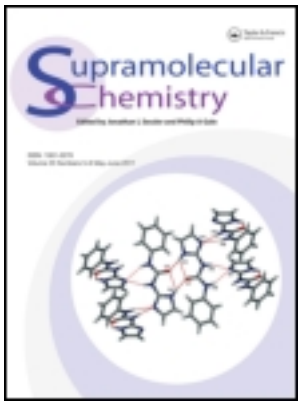

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Pattern-based discrimination of organic acids and red wine varietals by arrays of synthetic receptors

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Modern instrumental analytical techniques pervade chemical analyses in the authentication, quality control and research in wines. A potential alternative approach that does not involve the use of expensive analytical equipment, though dependent on availability of suitable chemical receptors, is array sensing. A 3 × 3 array of sensing ensembles that function *via* indicator displacement assays, and are composed of previously explored boronic acid receptors and indicators, was used for the discrimination of three organic acids commonly found in wine. This array, after finding it suitable for discriminating malate, tartrate and citrate, was used for the pattern-based discrimination of six wine varietals. Linear discriminant analysis of spectroscopic data obtained from the addition of the organic acids and wines to the array showed satisfactory discrimination of all analytes. Such a sensor array is envisioned to ultimately be able to classify wine varietals.

Keywords: array sensing; boronic acid and guanidinium receptors; 1,3,5-triethylbenzene scaffold; organic acids; red wines

Introduction

Fingerprinting of complex mixtures is common in the analysis of food, drug, biological and environmental samples (*1–4*). This is especially important in the wine industry, where authentication and quality assurance require fast, reliable methods. Current methods of analysis take advantage of advances in instrumental analytical techniques such as LC/MS-MS, GC-MS, NMR and NIR (*2*). Some studies have successfully combined these techniques with multivariate data analyses (*5*) that effectively produce metabolic profiles and fingerprints of wines that relate to geographic origin (*6*, *7*), viticultural practices and grape variety. while the instrumental techniques rely on the intrinsic properties such as exact masses of components of the wines (*6*, *8*), other techniques, such as electronic noses and similar thin film multisensor array, rely on the ability of the wine components to interact with sensors that can produce a measurable response (*9*). Recently, we have reported the use of arrays of peptidic colorimetric sensors in the differentiation of flavonoids and red wine varietals (*10*). In this paper, we employ a different set of sensors that target organic acids instead of flavonoids in red wines.

Malate and tartrate are major organic acid components of wine (*11*). These organic acids have been the analytes of interest by our group as part of our development of receptors involving the 1,3,5-triethylbenzene scaffold. This scaffold imparts preorganisation of covalently attached elements for receptor creation (*12*). The guanidinium group is known to bind carboxylates whereas the boronic acid group is known to bind 1,2-diols (*13*). Hence, compounds **1**, **2** and **3**, and related compounds (*14–20*), were previously synthesised and used in the analysis of acids and sugars. These studies focused on the ability of the individual receptors to bind tartrate, malate and citrate.

Herein, we report an array composed of these three synthetic receptors to discriminate organic acids in wines by employing pattern recognition techniques. The receptors are used in combination with colorimetric indicators in order to create a series of indicator displacement assays (*21–23*). The response is based on the change in optical properties of an indicator, which is displaced upon the addition of the analyte to a buffered solution of the receptor–indicator complex. Indicator displacement assays have been used in binding studies (*24*, *25*), as well as in quantitative determination of analytes (*26–28*). In array sensing, indicator displacement assays can be used by employing multiple receptor–indicator pairs that are differential (*29–31*) towards the target analytes.

Statistical pattern recognition protocols (*32*) are then employed to extract information from the data set obtained, i.e. the measured responses of the sensors

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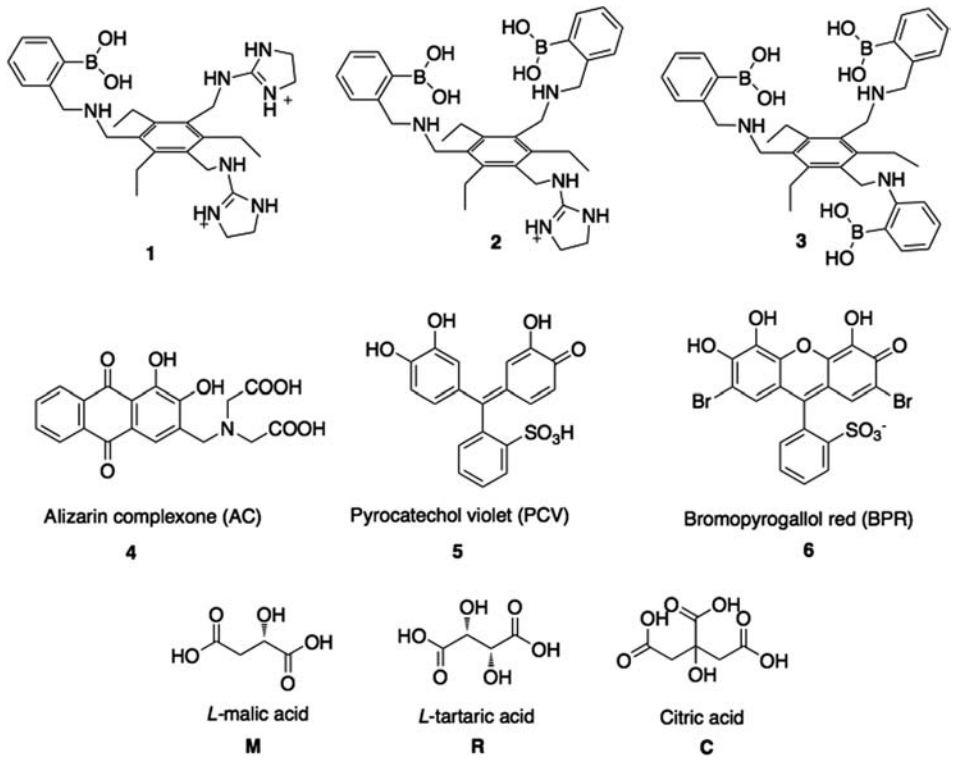

Figure 1. Structures of synthetic receptors, colorimetric indicators and analytes.

in the array from their interaction with the analytes. A common protocol used is principal component analysis (PCA), which gives a summary of the variance in the data set (*33*). Linear discriminant analysis (LDA) is a similar protocol but is a supervised technique, and simply results in maximum clustering and classification of observations.

Results and discussion

Receptor **1** (Figure 1) had previously been shown to bind the indicators alizarin complexone (AC) (*34*), pyrocatechol violet (PCV) (*35*) and bromopyrogallol red (BPR) (*35*). Furthermore, the receptors have been used in the indicator displacement assays of malate (**M**) and tartrate (**R**). Receptor **2** was shown to bind PCV (*35*, *36*) and BPR (*35*), also in the analysis of malate and tartrate. The ensemble of **2** and PCV was also used for the analysis of gallic acid in Scotch (*36*). Receptor **3** was found to bind AC in a study that used a sensing ensemble to analyse malate in Pinot Noir grapes (*37*).

To explore the feasibility of using all the possible ensembles from this collection of receptors and indicators for the pattern-based differentiation of the three organic acids, we first obtained binding isotherms of all receptor–indicator combinations (Figure 2). The 'binding ratios' (Table 1) were obtained for all the nine complexes by titrating the indicators with the synthetic receptors: the concentrations of the indicators were kept constant (AC, 0.180 mM; PCV, 0.060 mM and BPR, 0.030 mM), and the concentrations of the receptors were gradually increased until saturation, which was monitored by UV–vis spectrometry at the corresponding λ_{max} of the indicators at pH 7.4. In this paper, the binding ratio is arbitrarily defined as the ratio of the receptor to indicator concentrations at which the indicator is saturated with the receptor at the concentrations used in this study. This binding ratio should not be considered the stoichiometry, which has previously been determined to be 1:1 for these hosts and indicators (*34–37*). For example, the binding ratio of receptor **2** to AC in this paper was determined to be 2:1 since at this ratio, the binding curve begins to plateau [see Figure 2(a); the ratio is the *x*-axis in this plot]. The binding isotherms exhibited the differential nature of the receptors towards the indicators, as can be gleaned from the different extents of absorbance changes and a number of equivalents required to reach saturation.

Indicator displacement assays with **M**, **R** and **C** as analytes using the **1**:AC complex (Figure 3) also showed differential binding of the analytes to the receptors.

After verifying differential displacement, discrimination of the same organic acids using the suite of receptors and indicators was attempted. Changes in absorbance values at the λ_{max} of the indicators at pH 7.4 resulting from the addition of 0.5, 1.0 and 2.0 mM of the analytes to solutions of the ensembles buffered by HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) at pH 7.4

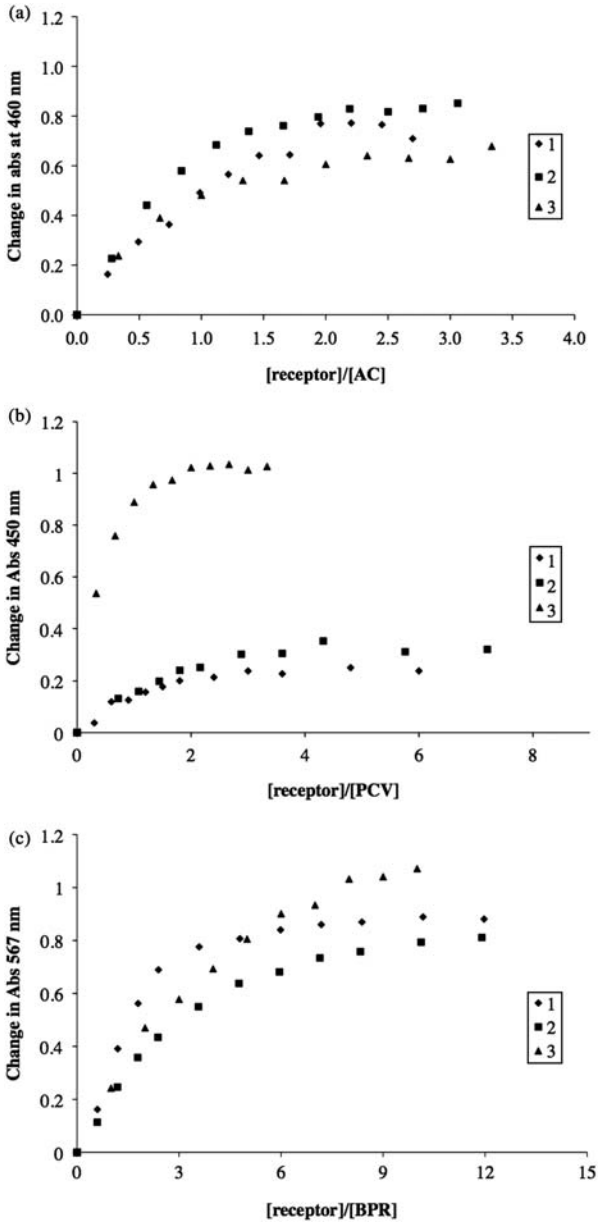

Figure 2. Binding isotherms of three indicators, (a) 0.180 mM AC, (b) 0.060 mM PCV and (c) 0.030 mM BPR with receptors **1**, **2** and **3** in 10 mM HEPES dissolved in 3:1 methanol/water at pH 7.4.

in 96-well plates were recorded and analysed by PCA (Figure S1 of the Supplementary Information, available online). The resulting classification of the analytes was poor

Table 1. 'Binding ratios' of the nine receptor–indicator ensembles used in this study.

	'Binding ratio'		
Indicator	Receptor **1**	Receptor **2**	Receptor **3**
AC	2:1	2:1	3:1
PCV	1:1	2:1	6:1
BPR	1.5:1	1:1	7:1

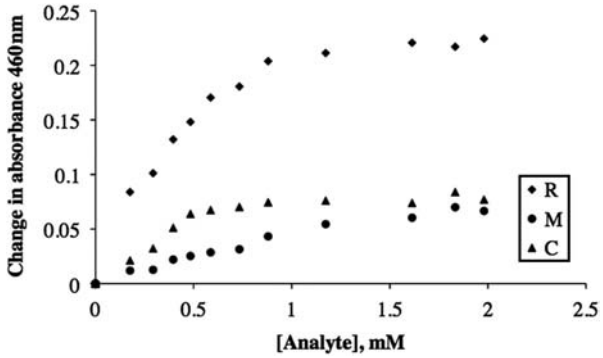

Figure 3. Indicator displacement assay of three analytes, tartrate (**R**), malate (**M**) and citrate (**C**) with receptor **1** (0.36 mM) and indicator AC (0.18 mM), in 10 mM HEPES dissolved in 3:1 methanol/water at pH 7.4.

from PCA but LDA was found to give moderate discrimination of the analytes [Figure 4(a)]. Despite the overlap of the analytes, it can be observed that higher concentrations of analytes were classified towards the right side of the plot, indicating that the first variable, F1, in the linear discriminant plot represents the concentration of the analytes, or the extent of displacement of the indicators by the analytes. The second variable, F2, reflects a different pattern, involving both a concentration and relative extent of indicator displacement by each organic acid. From these observations, tartrate induces a higher displacement of indicators than citrate, which in turn displaces the indicators more than malate. This observation can be clearly seen from the linear discriminant plot of the indicator displacement data at 0.5 mM acids [Figure 4(b)], which shows excellent discrimination of the acids that are classified into completely different quadrants of the plot.

The corresponding loading plot [Figure 4(c)], which shows the extent to which each sensing ensemble contributes to the scores, also shows cross-reactivity. The relationship between the analytes and the sensing ensemble can be obtained by comparing the relative positions of the analytes in the LDA score plot and the sensing ensembles in the loading plot (*33*). **1**:AC, **1**:PCV, **1**:BPR and **2**:BPR are grouped together in the upper right quadrant of the loading plot [Figure 4(c)]. This group is, therefore, considered to be positively correlated with citrate [Figure 4(a)], whereas tartrate is positively correlated with the group of sensing ensembles composed of **2**:AC, **3**:PCV and **3**:BPR, which are found in the lower right quadrant. The same can be said for malate and sensing ensemble 2:PCV. The correlation between an analyte and a sensing ensemble is attributable to the extent that the analytes displace the indicators in each sensing ensemble. The more the analyte changes the absorbance of the sensing ensemble, the more it will be found in the same quadrant as the sensing ensemble in the corresponding loading plot.

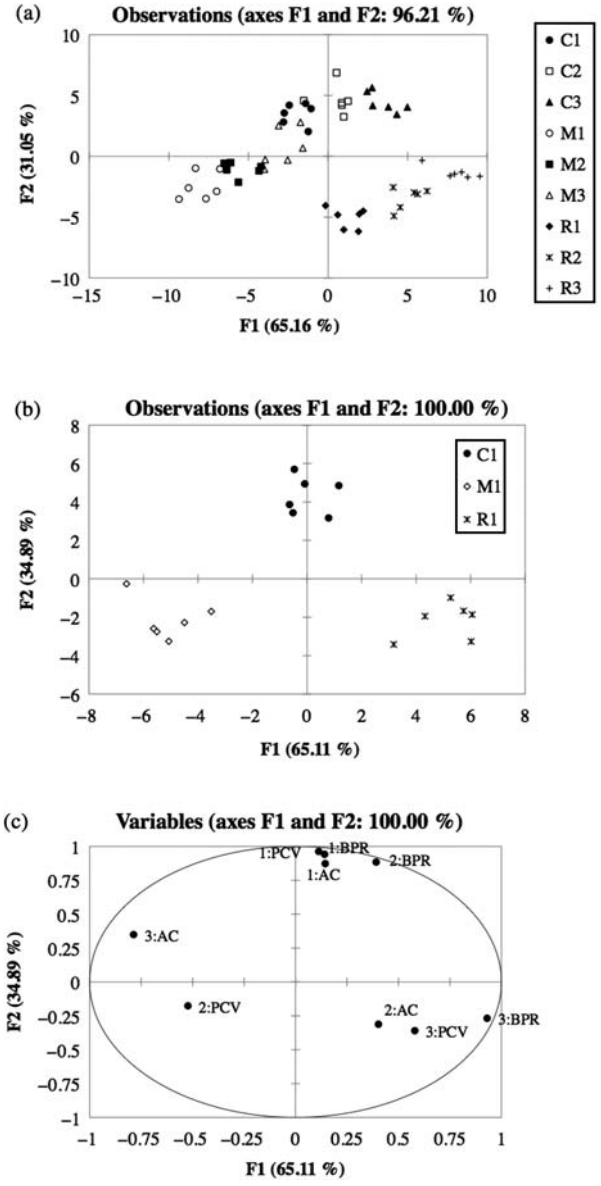

Figure 4. (a) LDA plot of spectroscopic data obtained from the addition of increasing concentrations (1–0.50 mM; 2–1.0 m; 3–1.5 mM) of malate (**M**), tartrate (**R**) and citrate (**C**) to solutions of indicator–receptor ensembles buffered with 10 mM HEPES dissolved in 3:1 methanol/water at pH 7.4 (Table 1); (b) LDA plot of spectroscopic data from the addition of 0.50 mM analytes to the ensembles; (c) loading plot for the analysis in (b).

Since we could classify the acids and discriminate concentrations to some extent, we next turned our attention to wines. First, indicator displacement assays using wine samples were explored using sensing ensemble **1**:AC (Figure 5), which was randomly chosen out of the possible nine ensembles. HEPES-buffered (10 mM) solutions of **1**:AC in 3:1 methanol/water containing increasing concentrations of wines were made at the determined optimal binding ratio (Table 1) in 96-well plates. The absorbance at 460 nm, the λ_{max} of AC at pH

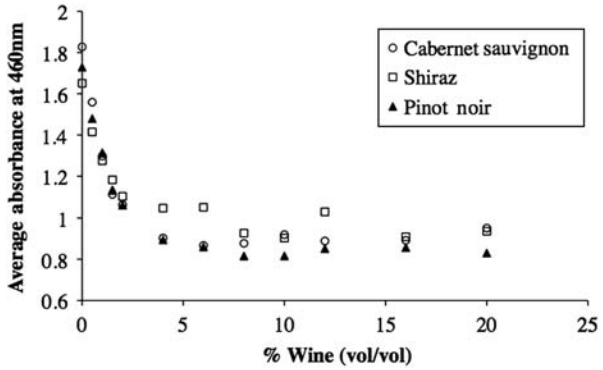

Figure 5. Binding isotherms obtained from the addition of wine samples (v/v) to solutions of 0.36 mM **1** and 0.180 mM AC in 10 mM HEPES dissolved in 3:1 methanol/water at pH 7.4.

7.4, was obtained. To remove background absorbance due to wines, the absorbance of solutions of wines alone at the same concentrations used in 10 mM HEPES dissolved in 3:1 methanol/water at pH 7.4 were determined and subtracted from the absorbance values obtained in the indicator displacement assay. Results showed the ability of the wines to displace the indicators from **1** (Figure 5). We then proceeded to the classification of wines using our 3 × 3 array of receptor–indicator pairs.

Using the same suite of receptor–indicator ensembles for the differentiation of acids, the differentiation of various wine varietals was explored. Six varietals, Merlot, Shiraz, Pinot Noir, Beaujolais, Cabernet Sauvignon and Zinfandel,[2] were evaluated at 1.0% (v/v) concentration in the final solution containing the ensembles at pH 7.4. Changes in absorbance at all three λ_{max} values (Table 2) of the sensing ensembles were obtained upon the addition of the wine (the absorbance values were corrected for the absorbance of the wines). Analysis of the resulting spectroscopic data showed clear classification of the wines. Linear discriminant plot (Figure 6) shows complete separation of the spectroscopic data according to wine samples. Jackknife analysis (*33*)[3] of the data shows 100% correct classification of the wines (Table S2 of the Supplementary Information, available online).

The discrimination is relatively high, as shown by the significant per cent weight along each axis (F1, F2 and F3; Figure 6, see axes). The cumulative variability to F3 is found to be 88% (Table S1 of the Supplementary Information, available online). This means that the classification is caused by, aside from the variations in

Table 2. Concentrations of indicators for the determination of binding ratios.

Indicator	λ_{max}	Concentration (mM)
AC	460	0.180
PCV	450	0.060
BPR	567	0.030

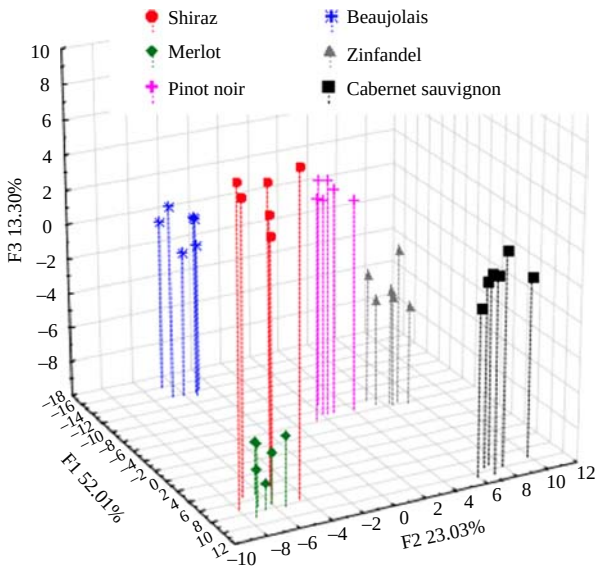

Figure 6. LDA plot of spectroscopic data from the evaluation of different wine varietals (Merlot, Shiraz, Pinot Noir, Beaujolais, Cabernet Sauvignon and Zinfandel; see note 2) using the receptor–indicator ensembles in Table 1.

the amounts of the interacting components of the wines with the receptors as shown by the results of the pattern-based differentiation of malate, tartrate and citrate in the first part of the paper, variations in a significant number of the analyte–receptor interactions during the indicator displacement assay of the wines.

Conclusions

An array of sensors comprising boronic acid and guanidinium group functionalised tripodal receptors, with various pH indicators, was shown to discriminate malate, tartrate and citrate. This array was also able to discriminate samples of red wines. The use of these receptors, in combination with the peptide-based receptors previously reported by our group (*10*), is currently being used for the discrimination of taste characteristics in wine, which will be reported in due course, and can potentially even predict wine age and spoilage during wine fermentation.

Experimental

General information

Receptors **1**, **2** and **3** were synthesised as previously reported (*13*). The indicators, organic acids and HEPES were purchased from Sigma-Aldrich (St. Louis, MO, USA) and/or Acros (Geel, Belgium) and used without purification. Methanol was purchased from Fisher (Fairlawn, NJ, USA). Deionised water was used in all assays. Wines were bought from a local grocery store (H-E-B, San Antonio, TX). Statistical analyses were carried out using XLSTAT.

Determination of receptor–indicator binding ratios

To determine the binding ratios between the receptors and indicators, absorbance values were recorded for well-plate solutions containing increasing amounts of receptors and constant concentrations of indicators in 10 mM HEPES dissolved in 3:1 methanol/water at pH 7.4. For example, for the determination of **1**:**AC** binding ratio, 300 μl solutions containing 0.18 mM **AC** and increasing amounts of **1** (0–0.55 mM in buffer) were prepared and their absorbance values were measured using a well-plate reader at 460 nm, the λ_{max} of **AC** at pH 7.4. The changes in absorbance were calculated and plotted against the ratio of the concentrations of **1** to that of **AC**. The binding ratios of the other receptor–indicator ensembles were determined in the same way. The concentrations of the indicators stayed constant (Table 2) while the concentrations of the receptors were increased. Absorbance values were determined at the corresponding λ_{max} of the indicators.

Indicator displacement assays with organic acids and wines

A series of solutions containing increasing concentrations of an organic acid or wine and constant concentrations of the receptors and indicators using the binding ratios determined (Table 1) were prepared. The absorbance values of the solutions were measured at the λ_{max} of the particular indicator used. For example, the indicator displacement assay of tartrate using **1**:**AC** was done by preparing a series of 300 μl solutions containing 0.180 mM of **AC** and 0.36 mM of **1**, but with increasing concentrations of tartrate (0–2.0 mM) in 96-well plates. The absorbance values of these solutions were measured at 460 nm (λ_{max} of **AC**). The binding isotherm for this assay was prepared by plotting the differences between the absorbance of the solutions containing the acid to that of the ensembles without the acid, against the concentration of the acid.

Array sensing of organic acids and wines

The assay for the classification of organic acids using the array of nine sensing ensembles involves preparing 300 μl solutions in well plates of the ensembles, following the ratios obtained in the binding assays (Table 1), with and without the organic acid, in 10 mM HEPES dissolved in 3:1 methanol/water at pH 7.4. The organic acids were evaluated at 0.5, 1.0 and 1.5 mM, and the changes in absorbance of the individual ensembles at all the λ_{max} of the indicators (Table 2) were calculated (absorbance of assay mixture with organic acid minus the absorbance of the assay mixture without the organic acid), and subjected to PCA and LDA.

The red wines were evaluated at 1.0% (v/v) concentration, following the optimal wine concentrations determined in a previous study (*10*), in the presence of the sensing ensembles,

in 10 mM HEPES dissolved in 3:1 methanol/water at pH 7.4. Absorbance values of the wines without the sensing ensembles at 1.0% (v/v) concentration in 10 mM HEPES, pH 7.4 were also obtained, and these absorbance values were subtracted from the absorbance values of the wines obtained with the sensing ensembles. Prior to evaluation, stock solutions of wines were prepared by changing the pH of the original wine sample by the addition of aqueous 10 M NaOH until the pH is 7.4. The wines were then filtered through a 0.45 μm filter disc and dissolved in 10 mM HEPES in 3:1 methanol/water pH 7.4 to make 6.0% (v/v) stock solutions that were used in performing the array sensing. Spectroscopic data from the evaluation of wines and organic acids were then composed of changes in absorbance values at three wavelengths (450, 460 and 567 nm, corresponding to the λ_{max} values of all the indicators used) using the nine sensing ensembles. These data were then analysed using LDA.

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Notes

1. These authors contributed equally to this work.
2. These wines were bought from the local grocery, H-E-B. Beaujolais – Louis Jadot, France 2008; Shiraz – Sterling Vintner's Collection, California 2005; Merlot – Blackstone, California 2007; Pinot Noir – Cono Sur, Chile, Valle Central 2008; Cabernet Sauvignon – Lindemans, Australia 2008; Zinfandel – Ravenswood Vintner's Blend, California 2006.
3. Jackknife analysis is a technique that examines the reliability of the statistical model by re-sampling the data.

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