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Efficient Fluorescence Detection of Aromatic Toxicants and Toxicant Metabolites in Human Breast Milk

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Abstract

Once chemical contaminants are released into the environment, there are a number of concerns that arise regarding the environmental persistence of the contaminants, their known and suspected toxicities, and their potential disruption to the ecosystem. One class of contaminants that is of continuing concern is polycyclic aromatic hydrocarbons (PAHs), persistent organic pollutants that are significant components of oil spills. PAHs have been found in the breast milk of nursing mothers living in oil spill affected regions, and can harm the nursing children. We report herein the sensitive and selective detection of 10 PAHs and PAH metabolites in human breast milk using fluorescence energy transfer from the PAH to a high quantum yield fluorophore, and array-based statistical analyses of the resulting fluorescence responses. This detection system was able to separate and identify the PAHs with 100% success in human breast milk and at concentrations as low as 0.17 μM. These results have significant implications in public health and in the monitoring and mitigation of environmental disasters.

Keywords

polycyclic aromatic hydrocarbons; fluorescence detection; cyclodextrin; energy transfer

Introduction

There are many challenges that result from anthropogenic or natural chemical spills, including the long term persistence of the chemical contaminants,[1] their toxic health effects to both humans[2] and animals,[3] and their harmful environmental effects.[4] One class of

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contaminants that is of significant concern is polycyclic aromatic hydrocarbons (PAHs), which are formed from the incomplete combustion of petroleum products,[5] and are introduced into the environment by both natural and anthropogenic events.[6] In one example, the 2010 Deepwater Horizon Spill released 4.9 million barrels of oil into the Gulf of Mexico;[7] this oil was composed of 16% aromatic hydrocarbons as well as a wide variety of other small molecule and macromolecular toxicants.[8]

PAHs can migrate in the environment in a number of ways, including through natural phenomena such as water runoff[9] and circulating air currents,[10] and they bioaccumulate and biomagnify in the food chain.[11] Human exposure to these compounds has been well-documented,[12] and occurs through the consumption of PAH-contaminated water and food, [13,14] and through exposure to PAH-contaminated air,[15] soil, [16] and sediments.[17] Individuals who have been exposed to PAHs contain oxidized PAH metabolites in their blood,[18] breast milk,[19] and urine.[20]

The ability to detect PAHs and PAH metabolites in biological media with high sensitivity and selectivity is significant from a public health perspective, as many PAHs are known or suspected carcinogens,[21] and exposure to such PAHs correlates with the risk of developing exposure-related disease.[22] Currently used methods for PAH detection require chromatographic purification using liquid chromatography[23] or gas chromatography,[24] followed by detection using mass spectrometry[25] or fluorescence spectroscopy.[26] Although these methods are highly sensitive, they require significant time and resources,[27] which can limit the ability to conduct high throughput assays of large populations to measure toxicant exposure. Moreover, because PAHs undergo rapid *in vivo* metabolism to a variety of highly oxygenated products, an ideal detection strategy should be able to detect both unmodified lipophilic PAHs as well as highly polar PAH metabolites; currently used methods focus on only one of these two classes.

Previous work by our group has focused on cyclodextrin-promoted energy transfer from aromatic toxicants to high quantum yield fluorophores as a method for sensitive, selective, and rapid toxicant detection.[28–29,30,31] We have shown that such detection operates successfully in human urine,[32,33] that it can be used for the detection of both non-polar aromatic toxicants as well as highly polar toxicant metabolites,[34] and that it can form part of oil spill remediation strategies using cyclodextrin-promoted PAH toxicant extraction followed by detection.[35–36,37,38]

One biological fluid that is particularly important is human breast milk, which is a vital source of nutrition for nursing children, and provides developmental, immunological and physiological benefits to both the child and mother.[39–40,41,42,43] Because PAHs are lipophilic, they are stored in maternal body fat, released into the breast milk with the onset of lactation, and passed directly to the child.[44] In fact, both non-polar PAHs and oxidized PAH metabolites have been found in the breast milk of individuals living in highly polluted areas. The presence of these contaminants in milk has been linked to adverse health effects in the child.[45] As such, their detection remains a high priority.

Reported herein is the sensitive, selective, and rapid detection of PAHs in breast milk using supramolecular cyclodextrin-promoted energy transfer to high quantum yield fluorophores and the practical relevance of this system in operating with high sensitivity (low limits of detection), selectivity (100% accuracy in identification of unknowns), and general applicability (for both unmodified lipophilic PAHs and highly polar oxidized PAH metabolites, as well as for samples collected from multiple lactating mothers).

Experimental

Materials and Methods

^{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-5301PC spectrophotofluorimeter. All GCMS measurements were obtained using a Shimadzu GCMS-QP2020 gas chromatograph-mass spectrometer. All toxicants and toxicant metabolites (compounds **1–10**, Figure 1) were purchased from Sigma Aldrich and used as received. Fluorophore **11** was synthesized following literature-reported procedures.[46] Fluorophores **12** and **13** were purchased from Sigma Aldrich and used as received.

Breast Milk Collection Procedure

Breast milk samples were provided for these experiments from five anonymous donors. The samples were collected in glass jars, which were sterilized prior to use. The samples were frozen and stored in a freezer until needed.

General Procedure for Sample Preparation

Frozen breast milk was thawed overnight in the refrigerator, after which time it separated into three layers. The breast milk was then allowed to come to room temperature on the lab bench, and shaken vigorously to recombine the layers and ensure a uniform suspension. 1875 μL of this suspension and 20 μL of an analyte solution (1 mg/mL analyte in tetrahydrofuran) were added to a centrifuge tube. The suspension was shaken and allowed to stand for 10 minutes. 938 μL of absolute ethanol and 938 μL 10 mM γ-cyclodextrin in phosphate buffered saline (PBS) were added to each tube, and the tubes were shaken and allowed to sit overnight in the refrigerator. The solution was then centrifuged at 3000 rpm for 10 minutes, after which time it separated again into three layers.

2.5 mL of the aqueous solution (middle layer) was transferred to a cuvette and the fluorescence of the solution was measured as a result of excitation at the analyte's excitation wavelength and a result of excitation at the fluorophore's excitation wavelength. 100 μL of a 0.1 mg/mL fluorophore solution (fluorophores **11–13**) was added to the cuvette, and the solution was again excited at the analyte's excitation wavelength and at the fluorophore's excitation wavelength.

The same procedure was followed but without the addition of an analyte to obtain data in undoped samples.

General Procedure for Energy Transfer Experiments

The efficiency of the energy transfer was quantified as the ratio of the integrated emission of the fluorophore via analyte excitation to the integrated emission of the fluorophore via direct excitation:

$$\text{Energy transfer efficiency } = I_{DA}/I_A \text{ x } 100\% \quad \text{(Eq. 1)}$$

Where I_{DA} is the integration of the fluorophore from analyte excitation and I_{A} is the integrated fluorophore emission from direct excitation.

Analyte comparison ratios were calculated for each experiment according to Equation 2:

$$\text{Analyte comparison } = I_{\text{blank}}/I_{\text{analyte}} \quad \text{(Eq. 2)}$$

where I_{blank} is the integrated emission of the fluorophore from excitation at the analyte's excitation wavelength in the absence of the analyte, and I_{analyte} is the integrated emission of the fluorophore from excitation at the analyte's excitation wavelength in the presence of the analyte. Analyte comparisons less than 1 indicate that the fluorophore emission via excitation at the analyte wavelength is higher in the presence of the analyte, indicating legitimate energy transfer is occurring, and analyte comparison ratios greater than 1 indicate that the fluorophore emission via excitation at the analyte wavelength is lower in the presence of the analyte, indicating analyte-driven fluorescence quenching. Analyte comparisons near 1 indicate little to no interaction of the analyte with the fluorophore, resulting in a fluorescence emission signal that is essentially unchanged with the addition of the analyte (the putative energy donor).

Donor decrease values were calculated as well, which measure the decrease in the analyte fluorescence emission as a result of introduction of the fluorophore. These ratios were calculated according to Equation 3:

$$\text{Donor decrease } = 1 - \left(F_{\mathrm{DA}}/F_{\mathrm{A}}\right) \quad \text{(Eq. 3)}$$

where F_{DA} is the integrated emission of the donor in the presence of the fluorophore and F_{A} is the integrated emission of the donor in the absence of the fluorophore.

General Procedure for Limit of Detection Calculations

The limit of detection (LOD) is defined as the lowest concentration of analyte at which a signal can be detected. To determine this value, the following steps were performed for each analyte-fluorophore combination.

1. Frozen breast milk was thawed overnight in the refrigerator. The breast milk was allowed to come to room temperature and shaken to combine the layers. 1875 μL of the suspension was added to a plastic centrifuge tube. The suspension was

shaken and allowed to stand for 10 minutes. 938 μL of a 10 mM y-cyclodextrin solution in phosphate buffered saline (PBS) and 939 μL absolute ethanol were added to each tube. The tubes were shaken and allowed to sit overnight in the refrigerator. The solution was centrifuged at 3000 rpm for 10 minutes, after which time it separated into three layers.

2. 2.5 mL of the aqueous middle layer was transferred to a cuvette. 100 μL of a 0.1 mg/mL solution of fluorophore **11** in tetrahydrofuran (THF) was added to the cuvette, and the solution was excited at the fluorophore's excitation wavelength and analyte's excitation wavelength and fluorescence spectra were recorded. Six repeat measurements were taken.

3. 5 μL of a 1 mg/mL analyte solution in THF was added to the cuvette and the solution was again excited at the fluorophore's excitation wavelength as well as at the analyte's excitation wavelength. Six repeat measurements were taken.

4. Step 2 was repeated, adding analyte in 5 μL increments until a final volume of 40 μL was added. In each case, the solution was excited at the fluorophore's excitation wavelength and analyte's excitation wavelength and the fluorescence emission spectrum was recorded six times.

5. All fluorescence emission spectra were integrated vs. wavenumber, and calibration curves were generated with the analyte concentration on the X-axis (in μM) and the energy transfer ratio (I_{DA}/I_A) on the Y-axis. The curve was then fitted to a straight line and an equation for the line was determined.

6. For each case, the solution before any analyte was added was also excited at the excitation wavelength for the fluorophore and excitation wavelength for the analyte and the fluorescence emission spectra were recorded (as per step 1). These measurements are referred to as the "blank."

7. The limit of detection is defined according to the following equation:

$$\mathrm{LOD} = 3(\mathrm{SD}_{blank})/m \quad \text{(Eq. 4)}$$

Where SD_{blank} is the standard deviation of the blank 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 LOD. In all cases, the LOD was calculated in μM.

General Procedure for Array Generation Experiments

Array-based analysis was performed using SYSTAT 13 statistical computing software with the following settings:

a. Classical Discriminant Analysis

b. Grouping variable: Analytes

c. Predictors: γ -cyclodextrin/BODIPY, γ -cyclodextrin/Rhodamine 6G, γ -cyclodextrin/Coumarin 6

d. Long-range statistics: Mahal

General Procedures for Characterization Experiments

Fluorescence Experiments—Defrosted breast milk samples were prepared following previously described sample preparation procedures. No analyte was added to these samples. 100 μL of fluorophores **11–13** were added, and the samples were excited at various excitation wavelengths.

GCMS Experiments—3 mL of each breast milk sample were added to 8 mL centrifuge tubes. Each sample was vortexed for one minute and allowed to settle for 15 minutes. 3 mL of acetonitrile were added, and each tube was shaken vigorously by hand. Then 1.2 grams of $MgSO_4$ and 0.3 grams of NaCl were added to each centrifuge tube and shaken for 1 minute. Samples were placed in the freezer for 10 minutes, followed by centrifugation at 3700 rpm for 10 minutes. The supernatant was removed from each sample and analysed by GCMS.

All GCMS measurements were obtained on a Shimadzu-QP2020 gas chromatograph-mass spectrometer with the following settings:

Column: Shimadzu SH-Rxi-5SilMS (30 m × 0.25 mm × 0.25 μm)

Oven temperature: 60°C, hold for one minute, increase 15°C/min until 325°C and hold for three minutes.

Injection temperature: 250°C

Splitting ratio: Splitless

MS ion source temperature: 230°C

Interface temperature: 150°C

Total run time: 22 minutes

Results and Discussion

Analyte Selection

The analytes were selected to include highly toxic PAHs (*i.e.* **2**, **6**, and **8**)[47] that are known components of large scale anthropogenic oil spills,[48] known metabolites of these PAHs (*i.e.* **1**, **3**, **4**, **7**, and **9**)[49,50] that have been found in a variety of biological fluids, compound **5** which is known to cause bladder cancer and has been found in human urine samples,[51] and tetrahydrocarbazole **10** which is more toxic than carbazole and a common motif in the pharmaceutical industry (Figure 1).[52]

Doped Energy Transfer Results

Table 1 shows energy transfer efficiencies from analytes **1–10** to fluorophore **11** in analyte-doped breast milk samples, with analyte comparison ratios shown in parentheses. The efficiency of the energy transfer was quantified as the ratio of the integrated emission of the fluorophore via analyte excitation to the integrated emission of the fluorophore via direct excitation, according to Equation 1. Even after sample preparation procedures were complete, significant amounts of analyte remained in the analysed aqueous layer (see ESI for more details).

Analyte comparison ratios were calculated for each experiment according to Equation 2. Analyte comparisons in most cases were less than 1, which means that the energy transfer peak was increased in the presence of the analyte and legitimate energy transfer from the analyte to the fluorophore occurred. These results represent significant improvements over our previous results obtained in purified buffer solution, where many of the putative results of cyclodextrin-promoted energy transfer were determined to be a result of directly exciting the analyte at a wavelength where it has noticeable absorption rather than a result of legitimate energy transfer.[28] These differences in the results between buffer and breast milk can be explained by differences in the media composition between the aqueous buffer and the lipophilic composition of breast milk.[53]Amphiphilic fatty acids in breast milk are known to form micelles with hydrophobic interiors.[54] These micelles can promote PAH inclusion and concomitant energy transfer, in accordance with literature precedent.[55,56]

Moreover, a comparison of the results obtained in the presence of 10 mM γ-cyclodextrin compared to the results obtained in its absence indicates that for many of the analyte-fluorophore combinations, the addition of cyclodextrin had **no effect** on the observed energy transfer efficiencies. These results indicate significant cyclodextrin-free association between the analyte and the fluorophore, which can occur either in self-assembled micelles (*vide supra*) or simply as a result of hydrophobic association between two hydrophobic small molecules in a complex aqueous-based environment. In such cases, when cyclodextrin is introduced into the complex environment it likely binds other small molecules in its interior instead of the analyte and fluorophore. A variety of hydrophobic small molecules have been found in the complex milieu of breast milk, [53] and some (or all) of these compounds likely form cyclodextrin host-guest complexes.

One example of a situation in which cyclodextrin addition has limited effect is for analyte **6** (Figure 2). In this case, energy transfer efficiencies without the cyclodextrin host (B and D) are essentially identical to the efficiencies observed with the cyclodextrin host (A and C). Analyte **6** is a particularly important detection target due to its known toxicity and high carcinogenicity.[57] This analyte has a large hydrophobic surface area that facilitates cyclodextrin-free association with the aromatic fluorophores;[57] the addition of cyclodextrin in this context does little to disrupt that strong association. Such association is further justified through careful consideration of electrostatic potential maps (see ESI) which were calculated for all analytes and fluorophores in their energy-minimized conformations. This electrostatic mapping indicates high degrees of complementarity for analyte **6** in particular (electron-rich surface) with fluorophores **11–13** (electron neutral/electron deficient surfaces).

Practical Considerations

The ability to use this fluorescence energy transfer for the development of practical detection systems requires (a) **sensitivity** for the detection of low concentrations of toxicants, at levels at or below literature-reported levels of concern; (b) **selectivity** in identifying toxic compounds and distinguishing them from structurally related analogues with markedly disparate toxicities; and (c) **general applicability** for broad classes of toxicants (non-polar PAHs and polar PAH metabolites) in multiple breast milk samples (obtained from multiple donors).

Sensitivity—The **sensitivity** of the system was determined by calculating limits of detection for all analytes using literature-reported procedures,[58] and some selected results are highlighted in Table 2. These results are comparable to the reported permissible exposure limits of 0.2 ppm for selected analytes.[59] Cases where the limit of detection is close to the literature-reported limits of concern showcase the practical applicability of this method. Current efforts in our laboratory are focused on lowering the detection limits for all analytes to obtain optimal sensitivity.

Selectivity—The **selectivity** of this system was determined using array-based statistical analysis to establish unique identifying fluorescence response patterns of structurally similar aromatic analytes.[60–61,62] Results using linear discriminant analysis of the response patterns generated from fluorescence-based detection of analytes **1–10** in the presence of 10 mM γ-cyclodextrin indicated **100% success** in generating unique response signals for the analytes (Figure 3), even among those that are remarkably structurally similar (analytes, **1**, **6** and **7** & analytes **3** and **4**) as well as those that are challenging to separate using other analytical techniques (*i.e.* analytes **1** and **6**).[63] One way to understand this high selectivity and differentiating capabilities, even between structurally similar analytes, is to look at the fluorescence spectra of each analyte-fluorophore combination, which shows clear differentiation and leads to a unique “fingerprint” for each analyte-fluorophore pair (see ESI for more details). These results highlight the uniqueness of this approach for achieving such successful differentiation.

Generality—The **generality** of this method was demonstrated by comparing energy transfer efficiencies for each analyte in four additional samples of breast milk (collected from four different anonymous volunteers). There is **no significant difference** observed in the energy transfer efficiencies and analyte comparison ratios for analytes **1–10** with fluorophores **11–13** in the four additional samples (Tables 3–5). The lack of significant differences shows the reliability of this method for broad-based population screening, and the arrays generated using the results from four independent donors still showed 100% success in differentiating even the most similar of analytes (Figure 4).

Undoped Sample Characterization Results

Sample characterization experiments using fluorescence spectroscopy and gas chromatography-mass spectrometry (GCMS) were performed on undoped samples (without any added analyte) to identify unique differences and variabilities between the five breast milk samples.[64,65]

GCMS—Differences between each of the breast milk samples were further confirmed by gas chromatography-mass spectrometry (GCMS) analysis (see ESI for details). All samples showed peaks corresponding to fatty acid compounds typically found in breast milk.[66] While the same fatty acids were identified in each sample, the amounts of fatty acids and other organic compounds differed significantly (Figure 5). There were no PAHs detected in any of the breast milk samples, likely as a result of the fact that samples were collected from women living in rural areas with limited known pollution and chemical contamination.

Fluorescence Experiments—Within each of the five breast milk samples, linear discriminant analysis of the emission of fluorophores **11–13** from excitation at different wavelengths generated unique pattern identifiers for nearly all of the wavelengths investigated. (Figure 6). Even as little as a 5 nm difference in excitation wavelength (320 vs. 325 nm, or 360 vs. 365 nm) led to complete differentiation in the fluorescence responses. Moreover, each individual breast milk sample demonstrated a visually different response pattern, as seen through differences in the relationships between the excitation wavelength-specific responses.

The high sample specificity is likely a result of differences in the fatty acid composition of the samples (see GCMS section, above), which leads to unique local environments for fluorophores **11–13**. As a result, excitation of the fluorophores at wavelengths at which they have noticeable absorption (see ESI for UV-Vis spectra of all fluorophores) leads to differential fluorescence responses. The uniqueness of each sample raises the possibility of profiling samples based on such identification and creating unique person-specific patterns for accurate sample identification.

Conclusion

Proximity-induced energy transfer from a variety of organic toxicants to high quantum yield fluorophores is a robust, highly efficient method with success in environments as complex as human breast milk. This supramolecular energy transfer leads to strong, turn-on fluorescence emission signals with high sensitivity (micromolar-level detection limits), selectivity (100% differentiation between analytes), and general applicability (no significant difference between multiple samples indicating reliability of method for broad-based population screening) with significant potential in practical detection systems. Efforts towards the development of such systems are currently underway in our laboratory, and results of these and other investigations will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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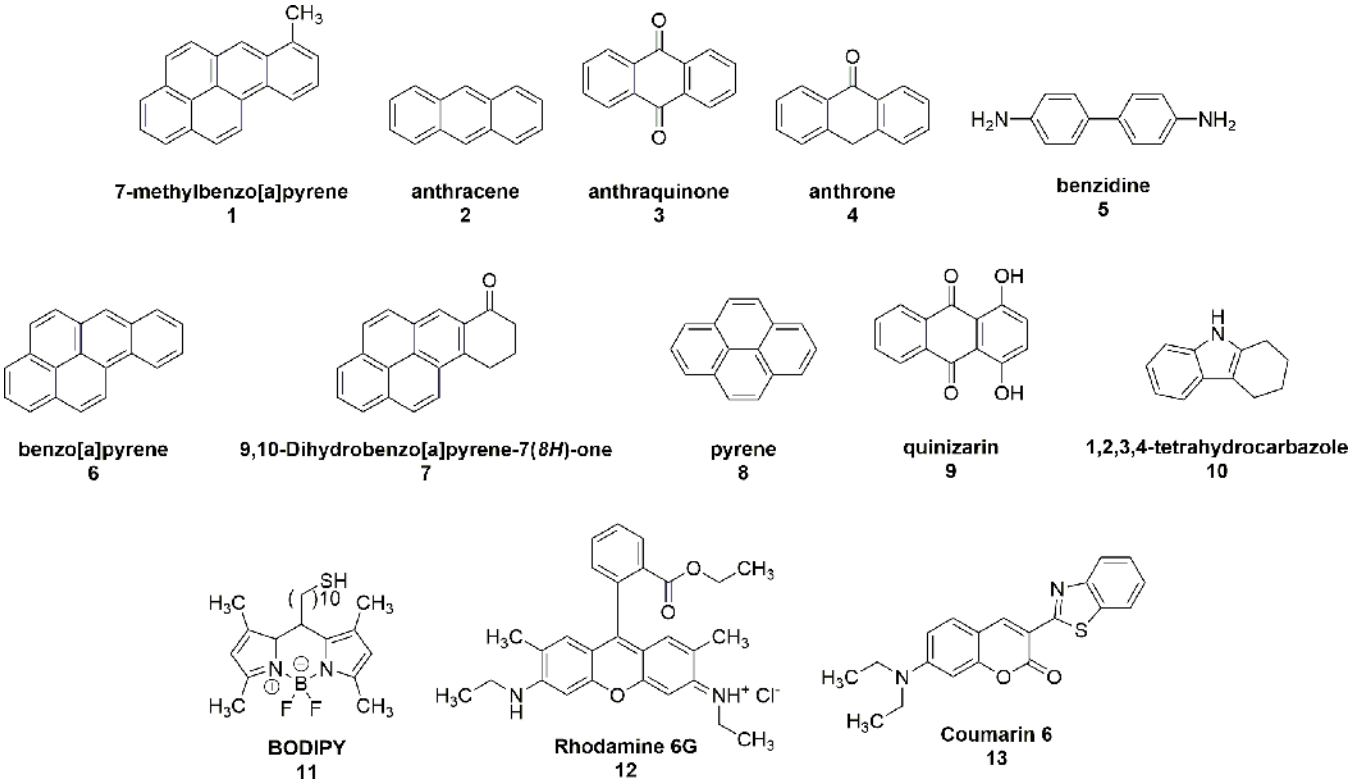

Figure 1.

Structures of analytes **1–10** and fluorophores **11–13**.

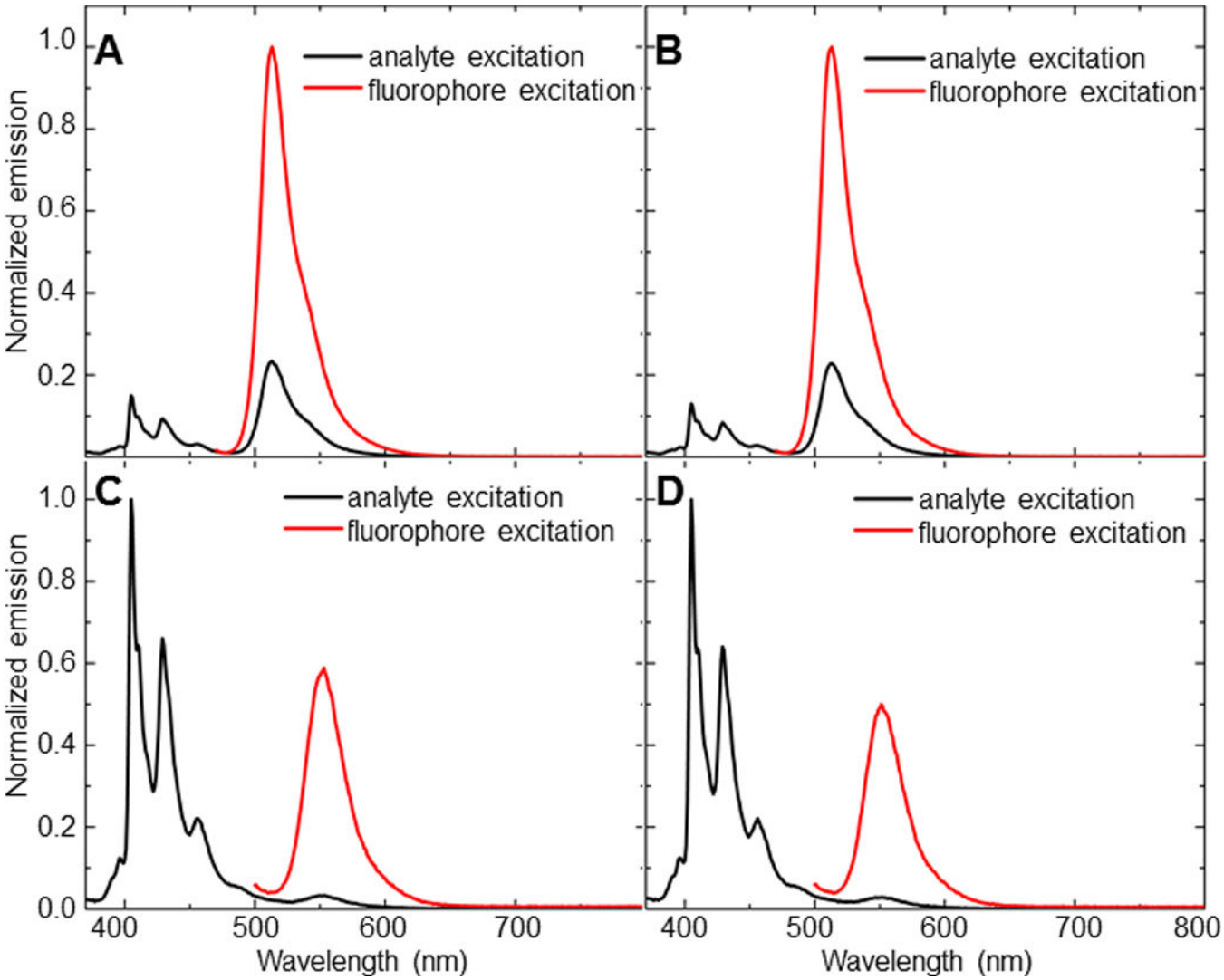

Figure 2.

Energy transfer from analyte **6** to fluorophores **11** and **12**. (A) Analyte **6** – fluorophore **11** in 10 mM γ-cyclodextrin; (B) Analyte **6** – fluorophore **11** in 0 mM γ-cyclodextrin; (C) Analyte **6** – fluorophore **12** in 10 mM γ-cyclodextrin; and (D) Analyte **6** – fluorophore **12** in 0 mM γ-cyclodextrin. The black line represents analyte excitation (360 nm) and the red line represents direct fluorophore excitation (analyte **11**- 460 nm; analyte **12** – 490 nm).

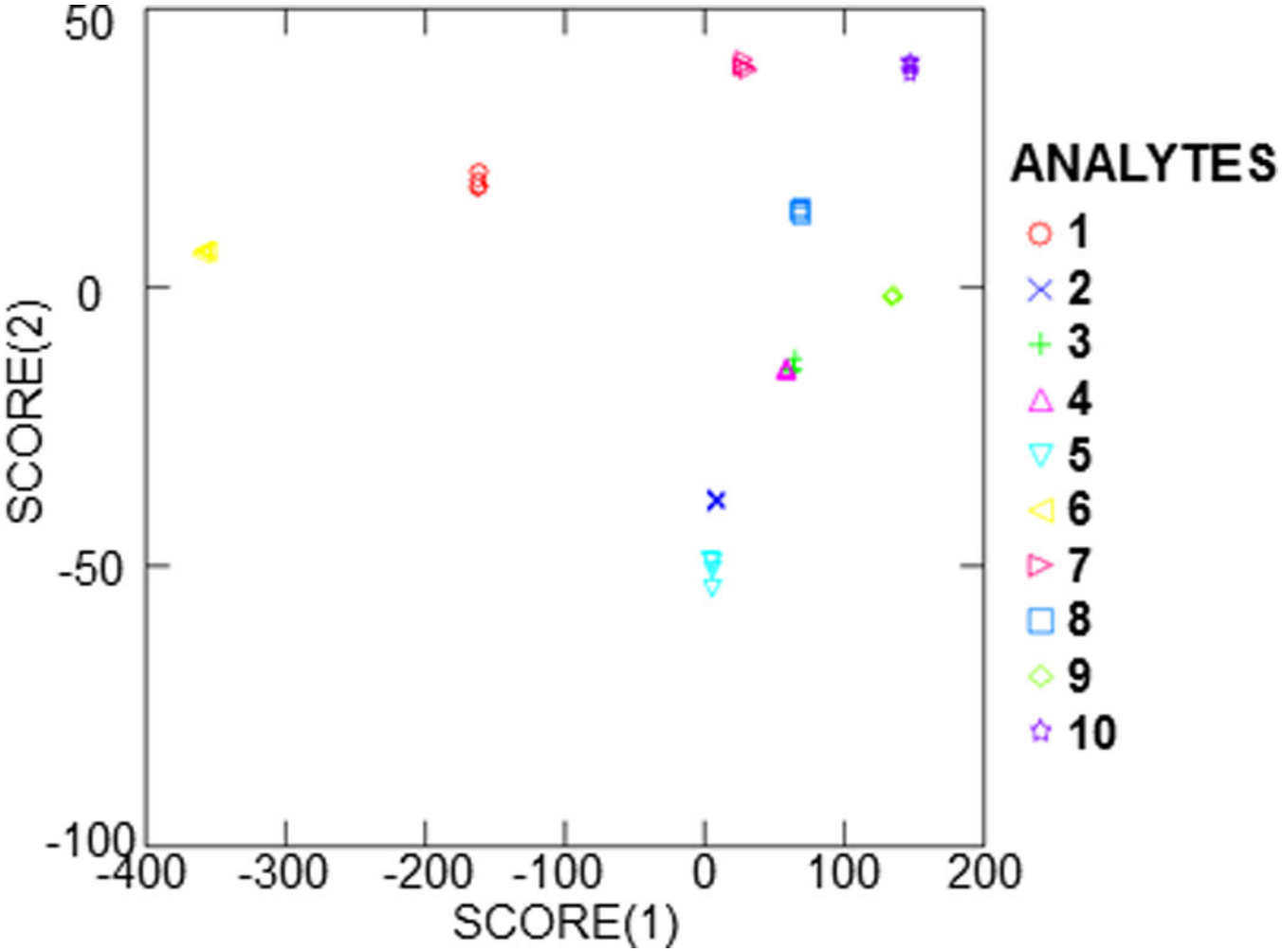

Figure 3.

Array-based detection of analytes **1–10** in one breast milk sample using cyclodextrin-fluorophore predictors.

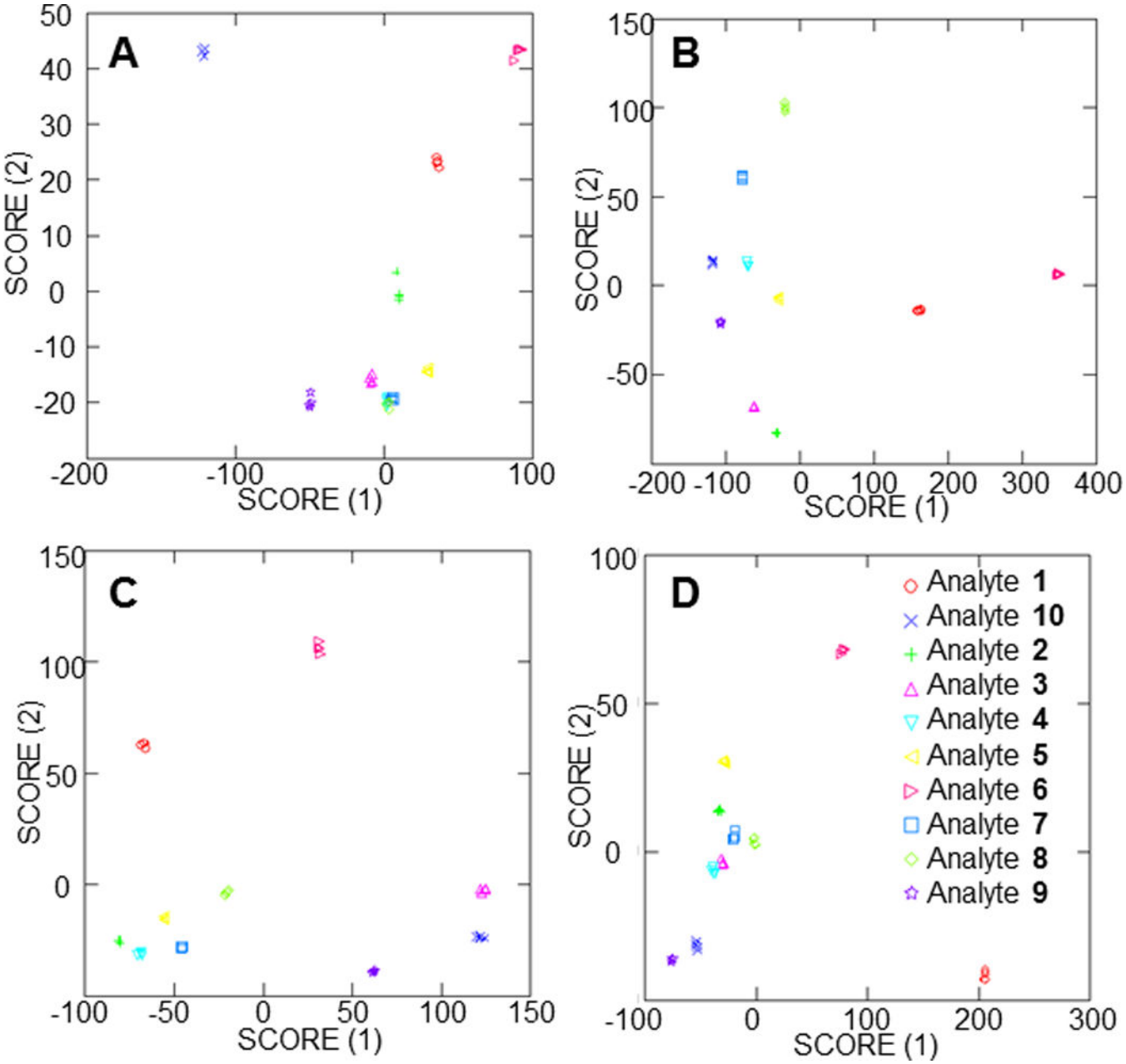

Figure 4.

Array-based detection of analytes **1–10** in (A) Sample 2, (B) Sample 3, (C) Sample 4, and (D) Sample 5, four additional, independent breast milk samples using cyclodextrin-fluorophore predictors.

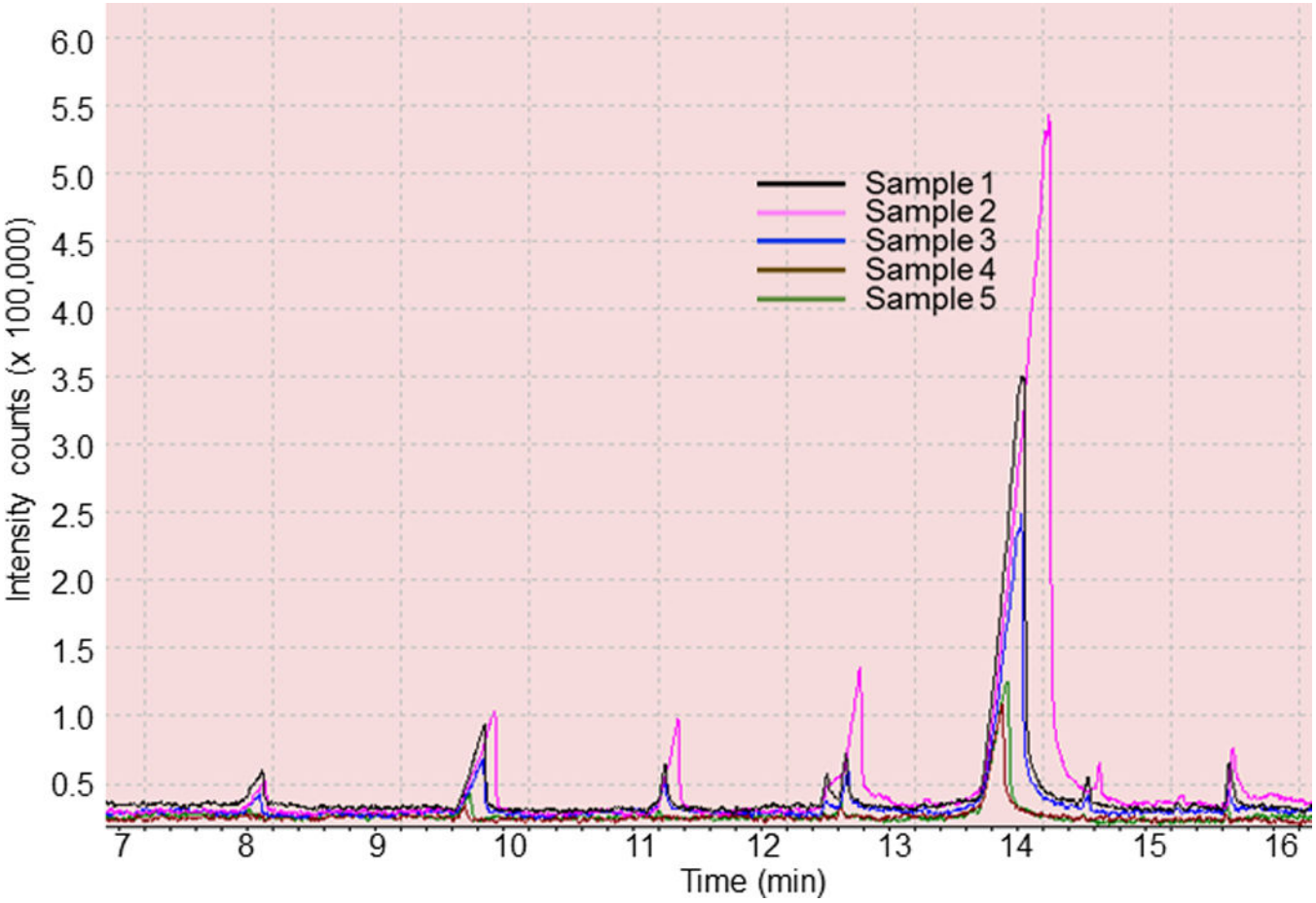

Figure 5.
Overlay of GCMS characterization of breast milk samples.

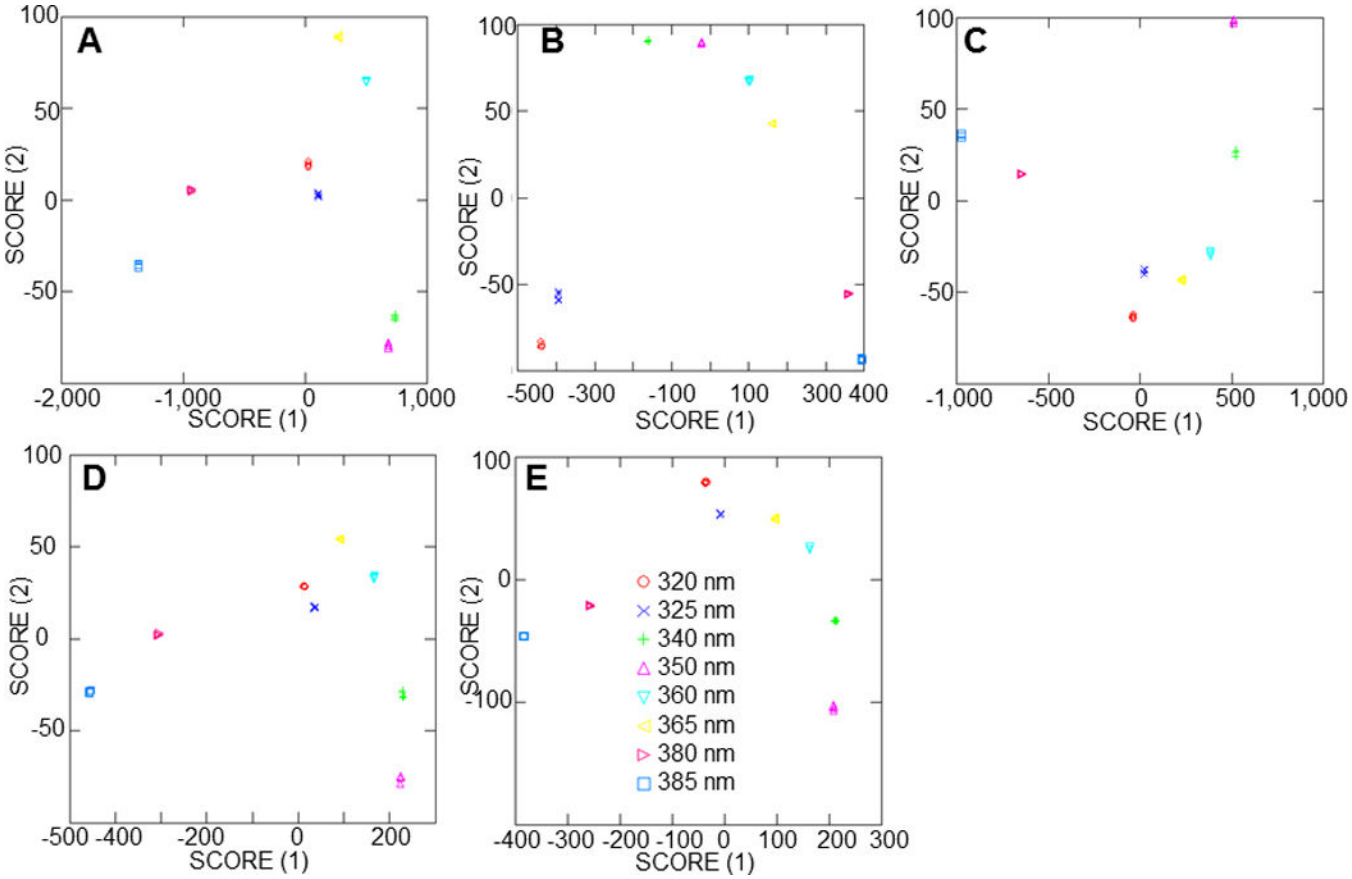

Figure 6.

Array response patterns using linear discriminant analysis from: (A) Sample 1; (B) Sample 2; (C) Sample 3; (D) Sample 4; and (E) Sample 5 using cyclodextrin-fluorophore predictors.

Table 1.

Energy transfer efficiencies to fluorophore **11** in 10 mM γ-cyclodextrin and in 0 mM γ-cyclodextrin. Analyte comparisons are shown in parentheses[a]

Analyte	10 mM γ-CD	0 mM γ-CD
1	21.2 ± 1.2 (0.84 ± 0.00)	21.7 ± 0.5 (0.88 ± 0.00)
2	20.0 ± 0.8 (0.89 ± 0.01)	18.9 ± 0.3 (1.07 ± 0.00)
3	18.6 ± 0.3 (0.86 ± 0.01)	18.5 ± 0.3 (1.11 ± 0.00)
4	18.8 ± 0.4 (0.84 ± 0.01)	18.6 ± 0.5 (1.04 ± 0.00)
5	18.7 ± 0.8 (0.96 ± 0.01)	17.8 ± 0.5 (1.09 ± 0.00)
6	22.5 ± 0.8 (0.84 ± 0.01)	22.2 ± 0.6 (0.93 ± 0.00)
7	19.0 ± 0.7 (0.97 ± 0.00)	18.7 ± 0.3 (1.11 ± 0.00)
8	19.7 ± 1.0 (0.87 ± 0.00)	19.0 ± 0.3 (1.02 ± 0.00)
9	19.8 ± 0.5 (1.16 ± 0.01)	19.1 ± 0.4 (1.51 ± 0.00)
10	16.0 ± 0.7 (1.24 ± 0.01)	15.6 ± 0.7 (1.29 ± 0.00)

[a]Energy transfer efficiencies were calculated using Equation 1 and analyte comparison ratios were calculated using Equation 2. Results presented in table represents an average of four trials.

Table 2.

Selected limits of detection for analytes 1–10 in breast milk[a]

Analyte	Fluorophore	Limit of Detection (μM)
1	**11**	0.32 ± 0.00
2	**11**	1.48 ± 0.12
3	**11**	27.53 ± 0.02[a]
4	**11**	59.52 ± 1.41[a]
5	**11**	2.36 ± 0.00[a]
6	**11**	0.17 ± 0.00
7	**11**	4.21 ± 0.00
8	**11**	8.5 ± 0.00
9	**11**	1.37 ± 0.00
10	**11**	14.7 ± 0.00[a]

[a]Limits of detection were calculated using the procedures in Cheng 2016; see Electronic Supporting Information for more information.

Table 3.

Results of fluorescence energy transfer in multiple breast milk samples with fluorophore **11**[a]

Analyte	Sample 2	Sample 3	Sample 4	Sample 5
1	25.9 ± 1.1	25.5 ± 1.4	24.3 ± 0.9	23.1 ± 1.1
2	20.9 ± 0.8	22.5 ± 1.1	21.7 ± 0.6	22.2 ± 0.7
3	21.2 ± 1.1	22.3 ± 1.0	21.0 ± 0.7	22.3 ± 0.4
4	19.4 ± 0.9	21.2 ± 1.3	21.1 ± 0.7	21.3 ± 0.3
5	25.2 ± 1.5	25.9 ± 1.6	24.7 ± 1.0	24.0 ± 1.0
6	18.9 ± 0.9	20.8 ± 1.1	19.4 ± 0.5	20.6 ± 0.5
7	20.5 ± 1.1	21.1 ± 0.9	20.2 ± 0.5	21.8 ± 0.8
8	21.2 ± 1.1	21.2 ± 1.9	24.2 ± 0.1	22.2 ± 0.9
9	22.0 ± 0.9	24.0 ± 1.8	22.4 ± 0.6	22.0 ± 0.7
10	16.6 ± 0.6	16.2 ± 0.1	16.4 ± 0.5	15.8 ± 0.7

[a]All results represent an average of results from four trials for each sample. Energy transfer values were calculated using Equation 1; analyte comparison ratios were calculated using Equation 2; donor decrease values were calculated using Equation 3.

Table 4.

Results of fluorescence energy transfer in multiple breast milk samples with fluorophore **12**[a]

Analyte	Sample 2	Sample 3	Sample 4	Sample 5
1	17.8 ± 0.1	14.5 ± 0.0	9.7 ± 0.0	27.1 ± 0.0
2	27.7 ± 0.3	8.5 ± 0.0	8.1 ± 0.0	14.7 ± 0.1
3	29.5 ± 0.1	8.3 ± 0.0	7.9 ± 0.0	15.1 ± 0.1
4	31.4 ± 0.0	7.2 ± 0.1	8.0 ± 0.1	14.8 ± 0.1
5	28.7 ± 0.1	5.2 ± 0.0	5.7 ± 0.0	13.8 ± 0.1
6	27.5 ± 0.1	6.7 ± 0.0	7.1 ± 0.0	15.6 ± 0.1
7	28.8 ± 0.1	6.9 ± 0.0	8.0 ± 0.0	15.3 ± 0.1
8	31.3 ± 0.0	7.6 ± 0.0	8.0 ± 0.0	15.8 ± 0.0
9	31.4 ± 0.2	7.6 ± 0.0	8.3 ± 0.0	12.6 ± 0.1
10	23.1 ± 0.1	8.9 ± 0.1	10.4 ± 0.1	15.7 ± 0.1

[a]All results represent an average of results from four trials for each sample. Energy transfer values were calculated using Equation 1; analyte comparison ratios were calculated using Equation 2; donor decrease values were calculated using Equation 3.

Table 5.

Results of fluorescence energy transfer in multiple breast milk samples with fluorophore **13**[a]

Analyte	Sample 2	Sample 3	Sample 4	Sample 5
1	27.2 ± 0.1	32.2 ± 0.8	24.6 ± 0.3	23.2 ± 0.1
2	23.8 ± 0.1	10.7 ± 0.0	10.4 ± 0.1	10.6 ± 0.1
3	22.2 ± 0.1	8.8 ± 0.0	8.8 ± 0.0	9.8 ± 0.1
4	23.4 ± 0.1	8.7 ± 0.0	9.1 ± 0.1	9.7 ± 0.1
5	27.4 ± 0.1	11.4 ± 0.1	11.8 ± 0.1	11.8 ± 0.1
6	32.4 ± 0.3	37.4 ± 0.2	30.6 ± 0.5	21.7 ± 0.3
7	23.6 ± 0.1	8.8 ± 0.0	9.4 ± 0.0	10.1 ± 0.1
8	22.7 ± 0.1	12.0 ± 0.1	11.3 ± 0.1	11.2 ± 0.1
9	26.6 ± 0.1	10.2 ± 0.0	9.8 ± 0.0	11.8 ± 0.0
10	12.4 ± 0.0	5.5 ± 0.0	6.2 ± 0.0	7.2 ± 0.1

[a]All results represent an average of results from four trials for each sample. Energy transfer values were calculated using Equation 1; analyte comparison ratios were calculated using Equation 2; donor decrease values were calculated using Equation 3.