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Efficient Detection of Phthalate Esters in Human Saliva via Fluorescence Spectroscopy

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SHORT TITLE: Fluorimetric Detection of Phthalates in Saliva

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ABSTRACT

The detection of phthalates in human biological fluids remains an important research objective because it provides an important measure of an individual's exposure to this class of compounds, which have known deleterious health effects. Moreover, the ability to accomplish such detection in fluids that are easy to collect, such as saliva and urine, provides additional practical advantages. Reported herein is the application of cyclodextrin-promoted fluorescence energy transfer and fluorescence modulation to accomplish precisely such detection: the development of sensitive and selective florescence-based detection methods for phthalates in saliva, an easily collectable human biological fluid. Such saliva-based detection methods occur with high levels of selectivity (100 % differentiation) and sensitivity (limits of detection as low as 0.089 μM), and provide significant potential in the development of practical phthalate detection devices.

Keywords: phthalate esters, saliva, cyclodextrin, fluorescence spectroscopy

INTRODUCTION

Phthalates are compounds that are of significant concern due to their toxic health effects, particularly as endocrine disruptors (Bowman and Choudhury 2016; Braun 2017). Dibutylphthalate, for example, has been shown to have anti-androgenic effects (Boberg et al. 2015), and diisononylphthalate increases the occurrence of reproductive malformations (Dekant and Bridges 2016). Exposure to phthalates can occur through the use of a broad variety of commercial products (Dodson et al. 2012), including fragrance cosmetics (Pinkas, Goncalves and Aschner 2017), plastics that have been softened by phthalates (Wensing, Uhde and Salthammer 2005), and a variety of vinyl (Wooten and Smith 2013) and food products (Anal and Singh 2007).

Current methods for phthalate detection generally rely on gas chromatography-mass spectrometry (GC-MS) (Russo et al. 2015; Kumar and Sivaperumal 2016) or liquid chromatography-mass spectrometry (LC-MS) (Gallart-Ayala, Nunez and Lucci 2013). While such methods have extremely high sensitivity for a broad variety of phthalates, they require significant time for sample preparation and analysis, as well as financial resources for the high cost instrumentation necessary to conduct such analyses and personnel resources for a highly trained instrument operator (Hartler et al. 2013; Gowda and Djukovic 2014).

Research in the Levine group has focused on the development of a fundamentally different detection method, using fluorescence-based detection in systems where cyclodextrin can promote favorable intermolecular interactions. In such systems, the presence of the toxicant in close proximity to a high quantum yield fluorophore (where the proximity is facilitated by the cyclodextrin) leads to efficient toxicant-to-fluorophore energy transfer (Serio, Miller and Levine 2013; Serio, Moyano et al. 2015; Serio, Roque, et al. 2015; DiScenza, Culton et al. 2017), in cases where the toxicant is photophysically active, and toxicant-specific, proximity-induced fluorescence modulation (DiScenza and Levine 2016a; DiScenza and Levine 2016b; DiScenza, Verderame and Levine 2016; DiScenza, Lynch and Miller et al. 2017), in cases where the toxicant is not photophysically active. In both cases, the system relies on favorable interactions between the cyclodextrin, toxicant, and fluorophore, and generates fluorescence response signals that are highly toxicant-specific for broad classes of toxicants in multiple complex environments, and highly sensitive in their ability to detect low concentrations of toxicants.

The detection of phthalates using cyclodextrin-promoted fluorescence modulation has not been reported to date, despite the fact that the phthalates have numerous structural features that are expected to facilitate their favorable interactions with cyclodextrin (Okoli et al. 2014). In particular, the hydrophobic components of the phthalates will bind in the hydrophobic cyclodextrin interior (Schneider 2015), whereas the carbonyl moieties will hydrogen bond with one of the cyclodextrin rims, likely the wider one with less steric congestion (Yeguas et al. 2011). Fluorescence modulation-based detection of phthalates would obviate many of the challenges associated with mass spectral detection, including the need for costly instrumentation (many portable and inexpensive fluorescence spectrometers exist), and the need to incorporate chromatographic purification prior to mass spectral detection (a result of the fact that every component in a complex mixture generates its own mass spectral signal).

Whether an individual has been exposed to toxicants, as well as the quantity (both at one time and over prolonged time periods), and identity of those toxicants, is important information for medical professionals in assessing that individual's risk of developing exposure-related disease (Mauriello et al. 2017). Such exposure is generally determined through detecting toxicants and/or toxicant metabolites in an individual's biological fluids, including in urine (Junghoon et al. 2017) and breast milk (Asamoah et al. 2018), and we have previously reported the ability of cyclodextrin-promoted detection to operate in these environments (DiScenza, Gareau et al. 2016; DiScenza, Lynch and

Verderame et al. 2018). The detection of toxicants in saliva using cyclodextrin-promoted detection has not been reported to date, despite the fact that saliva-based detection has a number of attractive features (Kintz and Samyn 2002), including the non-invasive nature of sample collection and the fact that ingested toxicants in saliva will have undergone limited metabolism and will be easier to detect in their native forms (Elmongy and Abdel-Rehim 2016). One potential complication is that cyclodextrins may undergo hydrolysis by the amylase present in saliva, although literature reports confirm that only γ-cyclodextrin is likely to undergo such hydrolysis on time scales that are relevant for these detection experiments (Saokham and Loftsson 2017).

Reported herein is the fluorescence detection of a variety of phthalates, in both purified buffer solution and in saliva. This system operates with high sensitivity (micromolar detection limits), selectivity (100% differentiation even between structurally similar analytes), and general applicability for a variety of phthalate esters as well as for binary mixtures of those esters).

EXPERIMENTAL

Materials and Methods

All phthalates and control analytes (compounds **1-5**, Figure 1) were purchased from Sigma-Aldrich chemical company and used as received, unless otherwise noted. All cyclodextrins were purchased from Tokyo Chemical Industry (TCI) and used as received. Fluorophore **6** was synthesized following literature-reported procedures (Shepherd et al. 2004). Fluorophores **7** and **8** were purchased from Sigma Aldrich and used as received. Single donor human saliva was purchased from Innovative Research, Inc. and stored in the freezer until use. ^{1}H NMR spectra were obtained using a Bruker 300 MHz spectrometer. UV-Visible spectra were obtained using an Agilent 8453 spectrometer equipped with a photodiode array detector. Fluorescence spectra were obtained using a Shimadzu RF-6000 spectrophotofluorimeter with 3.0 nm excitation and emission slit widths. GC-MS measurements were obtained using a Shimadzu GC-MS QP2020 gas chromatograph-mass spectrometer. Computational experiments were performed using Spartan 16 software using energy-minimized conformations.

General Procedure for GC-MS Characterization Experiments

GC-MS sample preparation was conducted following literature-reported procedures (Michelsen et al. 2008). In brief, 1 mL of saliva and 2 mL ethyl acetate were added to a glass vial. The vial was

shaken for 1 minute, and the organic extract was collected in a separate glass vial. This procedure was repeated two times, and the organic extracts were combined. The samples were concentrated using the rotary evaporator at 53 ºC until approximately 200 µL sample volume remained.

All GC-MS measurements were performed using a Shimadzu GC-MS QP2020 gas chromatograph-mass spectrometer following literature-reported procedures (Michelsen et al. 2008). The GC-MS operating conditions were as follows: column: Shimadzu SH-Rxi-5SilMS (30 m x 0.25 mm x 0.25 µm); carrier gas: helium at 1.0 mL/min; oven temperature: 50 ºC increase 50 ºC/min to 120 ºC (5 min) increase 10 ºC/min to 230 ºC increase 120 ºC/min to 280 ºC (1 min); injection temperature: 250 ºC, splitting ratio: splitless; electron impact ionization mode; MS ion source temperature: 230 ºC; interface temperature: 150 ºC; total run time: 30 min.

General Procedure for Fluorescence Modulation Experiments

For buffer experiments, 2.5 mL of a 10 mM cyclodextrin solution dissolved in phosphate-buffered-saline (PBS) was added to a quartz cuvette. For saliva experiments, 1.25 mL of a 10 mM cyclodextrin solution dissolved in PBS and 1.25 mL of the saliva sample were combined in a quartz cuvette. A small amount of fluorophore **6-8** (100 µL, 0.1 mg/mL in methanol) was added, and the solution was excited at the excitation wavelength of the fluorophore (460 nm for fluorophore **6**, 490 nm for fluorophore **7**, and 420 nm for fluorophore **8**). Analytes **1-4** (20 µL, 1.0 mg/mL solution in methanol) or control analyte **5** were added to the cuvette, and the resulting solution was excited at the excitation wavelength of the fluorophore. The fluorescence emission spectra were integrated versus wavenumber on the X-axis, and the fluorescence modulation was measured by the ratio of integrated fluorescence emission of the fluorophore in the presence of analyte divided by the integrated fluorescence emission of the fluorophore in the absence of the analyte, as shown in Eq. (1),

$$\text{Fluorescence modulation} = F/F_0 \quad (1)$$

where F is the integrated fluorescence emission of the fluorophore in the presence of analyte, and F_0 is the integrated fluorescence emission of the fluorophore in the absence of analyte. All experiments were performed at room temperature (~22 ºC). Control experiments were performed in which 0 mM cyclodextrin solution in PBS was used in place of 10 mM cyclodextrin in PBS.

For mixture experiments, the above procedure was repeated; however, 1:1 (vol/vol) mixtures of analytes (10 µL, 1.0 mg/mL in methanol) were added to the cyclodextrin or saliva-cyclodextrin solutions, and the fluorescence modulation values were calculated for fluorophores **6-8** following Equation 1.

General Procedure for Limit of Detection Experiments

Limit of detection experiments were performed following literature-reported procedures (Cheng et al. 2016). For buffer experiments, 2.5 mL of a 10 mM cyclodextrin solution dissolved in phosphate-buffered-saline (PBS) was added to a quartz cuvette. For saliva experiments, 1.25 mL of a 10 mM cyclodextrin solution dissolved in PBS and 1.25 mL of the saliva sample were combined in a quartz cuvette. 100 µL of fluorophore **6** (0.1 mg/mL in methanol) was added to the solution and excited six times at 460 nm.

Next, 5 µL of analyte (1.0 mg/mL in methanol) was added, and again the solution was excited at fluorophore **6**'s excitation wavelength. Six repeat measurements were taken. This step was repeated for 10 µL of analyte, 15 µL of analyte, 20 µL of analyte, 25 µL of analyte, 30 µL of analyte, 35 µL of analyte, and 40 µL of analyte.

All of the fluorescence emission spectra were integrated vs. wavenumber on the X-axis, and the calibration curves were generated. The curves plotted the analyte concentration in µM on the X-axis, and the fluorescence modulation ratio on the Y-axis. The curve was fitted to a straight line and the equation of the line was determined. The limit of detection was calculated according to Equation 2.

$$LOD = 3(SD_{blank})/m \quad (2)$$

Where SD_{blank} is the standard deviation of the blank sample and m is the slope of the calibration curve. In cases where the slope of the trend line was negative, the absolute value of the slope was used to calculate the LOD. In all cases, the LOD was calculated in micromolar.

General Procedure for Array Generation Experiments

Array analysis was performed using SYSTAT 13 statistical computing software with the following settings: (a) Classical discriminant analysis; (b) Grouping variable: analytes; (c) Predictors: fluorophores; and (d) Long-range statistics: Mahal.

RESULTS AND DISCUSSION

Saliva Characterization

The human saliva sample was characterized using GC-MS to determine the presence of inherent chemicals components, including those that are typically found in saliva and those that may be a result of toxicant exposure. Typical saliva components found in the sample include long-chain hydrocarbons typically found in human saliva (Figure 2) (Soini et al. 2010). Moreover, traces of caffeine, commonly found in a wide range of food and beverages (Carvalho et al. 2012; Gerald, Arthur and Adedayo 2014), and ditridecyl phthalate, commonly used as a plasticizer in housing insulation and automobile insulation (Net et al. 2015), were also found in the saliva, and indicate that the single anonymous donor may have consumed caffeine and been exposed to phthalate-containing insulation prior to donating his/her saliva.

Cyclodextrin Selection

Cyclodextrins selected for this research include α-cyclodextrin and β-cyclodextrin, dissolved in phosphate buffered saline (PBS, buffered at pH 7.4), as well as a control solution of PBS with no cyclodextrin present. These hosts were selected because they are known to bind at least one of the high quantum yield fluorophores, are readily available, have structural features that will facilitate their interactions with phthalates (via hydrophobic association and/or via intermolecular hydrogen bonding), and are not broken down by the amylase found in saliva. γ-Cyclodextrin, by contrast, was excluded from these studies due its strong propensity for rapid amylase-induced degradation, despite the fact that it has been shown to facilitate cyclodextrin-promoted fluorescence detection under a broad variety of conditions (Saokham and Loftsson 2017).

Fluorophore Selection

The fluorophores selected for this research include three common classes of fluorophores: BODIPY, Rhodamine, and Coumarin. Fluorophores **6-8** have been widely used by our group for cyclodextrin-promoted fluorescence-based detection. Moreover, all three of these fluorophores have high quantum yields, good photostability, and have been used in a wide variety of detection schemes (Katerinopoulos 2004; Beija, Afonso and Martinho 2009; Kim, Ren and Jong 2012).

Analyte Selection

The analytes targeted for detection include phthalate esters that are most commonly found in commercial products, including cosmetics, personal care products, and plasticizers (Koniecki et

al. 2011; Tarasov et al. 2015). Analyte **1** is commonly found in insect repellent (Karunamoorthi and Sabesan 2010). Analyte **2** has been widely used in perfume fragrances (Chingin et al. 2008). Analyte **3** can be found in nail lacquer (Kwapniewski et al. 2008). Analyte **4** is commonly found in polyvinyl chloride (PVC) pipes (Ma et al. 2014). Because individuals are exposed to these commercial products on a regular basis in their daily lives, there is significant benefit to the individuals, their physicians, and scientific researchers to monitoring and quantifying such exposure to better understand the complex relationship between toxicant exposure and the development of exposure-related disease. Measuring the presence of these highly common phthalates in saliva provides one important way to measure such exposure.

Fluorescence Modulation

Each cyclodextrin-fluorophore combination was used to enable the detection of specific phthalate esters in both purified buffer systems and human saliva samples. Micromolar concentrations of analytes **1-4** or control analyte **5** were added to each saliva-cyclodextrin-fluorophore or buffer-cyclodextrin-fluorophore combination, and the degree of fluorescence modulation of fluorophores **6-8** in the presence and absence of analyte was calculated using Equation 1. Each of the three fluorophores selected for analysis displays different behaviors and trends in the modulation results.

BODIPY (fluorophore **6**) shows the highest changes in fluorescence modulation with the introduction of the target analytes (Table 1). This is likely due to the fact that the predominant way in which BODIPY interacts with cyclodextrin is also one of the key ways in which phthalate esters interact with cyclodextrin, via intermolecular hydrogen bonding of the small molecule with the cyclodextrin rim (Gu et al. 2010; Zhang et al. 2016). Introduction of the analyte therefore results in significant disruption of the BODIPY-cyclodextrin association, which is manifested in the fact that the BODIPY emission spectra changes so dramatically.

Similar intermolecular interactions of BODIPY and phthalates with the cyclodextrin hosts also explains why unique emission characteristics are observed for each of the different cyclodextrin hosts: because the BODIPY-cyclodextrin association and the disruption of such association is so key for determining the unique modulation responses, changes to the identity of the cyclodextrin result in significant changes in the BODIPY's microenvironment, which strongly affects the resulting observable fluorescence emission before and after analyte addition (Figure 3).

The fluorescence emission spectrum of Rhodamine 6G (fluorophore **7**) displayed little change with the addition of analyte, for all analytes and all cyclodextrin combinations tested (Table 2). These results indicate a limited association between the Rhodamine 6G and the analytes, likely as a result of the twisted biphenyl axis that precludes close-range intermolecular interactions (Martinez et al. 2006). Some association between fluorophore **7** and the cyclodextrin hosts is likely, however, as indicated by the fact that the fluorescence intensity of fluorophore **7** is higher in the presence of β-cyclodextrin compared to the emission in cyclodextrin-free (i.e. PBS) solution (Figure 4). Intermediate values of fluorescence emission were observed in the presence of α-cyclodextrin, indicating some (albeit limited) association likely occurs.

The fluorescence emission spectrum of Coumarin 6 (fluorophore **8**) displays excimer-like emission peaks, both in the presence of α-cyclodextrin and in the absence of any cyclodextrin (pure PBS) (select results shown in Figure 5).

This indicates that fluorophore **8** does not bind in the α-cyclodextrin cavity, and instead self-associates in a mostly aqueous solvent environment, resulting in the observed excimer emission. In the presence of β-cyclodextrin, by contrast, no excimer peaks are observed, which is a direct result of the strong binding of fluorophore **8** in the β-cyclodextrin cavity (binding affinity ≈ 10,000 M^{-1}) (Edetsberger et al. 2011). Moreover, the strong binding also results in limited changes to the fluorescence of fluorophore **8** in β-cyclodextrin with the introduction of phthalate analytes, which is a result of the inability of the phthalates to displace the strongly bound fluorophore from the cyclodextrin cavity.

In addition to the fluorophore-specific trends discussed above, analyte-specific trends were also observed. In particular, the addition of diisononyl phthalate (analyte **4**) resulted in the highest degree of fluorescence modulation with fluorophores **6-8** and most dramatic changes in fluorescence emission compared to the other analytes (Figure 6). This is likely a result of the long, hydrophobic isononyl chains binding in the cyclodextrin cavity and promoting strong cyclodextrin-analyte association, as well as significant disruption of the cyclodextrin-fluorophore association. In support of this explanation, literature-reported binding constants of the analytes in β-cyclodextrin follow the trend that longer alkyl chains result in strong binding affinities, due to the ability of the alkyl chains to bind in the interior cyclodextrin cavity via hydrophobically-induced complexation (Table 3).

Of note, we also tested for any observable effects that could be attributed to the solvent used to dissolve the phthalate analytes, methanol, in the absence of any added analyte. As expected, only minimal changes in the fluorescence spectra with the introduction of small amounts of methanol were observed. Due to the ability of methanol to engage in substantial hydrogen bonding with the heteroaromatic fluorophores and to perturb the local microenvironment, we observed small changes in the fluorescence emission of the fluorophore with the addition of methanol as the control analyte (Jung, Gerharz and Schmitt 2009; Chai et al. 2015). These interactions are fundamentally different from the interactions between the phthalate analytes and the fluorophores, both in terms of the intermolecular forces that underlie such interactions as well as in the magnitude of the fluorescence emission changes. Our use of the solvent methanol, rather than tetrahydrofuran which was used as the solvent in our previous fluorescence studies, was driven by the need to ensure full solubility of all analytes, while requiring full miscibility with the majority aqueous solvent system that is required for the requisite hydrophobically-driven cyclodextrin binding (Nyssen et al. 1987).

Limit of Detection

The sensitivity of the system was determined by calculating LODs for all buffer-β-cyclodextrin-fluorophore **6**-analyte and saliva-β-cyclodextrin-fluorophore **6**-analyte combinations following literature-reported procedures (Cheng et al. 2016), and selected results of these studies are highlighted in Table 4.

In general, LODs for analytes in saliva were slightly higher, reflecting slightly worse sensitivities, than those measured in purified buffer systems. This is likely due to the competitive binding of saliva components, such as long-chain alkanes, caffeine, or the inherent phthalate compound ditridecyl phthalate, with cyclodextrin. In every case, the calculated limits of detection were significantly below literature-reported limits of concern for these compounds. These low limits of detection highlight the extreme sensitivity of this cyclodextrin-promoted fluorescence modulation-based detection method in environments containing ppm-levels of phthalates. However, phthalates, if found in saliva, exist at ppb-range concentrations (Hines et al. 2009). Current work in our laboratory is dedicated towards optimizing our detection method to detect phthalates at even lower concentrations.

Array Generation

The selectivity of this system was determined by creating statistical arrays using fluorescence modulation results to differentiate between structurally similar analytes in both purified buffer systems and in saliva samples. This array-based analysis showed 100% differentiation between analytes **1-5** in buffer and saliva for all cyclodextrin-fluorophore combinations, with a specific example of such differentiation highlighted in Figure 7.

The response patterns show well-separated signals between the same analytes in buffer and saliva (i.e. analyte **1** in buffer has a signal that is well separated from analyte **1** in saliva), and overall, analytes in saliva are grouped separately from those measured in buffer. A specific example includes the grouping of analyte **2** in saliva with analyte **3** in saliva and analyte **2** in buffer with analyte **3** in buffer, rather than analyte **2** in both samples grouping together.

Additionally, we can also use our statistical arrays to differentiate between different concentrations of analytes (Figure 8).

Figure 8 shows an array generated using different concentrations of analyte **3** with β-cyclodextrin in buffer and saliva. Interestingly, both the buffer and saliva quantitative arrays led to 100% differentiation between different concentrations of analyte **4**. This selectivity builds on the high sensitivity of our system to be able to induce measurable changes in fluorescence emission of fluorophores with very small changes in analyte concentration. This quantitative array provides both the concentration and the identity of the analyte and highlights the potential for the use of statistical arrays to identify and quantify analytes in unknown samples.

Mixture Experiments

In complex biological fluids, such as saliva, oftentimes there are several toxicants present in one sample, which can complicate the accurate detection of analytes. To address the question of toxicant detection in complex mixtures, we tested binary mixtures of phthalates using cyclodextrin-promoted fluorescence modulation and found that 100% differentiation between binary mixtures of analytes was obtained (Figure 9).

Of note, the visual response patterns for analyte mixtures varied depending on the identity of the cyclodextrin hosts. Grouping of analytes is dependent upon cyclodextrin host because the larger β-cyclodextrin cavity may allow binding or association of both analytes, while the smaller α-cyclodextrin cavity may only allow limited association of one analyte in the mixture. In the

presence of α-cyclodextrin (Figure 9A), analyte mixtures of analyte **1** and analyte **2**, analyte **1** and analyte **3** and analyte **2** and analyte **3** group closely together. Analytes **3** and **4** are extremely well-separated from the other analyte mixtures in the presence of α-cyclodextrin. In β-cyclodextrin (Figure 9B), analyte mixtures containing analytes **1** and **2** and analytes **1** and **4** group closely together. Analytes **1** and **3**, analytes **2** and **3**, and analytes **3** and **4** are well-separated in the presence of β-cyclodextrin. In the presence of PBS (Figure 9C), no two analyte mixtures are particularly close, each analyte mixture signal is well-separated. Current work in our laboratory is focused on expanding this array-based detection to include ternary and quaternary mixtures of analytes.

CONCLUSIONS

In conclusion, reported herein is the use of cyclodextrin-promoted fluorescence modulation for the detection of phthalate esters in human saliva. This method is selective (100% successful in differentiating structurally similar compounds), sensitive (sub-micromolar detection limits), and generally applicable (for mixtures of analytes). The high selectivity, sensitivity, and general applicability show potential for the development of rapid, on-site detection devices for phthalate esters.

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ASSOCIATED CONTENT

Electronic supplementary information is available, containing synthesis of fluorophore **6** and summary tables and summary figures for all experiments.

The authors declare no conflict of interest.

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TABLES

Table 1. Fluorescence modulation values for analytes with BODIPY[a]

Analyte	Buffer			Saliva		
	Alpha-cyclodextrin	Beta-cyclodextrin	Phosphate Buffered Saline	Alpha-cyclodextrin	Beta-cyclodextrin	Phosphate Buffered Saline
Dimethyl phthalate	0.98 ± 0.02	1.15 ± 0.02	1.43 ± 0.03	1.15 ± 0.02	1.09 ± 0.02	1.21 ± 0.04
Diethyl phthalate	1.12 ± 0.04	1.01 ± 0.00	1.07 ± 0.01	1.06 ± 0.06	0.99 ± 0.02	1.11 ± 0.02
Dibutyl phthalate	1.15 ± 0.04	1.27 ± 0.0	1.77 ± 0.05	1.12 ± 0.02	1.11 ± 0.03	1.20 ± 0.03
Diisononyl phthalate	2.62 ± 0.06	3.96 ± 0.12	2.16 ± 0.04	1.95 ± 0.02	1.69 ± 0.15	1.77 ± 0.15
Methanol	0.96 ± 0.01	1.19 ± 0.08	1.14 ± 0.04	0.99 ± 0.01	1.13 ± 0.03	0.96 ± 0.08

[a]All results represent an average of results from four trials for each sample. Fluorescence modulation values were calculated using Equation 1.

Table 2. Fluorescence modulation values for analytes with Rhodamine 6G[a]

Analyte	Buffer			Saliva		
	Alpha-cyclodextrin	Beta-cyclodextrin	Phosphate Buffered Saline	Alpha-cyclodextrin	Beta-cyclodextrin	Phosphate Buffered Saline
Dimethyl phthalate	0.99 ± 0.00	1.00 ± 0.00	0.98 ± 0.00	0.98 ± 0.00	1.01 ± 0.00	0.98 ± 0.00
Diethyl phthalate	0.99 ± 0.00	0.99 ± 0.00	0.98 ± 0.00	0.99 ± 0.00	1.00 ± 0.00	0.99 ± 0.00
Dibutyl phthalate	1.00 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	1.00 ± 0.00	1.00 ± 0.01	0.98 ± 0.00
Diisononyl phthalate	1.00 ± 0.00	1.00 ± 0.00	0.98 ± 0.00	1.00 ± 0.00	1.02 ± 0.00	0.98 ± 0.00
Methanol	0.97 ± 0.00	1.00 ± 0.00	0.97 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00

[a]All results represent an average of results from four trials for each sample. Fluorescence modulation values were calculated using Equation 1.

Table 3. Literature-reported binding constants for analytes with β-cyclodextrin[a]

Analyte	Binding Constant (M^{-1})
Dimethyl phthalate	82
Diethyl phthalate	107
Dibutyl phthalate	1160
Methanol	*b*

[a]Binding constant values obtained from Hattori 1999. [b]No literature-reported binding constant available.

Table 4. Limits of detection for analytes with BODIPY and β-cyclodextrin in buffer and saliva[a]

Analyte	LOD in Buffer (mg/L)	LOD in Saliva (mg/L)	Exposure Limit (mg/L)[b]
Dimethyl phthalate	1.68 ± 0.32	3.31 ± 0.21	160
Diethyl phthalate	0.49 ± 0.01	0.62 ± 0.04	60
Dibutyl phthalate	0.28 ± 0.01	0.41 ± 0.04	5
Diisononyl phthalate	0.037 ± 0.001	0.18 ± 0.04	*c*

[a] Limits of detection were calculated using the procedures in Cheng 2016; see Electronic Supporting Information for more details. Errors are shown with enough significant figures to accurately capture the errors [b] Exposure limits from National Service Center for Environmental Publications 1978; [c] No established exposure limit currently exists.

FIGURE CAPTIONS

Figure 1. Structures of analytes **1-4**, control analyte **5**, and fluorophores **6-8**

Figure 2. GC-MS spectrum of the human saliva sample

Figure 3. Fluorescence changes of BODIPY upon introduction of (A) no analyte in buffer, (B) dimethyl phthalate in buffer, (C) no analyte in saliva, and (D) dimethyl phthalate in saliva. The black line represents α-cyclodextrin, the red line represents β-cyclodextrin, and the blue line represents phosphate buffered saline (PBS). Analytical conditions: BODIPY (100 µL, 0.1 mg/mL in THF); dimethyl phthalate (20 µL, 1.0 mg/mL in THF); excitation wavelength: 460 nm; emission range: 470-800 nm; excitation and emission slit widths: 3.0 nm.

Figure 4. Fluorescence emission of Rhodamine 6G upon introduction of (A) no analyte in buffer, (B) diethyl phthalate in buffer, (C) no analyte in saliva, and (D) diethyl phthalate in saliva. The black line represents α-cyclodextrin, the red line represents β-cyclodextrin, and the blue line represents phosphate buffered saline (PBS). Analytical conditions: Rhodamine 6G (100 µL, 0.1 mg/mL in THF); diethyl phthalate (20 µL, 1.0 mg/mL in THF); excitation wavelength: 490 nm; emission range: 500-800 nm; excitation and emission slit widths: 3.0 nm.

Figure 5. Fluorescence changes of Coumarin 6 upon introduction of dibutyl phthalate with (A) α-cyclodextrin, (B) β-cyclodextrin, and (C) phosphate buffered saline (PBS). The black line represents buffer and the red line represents saliva. Analytical conditions: Coumarin 6 (100 µL, 0.1 mg/mL in THF); dibutyl phthalate (20 µL, 1.0 mg/mL in THF); excitation wavelength: 420 nm; emission range: 430-800 nm; excitation and emission slit widths: 3.0 nm.

Figure 6. Fluorescence changes of (A) BODIPY, (B) Rhodamine 6G, and (C) Coumarin 6 upon introduction of diisononyl phthalate in saliva. The black line represents α-cyclodextrin, the red line represents β-cyclodextrin, and the blue line represents phosphate buffered saline (PBS). Analytical conditions: BODIPY, Rhodamine, and Coumarin 6 (100 µL, 0.1 mg/mL in THF); diisononyl phthalate (20 µL, 1.0 mg/mL in THF); excitation wavelength: BODIPY at 460 nm, Rhodamine 6G at 490 nm, and Coumarin 6 at 420 nm; emission range: BODIPY from 470 nm to 800 nm, Rhodamine 6G from 500 nm to 800 nm, and Coumarin 6 from 430 nm to 800 nm; excitation and emission slit widths: 3.0 nm.

Figure 7. Array-based detection of analyteswith β-cyclodextrin in buffer and saliva using BODIPY, Rhodamine 6G, and Coumarin 6 as predictors

Figure 8. Array-based detection of various concentrations of dibutyl phthalate with β-cyclodextrin in (A) buffer and (B) saliva using BODIPY, Rhodamine 6G, and Coumarin 6 as predictors

Figure 9. Array-based detection of mixtures of analytes in the presence of (A) α-cyclodextrin; (B) β-cyclodextrin, and (C) PBS using fluorophores BODIPY, Rhodamine 6G, and Coumarin 6 as predictors