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Citrate and calcium determination in flavored vodkas using artificial neural networks

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Abstract—The development of multianalyte sensing schemes by combining indicator-displacement assays with artificial neural network analysis (ANN) for the evaluation of calcium and citrate concentrations in flavored vodkas is presented. This work follows a previous report where an array-less approach was used for the analysis of unknown solutions containing the structurally similar analytes, tartrate and malate. Herein, a two component sensor suite consisting of a synthetic host and the commercially available complexometric dye, xylenol orange, was created. Differential UV–Visible spectral responses result for solutions containing various concentrations of calcium and citrate. The quantitation of the relative calcium and citrate concentrations in unknown mixtures of flavored vodka samples was determined through ANN analysis. The calcium and citrate concentrations in the flavored vodka samples provided by the sensor suite and the ANN methodology described here are compared to values reported by NMR of the same flavored vodkas. We expect that this multianalyte sensing scheme may have potential applications for the analysis of other complex fluids.

1. Introduction

Instead of using molecular sensors specifically designed to target one analyte, trends are now shifting towards utilizing an array of differential sensors responsive to multiple analytes where the combination of the signals from all the sensors in the array generates a fingerprint response that is unique to the composition of the mixture being analyzed. Despite the explosion of reports in the literature of array-based systems used for the analysis of multiple components in an unknown mixture,[1–5] the number of research groups with access to an array platform with which to perform their analyses is still quite limited. An instrument with much more visibility and often present in most college undergraduate laboratories is a UV–Visible spectrophotometer. We expect that a multianalyte sensing scheme dependent on the analysis of homogeneous solutions in a UV–Visible spectrophometer would hold greater potential for utility among chemists due to the relative availability of this type of instrumentation.

Recently, we demonstrated simultaneous detection of two structurally similar analytes, tartrate and malate, via spectrophotometric analysis of two indicator displacement assays utilizing two cross reactive synthetic hosts and two different indicators.[6] Application of an artificial neural network (ANN) allowed for the evaluation of the relative amounts of each analyte in unknown solutions. The power behind this simple approach is that it creates multi-analyte sensing protocols in the absence of an array setting. Despite the success of this approach, we sought to incorporate two additional levels of complexity. First, we wanted to further demonstrate the versatility of this method by extending it to the use of a cross reactive indicator, that leads to synergistic binding events between hosts and analytes. Second, we wanted to employ this method for practical uses through the analysis of commercially available flavored vodkas.

2. Materials and methods

Reagents. All the solvents used in spectrophotometric studies were of spectroscopic grade and purchased from Aldrich. Xylenol orange was obtained from Aldrich and used without further purification. Calcium nitrate was obtained from Fisher Scientific and used without further purification. Buffer components were of reagent grade. The synthesis of **1** has been previously reported.[7]

Absorption studies. The absorption titrations (generation of the matrix for the ANN) were performed by keeping the concentration of the H_2:I solution constant (0.24 mM **1** and 0.01 mM **2**) and adding both $Ca(NO_3)_2$ and **3** in varying

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Table 1. (A) Concentration of citrate (mM) and calcium (mM) in the validation test points samples determined by the two-component sensing system and ANN analysis. The reported values shown are the average value±standard deviation of three measurements with the percent different shown in parenthesis. (B) Concentration of citrate (mM) and calcium (mM) in various flavored Smirnoff® vodkas determined by ANN, and NMR

(A) Prepared samples

Real values		Predicted values	
[citrate] (mM)	[calcium] (mM)	[citrate] (mM)	[calcium] (mM)
0.00	0.10	0.03±0.01	0.12±0.04
0.20	0.40	0.15±0.03	0.37±0.01
0.40	0.15	0.35±0.005	0.12±0.002
0.60	0.20	0.56±0.01	0.18±0.001
0.80	0.35	0.87±0.05	0.30±0.01

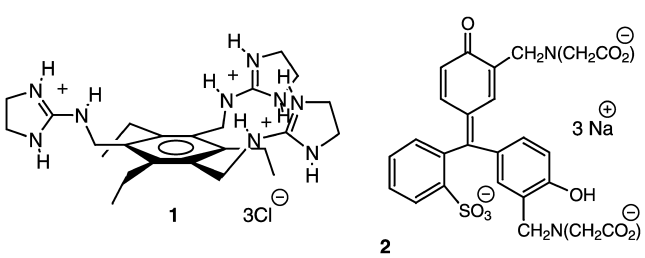
(B) Vodkas

Flavor	Predicted values		NMR determined values
	[citrate] (mM)	[calcium] (mM)	[citrate] (mM) (15% error)
Citrus twist	0.98	0.72	0.90
Orange	0.31	0.21	0.44
Vanilla	1.18	0.74	1.16
Green apple	1.55	0.02	1.04
Raspberry	1.37	0.11	1.30

concentrations to separate solutions of **1**:**2**. All solutions were buffered at pH 7.5 with HEPES buffer (10 mM) in 75% Smirnoff Vodka in water (v/v). Nine separate solutions were made that contained 0.5 mL of the H_2I solution, 100 μL of a 10 mM $Ca(NO_3)_2$, and aliquots of a 20 mM Citrate solution brought to a volume of 1.00 mL with the solvent system described above. Next, nine other solutions were prepared in the same manner without the addition of the $Ca(NO_3)_2$ solution. For the 'leave-one-out' strategy, four test solutions were prepared to evaluate the network's ability to extrapolate and are shown in Table 1.

For the analysis of flavored vodkas, sample preparation proceeded by taking an aliquot (25.0 mL) of each flavored vodka and evaporating any ethanol. To each residue, 100 mL of deionized water was added and each sample was lyophilized to remove the water. The residue was then dissolved in 25% (v/v) water in vodka (5.0 mL). An aliquot (100 μL) of this beverage solution was added to the sensing ensemble (0.2 mM **1** and 0.01 mM **2**) and brought to a total volume of 1.00 mL. An absorbance value was recorded three times for each sample and the corresponding citrate concentration and calcium value were obtained from the matrix (Table 1A). The final values reported in Table 1A were obtained by multiplying the value determined from the ANN by the dilution factor.

Artificial neural network processing. Processing of the UV–Vis measurements was accomplished using Statistica Artificial Neural Network software (version 5.5) for the multi-layered perceptron (MLP) analysis. No pre-processing of the data was attempted.



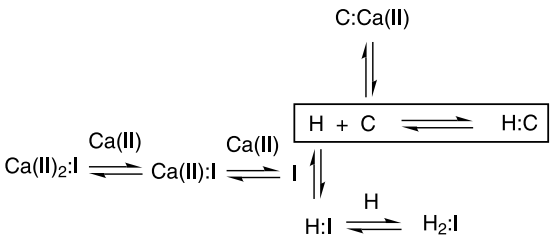
Scheme 1. General molecular structure of the host **1** and the indicator (xylenol orange) **2**.

3. Results and discussion

The host (**1** or **H**) and indicator (**I**), xylenol orange (**2**), were chosen for this study due to their established affinities for citrate (**3**) and calcium (Ca(II)), respectively (Scheme 1).[8] The presence of the guanidinium groups on **1** is known to impart affinity to carboxylates,[7,9–12] while the iminio-diacetic acid moieties on **2** have been shown to bind divalent cations like calcium.[13–16] Also, the indicator was chosen due to the characteristic color change or λ_{max} shift observed earlier in similar systems resulting in a larger dynamic range with which to work.[8] The assay relies upon the differential binding characteristics of the indicator, which binds both host **1** and Ca(II). Further, the binding is synergistic. The binding of citrate to **1** releases **2** allowing it to bind Ca(II). In addition, the binding of Ca(II) to **2** frees up **1** to bind citrate. All these events are in equilibrium in solution, as described below.

The signaling mechanism envisioned is an indicator-displacement assay which has often been utilized by the Anslyn group.[7,9–12] The signaling scheme is executed by adding a host molecule to an indicator adorned with binding groups similar in charge or geometry to the target analyte. Once a host–indicator complex is formed, the analyte of interest is introduced and the host–indicator equilibrium is disrupted. As the analyte preferentially binds to the host, an optically measurable response is observed and is attributed to the changes in the microenvironment of the indicator in the solution.

The training set (matrix) needed for the identification and quantification of calcium and citrate was carried out by obtaining UV–Visible spectra of a two-component sensing ensemble of **1** and **2** in solution (Scheme 2). With this



Scheme 2. Equilibria of the systems studied. H=host, C=citrate, I=indicator. The primary equilibria between the host and citrate is boxed for emphasis, but it is clear that a variety of interrelated equilibria are also present in the solution.

approach, several absorbance measurements at different wavelengths from a solution containing this sensing ensemble can serve as unique data inputs (or a fingerprint response) allowing for identification and quantification of components present in the solution. In this regard, the concentration of the host and indicator were kept constant while separate UV–Visible spectra were obtained for each addition of various amounts of calcium and citrate. Figure 1 is a general outline for the matrix of spectra obtained for the training set.

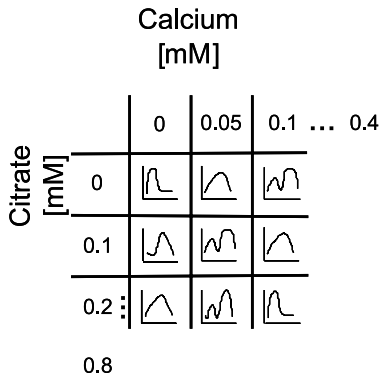


Figure 1. The approach taken to obtain data via UV–Vis spectroscopy for the two-component sensing ensemble, where spectra were recorded at various concentrations (mM) of calcium and citrate.

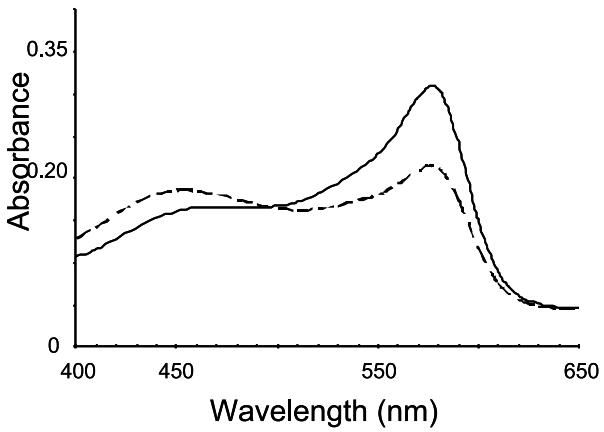


Figure 2. Representative UV–Vis spectra of the indicator-displacement assays created when a solution containing **1** (240 μM) and **2** (10 μM) is titrated with various concentrations of calcium and **3** in 25% (v/v) vodka in water with 10 mM HEPES buffer at pH 7.5. [(–) calcium at 400 μM and citrate at 100 μM] and [(- -) calcium at 50 μM and citrate at 600 μM] Inside tick marks on the *x*-axis illustrate the 25 wavelengths chosen to use in the ANN analysis.

Eighty discrete spectra resulted by systematically changing the concentrations of calcium in 50 μM increments ranging between 0 and 400 μM and also changing the concentrations of citrate in 100 μM increments ranging between 0 and 800 μM. An example of the spectral difference between the binding of varying ratios of calcium and citrate to **1** and **2** is illustrated in Figure 2. These two spectra show that a unique response is detected when a complexometric dye is combined with a synthetic receptor upon exposure to mixtures of the two different analytes. The solvent used for these solutions was a 25% (v/v) vodka in aqueous buffered solutions so that possible interfering components in the unflavored vodka would be accounted for within the training set.

As shown in Scheme 2, the equilibria of this system are complex. The indicator binds two hosts and two calciums, and citrate and calcium also interact. Detection of these complexes would normally prove to be difficult because many of the species that may exist in the solution do not contain chromophoric groups for optical monitoring by UV–Visible or fluorescence spectroscopy. In fact, only the presence of the complexometric dye allows for optical monitoring of the events occurring in the solution. However, the spectrophotometric changes that occur upon introduction of different analytes to the solution are subtle (Fig. 2) and these subtle differences can be extracted quantitatively through the application of pattern recognition.

Supervised learning with pattern recognition protocols was accomplished using multi-layer perceptron (MLP) artificial neural networks (ANN).[17–19] The diagram in Figure 3 illustrates the typical organization of the components within a multilayer ANN. The structure of an ANN can be tailored to the problem being solved. For our purposes, the number of units in the input layer is equal to the number of wavelengths taken from each UV–Visible spectrum in the training set. The output layers are chosen to be equal to the number of predictions desired from the network. For our studies, the network is expected to predict the concentrations of calcium and citrate. The intermediate layers ('hidden layers') positioned between the input layers and the output layers were selected to be half the number of the input layers. The connections between each of the layers (Fig. 3) allow for each component (or neuron) to interact with each other and extend the ability of the network to

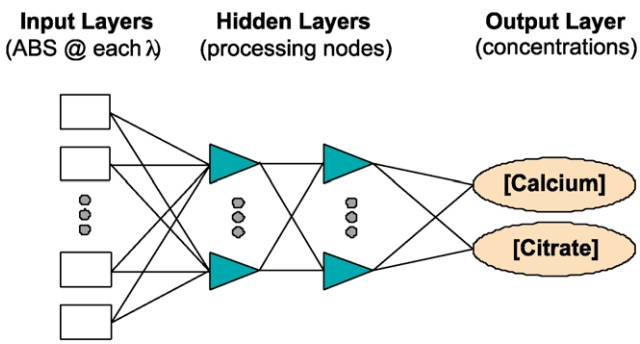


Figure 3. General representation of the multilayer ANN used for the analysis of flavored vodkas. The ANN is composed of an input layer, a variable number of hidden layers, and an output layer.

generate more complex algorithms for a range of difficult applications.

With the network trained, 'unknowns' or inputs not present in the matrix can be supplied into the trained network so that the previously formed algorithms can attempt to calculate an output value. All but five of the spectra were used to train the ANN using 25 wavelengths from each titration. The five traces that were omitted from the training set were utilized as test points to evaluate the network's ability to extrapolate and therefore gage the performance of the ANN matrix. The percent difference within the matrix for the output values of the test points ranged from 3.4 to 30% (Table 1A).

Despite the fact that the relative error associated with the output values for each test point was less than 30%, independent verification of the individual citrate concentrations of the unknowns reported for the flavored vodka samples was carried out by other techniques. Given the good correlation between citrate and Ca(II) found in Table 1, we focus here on one component for verification: citrate. The amount of citrate present in each flavored vodka sample was accomplished by a NMR analysis. As shown in Table 1B, verification of citrate concentrations by NMR gave values with a percent difference ranging from 1.7% for the vanilla flavor to 33% for the green apple flavor. These values are in good agreement, but it is believed that increasing the number of spectra obtained for the training matrix would improve the accuracy and reduce the error of the methodology presented here. In addition, it was surprising to us that the values found by both the ANN and the NMR methods indicate more citrate in the vanilla, green apple, and raspberry flavored vodkas compared to the orange and citrus twist flavors.

In conclusion, the combination of indicator-displacement assays and a synthetic host with pattern recognition algorithms generates a useful sensing strategy. It allows for an analysis of a system involving multiple equilibria to be performed, because the patterns present is the UV–Vis spectra are the sole data needed for the training set. Also, we successfully extended our differential sensing methods to encompass a practical application for the analysis of commercially flavored vodkas by using a synergistic assay that relies on the cross reactivity of the indicator. This detection method demonstrated that concentrations of more than one analyte can be determined in a single analysis without the need for an array or sophisticated instrumentation.

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References

1. Albert, K. J.; Lewis, N. S.; Schauer, C. L.; Sotzing, G. A.; Stitzel, S. E.; Vaid, T. P.; Walt, D. R. *Chem. Rev.* **2000**, *100*, 2595.
2. Drew, S. M.; Janzen, D. E.; Mann, K. R. *Anal. Chem.* **2002**, *74*, 2547.
3. Goodey, A.; Lavigne, J. J.; Savoy, S. M.; Rodriguez, M. D.; Curey, T.; Tsao, A.; Simmons, G.; Wright, J.; Yoo, S. J.; Sohn, Y.; Anslyn, E. V.; Shear, J. B.; Neikirk, D. P.; McDevitt, J. T. *J. Am. Chem. Soc.* **2001**, *123*, 2559.
4. Lonergan, M. C.; Severin, E. J.; Doleman, B. J.; Beaber, S. A.; Grubb, R. H.; Lewis, N. S. *Chem. Mater.* **1996**, *8*, 2298.
5. Toko, K. *Biosens. Bioelectron.* **1998**, *13*, 701.
6. Wiskur, S. L. F.; Anslyn, E. V.; McDevitt, J. T. *Angew. Chem., Int. Ed.* **2003**, *42*, 2070.
7. Metzger, A.; Lynch, V. M.; Anslyn, E. V. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 862.
8. McCleskey, S. C.; Metzger, A.; Simmons, C. S.; Anslyn, E. V. *Tetrahedron* **2002**, *58*, 621.
9. Metzger, A.; Anslyn, E. V. *Angew. Chem., Int. Ed.* **1998**, *37*, 649.
10. Niikura, K.; Metzger, A.; Anslyn, E. V. *J. Am. Chem. Soc.* **1998**, *120*, 8533.
11. Lavigne, J. J.; Anslyn, E. V. *Angew. Chem., Int. Ed.* **1999**, *38*, 3666.
12. Cabell, L. A.; Best, M. D.; Lavigne, J. J.; Schneider, S. E.; Perreault, D. M.; Monahan, M. K.; Anslyn, E. V. *J. Chem. Soc., Perkin Trans. 3* **2001**, 315.
13. Bremer, C.; Grell, E. *Inorg. Chim. Acta* **1996**, *241*, 13.
14. Hulanicki, A.; Glab, S.; Ackermann, G. *Pure Appl. Chem.* **1983**, *55*, 1137.
15. Green, F. J. *The Sigma-Aldrich Handbook of Stains Dyes and Indicators*; Aldrich Chemical Company: Milwaukee, 1990; pp 468–740.
16. A.H.A. Wardi, W. S.; Varma, R. *Anal. Chem.* **1974**, *46*, 919.
17. Beebe, R. J.; Seasholtz, M. B. *Chemometrics*; Wiley: New York, 1998.
18. Jansson, P. A. *Anal. Chem.* **1991**, *63*, A357.
19. Burns, J. A.; Whitesides, G. M. *Chem. Rev.* **1993**, *93*, 2583.