Sensitivity and Specificity of the Cozart Microplate EIA Cocaine Oral Fluid at Proposed Screening and Confirmation Cutoffs

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**Background:** Oral fluid is currently being evaluated as an alternative matrix for monitoring illicit drugs in federally mandated workplace drug testing, for addiction treatment programs, and for driving under the influence testing. The sensitivity, specificity, and efficiency of the Cozart Microplate EIA Cocaine Oral Fluid Kit (COC ELISA) were determined by comparison with gas chromatography–mass spectrometry (GC/MS) results at screening and confirmation cutoffs proposed in the US and UK.

**Method:** Oral fluid was collected by expectoration after citric acid candy stimulation or with Salivette® neutral cotton swabs or Salivette citric acid-treated cotton swabs before and after cocaine (COC) administration. Specimens (n = 1468) were analyzed with the COC ELISA for screening and with solid-phase extraction followed by GC/MS for confirmation. Three screening cutoffs (10, 20, and 30 μg/L) and four GC/MS cutoffs (2.5, 8, 10, and 15 μg/L COC, benzoylecgonine, and/or ecgonine methyl ester) were evaluated. GC/MS limit of quantification was 2.5 μg/L for all analytes.

**Results:** COC ELISA interassay imprecision (CV; n = 19) was 16% at 16.7 μg/L and 12% at 81.8 μg/L. With the 2.5, 8, 10, and 15 μg/L GC/MS cutoffs, 59.0%, 54.7%, 52.7%, and 48.7% of the oral fluid specimens were positive, respectively. Sensitivity, specificity, and efficiency were 92.2%, 84.7%, and 88.8%, respectively, for the suggested Substance Abuse and Mental Health Services Administration (SAMHSA) cutoffs and 90.2%, 89.2%, and 89.7% for cutoffs currently used in the UK.

**Conclusions:** COC ELISA had suitable sensitivity, specificity, and efficiency for identifying COC exposure at both the proposed SAMHSA and UK cutoffs. Sensitivity, specificity, and efficiency were >84% for both cutoffs, but 92 additional true-positive samples were identified with the SAMHSA cutoffs.

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Oral fluid testing provides an alternative matrix for drug monitoring in treatment, workplace, criminal justice, and driving under the influence testing programs. Advantages include less invasive specimen collection and reduced opportunity for specimen adulteration. Oral fluid collection devices and simple and sensitive methods for the detection of drugs in oral fluid have been developed. Microplate enzyme immunoassays for drugs of abuse have been used for qualitative and semiquantitative analysis of drugs of abuse in whole blood, hair, sweat, and oral fluid (1–4).

Cocaine (COC), a widely abused drug, rapidly appears in oral fluid after intravenous injection, inhalation, and intranasal administration (5, 6). The concentration of COC is usually higher in oral fluid than in plasma because of ion trapping (5, 7–10). Benzoylecgonine (BE) and ecgonine methyl ester (EME), major metabolites of COC, have longer half-lives in plasma and oral fluid than does the parent drug (5, 9, 11–14).

Recently, the Substance Abuse and Mental Health Services Administration (SAMHSA) has proposed an oral...
fluid screening cutoff of 20 μg/L with BE as the target analyte and confirmation cutoffs of 8 μg/L COC and/or BE. In the UK, a screening cutoff of 30 μg/L and a confirmation cutoff of 15 μg/L for COC, BE, and/or EME in oral fluid have been used. Additional cutoffs, including a cutoff at the limits of quantification (LOQ) of the screening (10 μg/L) and confirmation assays (2.5 μg/L), and a 10 μg/L screen, and a 10 μg/L confirmation cutoff, were evaluated.

The purpose of this study was to evaluate the sensitivity, specificity, and efficiency of the Cozart® Microplate Elisa Cocaine Oral Fluid Kit (COC ELISA) as a routine screening method for detecting COC use and to evaluate proposed cutoffs for immunoassay and gas chromatography–mass spectrometry (GC/MS) analysis of COC and its metabolites in oral fluid.

**Materials and Methods**

**HUMAN PARTICIPANTS**

Eleven male and 7 female healthy volunteers participated in a controlled drug administration protocol. Of the 18 participants, 14 were African American, 2 were Caucasian, and 2 were Hispanic. Their mean (SD) age was 35.3 (4.5) years (range, 23–43 years), and their mean weight was 75.7 (12.5) kg (range, 56.6–96.2 kg). The protocol was approved by the National Institute on Drug Abuse Institutional Review Board. Participants provided written informed consent and were paid for their participation. Screening procedures included comprehensive physical and psychologic examinations. Participants reported a history of COC and opioid use, and were not physically dependent on drugs or medications, with the possible exception of nicotine and caffeine. During the 10-week study, all participants resided on the closed research unit of the Intramural Research Program, National Institute on Drug Abuse.

**DRUG ADMINISTRATION**

COC hydrochloride for human administration was obtained from Mallinkrodt and was prepared in saline for subcutaneous injection. Participants received three low doses (75 mg/70 kg) of COC hydrochloride within 7 days and, after a 3-week interval, three high doses (150 mg/70 kg) within 7 days.

**SPECIMENS**

Oral fluid was collected before and up to 72 h after COC administration by citric acid candy stimulation and expectoration (n = 1100). Smaller subsets were collected with Salivette® neutral cotton swabs (n = 165) or Salivette citric acid-treated cotton swabs (n = 203). A mean (SD) of 82 (45) oral fluid specimens were collected (range, 26–169) per participant. The Salivette neutral and citric acid-treated cotton swabs were placed between the cheek and gum or under the individual’s tongue until saturated with oral fluid. The cotton swab was sometimes chewed to stimulate oral fluid production. Saturation could occur in 30–45 s but always was completed within 3–4 min. After collection, the swab was centrifuged to release ~1–1.5 mL of clear oral fluid. Oral fluid collection was supervised by trained research associates and medical staff. Subsequently, oral fluid was transferred to polypropylene cryotubes and frozen at −20 °C until analysis. A total of 602 negative specimens were included in the study. The limit of detection of the GC/MS method (2.5 μg/L) was used to differentiate positive and negative specimens. Specimens with COC, BE, EME, and cocaethylene (CE) <2.5 μg/L were considered negative.

**COZART MICROPLATE ELISA COCAINE ORAL FLUID**

The COC ELISA is a competitive enzyme immunoassay for the detection of COC in human oral fluid, developed by Cozart Bioscience Ltd. The assay was performed according to manufacturer’s directions. Briefly, 25 μL of oral fluid specimen, calibrator, or quality-control sample was added to a microtiter plate well coated with anti-BE antibody, and 100 μL of enzyme conjugate was added. Each microtiter plate was calibrated in duplicate with 0, 10, 25, 50, and 100 μg/L BE calibrators. Duplicate quality-control samples were interspersed within each plate. Participant oral fluid samples were assayed in singlicate. After a 30-min incubation, the plate was washed, and 100 μL of substrate solution (3,3’,5,5’-tetramethylbenzidine) was added, followed by another 30-min incubation. Finally, 100 μL of stop solution (1 mol/L sulfuric acid) was added, and the absorbance at 450 nm measured within 30 min with a microplate reader (Dynex Technologies).

For the pilot study of pH effect on COC ELISA, oral fluid was collected from healthy volunteers with Salivette citric acid-treated cotton swabs (n = 12) and verified to be drug free, and the pH was determined. Oral fluid specimens were assayed with the COC ELISA, and absorbance was recorded at 450 nm as described above.

**CHEMICALS AND REAGENTS**

Chemicals were obtained from the following sources: BE, Research Biochemicals International; [3H]COC, [3H]BE · 4 H2O, EME hydrochloride, and [3H]EME hydrochloride, Sigma Chemicals; CE, Research Triangle Institute; [3H]CE, Cerilliant Corp.; N,O-bis(trimethyl)trifluoroacetamide with 1% trimethylchlorosilane and N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide with 1% tert-butyldimethylchlorosilane, Pierce Chemical; and solid-phase extraction columns (Clean Screen 25DAU020) and filtration columns (RFV02F4P), United Chemical Technologies. HPLC-grade solvents and ACS reagent-grade chemicals were used in the analysis.

**GC/MS PROCEDURE**

Calibrators, quality-control samples, and oral fluid samples were analyzed for COC and its metabolites by solid-phase extraction according to a previously published procedure (15–18). COC, BE, EME, and CE calibra-
tors were prepared at drug concentrations of 1.25–1000 μg/L in drug-free oral fluid. Different stock solutions of COC, BE, EME, and CE at 0, 10, 100, and 500 μg/L were added to drug-free oral fluid to prepare quality-control samples. Specimens were analyzed with a low (2.5–50 μg/L) or high calibration curve (50–500 μg/L). Split curves were required to obtain suitable sensitivity and linearity (r = 0.98) for the four analytes.

GC/MS analysis was performed on a Hewlett-Packard 5890A or 5973 gas chromatograph interfaced with a Hewlett-Packard 5972 or 6890 mass-selective detector in splitless mode. Chromatographic conditions have been published previously (16). Three ions were monitored for each analyte (quantitative ion indicated in parenthesis): [1H3]COC, m/z (185), 306, and 85; COC, m/z (182), 303, and 82; [1H3]BE, m/z (285), 349, and 406; BE, m/z (282), 346, and 403; [1H3]EME, m/z (185), 259, and 99; EME, m/z (182), 256, and 96; [1H3]CE, m/z (199), 320, and 85; CE, m/z (196), 317, and 82. Ion ratios of quality-control samples and participant samples were required to be within ± 20% of those observed for the 10 and 100 μg/L calibrators for the low and high calibration curves, respectively. The limit of detection and the LOQ of the method were 2.5 μg/L for all analytes.

**IMPRECISION (CV), SENSITIVITY, SPECIFICITY, AND EFFICIENCY**

Interassay precision for the low and high quality-control specimens was calculated from 35 and 37 sample results, respectively, assayed in 19 batches. Duplicate quality-control samples were included in each full batch. The numbers of true-positive (TP), false-negative (FN), false-positive (FP), and true-negative (TN) specimens were determined by comparison of immunoassay with GC/MS results. For the purposes of this comparison, three different screening test cutoffs (10, 20, and 30 μg/L) and four GC/MS cutoffs (2.5, 8, 10, and 15 μg/L COC, BE, and/or EME) were evaluated. Screening cutoffs were selected because the lowest calibrator for the COC ELISA screen is 10 μg/L, the proposed SAMHSA cutoff is 20 μg/L, and the cutoff used in the UK is 30 μg/L. Confirmation cutoffs were chosen because 2.5 μg/L is the LOQ of the GC/MS method, the proposed SAMHSA cutoff is 8 μg/L, Niedbala et al. (19) evaluated 10 μg/L with a different ELISA for COC in oral fluid, and the UK cutoff is 15 μg/L. A sample was considered TP if both the immunoassay and GC/MS were positive for COC, BE, and/or EME and TN if both results were negative. A sample was considered FP if the immunoassay result was positive and the GC/MS result was negative for COC, BE, and/or EME. A sample was considered FN if the immunoassay result was negative and the GC/MS result was equal to or above the specified cutoff for any of the three analytes. Sensitivity was calculated as TP/(TP + FN) × 100 and specificity as TN/(TN + FP) × 100. Efficiency was calculated as (TP + TN)/total number of specimens × 100.

**Results and Discussion**

Interest in the use of alternative matrices for drug testing has led to development of many new assays. Immunoassays for drugs of abuse can be used to screen serum, whole blood, oral fluid, sweat, and hair as well as urine (1–3, 19–21). Advantages of these screening tests include their simplicity, rapidity, sensitivity, and reduced cost.

The COC ELISA was developed for the qualitative and semiquantitative determination of COC and metabolites in oral fluid.

**Table 1. Sensitivity, specificity, and efficiency of the COC ELISA for detection of COC and metabolites in oral fluid at different immunoassay and GC/MS cutoffs after controlled COC administration (n = 1468).**

<table>
<thead>
<tr>
<th>Cutoff, μg/L</th>
<th>10/2.5</th>
<th>10/10</th>
<th>20/8</th>
<th>SAMHSA</th>
<th>UK</th>
</tr>
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<tbody>
<tr>
<td>TP, a n</td>
<td>846</td>
<td>765</td>
<td>737</td>
<td>734</td>
<td>642</td>
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<tr>
<td>FP, n</td>
<td>205</td>
<td>286</td>
<td>100</td>
<td>103</td>
<td>78</td>
</tr>
<tr>
<td>TN, n</td>
<td>397</td>
<td>409</td>
<td>565</td>
<td>569</td>
<td>675</td>
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<td>20</td>
<td>8</td>
<td>66</td>
<td>62</td>
<td>73</td>
</tr>
<tr>
<td>Sensitivity, %</td>
<td>97.7</td>
<td>99.0</td>
<td>91.8</td>
<td>92.2</td>
<td>89.8</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>65.9</td>
<td>58.8</td>
<td>85.0</td>
<td>84.7</td>
<td>89.6</td>
</tr>
<tr>
<td>Efficiency, %</td>
<td>84.7</td>
<td>80.0</td>
<td>88.7</td>
<td>88.8</td>
<td>89.7</td>
</tr>
</tbody>
</table>

a Cozart COC ELISA screening cutoff for COC, BE, and/or EME: confirmation cutoff by GC/MS.

b Proposed SAMHSA cutoffs for COC and metabolites in oral fluid: 20 μg/L screening cutoff and 8 μg/L GC/MS cutoff for COC and/or BE.

c Cutoffs currently used in the UK for COC and metabolites in oral fluid: 30 μg/L screening cutoff and 15 μg/L confirmation cutoff for COC, BE, and/or EME.

d TP, FN, TN, and FN were determined for the specified cutoffs by comparison of the immunoassay result with the GC/MS result (see text).

e Sensitivity, specificity, and efficiency were calculated using the following formulas:

Sensitivity = TP/(TP + FN) × 100%.

Specificity = TN/(TN + FP) × 100%.

Efficiency = (TP + TN)/(TP + TN + FP + FN) × 100%.
the Salivette cotton roll were estimated to be at least 80–90% based on results published by Samyn et al. (22).

TN, FP, FN, and TP results at specified screening and confirmation cutoffs are shown in Table 1. All evaluated cutoffs had sensitivities ≥89% and efficiencies ≥80%.

With the proposed SAMHSA criteria, which required GC/MS analysis of only COC and BE, we found a sensitivity of 92.2%, a specificity of 84.7%, and an efficiency of 88.8%. Adding EME to the GC/MS confirmation identified only 3 additional TP results (from 734 to 737) with little change in test efficiency (from 88.8% to 88.7%).

The use of the proposed SAMHSA cutoffs gave improved results for specificity and efficiency, increases of 18.8% and 4.1%, respectively, with a decrease in sensitivity of <5.5% compared with the lowest (10/2.5 µg/L) cutoffs. The sensitivity, specificity, and efficiency of the UK cutoff were similar to those of the SAMHSA cutoffs, with the highest efficiency among the evaluated cutoffs. However, there were 92 fewer TP samples (642) compared with SAMHSA. The highest sensitivity (99.0%) and lowest specificity (58.8%) were obtained at the 10/10 µg/L cutoffs, which considered a sample as positive with COC, BE and/or EME ≥ 10 µg/L. Niedbala et al. (19) reported sensitivity (95%), specificity (82%), and efficiency (88%) data for the STC Cocaine Metabolites MICRO-PLATE EIA with a 10 µg/L screening cutoff and a 10 µg/L combined cutoff concentration for COC and BE by GC/MS. The LOQ for COC and BE were 4.0 and 7.0 µg/L, respectively. Sensitivity results were comparable to those reported in our study, but there was a major difference in specificity because of the larger number of FP results in our data. This was most likely attributable to differences in GC/MS cutoffs and to possible differences in the cross-reactivities of the respective antibodies.

As observed for the total specimen set of 1468 oral fluid specimens (Table 1), the number of FP specimens varies depending on the screening and confirmation cutoffs used (14% for the 10/2.5 µg/L cutoffs, 19% for the 10/10 µg/L cutoffs, 6.8% for the 20/8 µg/L cutoffs, 7.0% for the SAMHSA cutoff, and 5.3% for the UK cutoffs). In addition, the percentage of FP specimens also varies with the type of oral fluid collection device, as seen in Table 2.

### Table 2. Comparison of the sensitivity, specificity, and efficiency of Cozart COC ELISA for oral fluid obtained with different oral fluid collection methods at specified immunoassay and GC/MS cutoffs after controlled COC administration.

<table>
<thead>
<tr>
<th>Collection method</th>
<th>Cutoff, µg/L</th>
<th>10/2.5&lt;sup&gt;a&lt;/sup&gt;</th>
<th>10/10&lt;sup&gt;a&lt;/sup&gt;</th>
<th>20/8&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SAMHSA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>UK&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid candy stimulation (n = 1100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP, n</td>
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<td>690</td>
<td>620</td>
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</tr>
<tr>
<td>FP, n</td>
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<td>115</td>
<td>185</td>
<td>43</td>
<td>45</td>
<td>43</td>
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<tr>
<td>TN, n</td>
<td></td>
<td>280</td>
<td>289</td>
<td>407</td>
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<td>15</td>
<td>6</td>
<td>58</td>
<td>55</td>
<td>62</td>
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<tr>
<td>Sensitivity, %</td>
<td></td>
<td>97.9</td>
<td>99.0</td>
<td>91.1</td>
<td>91.5</td>
<td>89.2</td>
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<td>Specificity, %</td>
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<td>61.0</td>
<td>90.4</td>
<td>90.1</td>
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<td>88.2</td>
<td>82.6</td>
<td>90.8</td>
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<td>90.5</td>
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<td>Salivette with citric acid- treated cotton swab (n = 203)</td>
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<td>TP, n</td>
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<td>104</td>
<td>106</td>
<td>105</td>
<td>93</td>
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<tr>
<td>FP, n</td>
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<td>68</td>
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<td>TN, n</td>
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<td>Salivette with neutral cotton swab (n = 165)</td>
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<td>82.4</td>
<td>85.5</td>
<td>86.1</td>
<td>90.9</td>
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<sup>a</sup> Cozart COC ELISA screening cutoff for COC, BE, and/or EME: confirmation cutoff by GC/MS.

<sup>b</sup> Proposed SAMHSA cutoffs for COC and metabolites in oral fluid: 20 µg/L screening cutoff and 8 µg/L confirmation cutoff for COC and/or BE.

<sup>c</sup> Cutoffs currently used in the UK for COC and metabolites in oral fluid: 30 µg/L screening cutoff and 15 µg/L confirmation cutoff for COC, BE, and/or EME.

<sup>d</sup> TP, FP, TN, and FN were determined for the specified cutoffs by comparison of the immunoassay result with the GC/MS result (see text).

<sup>e</sup> Sensitivity, specificity, and efficiency were calculated using the following formulas:

- Sensitivity = TP/(TP+FN) × 100%.
- Specificity = TN/(TN+FP) × 100%.
- Efficiency = (TP+TN)/(TP+TN+FP+FN) × 100%.
Therefore, when evaluating the FP results produced by an immunoassay, these factors need to be taken into consideration. Comparison of percentages of FP results of different immunoassays with the same cutoffs and the same collection system would be the best means of determining differences attributable to antibody cross-reactivity. We have shown that the low pH produced in specimens collected with the Salivette with citric acid-treated cotton swab yielded a higher percentage of FP results compared with specimens collected after citric acid candy expectoration (20.2% vs 4.1% at the SAMHSA cutoff). The primary purpose of the screening assay is to identify potential positive specimens (high sensitivity is needed), and the primary purpose of the confirmation test is to eliminate negative specimens (high specificity is needed). Although GC/MS confirmation of FP specimens is time-consuming and costly, lowering the number of presumptive positive specimens in the immunoassay usually increases the number of FN specimens. FN specimens tend to undermine the deterrent effect of drug testing in workplace and treatment programs and could have public health consequences if individuals driving under the influence of drugs fail to be identified. Our results, obtained after controlled COC administration and analysis of oral fluid specimens by immunoassay and GC/MS, provide objective data to evaluate these factors and to select screening and confirmation cutoffs to meet the goals of the individual drug testing programs.

Cross-reactivities of the antibodies in the ELISA screen were reported to be 72% for COC, 100% for BE, 0.1% for EME, and 22% for CE (23). The high cross-reactivity to EME supports the finding that the addition of EME to the proposed SAMHSA cutoffs had a minimal effect on the sensitivity and efficiency of this COC ELISA. EME is a major COC metabolite in oral fluid along with BE; however, almost all EME-positive specimens were also positive for COC and/or BE. CE may be found in body fluids of individuals who concurrently use COC and ethanol (24–28). CE concentrations in seven human postmortem blood samples ranged from 73 to 1447 μg/L, with four of the seven samples having higher CE than COC concentrations (25). Bailey (28) also reported a CE:COC ratio in plasma ranging from 0.1 to 4.7 in 41 patients [mean (SD), 1.3 (1.1)] and in urine ranging from 0.03 to 8.0 [1.4 (1.9)]. The high cross-reactivity of CE with the COC ELISA suggested that some of the FP immunoassay results could be attributable to the presence of CE. We found CE concentrations of 2.5–104.4 μg/L, always with high concentrations of COC, BE, and/or EME. The mean ratios of CE to COC, BE, and EME in oral fluid were 0.020 (SD, 0.029; range, 0.002–0.214), 0.358 (SD, 0.422; range, 0.014–1.931), and 0.332 (SD, 0.444; range, 0.018–2.612), respectively. There were no cases where CE was present without concurrent COC, BE, and/or EME concentrations greater than the cutoff. Therefore, the presence of CE did not produce FP results and did not lower the specificity of the COC ELISA.

Comparisons of the sensitivities, specificities, and efficiencies for the different oral fluid collection methods are shown in Table 2. In specimens collected with citric acid candy expectoration (n = 1100) and with the Salivette with neutral cotton swab (n = 165), the sensitivities, specificities, and efficiencies were similar. In the oral fluid specimens obtained with the Salivette with citric acid-treated cotton swabs (n = 203), sensitivity (≥95%) compared closely with that of the other collection methods, but specificity was low at all cutoffs because of the high number of FP samples. Specimens obtained with all three methods were assayed together on the same ELISA plates. Furthermore, quality-control samples included on the plates produced accurate semiquantitative results, leading to the conclusion that analytical errors were not the source of these discrepancies. We reported earlier that the mean (SD) pH of oral fluid specimens collected with the Salivette with citric acid-treated cotton swabs [pH 2.8 (0.3)] was lower than that observed after citric acid candy expectoration [pH 4.3 (0.8)] (18). The latter was also 1.7 pH units lower than that of the mean pH of specimens collected with the Salivette with neutral cotton swabs [pH 6.0 (0.6)]. Niedbala et al. (19) reported that there was no significant effect of oral fluid pH on the STC Cocaine Metabolite MICRO-PLATE EIA when the sample pH was between 5.0 and 9.0. Schwartz et al. (29) reported that lemon-lime-flavored Crystal Light® powdered drink mix crystals lowered oral fluid pH and increased cortisol concentrations by RIA. The lower the pH (≤4), the more the cortisol concentration was increased. In our pilot study of pH effects on the COC ELISA, we found a correlation between oral fluid pH and absorbance (Fig. 1). There was substantial intersubject variability in pH (range, 2.79–7.18) in drug-free oral fluid specimens collected with the Salivette with citric acid cotton swabs (n = 12). Oral fluid samples with pH <4 had absorbances close to the COC ELISA cutoff of 10 μg/L or lower and,
therefore, sometimes yielded FP results. The low pH in oral fluid samples collected with the Salivette with citric acid cotton swabs is most likely the source of the FP results observed in this group of samples. It appears that low salivary pH interferes with enzyme-labeled BE binding to anti-BE antibody to produce FP results with the COC ELISA. These data provide important information about the effect of pH on oral fluid immunoassays and suggest caution in selection of an oral fluid collection method.

In conclusion, based on the results of the present study, COC, BE, and EME are appropriate oral fluid analytes for screening and confirming COC use. This controlled COC administration study demonstrates that the Cozart Microplate EIA Cocaine Oral Fluid Kit provides a suitable screening procedure for the identification of COC exposure. The proposed SAMHSA and UK cutoffs provided similar sensitivity, specificity, and efficiency, but with the SAMHSA cutoffs, 92 additional TP samples were identified.

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