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Colorimetric Identification of Carbohydrates by a pH Indicator/pH Change Inducer Ensemble**

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Carbohydrates are one of the primary biological materials, along with proteins and nucleic acids. Although automatic analyses have been fully developed and routinely used for proteins and nucleic acids, carbohydrate analysis remains a highly challenging process.[1] Unlike other biomolecules, most carbohydrates have only one kind of functional group (hydroxy) with different stereochemistry, making the recognition of different carbohydrates extremely difficult. While biosensors[2] and chemical receptors[3] for carbohydrate recognition have been extensively studied, most research has been focused on a target-oriented approach to discover a selective sensor/probe for a given carbohydrate target. As a result of the similarity of the chemical nature between different carbohydrates, many of these selective sensor molecules suffer from cross-reactivity. With such technical difficulties, a systematic study to distinguish a broad range of carbohydrates is even more challenging.

The human tongue uses an array of receptors combined with a pattern analysis to distinguish, in principle, unlimited numbers of different tastes.[4] Inspired by nature's combinatorial sensing approach, many chemosensor arrays have been developed as artificial sensory systems.[5,6] Recently, an indicator displacement assay (IDA) based on organometallic receptors was reported for amino sugar discrimination,[7] but no practical sensing method for the discrimination of large numbers of conventional carbohydrates (with only hydroxy groups) is available yet. Herein, we report the successful recognition of 23 different carbohydrates by using a new strategy based on a pH indicator/pH change-inducer ensemble.

We recently developed an off-the-shelf dye array (NYT-1 with 47 dyes) for the discrimination of 44 metal ions both in identity and quantity.[8] Although NYT-1 can discriminate many different analytes, when we tried carbohydrates in this

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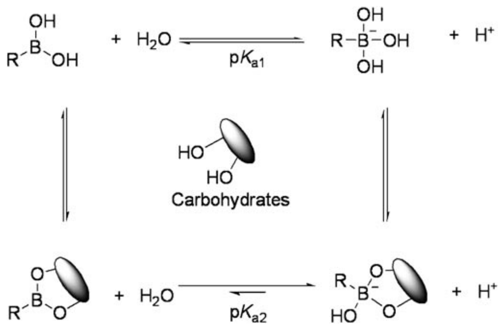
system, the discriminating power was not satisfactory, reflecting the challenging nature of this work.

Among the known chemical recognition motifs for carbohydrates, boronic acids have been the most popular and successful since they were first reported by Lorand and Edwards in 1959.[9a] Most of these types of carbohydrate sensors are dye-conjugated boronic acid molecules. Upon carbohydrate binding, the dye generates readouts as changes in absorbance or fluorescence. Alternatively, diol-containing dyes have been used as carbohydrate competitors against boronic acid receptors to measure glucose concentrations[10] or to study carbohydrate–boronic acid binding.[11] Boronic acid forms reversible cyclic ester complexes with diols, which are abundant in carbohydrates, and undergoes an acid–base reaction with water as a conjugate acid (Scheme 1). Typically, boronic ester is more acidic than boronic acid ($pK_{a1}-pK_{a2} \approx 3$),[12] and ester formation is preferred at higher pH values by an equilibrium shift to the conjugate base releasing a proton.[13]



Scheme 1. Boric/boronic acid equilibria in water in both the absence and presence of carbohydrate.

Utilizing the well-known fact that different carbohydrates exhibit different binding constants to boronic acid, thus generating diverse changes in pH, two key components were devised to maximize the sensitivity and the discrimination power. To enhance the resolution of the pH change readout, we used an array of pH indicators instead of a pH meter. Another key component is the diversity of the boronic acid partner. Although the general binding constant of carbohydrates and boronic acid mainly depends on the pK_a value of boronic acid and the pH value of the solution, it is also affected by other unknown factors.[13] Thus, diverse boronic acids would further discriminate the carbohydrates by generating more unique response patterns per carbohydrate.

We tested more than 100 organic indicator dyes and over 10 boronic acids at various pH values with various buffers using conventional carbohydrates, and narrowed the choice down to 12 dyes and phenylboronic acid/boric acid as the best probe set. The optimized buffer was a sodium phosphate buffer (10 mM) with boric acid ($pK_a = 9.2$)[14] or phenylboronic acid ($pK_a = 8.8$).[13] In the absence of carbohydrate (water control), the final pH values were 8.0 and 7.9, respectively.

We tested 23 different carbohydrates, including 14 mono-, seven di-, and two trisaccharides, at a concentration of 100 mM. The absorbance spectra were collected using a 384-well microtiter plate reader, and the change in absorbance was calculated for further analysis following the previously reported procedure.[8] Ten independent measurements were performed for the statistical data process.

First, we visualized the probe responses by using a heat map plot (Figure 1). In general, a greater fold change of absorbance is correlated to a larger change in pH induced by easier formation of the ester.[13] It seems that unrestrained vicinal diols that contain anomeric hydroxy groups (e.g. in L-sorbose, palatinose, and D-fructose) form the strongest esters, followed by the vicinal diol of furanose (D-ribose). While full analysis of the molecular recognition between each sugar and boric/boronic acids is complicated, several case studies have cast molecular bases for the structural discrimination.[15]

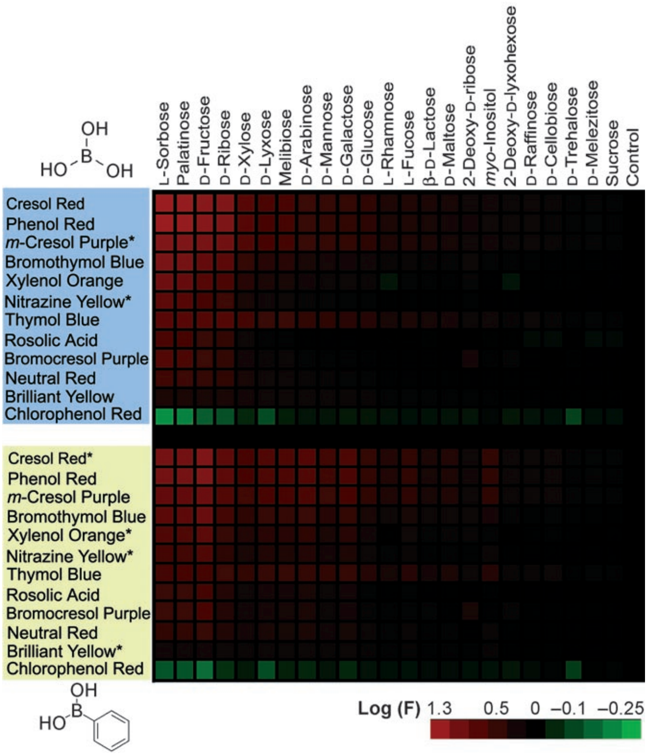
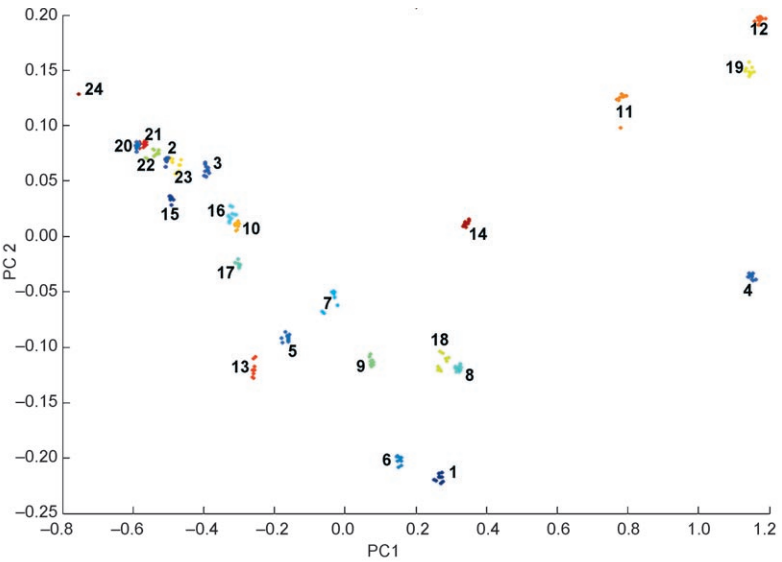


Figure 1. Heat map plot of the 23 carbohydrates (red: increase; green: decrease). F = fold change of absorbance (see the Supporting Information for the full definition). The dyes highlighted on a blue background were tested with boric acid, while those highlighted on a yellow background were tested with phenylboronic acid. Asterisks (*) indicate the six probes selected for minimally requested probe sets.

For pattern analysis and predictive ability determination, we used linear discriminant analysis (LDA) combined with a "jackknifed" classification matrix.[16] Surprisingly, 100% accurate discrimination was achieved for all 23 carbohydrates from this cross-validation routine, which we had expected to be highly challenging to achieve.

Encouraged by this result, we next examined how many probes are minimally required for full discrimination out of a total of 24 probe pairs (12 pH indicators and two pH-change inducers). Selection was performed by a variable selection



1:	D-Arabinose	7:	D-Glucose	13:	*myo*-Inositol	19:	Palatinose
2:	2-Deoxy-D-lyxohexose	8:	D-Lyxose	14:	D-Xylose	20:	Sucrose
3:	2-Deoxy-D-ribose	9:	D-Mannose	15:	D-Cellobiose	21:	D-Trehalose
4:	D-Fructose	10:	L-Rhamnose	16:	β-D-Lactose	22:	D-Melezitose
5:	L-Fucose	11:	D-Ribose	17:	D-Maltose	23:	D-Raffinose
6:	D-Galactose	12:	L-Sorbose	18:	Melibiose	24:	Control

Figure 2. Principal component analysis (PCA) plot for the identification of the 23 carbohydrates using six probe pairs. PC1 variance = 95.4%; PC2 variance = 3.4%.

algorithm using SYSTAT (version 11.0). Six probe pairs (marked with asterisks in Figure 1) were selected as a minimum probe set for full discrimination of the 23 carbohydrates by using stepwise forward algorithms with restricted iteration number. Note that probe sets using only boric acid or only phenylboronic acid could not distinguish all 23 carbohydrates at any combination, which clearly demonstrated the critical synergy effect of the boric/phenylboronic acid combination. The discrimination of 23 carbohydrates using the six probe pair minimum set were visualized by a principal component analysis (PCA; Figure 2). Within the two principal components, over 98% of all discriminatory information is contained and most of the carbohydrates are visually distinguishable in this two-dimensional plot.

In conclusion, a colorimetric discrimination of 23 carbohydrates was successfully demonstrated by using a pH indicator/pH change inducer array. The practical application of this new approach in to real samples is under study.

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