discussed in the robustness part of theophylline and theobromine detection.

4. Conclusions

As previously reported [30–32], taste sensors can detect non-charged substances such as caffeine with lipid/polymer membranes modified with HBAs. The mechanism of caffeine detection by taste sensors was identified to be an allosteric one. In this study, we used a three-step process to clarify the molecular structure conductive to the allosteric mechanism of detecting caffeine. Based on the clarified allosteric mechanism of detecting caffeine, we improved the sensitivity of taste sensors to caffeine. The detection of the caffeine analogs theophylline and theobromine was also analyzed. In Step 1, we found that 2,6-DHBA has a structure conductive to the allosteric mechanism of detecting caffeine, and the elements of the allosteric mechanism and caffeine detection were also identified. We propose that the allosteric site is the hydroxyl group that can form H bonds with caffeine; the active site is the carboxyl group; the ligand is caffeine, and the substrate is H^+ ionized by the carboxyl group. In Step 2, we further validated this structure of the allosteric mechanism with three other modifiers, such as 1,3-DHNA, 2,6-DHTA, and 3-Br-2,6-DHBA. The results indicate that the sensitivity of the taste sensor to caffeine could be improved by using a modifier with a structure conductive to the allosteric mechanism and smaller pKa than that of 2,6-DHBA. Although we confirmed that using three new modifiers with the allosteric mechanism of detecting can make the taste sensors sensitive to caffeine, we found that for these modifiers, appropriate membrane conditions (for example, modifier concentration and lipid concentration) are required for the modified taste sensors to respond to caffeine effectively. Therefore, more research into the connection between modifiers and the proper membrane conditions should be a part of future study. In Step 3, the results reveal that the use of the sensor whose sensitivity to caffeine is improved by the modification with 2,6-DHTA or 3-Br-2,6-DHBA improves the sensitivity to caffeine analogs. This helps improve the sensitivity of taste sensors to other non-charged substances detected by using the allosteric mechanism. By using the allosteric mechanism, we can develop taste sensors for other non-charged pharmaceutical substances, such as dexamethasone, prednisolone, and naringin, in the future.

Author Contributions: The work presented here was carried out as a collaboration among all authors. H.X., Z.Z. and K.T. defined the research theme; H.X. and Z.Z. carried out the experiments and analyzed the data; H.X. and Z.Z. interpreted the results and wrote the paper; K.T., T.O. and S.K. provided directions for the experimental methods, the analysis of data, the interpretation of the results, and the writing of the paper. All authors have contributed to, seen, and approved the manuscript. All authors have read and agreed to the published version of the manuscript.

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