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Threshold Detection Using Indicator-Displacement Assays: An Application in the Analysis of Malate in Pinot Noir Grapes

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Abstract: The mathematics for modeling indicator-displacement assay isotherms is presented and contrasted to the classical host−guest binding isotherm. It is shown that the signal response can be tuned to occur closer to 1 equiv of guest relative to a standard binding algorithm. This delay in response leads to a better triggering protocol for threshold detection schemes. The determination of malate in Pinot Noir must was calculated using this new mathematical model, which demonstrates how a color change can be tuned to occur near a desired concentration of analyte.

Introduction

The development of colorimetric analyses for small organic and inorganic species is a current focus in the field of molecular recognition.[1] The simplest example of a colorimetric analysis is the use of the human eye as an optical detector.[2] Because the human eye has a high ability to distinguish different colors, "naked eye" detection of analytes is the oldest method of qualitative analysis. For example, it has been known for centuries that certain plant extracts could be used as optical indicators of solution acidity.[3] Such natural plant extracts are the ancestors of today's colorimetric pH indicators.[4] Because of their "naked eye" response, pH indicators are still used in many analytical methods, such as titrimetric analysis.[5]

pH indicators that have two protonation states (protonated and not protonated) are characterized by a sigmoidal titration curve. For example, 2,6-dichlorophenolindophenol is red in acidic media (pH 0−4.8), changes to blue in the pH 4.8−6.5 range, and is blue from pH 6.5−14, and has been extensively used for the determination of ascorbic acid.[6] Hence, the indicator color remains almost constant i.e., no signal, but above a threshold concentration of H_3O^+ the difference in pH can be clearly seen by a difference in the color of the solution. This kind of system behavior can be described as threshold detection and is a classic example of an assay that can be modeled mathematically with standard equations.[7]

The mathematics used to model pH titration curves is identical to that of standard host−guest chemistry, where [H] and [G] represent the concentrations of the host and guest, respectively. One can regard the indicator as a host for the proton guest. This leads to the well recognized guideline that when $[H] = K_d$, [G] = [HG]. This equality is the direct analogue of the statement that when the $pH = pK_a$, [HA] = [A]. For molecular sensing purposes, the covalent attachment of an indicator to the host follows the same binding algorithm.

An alternative method to a covalently attached indicator is a competition between an indicator (I) and analyte (guest, G) for the binding pocket of a receptor (host, H).[8] The bound indicator is displaced from the receptor upon addition of a guest, causing a signal modulation. We and others[9] have applied this approach to sensing a variety of analytes, some of which are present in wines, sodas, saliva, etc. In this type of competition assay, there are three different chemical species that participate in the reaction: indicator (I), host (H), and guest (G). Because the host and indicator can be introduced to the system in variable concentrations, advantages as described herein occur for threshold recognition.

In this article, we develop the mathematics for indicator-displacement assays. We show that the binding algorithm can lead to a more responsive color change at a predetermined concentration of analyte than with standard host−guest chem-

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istry. We exploit a previously published sensing ensemble for tartrate/malate[9a] and use it to analyze grape must as a demonstration of the principle. First, however, we summarize the well-known mathematics of standard host−guest chemistry simply as a comparison to that for an indicator-displacement assay.

The majority of "classical" colorimetric molecular sensors are composed of a binding site and a covalently attached chromophore.[10] Upon binding of the analyte, the electronic transitions of the chromophore are perturbed. A model of the reaction between an analyte (i.e., guest (G)) and a recognition moiety (i.e., the host (H)), in a 1:1 stoichiometry, is as follows:

$$H + G \rightleftharpoons HG \tag{1}$$

The equilibrium constant for this process is expressed as the ratio of product over reactants (eq 2):

$$K_{HG} = \frac{[HG]}{[H][G]} \tag{2}$$

The absorbance in this process is defined by Beer's Law (eq 3).

$$A = \epsilon_{HG} b[HG] + \epsilon_{H} b[H] \tag{3}$$

The K_{HG} equilibria expression (eq 2) can be solved for [HG] as a function of total host concentration $[H]_T$ and total guest concentration $[G]_T$ (eq 4), giving:

$$K_{HG}[HG]^2 - [HG](K_{HG}[H]_T + K_{HG}[G]_T + 1) + K_{HG}[H]_T[G]_T = 0 \tag{4}$$

After solving this quadratic equation, [H] can be calculated from eq 5:

$$[H] = [H]_T - [HG] \tag{5}$$

Putting both values ([HG] and [H]) into eq 3 allows one to determine the absorbance at each total guest concentration; the resulting binding isotherm has a hyperbolic shape.

Result and Discussion

The Indicator-Displacement Isotherm. As we mentioned above, the indicator-displacement assay process is based on an equilibrium when an indicator (I) is bound to a receptor (H) by noncovalent interactions.[8a] When the analyte (G) is added, the indicator is displaced from the binding cavity, producing a measurable change in the indicator's optical properties (eq 6).[11]

$$HI + G \rightleftharpoons HG + I \tag{6}$$

The binding constant K for the indicator-displacement process can be expressed as the ratio of products over substrate concentrations (eq 7).

$$K = \frac{[HG][I]}{[HI][G]} \tag{7}$$

The following two equilibriums also occur during this process:

$$H + I \rightleftharpoons HI \tag{8}$$

and

$$H + G \rightleftharpoons HG \tag{9}$$

The binding constant K_{HG} for the process in eq 9 is given in eq 2. The absorbance in an indicator-displacement assay is defined by Beer's Law (eq 10):

$$A = \epsilon_{HI} b[HI] + \epsilon_{I} b[I] \tag{10}$$

If one solves for [HG] from eq 2 as a function of total host $[H]_T$, total guest $[G]_T$, and total indicator $[I]_T$ concentrations, eq 11 is obtained.

$$K_{HG}(1-K)[HG]^3 + (K_{HG}K[G]_T + K_{HG}[I]_T - (1-K) - K_{HG}[G]_T(1-K) - K_{HG}[H]_T(1-K))[HG]^2 + (K_{HG}[H]_T[G]_T(1-K) - K_{HG}K[G]_T^2 - K_{HG}[I]_T[G]_T - K_{HG}K[H]_T[G]_T - K[G]_T)[HG] + K_{HG}K[H]_T[G]_T^2 = 0 \tag{11}$$

Equations 12 and 13 give [I] and [HI], respectively, as a function of [HG]

$$[I] = [I]_T - [HI] \tag{12}$$

$$[HI] = \frac{[HG][I]_T}{[HG](1-K) + K[G]_T} \tag{13}$$

The cubic eq 11 was solved by the method of Viète,[12] and the resulting three [HG] roots were used to solve eqs 12 and 13. The resultant [HI] and [I] were substituted into eq 10. This mathematical development gave a theoretical isotherm for an indicator-displacement assay, which was compared to the binding isotherm for a classical sensor system (Figure 1).

We began by writing a computer program that simulates the binding isotherm (concentration of guest vs absorbance) for classical and indicator-displacement assays. This program allowed us to manipulate the system parameters (i.e., K's, ϵ's, concentrations). To test the program, we first compared a binding isotherm generated by the program with independently generated experimental data. We chose an indicator-displacement system for tartrate, which was recently developed in our group, using the experimentally determined K and ϵ values.[13] Our assay for tartaric acid employed an ensemble of alizarin complexone (**3**) and host **1** in 25% water solution in methanol by volume (10 mM HEPES buffer at pH 7.4) (Scheme 1). The previously determined binding constant for **1·3** ($K_{HI} = 5.2 \times 10^4$ M^{-1}), and tartarate·**1** ($K_{HG} = 4.0 \times 10^4$ M^{-1}) were used. The change in absorbance of **3** was monitored by UV/vis during the titration of tartaric acid into a solution of host **1** (185 μM) and dye **3** (150 μM). All these parameters were introduced to the program, and the binding isotherm was simulated. Both binding isotherms (experimental and theoretical) were plotted on the same plot (Figure 2), and the experimental and theoretical

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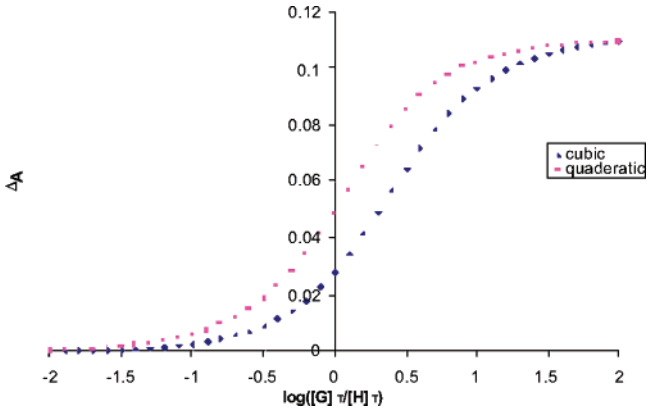
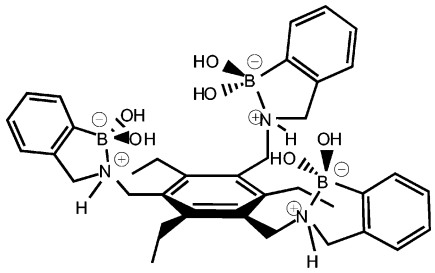


Figure 1. Comparison of the theoretical isotherm binding curve for an indicator-displacement assay (blue ◆) process with the theoretical binding isotherm for a classical sensor system (pink ■).

Scheme 1



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data are in excellent agreement. Plot A (Figure 2) depicts the most common way one presents the binding isotherm curve (concentration vs ΔA), whereas plot B presents the same absorbance data vs a logarithm scale. This mathematical operation visually enhances the inflection point of the isotherm.

After checking the accuracy of our computer program, we manipulated the value of the equilibrium constant (K) for the indicator-displacement assay, as well as ϵ and K_{HG} values, with an eye toward applications in threshold detection. When keeping the ϵ and K_{HG} values constant, the variation of K does not dramatically change the shape of the binding isotherm (Figure 3). However, it is noteworthy to mention that the roots of the cubic equation changes. When $K < 1$, one of the roots is correct; however, when $K > 1$, a different one of the three roots is correct.

The next step was to vary the K_{HG} values by keeping the rest of the parameters constant. The study showed that raising the host−guest binding constant (K_{HG}) causes a hyperchromic shift in absorbance at the λ_{max} for I as well as a response of the system to the addition of guest (Figure 4) closer to 1 equiv. Next, we examined the effect of the molar absorptivity of free indicator (ϵ_I) and host−indicator complex (ϵ_{HI}). When both ϵ parameters are similar, the change in absorbance is negligible. The difference between ϵ_I and ϵ_{HI} has a critical influence on the binding isotherms. As may be expected, the larger the difference of ϵ_I and ϵ_{HI} at the wavelength of observation, the greater the change in color over the titration (Figure 5).

In the last part of our theoretical exercise, we varied the difference between host and indicator concentrations (Figure 6). We started from a system in which both of these concentrations were equal (180 μM). Then we kept the indicator concentration constant and increased the host concentration.

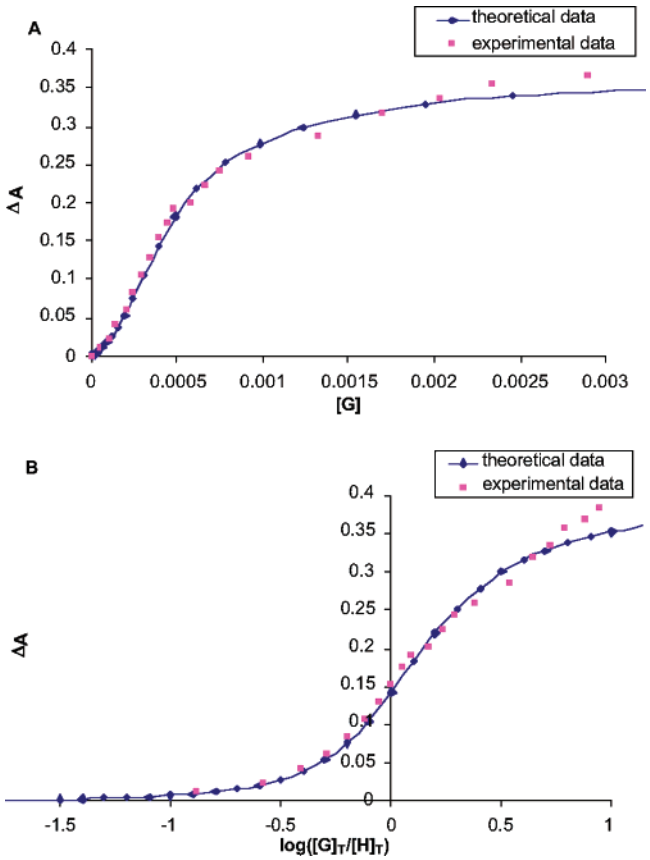

Figure 2. Comparison of theoretical and experimental binding isotherms for an indicator-displacement assay; guest concentration (1.58 mM), host concentration (185 μM), indicator concentration (150 μM). (A) ΔA vs guest concentration. (B) ΔA vs logarithm of total guest concentration over total host concentration.

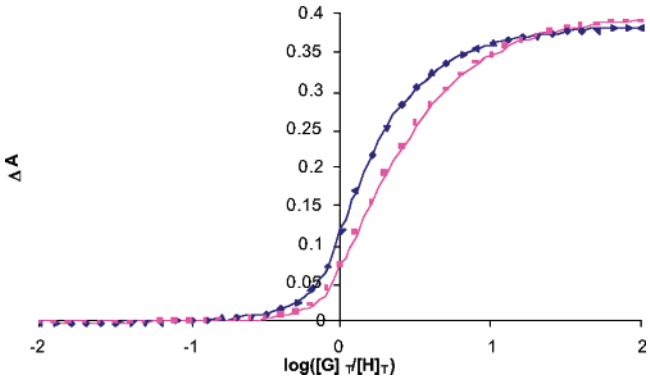

Figure 3. Variation of association constants for indicator-displacement assay. Pink ■ $K = 0.77$, $K_{HG} = 48\,000$, $[H]_T = 8 \times 10^{-4}$ M, $[I]_T = 1.8 \times 10^{-4}$ M, $\epsilon_I = 2400$, $\epsilon_{HI} = 4670$. Blue ◆ $K = 1.77$, $K_{HG} = 48\,000$, $[H]_T = 8 \times 10^{-4}$ M, $[I]_T = 1.8 \times 10^{-4}$ M, $\epsilon_I = 2400$, $\epsilon_{HI} = 4670$.

Simulated binding isotherms show that increases of the host concentration cause significant changes in the early absorbance values. Moreover, these isotherms show that relative to a standard isotherm, the absorbance changes can be tuned to occur at higher concentrations of guest, and the slope of the isotherm is steeper near 1 equiv of guest in the titration. These results indicate that variation of the ratio of host versus indicator concentration allows us to create a sensing ensemble which is characterized by a delayed response to guest concentration followed by a steep response, thereby giving an improved approach to a threshold detection motif.

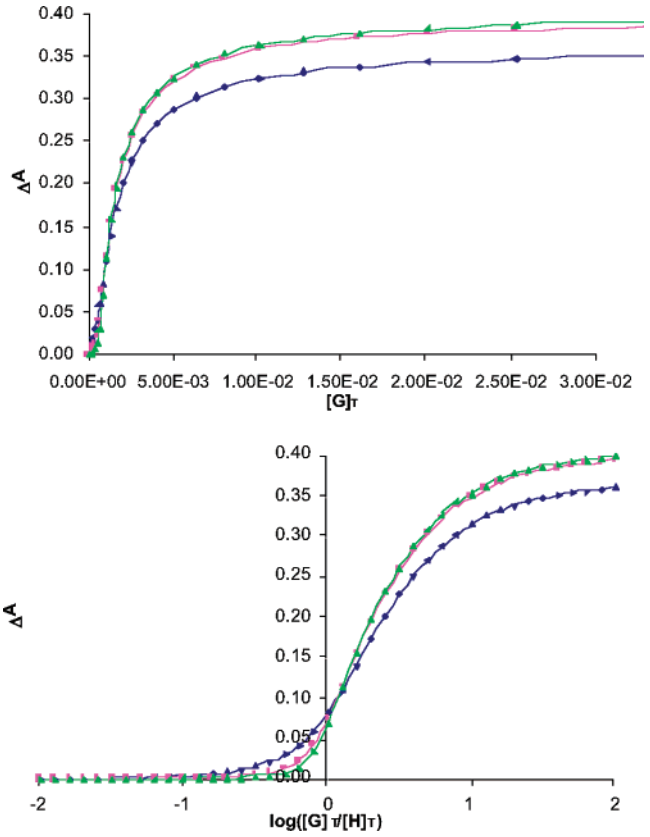

Figure 4. Variation of host−guest association constant (K_{HG}) for indicator-displacement assay (blue ◆ K_{HG} = 10 000, pink ■ K_{HG} = 50 000, green ▲ K_{HG} = 100 000). The rest of the parameters are constant: K = 0.77, $[H]_T = 8 \times 10^{-4}$ M, $[I]_T = 1.8 \times 10^{-4}$ M, ϵ_I = 2400, ϵ_{HI} = 4670.

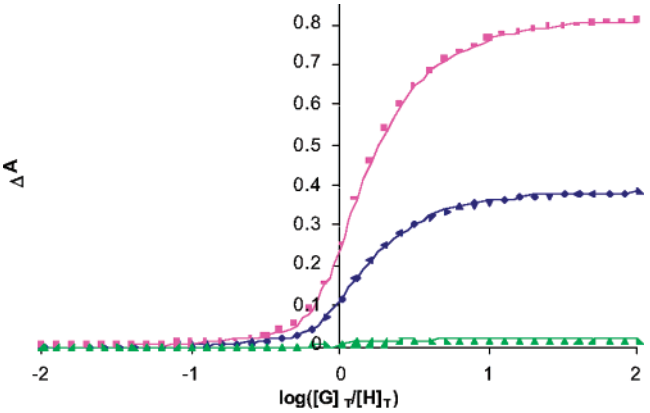

Figure 5. Variation of molar absorptivity for host−indicator complex and free indicator (pink ■ ϵ_I = 1200, ϵ_{HI} = 6000; blue ◆ ϵ_I = 2400, ϵ_{HI} = 4670; green ▲ ϵ_I = 2000, ϵ_{HI} = 2100). The rest of the parameters are constant: K = 1.77, K_{HG} = 48 000, $[H]_T = 8 \times 10^{-4}$ M, $[I]_T = 1.8 \times 10^{-4}$ M.

In summary, the modeling of the indicator-displacement isotherm, as well as its comparison to a classical isotherm, shows one how to manipulate experimental parameters to achieve a desired colorimetric response. In particular, by adjusting $1/K_{HG}$ to be near the concentration of the analyte of interest, having K near 1, maximizing the difference between ϵ_I and ϵ_{HI}, and having an excess of host, leads to a sharp color change near 1 equiv of guest.

An Application in Analysis of Pinot Noir Grapes. A practical application of the indicator-displacement assay discussed above for threshold detection was explored by monitoring

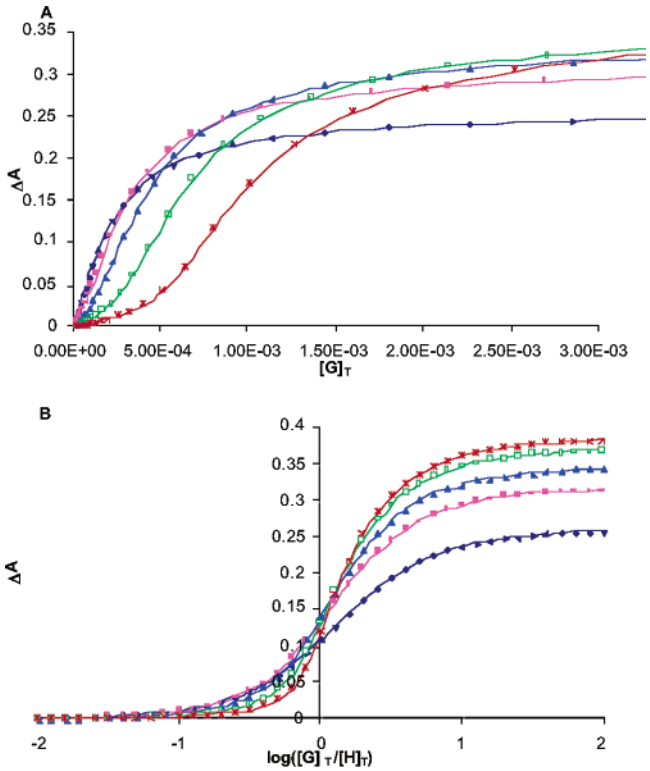

Figure 6. (A,B) Variation of host concentration (blue ◆ $[H]_T = 180$ μM, pink ■ $[H]_T = 270$ μM, blue ▲ $[H]_T = 360$ μM, green □ $[H]_T = 540$ μM, red * $[H]_T = 800$ μM). The rest of the parameters are constant: K = 1.77, K_{HG} = 48 000, $[I]_T = 1.8 \times 10^{-4}$ M, ϵ_I = 2400, ϵ_{HI} = 4670.

the grape maturity process. It is important to define the optimal grape maturity for wine production and to develop clear chemical or biochemical tests that can be used to define the peak of ripeness.[14] The grape maturity can be defined based on many different factors such as sugar level, acidity, and balance of sugar and acidity.[15] Further, tartaric and malic acids are present when the grapes are picked, and they are carried over throughout the fermentation process into the finished wine. The concentration of tartaric acid in grapes remains relatively constant throughout the ripening period, but the malic acid concentration decreases because it is consumed as an energy source in the berry during véraison.[16]

The Anslyn group has recently been exploring a colorimetric assay for tartrate and malate using the synthetic receptor **2** (Scheme 2).[9a] The incorporation of a boronic acid-binding site imparts affinity to vicinal diols,[17] while the use of guanidinium groups imparts affinity to carboxylates.[18] Receptor **2** was used for the analysis of tartrate in beverages. It was found that tartrate and malate both have high affinity for the host **2** ($K_{tartrate} = 5.5 \times 10^4$ M^{-1}, $K_{malate} = 4.8 \times 10^4$ M^{-1}) when an indicator-displacement assay experiment with alizarin complexone was used as a dye. It was anticipated that such a system can be used as a test for a threshold detection assay using an indicator-

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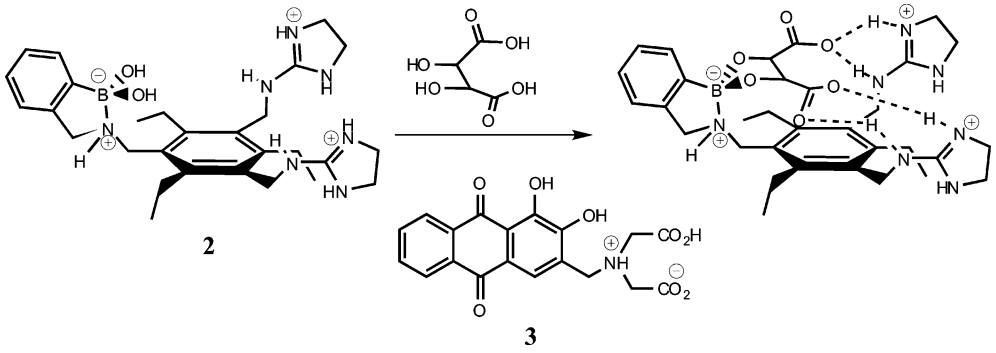
Scheme 2



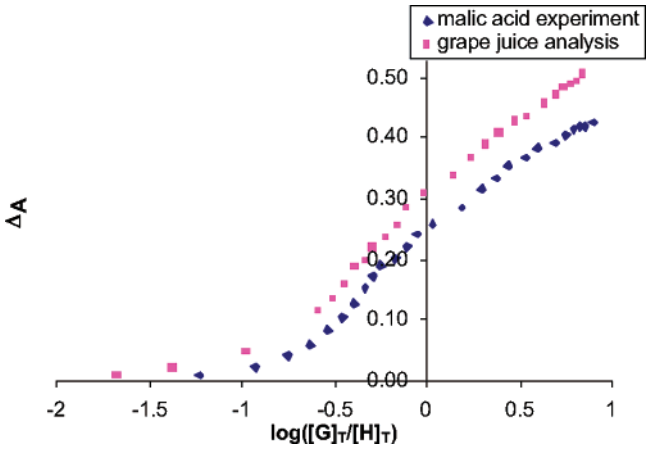


Figure 7. Binding isotherms for indicator-displacement titration of host (**2**) and indicator (**3**) with malic acid. The blue plot is without addition of must and tartaric acid. The pink plot is with added tartrate and grape must.

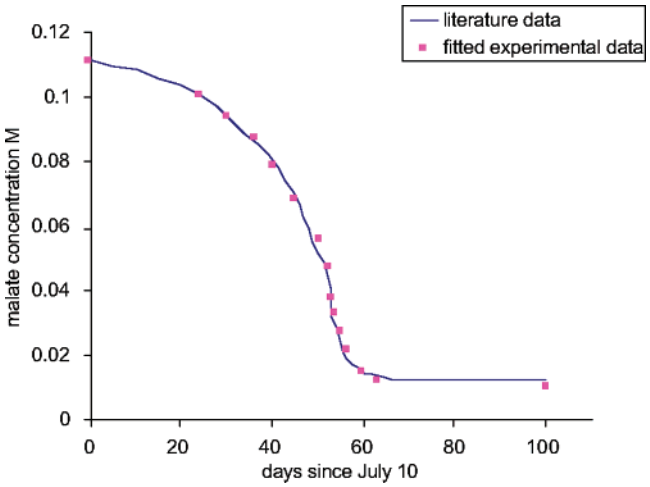


Figure 8. Literature over experimental plots of malate concentrations versus days of ripening.

displacement model for determining the grape maturity based on the concentration of malic acid.

On the basis of the previously discussed theoretical study, the parameters for the experiment were optimized. Because some of the values are set by the host and indicator used (K, K_{HG}, ϵ), only the host and indicator concentrations were manipulated. However, from the previously discussed theoretical experiments, it is known that these two parameters have a large impact on the indicator-displacement assay system. On the basis of modeling using the previously discussed program, it was found that the steepest inflection point on the plot for triggering a concentration of malate near the point with the greatest rate of concentration change as a function of days of ripening (approximately 56 days, look ahead to Figure 8) will be when a ratio of host **2** to indicator **3** in our experiments is 5.6:1. UV/vis spectroscopy was used for monitoring the titration process at 450 nm. The malic acid solution was titrated into a buffer solution (10 mM HEPES in 25% water in methanol by volume, pH 7.4) of the host **2** (857 μM) and the indicator **3** (151 μM), the binding isotherm obtained is shown in Figure 7 (Scheme 2).

Next, the content of grape juice obtained from grape must was examined. We chose Pinot Noir grapes as a subject of our study. To determine the tartaric and malic acid concentrations in the must, we made a preliminary ^{1}H NMR study. The ^{1}H NMR spectra showed that the content of both acids is negligible because the grapes have already undergone the ripening period.

We next modeled the grape ripening process by adding tartaric acid to the grape must to achieve a concentration consistent with literature (5 g/L).[14b] Then, to a solution of host **2** (904 μM) and indicator **3** (143 μM) in buffer solution (10 mM HEPES in 25% water in methanol by volume, pH 7.4) was added the must containing tartaric acid (12.8 μL of grape juice, concentration of tartaric acid was 85 μM). Different concentrations of malic acid were added to this solution, and the absorbance was determined. The resulting binding isotherm was plotted alongside an isotherm derived from an experiment in which the **2**·**3** complex was titrated with malic acid only (no grape must), and very similar isotherms were observed (Figure 7). However, the isotherm for juice analysis is shifted up because of the addition of tartaric acid to the system.

We then took the concentrations we could determine for malate in grape must and placed them on a curve from the literature that plots malate concentration versus days of ripening (Figure 8). This analysis shows that the technique could potentially be used with grape must to colorimetrically indicate the state of ripeness by following a color change tuned to occur near or at the greatest rate of change of malate concentration. The general strategy of using indicator-displacement assays to give a rapid color change close to a particular concentration of an analyte has been demonstrated and should present a general strategy with the discussed advantages over traditional binding isotherms.

Summary

It has been demonstrated that the indicator-displacement assay presents a benefit over the classical colorimetric sensors for threshold detection. Our theoretical approach has shown how

all parameters influence the indicator-displacement system and which parameters have a crucial influence. By adjusting the host−indicator ratio, one can cause a color change to occur closer to the point at which 1 equiv of analyte has been added. This technique was successfully applied to grape must, showing that a detector ion system for grape maturity can be easily created.

Experimental Section

General Consideration. The chemicals were obtained from Aldrich and used without further purification. Flash chromatography was performed on Whatman 60 Å 230−400 mesh silica gel. ^{1}H (300 MHz) and ^{13}C (75 MHz) spectra were measured by a Varian Unity Plus spectrometer. Mass spectra were recorded on a Finnigan VG analytical ZAB2-E spectrometer. UV/vis spectra were collected on a Beckman DU-640 at 25 °C unless noted otherwise.

UV/Vis Titrations. The titrations were performed on a Beckman DU-640 UV/vis instrument.

UV/Vis Titrations of Indicator and Receptor. All solutions were buffered at pH 7.4 with HEPES (10 mM) in 25% water in methanol (v/v). A solution of alizarin complexone (150 μM) was prepared in the cuvette, and into this was titrated a stock solution of **1** (1.2 mM) and alizarin complexone (150 μM), thereby keeping the host concentration constant. The data were taken at 525 nm to determine the association constant. The association constants for **2** were determined in a similar manner with differences in concentrations; alizarin complexone−**2** (10 mM HEPES, pH = 7.4, 150 μM indicator, 2.1 mM **2**, 450 nm).

UV/Vis Titration of Receptor/Indicator Ensemble and Guest. All solutions were buffered at pH 7.4 with HEPES buffer (10 mM) in 25% water in methanol (v/v). A solution of indicator (**3**, 150 μM) and receptor (**1**, 185 μM) was prepared in the cuvette, and into this was titrated a stock solution of indicator, host, and guest, keeping the indicator and host concentrations constant. The data were taken at the appropriate wavelength to determine the association constants. The guest concentration in the stock solution was 1.58 mM. The association constants for **2** were determined in a similar manner with differences in concentrations; alizarin complexone−**2** (10 mM HEPES, pH = 7.4, 151 μM indicator, 857 μM **2**, 10.16 mM guest). The association constants for **2** with grape must were determined in a similar manner with differences in concentrations; alizarin complexone−**2** + grape juice (10 mM HEPES, pH = 7.4, 143 μM indicator, 904 μM **2**, 9.58 mM guest). To the stock solution was additionally added tartaric acid (85 μM) and grape juice (12.8 μL).

NMR Determination of Tartrate/Malate in Grape Juice. Pinot Noir must was poured into a cheesecloth, and the juice was squeezed into a flask. The resulting 60 mL of grape juice, pH = 4, was lyophilized. The residue was dissolved in D_2O, and an ^{1}H NMR spectra was taken. To the NMR tube were added malic acid and then tartaric acid. The appearance of a new set of signals, assigned to both the acids, confirmed that the juice does not contain significant levels of these acids.

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Supporting Information Available: The two mathematical pathways to solve quadratic and cubic equations for classical colorimetric and indicator-displacement models, respectively (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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