FORENSIC TOXICOLOGY:
FROM CRIME SCENE TO VIRTUAL LAB

MODULE 2

CHAPTER 2: Presumptive Testing

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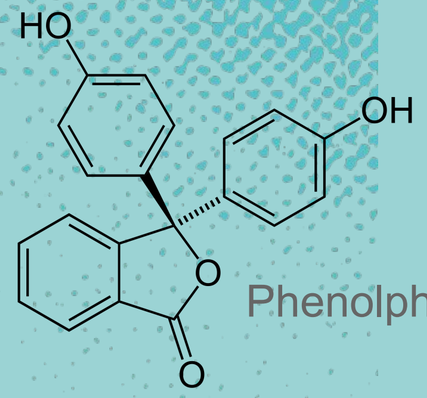
01.
INTRODUCTION

01. Is this a biological sample?

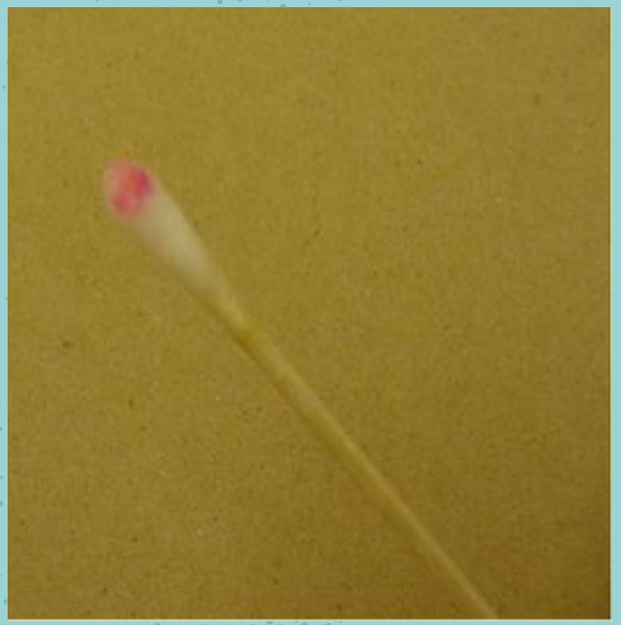
WHAT:
Phenolphthalein is a presumptive test that reacts with the heme molecule present in blood. Phenolphthalein tests are typically conducted on suspected bloodstains prior to collection.

HOW:
A positive reaction gives a pink color. While bloodstains normally appear red-brown in color, the color of the substrate or the age of a stain may affect the appearance or visibility of the stain.



Phenolphthalein

LIMITATIONS:
While a positive phenolphthalein reaction is indicative of blood, it is only a presumptive test and false positives are possible. Additionally, the reaction is not species specific. Positive reactions are not limited to human blood.



The swab shows the characteristic color of a positive reaction with the phenolphthalein test, indicating the presence of blood.

01.

BASIC SCREEN

Blood is the primary specimen used for basic screen.

If two bloods are submitted the most peripheral with sufficient volume will be scheduled for testing.

Blood is tested for the presence of:

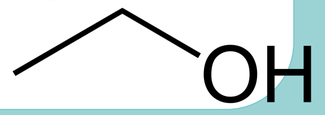
a. Volatiles (ethanol, methanol, isopropanol, acetone) by headspace gas chromatography (HS/GC)

b. Opiates, benzoylecgonine, barbiturates, benzodiazepines, amphetamines, oxycodone and methadone by enzyme immunoassay (ELISA). Positive results for a given test will initiate confirmation testing.

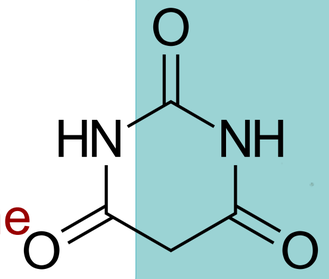
c. Cannabinoids by ELISA as appropriate

01. If blood and urine are available blood is tested for the presence of:

a. Volatiles (ethanol, methanol, isopropanol, acetone) by headspace gas chromatography (HS/GC)

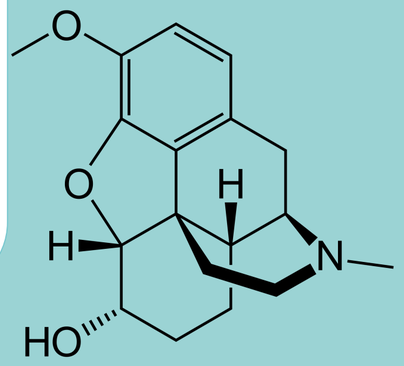


c. Opiates benzoylecgonine, barbiturates, benzodiazepines, amphetamines and cannabinoids, by enzyme immunoassay (ELISA).

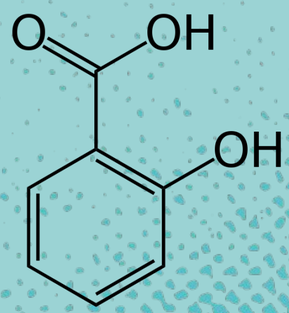


e. Basic drugs by gas chromatography using a nitrogen-phosphorus detector (GC). Drugs included in this screening procedure are listed under “Agents included in routine screening”.

b. Opiates by enzyme immunoassay (ELISA) if case history indicates acute overdose. Urine is tested for the presence of:



d. Salicylates and acetaminophen by color test (CT).



01.

What is a non-routine test?

Certain tests are performed in addition to the above initial testing if they are indicated by the case history or they are specifically requested by the pathologists.

Acetaminophen - Routinely done if codeine, oxycodone, hydrocodone, propoxyphene or tramadol are detected or if indicated by case history.
Carbon monoxide - Routinely done in all fire death with no or minimal survival time, if history indicates driver with no significant survival time or if otherwise indicated by case history.
Cyanide - By request or if indicated by case history.
Ibuprofen - By request or if indicated by case history.
Heavy metals - By request or if indicated by case history.
Ethylene glycol Propylene glycol - Routinely done if history indicates ingestion of antifreeze, otherwise by request. Warfarin - By request or if indicated by case history

02.

COLOUR TESTS

02. COLOUR SPOT TEST

With color spot tests, a portion of an unknown powder/residue/crushed tablet is combined with a chemical reagent which may produce a color change.

The color change is a presumptive indication of what the unknown could be.

For example, the color test for cocaine is Cobalt Thiocyanate; addition of cocaine will produce a rapid blue color change.

Another example of a color spot test is the Marquis test, which will produce a purple color with the addition of certain drugs such as Heroin.

02.

HISTORY OF COLOUR SPOT TESTS

1800’s

Colour and precipitation tests became essential in **early forensic toxicology** studies for the identification of **plant alkaloids**

A **series of tests** named after their chemist inventors appeared in the mid to late 1800s, including **Dragendorff, Marquis, Mandolin, Mecke,** and **Froehde**

1900’s

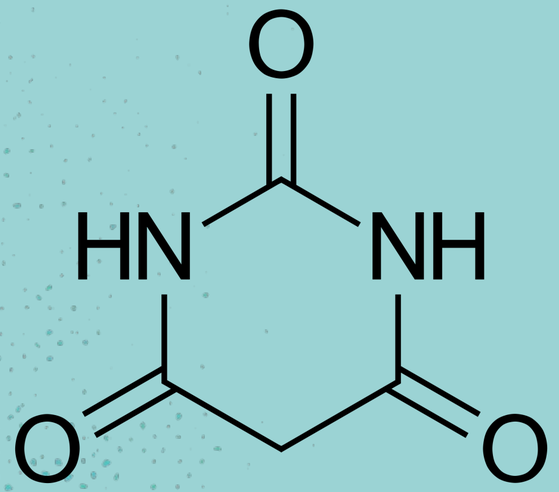
The discovery of **new alkaloids** and the enhanced knowledge of their **chemical structures** led to the arrival of **new colour tests** including **Chen’s test for ephedrine** and **Scott’s test for cocaine**

The arrival of **synthetic drugs** of abuse saw **further successful applications** of these colour test methods

02.

Dille-Koppanyi Reagent

What?
A simple spot test to presumptively identify barbiturates



Barbiturate acid: the parent structure of all barbiturates

How?
Solution A: Dissolve 0.1 g of cobalt (II) acetate dihydrate in 100 mL of methanol. Add 0.2 mL of glacial acetic acid and mix.

Solution B: Add 5 mL of isopropylamine to 95 mL of methanol. Procedure: Add 2 volumes of solution A to the drug, followed by 1 volume of solution B.

Result
If barbiturates are present, the solution will turn light purple by the complexation of cobalt with the barbiturate nitrogens

02. Duquenois-Levine Reagent

METHOD

Solution A: Add 2.5 mL of acetaldehyde and 2.0 g of vanillin to 100 mL of 95 percent ethanol.
Solution B: Concentrated hydrochloric acid.
Solution C: Chloroform.

Procedure: Add 1 volume of solution A to the drug and shake for 1 min. Then add 1 volume of solution B. Agitate gently, and determine the color produced. Add 3 volumes of solution C and note whether the color is extracted from the mixture to A and B.

RESULTS

- A **blue to violet color** after the addition of HCl to the mixture of Duquenois reagent and plant material or extract is a positive reaction and indicates the **possible presence of cannabinoids.**
- After adding chloroform and mixing, a **purple color in organic (lower) layer** is a **positive reaction** for the possible presence of **cannabinoids.**
- A **positive result** indicates that **components (cannabinoids, including THC) unique to marijuana,** marijuana residue, or hashish **are present.**
- A positive (or +) indication on the worksheet means test resulted in a **purple/violet color** after **addition of hydrochloric acid and Duquenois reagent,** and that the **chloroform layer also yields purple color.**

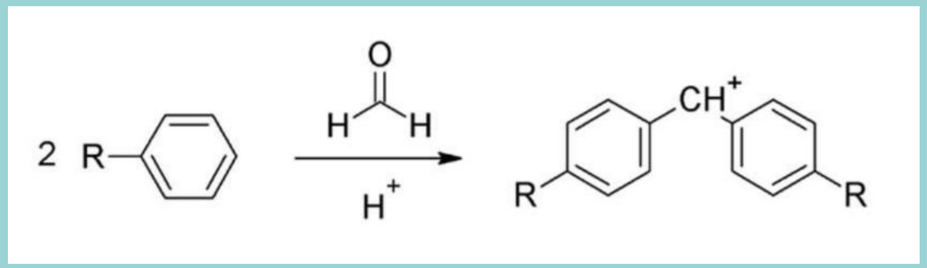
02.

MARQUIS REAGENT

The marquis reagent is the most frequently used colour test for screening unknown substances and is the first to be performed in test sequences.

Preparation:
Add 100 mL concentrated (95-98%) sulfuric acid to 5 mL 40% (v/v) formaldehyde

The test is performed by scraping a small amount of a substance and adding a drop of the reagent (initially clear and colourless). The results are based on the colour and time required for a colour change



Amphetamine reacts with only one molecular of formaldehyde to form an orange carbenium ion product

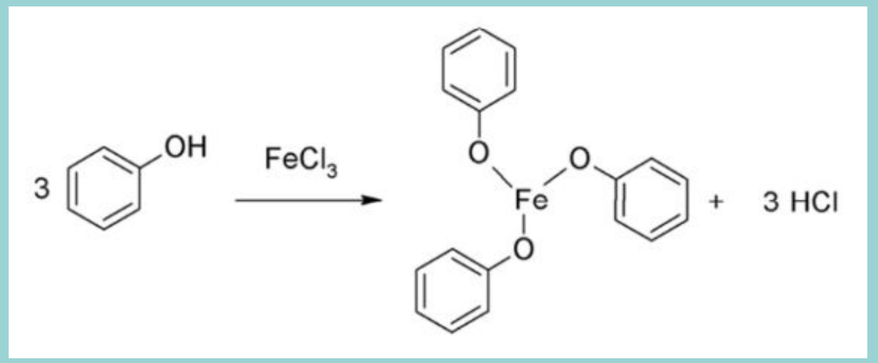
Substance	Final Colour
Amphetamine	Intense red-orange to dark red-brown
Aspirin	Deep red
Codeine	Very deep purple
Heroine	Purple
LSD	Olive black
Methamphetamine	Deep red-orange to dark red-brown
Morphine	Intense red-purple
Opium	Dark greyish reddish brown
Sugar	Dark brown

02.

FERRIC CHLORIDE REAGENT

A simple functional group identification test for the presence of phenols

Ferric Chloride Test:
Step 1: Dissolve the sample in water and ethanol
Step 2: Add drops of a dilute solution of ferric chloride (FeCl3)
Step 3: If the sample turns to red, green, purple, or blue coloration then it indicates the presence of phenols



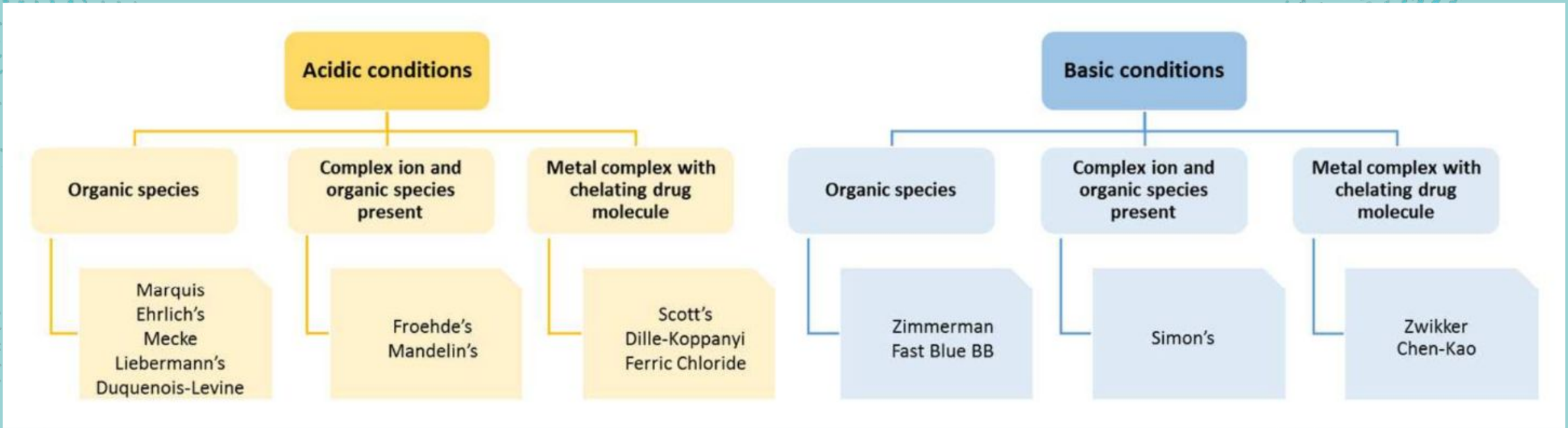
The structure of the resulting purple coloured product is due to the iron(III)-phenol complex

This test can be used to detect aspirin and salicylic acid

Preparation of Ferric Chloride Solution:

1. **Dissolve iron ore in HCl:**
   $Fe_3O_4 + 8HCl \rightarrow FeCl_2 + 2FeCl_3 + 4H_2O$
2. **By oxidizing iron (II) chloride with Cl:**
   $2FeCl_2 + Cl_2 \rightarrow 2FeCl_3$
3. **By oxidizing iron (II) chloride with oxygen:**
   $4FeCl_2 + O_2 + 4HCl \rightarrow 4FeCl_3 + 2H_2O$

02.



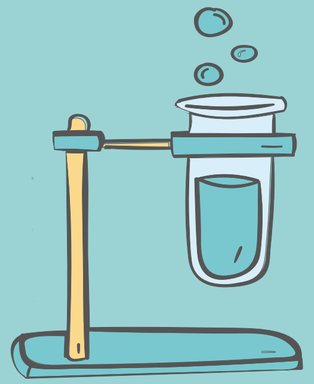
Classification of common colour test reagents according to pH of the test solution and compounds detected

02.

Advantages and Disadvantages

Advantages

- Exist for **most drugs of abuse**
- Requires **small amount of sample**
- Can be **sensitive** with **LOD in µg range** depending on test and analyte
- Can be **specific** with proper standards
- **Easy to interpret**
- **Fast**



Disadvantages

- **Destructive**
- Requires **multiple analyses** to be specific
- Knowledge about **what the substance is** supposed to be can **increase specificity**
- Only tests for **presence/absence** of a certain compound (**not quantitative**)
- Not sufficient for **mixture analysis**
- Colour results dependent on **concentration,** whether the drug is in **salt or free base** form, additional **diluents** or **contaminants**
- Positive results may only indicate the **class of drug**

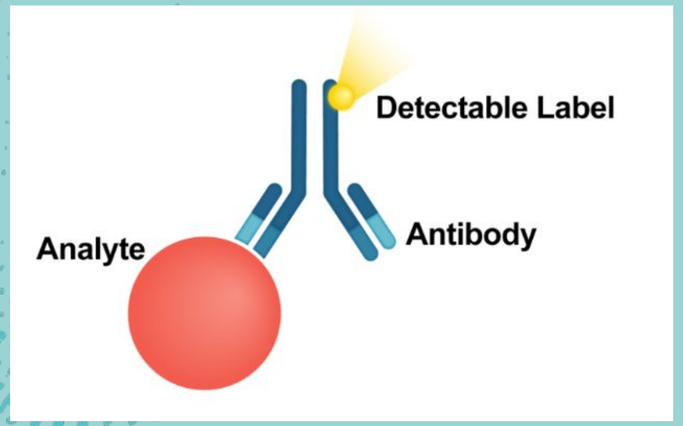
03.
IMMUNOASSAYS

03.

IMMUNOASSAYS

WHAT

Involve the binding of an **antibody** that is **selective** for the drug or drug group of interest **(antigen)** and a **label** that will be part of the **antibody-antigen complex** that can be detected using some means (such as fluorescence)



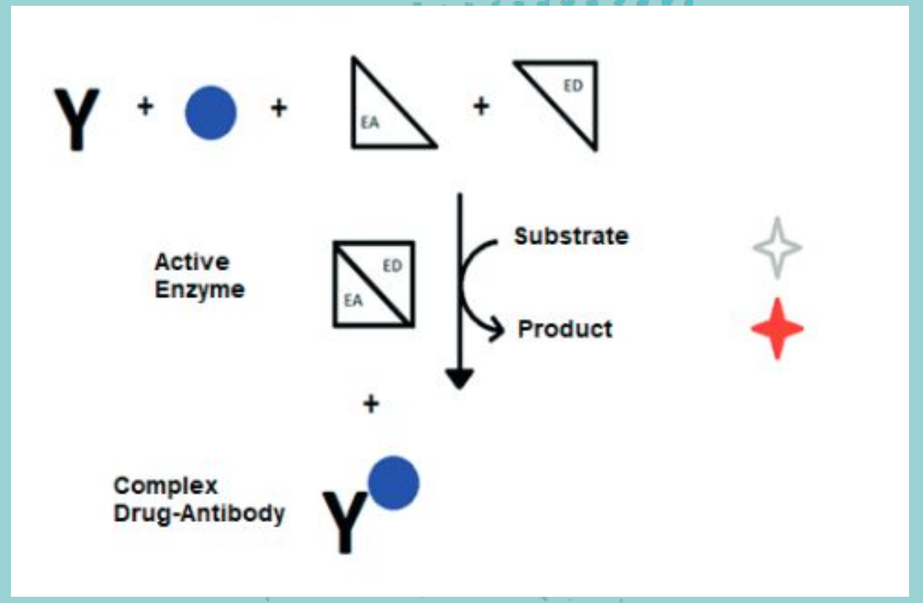
HOW

- Measure the concentration of a substance in biological fluids using a reaction between an antibody or antibodies toward an antigen (drug or drug metabolite).
- Antibodies are prepared to recognize specific drugs/metabolites or drug classes based on their shape and charge
- Detection of the amount of drug present is based on competition between the drug being analyzed and a drug tracer
- The tracer tag is an enzyme, fluorescent label or a particle
- When these drug/metabolites interact with these antibodies, one obtains a measurable chemical response from the tracer tag which is proportional to the amount of drug/metabolite present in the sample

03.

CEDIA

Cloned Enzyme Donor Immunoassay **(CEDIA)** is a platform designed by Thermo Scientific™ which involves a wide range of immunoassays for **drugs of abuse screening and toxicology testing.**
This homogeneous enzyme immunoassay has allowed for the **automation** of the immunoassay test and, was important for facilitating transition from **specialty laboratories to general labs.**



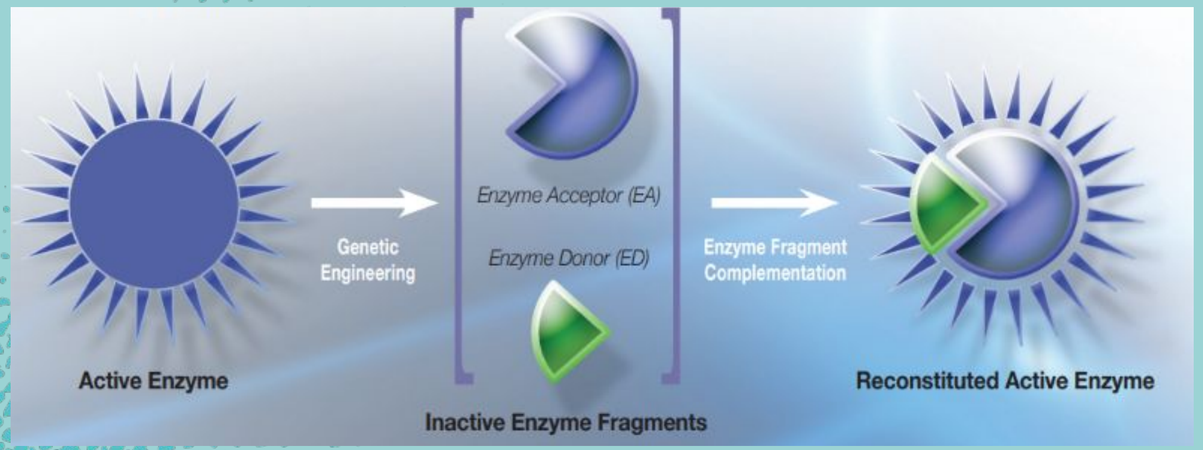
The CEDIA method involves a **competitive binding process** that combines **analyte-specific antibodies** and two genetically engineered fragments of the **bacterial enzyme β-galactosidase**. This can then be used to accurately and reliably detect the presence of **drugs and their metabolites** as well as other substances in **serum, plasma, whole blood, urine** and **oral fluids**

03.

CEDIA

1. The foundation of CEDIA is the use of two polypeptides that are created by separating B-galactosidase into two inactive fragments: the enzyme acceptor (EA) and the enzyme donor (ED).

2. These two fragments can recombine to create an active enzyme. To detect an analyte in a sample, the ED fragment is first coupled to the target analyte. The labelled ED fragment is referred to as an ED-ligand conjugate (ED-LC).



3. If the analyte is not present, the ED-LC will bind to the antibody, allowing fewer active enzymes to form.

4. If the analyte is present, it will successfully compete for analyte binding sites, resulting in greater complementation of ED-LC and EA fragments.

5. When the reagents and the sample fluid are brought together, the labeled fragments (ED-LC) and free analyte in the sample fluid compete in binding to a limited number of analyte specific antibody binding sites.

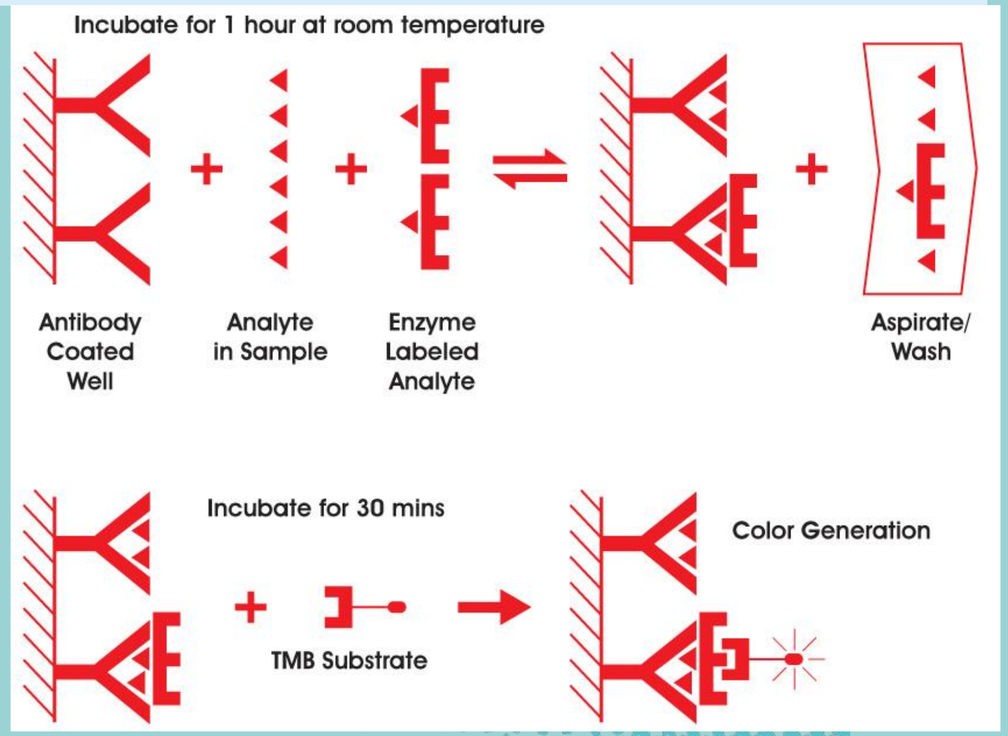
6. When these fragments recombine, the active enzymes cleave the assay substrate, generating a color change that can be measured spectrophotometrically. The extent of the color change is proportional to the analyte concentration in the sample.

03.

ELISA

Enzyme Linked Immunosorbent Assay (ELISA) screening techniques are widely utilized by forensic toxicologists to screen samples for drugs or other forensically relevant molecules

1. **Antibody is immobilized** on the microplate well
2. Competition between **drug in forensic sample** and **enzyme labeled drug** for **antibody binding sites**
3. The **unbounded material** is **washed out**
4. Chromogenic substrate added to **develop colour** with **bound enzyme**
5. Resulting colour is read in a **spectrophotometer**



03.

ELISA

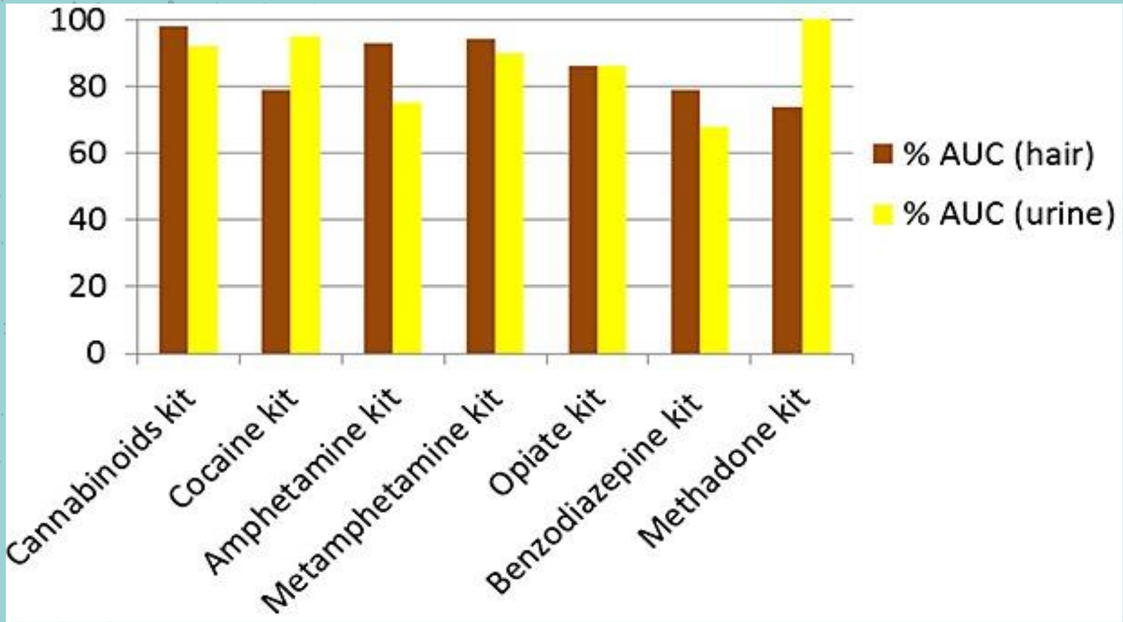
Advantages:

- **Heterogeneous assays** maximize **sensitivity**
- **Oriented** antibody
- Optimal **enzyme** conjugates
- **Low** sample volume
- **Long shelf life** (minimum one year)
- “**Ready to use**” reagents
- **No azide** or **mercury** preservatives
- Different levels of **automation**

Disadvantages:

- **Labour-intensive** & **expensive** antibody preparation
- **Sophisticated techniques** required
- High possibility of **false positive/negative**
- Antibody **instability**
- **Refrigerated transport** required

03. USE OF ELISA FOR MONITORING ABSTINENCE FROM ILLEGAL AND LEGAL DRUGS IN HAIR AND URINE



AUC for the seven LUCIO-Direct ELISA kits validated for the 6-drug MPA profile in hair and urine at the MPA cut-offs

The Study

Amphetamines, cannabinoids, cocaine, opiates, methadone, and benzodiazepines in hair and urine samples with drug concentrations around medical and psychological assessment (MPA) guidelines cutoffs were screened by LUCIO-direct ELISA kits

The Findings

- The AUC for almost all screening tests was greater than 0.8, indicating good to excellent performance
- The AUC calculated for the detection of drugs in hair did not differ significantly to the AUC calculated for the detection of the same drug classes in urine, thus showing a comparable screening performance to previously published studies

Area under the curve (AUC): an objective parameter that includes both **sensitivity and specificity**, and therefore directly measures the **diagnostic power** of the test. The AUC results here **indicate the effective use of all tested immunoassay kits for both matrices.**

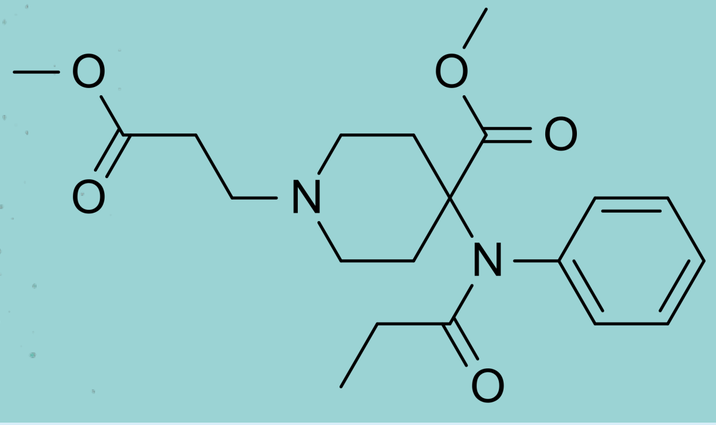
04.

TEST STRIPS

04.

FENTANYL TEST STRIPS

A sample of the drug sample is dissolved in water, and if the drug contains fentanyl in a concentration above the cut-off levels, an indicator on the strip will appear



Method works via chromatographic immunoassay, and in the presence of an appropriate analyte, a strip on the indicator stick appears/changes colour

+ Positive test

One line means fentanyl detected.

− Negative test

Two lines means no fentanyl detected.

? Inconclusive / Invalid test

The top red line (closest to the solid blue) should always show up on the strip. If it doesn't, the test is invalid. If no red lines show up, test is invalid. Stirring or wetting above the solid line may result in an invalid test.

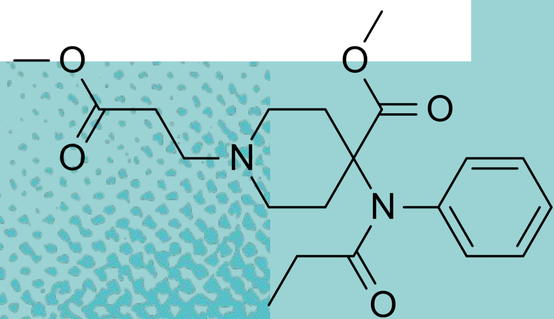
04. EVALUATION OF A LATERAL FLOW IMMUNOASSAY FOR THE DETECTION OF THE SYNTHETIC OPIOID FENTANYL

This study evaluates the effectiveness of commercially available competitive LFIs designed to detect the synthetic opioid fentanyl in either urine or saliva

Aim: to address the technology gap of the **limited number of tests** available for **fentanyl detection**.

How: examined the newly developed **lateral flow immunoassays (LFIs)** designed to detect **fentanyl and its derivatives.** These LFIs were evaluated for effectiveness in **different biofluid matrices,** following an *in vivo* exposure, cross-reactivity with fentanyl analogs, and in **case samples.**

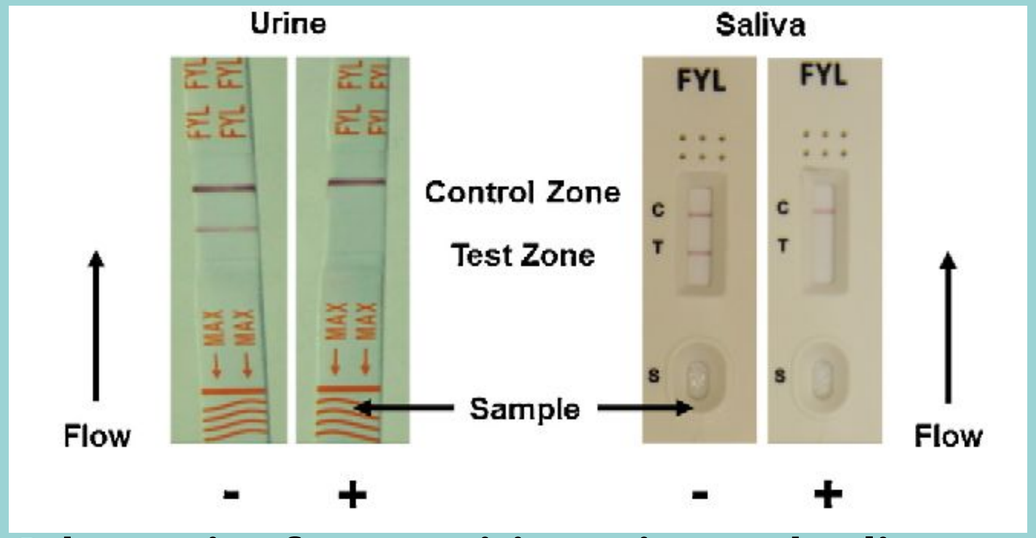
Method: LFIs are widely used for the detection of specific substances. These assays are typically **paper-based matrices** in which **antibodies directed against compounds of interest** have been incorporated. **Liquid sample**, typically in the form of a buffered solution containing a **specific analyte**, is placed on the absorbent sample pad which allows **capillary action** to move the liquid up the strip to the test and **control lines containing the capture reagents (i.e. antibodies).**



04. EVALUATION OF A LATERAL FLOW IMMUNOASSAY FOR THE DETECTION OF THE SYNTHETIC OPIOID FENTANYL

1. A liquid sample is placed on the absorbent sample pad by either dipping (urine) or pipetting (saliva)

2. This allows capillary action to move the liquid up the strip to the test and control lines containing the capture antibodies.



Schematic of competitive urine and saliva fentanyl LFIs.

(-): negative result
(+): positive result
C: control zone
FYL: fentanyl
T: test zone
S: sample

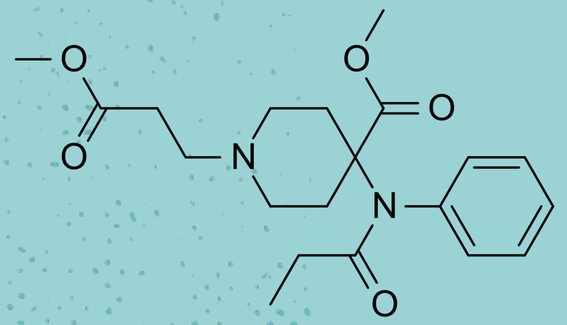
The LFIs are competitive, meaning:

- the appearance of a **single band** in the control zone is a **positive result**
- the appearance of bands in **both the control and test zones** is a negative result

04. EVALUATION OF A LATERAL FLOW IMMUNOASSAY FOR THE DETECTION OF THE SYNTHETIC OPIOID FENTANYL

The limit of detection (LOD) and sensitivity values for commercial off the shelf (COTS) and custom urine and saliva LFIs were compared

LFI	LOD (ng/mL)	Sensitivity (ng/mL)
COTS Urine	8	5
COTS Saliva	100	75
Custom Urine	25	8
Custom Saliva - Saliva	100	50
Custom Saliva - PBS	75	25



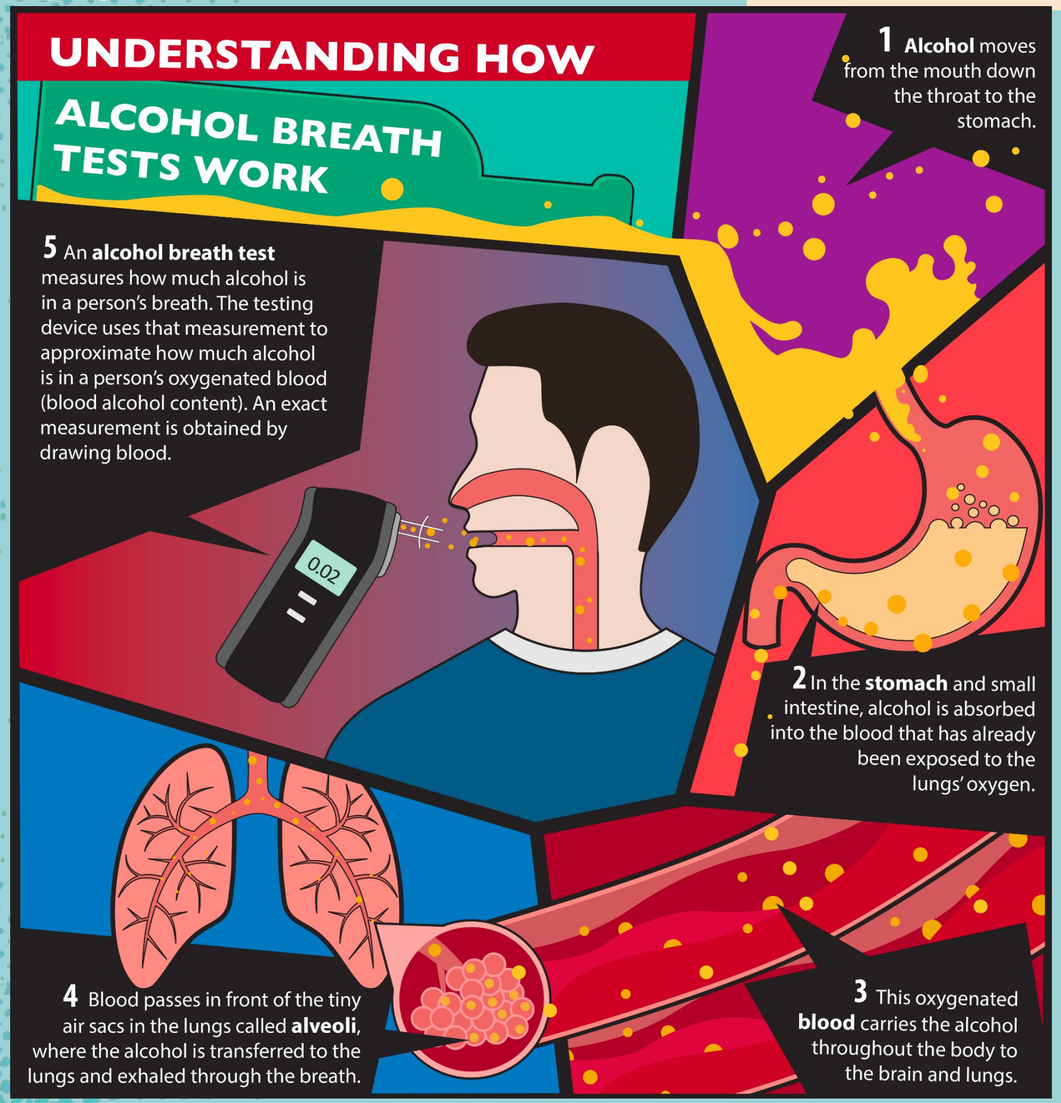
Conclusions: Data associated with the fentanyl directed LFIs have demonstrated the potential to be used in the field by law enforcement officers as presumptive fentanyl tests

05.

IN-FIELD TESTING

05.

Breathalyzer Testing

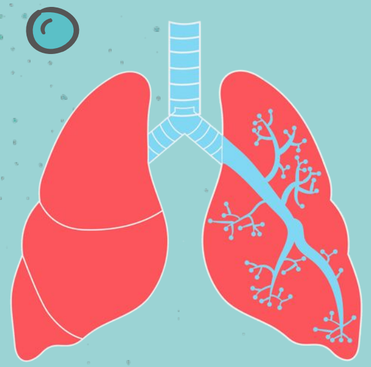


When you blow into a breathalyzer, the ethanol in your breath reacts with water from the air at the anode and is oxidized to form acetic acid (like in vinegar).

$$\underset{\text{ethanol}}{CH_3CH_2OH} + H_2O \rightarrow \underset{\text{acetic acid}}{CH_3CO_2} + 4H^+$$

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

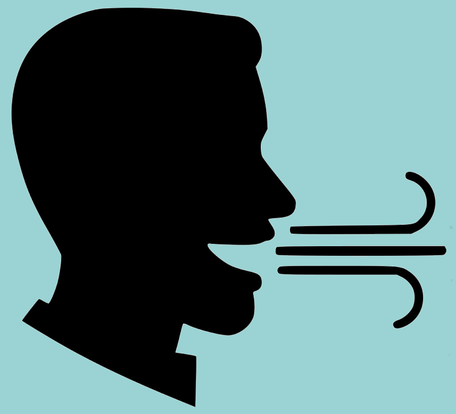
05. BREATH ALCOHOL TESTING

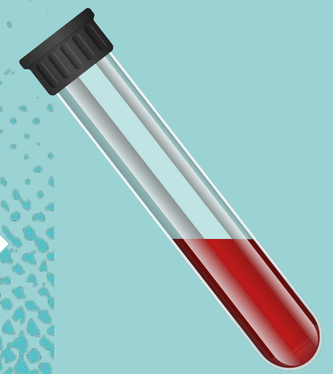


The lungs are specially adapted for **rapid and efficient gas exchang**e between the blood and breath, largely due to the presence of **alveoli.** This allows for the **rapid exchange** of volatile gases.

When blood containing the volatile drug, alcohol, passes through the **lungs**, the amount of alcohol in the blood is in a **fixed ratio** with the amount of alcohol in the breath. By analysis of **deep lung air**, the **BAC can be determined**.

The **blood:breath ratio** (BBR) for alcohol is **2100:1**. This means that **2100 mL** of breath contains the **same amount of alcohol** as **1 mL** of blood.





05. ACCURACY OF CONSUMER-MARKETED SMARTPHONE-PAIRED BREATH TESTING DEVICES: A LABORATORY VALIDATION STUDY

Background: Alcohol breath testing devices that pair with smartphones have been promoted to prevent impaired driving. However, the accuracy of these tests has not been established.

Methods: Weight-based doses of ethanol were administered to two groups of 10 healthy, moderate drinkers, with the goal of achieving a target peak blood alcohol concentration (BAC) of 0.10%. Once the peak phlebotomy BAC was obtained, the BrAC was measured with a police-grade device (Intoxilyzer 240) and 6 consumer smartphone-paired devices with measurements every 20 minutes until the BrAC reached <0.02% on the police device.

Conclusions: The smartphone-paired devices were found to vary widely in accuracy in this study. Although some devices were found to be suitable for clinical and research purposes, others significantly underestimated BAC. This has the potential to mislead intoxicated users into thinking they are fit to drive. Therefore, these devices should not be heavily relied upon in determining state of intoxication.

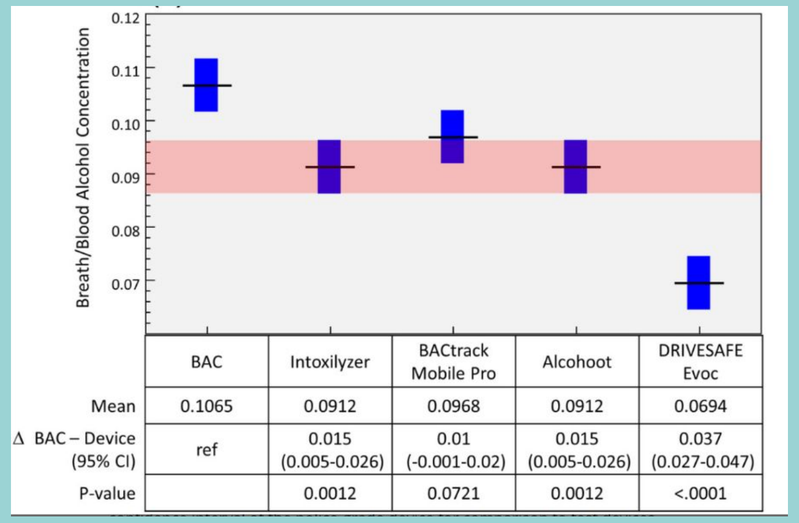
05. ACCURACY OF CONSUMER-MARKETED SMARTPHONE-PAIRED BREATH TESTING DEVICES: A LABORATORY VALIDATION STUDY

1 All breath testing devices, including the police-grade device, underestimated the phlebotomy BAC by a mean of 0.01% or more

2 In contrast, 95% of the Drinkmate and DRIVESAFE Evoc measurements underestimated the BAC by at least 0.02% or more, with the mean estimates being 0.04% below the peak BAC.

3 The devices closest to the phlebotomy BAC were the BACtrack Mobile Pro and police-grade device with 95% of measurements underestimating the BAC by no more than 0.02%

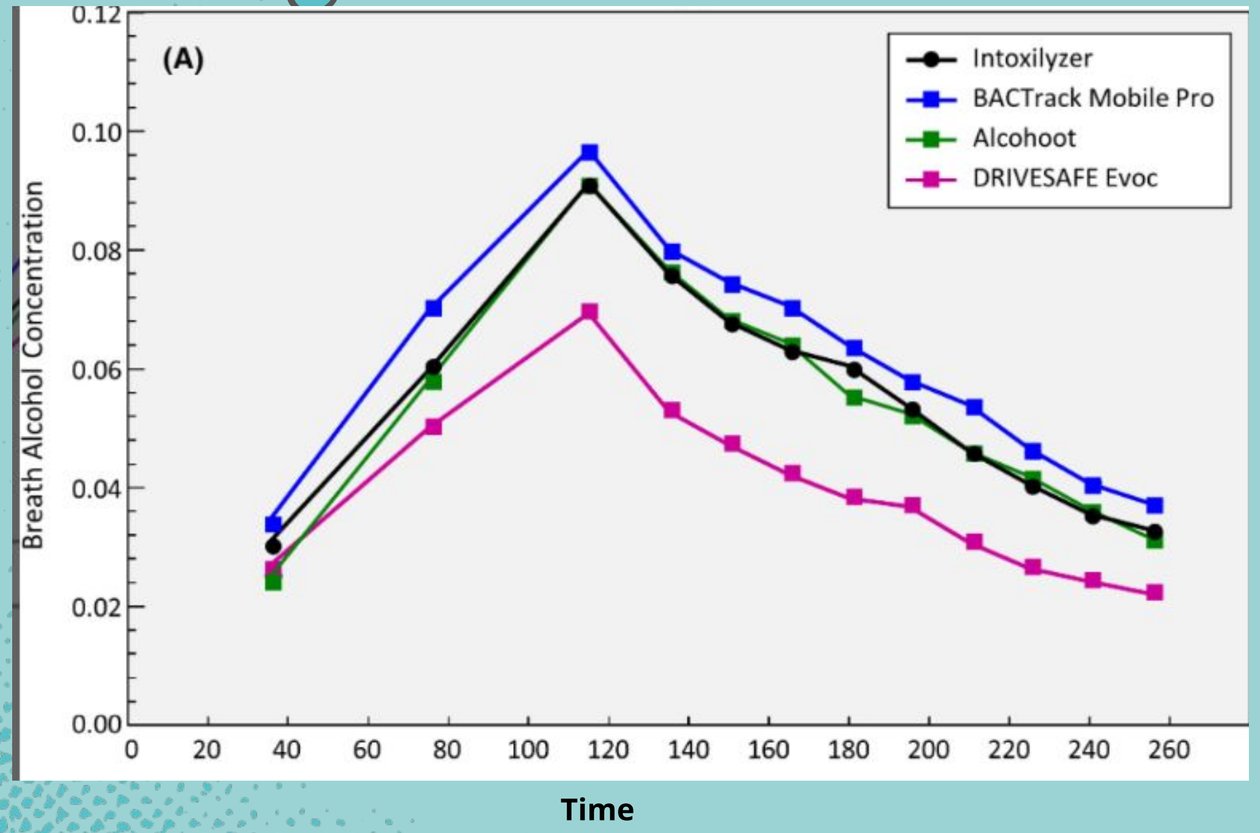
Horizontal black line = point estimate
Blue bars = 95% confidence interval
Pink band = 95% of confidence interval of the police-grade device for comparison to test devices.



	BAC	Intoxilyzer	BACtrack Mobile Pro	Alcohoot	DRIVESAFE Evoc
Mean	0.1065	0.0912	0.0968	0.0912	0.0694
Δ BAC – Device (95% CI)	ref	0.015 (0.005-0.026)	0.01 (-0.001-0.02)	0.015 (0.005-0.026)	0.037 (0.027-0.047)
P-value		0.0012	0.0721	0.0012	<.0001

Difference in breath alcohol concentration (BrAC) from police-grade (Intoxilyzer 240) and consumer smartphone-paired breath testing devices relative to BAC.

05. ACCURACY OF CONSUMER-MARKETED SMARTPHONE-PAIRED BREATH TESTING DEVICES: A LABORATORY VALIDATION STUDY



Mean paired difference in BrAC between the police grade and smartphone-paired devices across all time point. Increasingly negative Y-axis values indicate a greater underestimation of BrAC relative to the police grade device

The **BACtrack Vio** and **Alcohoot** measurements were **similar** to the Intoxilyzer. Only the Bactrack Vio was **significantly lower** than the police-grade device at **115 min.**

BACtrack readings were only **significantly higher** than the Intoxilyzer at **75** and **210 min.**

05. TOUCHLESS NARCOTICS AND CHEMICAL IDENTIFICATION SAFELY AND QUICKLY

05.

FIELD-BASED RAMAN SPECTROSCOPY

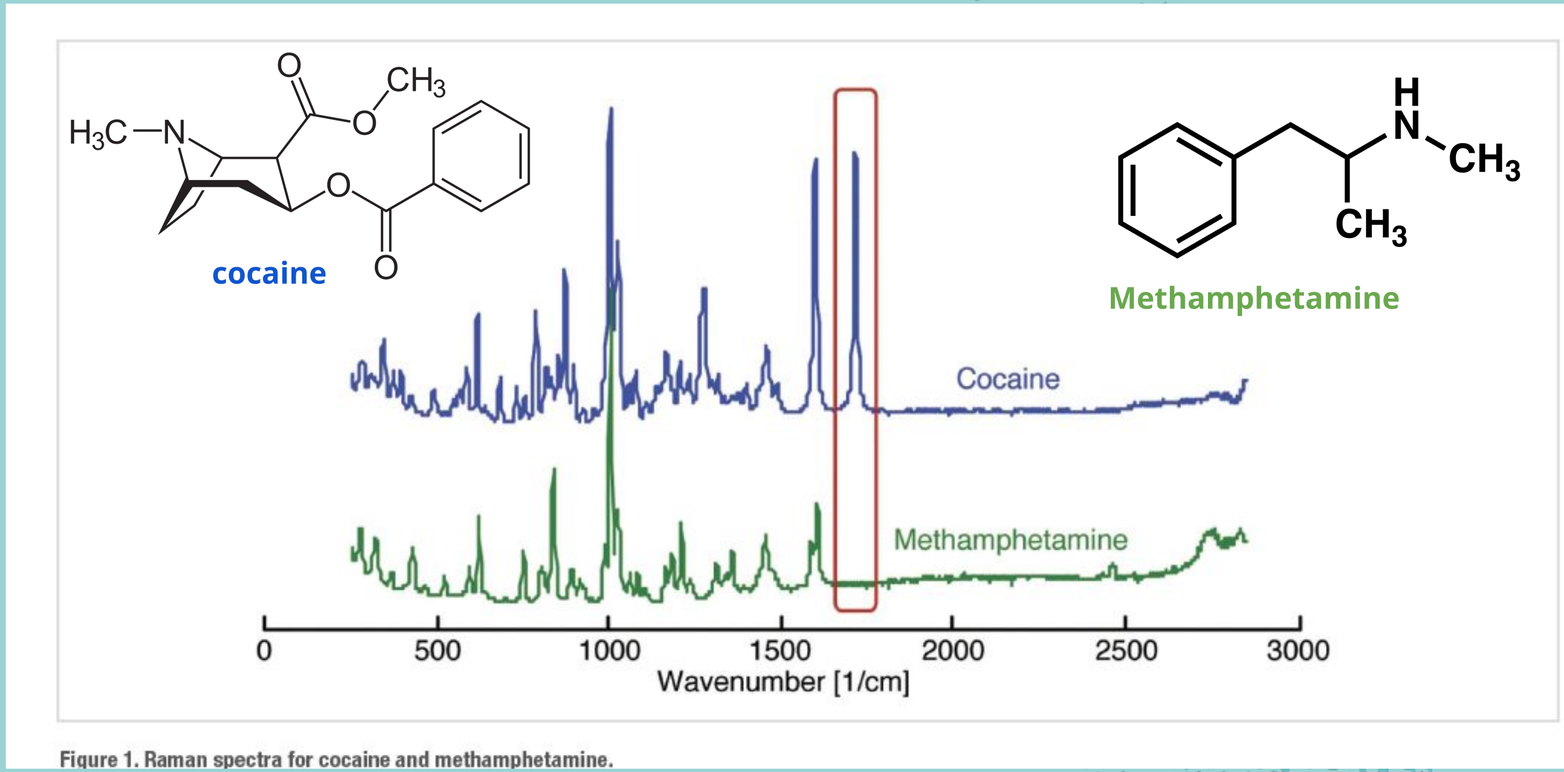


Figure 1. Raman spectra for cocaine and methamphetamine.

06.

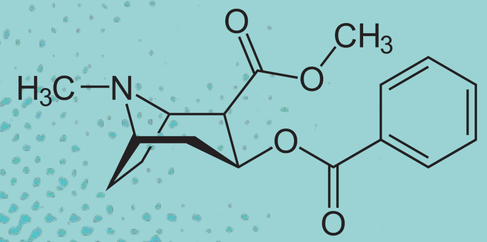
CANLII

06.

R.v. BOYCE

Background

Dexter Boyce was charged with conspiracy to import a controlled substance into Canada. He was alleged to be involved in a conspiracy to import ~356g of cocaine from Costa Rica to Toronto. The cocaine was hidden inside two packages of travel brochures, and was discovered when the packages were examined in Panama, a transit point for shipments.



Expert report from Panama

- The samples underwent a **Marquis Test** for the detection of opiates, and a modified **Scott test** for the detection of cocaine and microcrystals with a 5% aqueous gold chloride reagent
- Tests were also conducted for solubility, and an extraction was performed with Hexane in basic Sodium Hydroxide
- **IR spectroscopy** was used as a confirmatory technique
- **Concluded that the analyzed samples were positive for cocaine in the amount of 355.86g**

Examination by Health Canada Drug Analysis Services (DAS)

- Used **GC-FID and TLC** as non confirmatory tests
- Used **GC-MS and IR** as confirmatory tests
- Reached the same conclusion as the panamanian examination

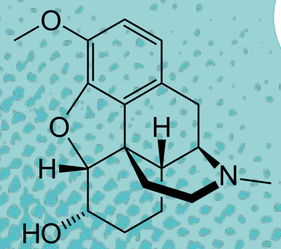
06.

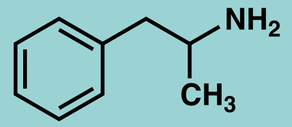
R. v. HUMPHREY

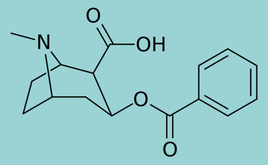
Issue: Did Mr. Kent have morphine in his system?

1. **A sample of urine was collected from Mr. Kent and tested using a Urine Toxicology Screen, Immunoassay, and Chromatography**

2. **The immunoassay screen detected amphetamines, cocaine metabolites, and opiates**







3. **The chromatography test detected cocaine and cocaine metabolites, but amphetamines, morphine and morphine metabolites were not detected**

Finding

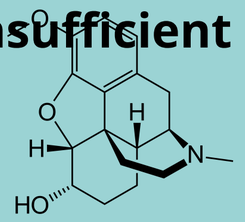
Although the immunoassay screen gave positive results for opiates, this was not confirmed by the Chromatography and that there was insufficient concentration of any one opiate to be detected by the Chromatographic method

06.

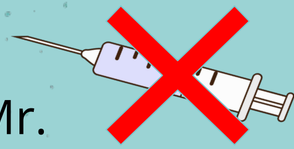
R. v. HUMPHREY

Expert Report Findings

1. Opiate immunoassay **measures total opiates**, for example morphine and codeine and their respective metabolites hence the **enhanced sensitivity**
2. Even **poppy seeds** can cause a positive opiate immunoassay result
3. The immunoassay screen detected the amphetamines and opiates because it **considered them together**
4. They could not be separately detected in the Chromatography test given their **insufficient concentration**



Findings



Mr. Mitchell (toxicologist) submits that Mr. Kent **did not take morphine intravenously** and that this contradicts the evidence of Mr. Humphrey

No evidence was presented on **how fast morphine and its metabolites dissipate in the urine**

Concluded that Mr. Kent did not inject morphine as alleged by Mr. Humphrey

07.
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