EFFICIENCY EVALUATION OF DIASYSTEM AUTOMATED IMMUNOASSAY IN DETECTION OF 11-NOR-9-CARBOXY-D9-TETRAHYDROCANNABINOL AND TRAMADOL IN URINE SAMPLES OF DRUG ABUSERS CHEATED BY HOUSEHOLD PRODUCTS

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ABSTRACT

Background: In Egypt, incidents of road and train accidents caused by drivers under the influence of drugs have led to the introduction of a law that mandates drug testing for public sector employees. **Aim of the work:** This study aimed to explore various methods for the detection of subversion of 11-nor-9-carboxy-D9-tetrahydrocannabinol (THC–COOH) and tramadol-positive urine samples. **Subjects and Methods:** After confirming the negativity and positivity of urine samples for THC–COOH and tramadol by using GC-MS, the urine samples were divided into negative control, THC–COOH-positive, and tramadol-positive samples. Positive samples were further sub-divided into six groups, one group without adulterants and five groups were adulterated with carbonated water, laundry detergent gel, tea, hydrogen peroxide, and drinking water at 20% and 40% concentrations (conc.) in each subgroup. **Results:** The integrity strips showed normal pH, specific gravity (SG), and negative nitrites that were corroborated by a pH meter and refractometer. As well as the creatinine levels were in the reference ranges. The rapid drug immunoassay strips yielded false-negative results for THC–COOH in all adulterated and diluted urine samples at both concentrations. Meanwhile, the 40% concentration of urine samples manipulated with tea, hydrogen peroxide, and drinking water displayed false negative results for tramadol. **Conclusion:** THC–COOH and tramadol-positive urine samples adulterated with at 20%, 40% conc. of laundry detergent gel and 40% conc. of tea displayed false negative results by automated immunoassay and GC-MS. The automated immunoassay provided accurate results for drug screening of most adulterated and diluted urine samples that tested positive for THC–COOH and tramadol compared with GC-MS.

Keywords: *THC–COOH, Tramadol, GC-MS, Immunoassay, Adulteration, Integrity Strips.*

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INTRODUCTION

National Institute on Drug Abuse ***(NIDA, 2020)*** defined drug abusers as individuals who utilize illegal substances to attain some degree of euphoria or hallucination, or those who utilize a legally prescribed drug without its medical guidelines**.** Substance abuse widespread among different socioeconomic classes has turned it into a public health major concern ***(Mathew et al., 2011; Kassew et al., 2023)***.

There was a sudden surge in Egypt, beginning in the 1970s, in the number of substances abused by individuals ***(Yassa and Badea, 2019)***.

Drug addiction is a significant concern for the Egyptian government, particularly as it affects employees in their work places. This issue can result in numerous complications, including poor social adaptation, reduced work productivity, and potential job loss ***(Smook et al., 2014)***.

The prevalence of recreational drug use among drivers is a major cause of traffic accidents, as they believe these substances enhance their performance and reduce fatigue ***(El-Gendy et al., 2015)***.

Whereas several biological samples are included in screening for substance abuse such as (blood, saliva, urine, hair, and nails), nevertheless, the most favorite choice is the urine sample. The easy urine collection, longer detection windows for substances of abuse in urine, and higher drug concentrations

compared to blood are the factors that contribute to this choice ***(Mizrak, 2019; Aydogdu and Akgu¨r, 2021)***.

The immunochromatographic test strips for urine drug screening (UDSTs) have many benefits as a quick, cheap, non-invasive, and on-site user-friendly method ***(Rajšić et al., 2020)***.

However, they have some limitations. The most common one is that they may yield false-positive results because of their potential to react with other non-targeted drugs having similar chemical structures or certain food components. They may yield false negative results if the collected samples have been diluted—additionally, their low accuracy and specificity ***(Riahi-Zanjani, 2014)***.

Drug abusers dilute their urine samples to get negative results using different methods. Either by *in vivo* adulteration which is a huge fluid drinking and or diuretics used to make the drug in the urine sample undetectable, or substituting the urine sample with a drug-free sample ***(Dasgupta, 2007)***.

In cases of unexpected testing, drug abusers tend to use the *in vitro* adulteration method which is the addition of urine adulterating substance for getting false negative results ***(Standridge et al., 2010)***.

Numerous chemical substances are available around us to evade the drug test results involving acids, alkalis, surfactants, and oxidizing agents ***(Schulberg and Gerostamoulos, 2013)*** or vinegar, table salt, hypochlorite bleach, laundry detergent. Therefore, urine integrity assessments are employed to avoid inaccurate negative outcomes ***(Mizrak, 2019)***.

THE AIM OF THE WORK

This research aimed to investigate how various household products such as carbonated water, tea, laundry detergent, and hydrogen peroxide, as well as dilution with drinking water, affects positive urine samples for 11-nor-9-carboxy-D9-tetrahydrocannabinol (THC–COOH) and tramadol. Additionally, the study sought to compare the results obtained from urine integrity tests, rapid screening strips, and DiaSystem automated immunoassay after confirmation by GC-MS.

SUBJECTS AND METHODS

This case-control study was done in the Forensic and Clinical Toxicology Research Laboratory, Faculty of Medicine, Zagazig University. The research was approved by Ethics Committee of Faculty of Medicine, (Institutional review boards (IRB), Zagazig University, Egypt and the reference number is **(ZU-IRB#11106-10/9-2023)**. The Ethics Committee guidelines are in accordance with the Declaration of Helsinki.

Sample size: Based on a 95% confidence interval, with a power of 80% and a sensitivity of 70% for the immune assay in diagnosing THC–COOH and tramadol, the sample size was determined to be 27 samples, divided equally into three groups, with 9 samples in each group (negative control, positive for THC–COOH group, and positive for tramadol group), maintaining a 1: 1:1 ratio.

Subject and samples

A. The urine samples used in this study were collected from patients admitted to the intensive care unit (ICU), Poisoning Treatment Unit, and from patients who presented to the Forensic Medicine and Clinical Toxicology Research Laboratory for drug screening after obtaining their consent. The urine samples of 50-100 mL were collected in clean dry labeled plastic containers.

Inclusion criteria:

- 18 - 60 years old of male patients.
- History of hashish, bang intake, or tramadol intake

Exclusion criteria:

- Patients with chronic disease
- Patients with a history of drugs that give false positive THC–COOH results as non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors, antiviral, antihistaminic, vitamins: riboflavin, diuretics: ethacrynic acid or synthetic cannabinoid ***(Gragnolati, 2022)***.
- Patients with a history of drugs that give false positive tramadol results as antitussive (dextromethorphan), anti-histaminic (fexofenadine, diphenhydramine), hallucinogens

(phencyclidine), antibiotic (rifampin) ***(Gragnolati, 2022)***.

B. Additional urine samples of 50 to 100 mL were collected from healthy volunteers for drug-free urine samples after obtaining their consent and using them as negative control group.

Adulterants and dilutants

1. Carbonated water (fayrouz) was obtained from a local market.
2. Tea: a single tea bag was added to 100 mL of boiling water for 1 minute. Subsequently, the bag was removed, and the extract was left to cool to room temperature.
3. Laundry detergent gel: was obtained from a local market.
4. Hydrogen peroxide 20 %: was obtained from a local pharmacy.
5. Drinking water: was obtained from a local market

The adulterants were selected based on their availability, sourced either from the house or from the market. Moreover, some of these adulterants were used by abuser such as carbonated water (fayrouz) and tea during the drug testing for public sector employees.

Design protocol

All urine samples were tested for positivity and negativity for THC–COOH and tramadol by GC-MS. The urine samples were divided into the following three main groups (9 samples in each group) **(Figure 1):**

Group A (Negative control group): Drug free urine samples.

Group B (Positive urine samples for THC–COOH): Each urine sample was equally subdivided into 6 groups:

- ***Group B1 (THC–COOH -positive control group):*** 10 mL urine without adulteration.
- ***Group B2 (Carbonated water group):***10 mL urine was divided equally into 2 subgroups:
  - ***Group B2L (Carbonated water 20% group);*** 1mL was prepared by adding 800 µL of urine to 200 µL of carbonated water.
  - ***Group B2H (Carbonated water 40% group);*** 1 mL was prepared by adding 600 µL of urine to 400 µL of carbonated water.
- ***Group B3 (Laundry detergent gel):*** 10 mL urine was divided equally into 2 subgroups:
  - ***Group B3L (Laundry detergent gel 20% group);*** 1 mL was prepared by adding 800 µl of urine to 200 µl of laundry detergent gel.
  - ***Group B3H (Laundry detergent gel 40% group);*** 1 mL was prepared by adding 600 µL of urine to 400 µL of laundry detergent gel.
- ***Group B4 (Tea group):*** 10 mL urine was divided equally into 2 subgroups:
  - ***Group B4L (Tea 20% group);*** 1 mL was prepared by adding 800 µL of urine to 200 µL of tea drink.
  - ***Group B4H (Tea 40% group);*** 1 mL was prepared by adding 600 µL of urine to 400 µL of tea drink.
- ***Group B5 (Hydrogen peroxide group):*** 10 mL urine was divided equally into 2 subgroups:
  - ***Group B5L (Hydrogen peroxide 20% group);*** 1 mL was prepared by adding 800 µL of urine to 200 µL of hydrogen peroxide.
  - ***Group B5H (Hydrogen peroxide 40% group);*** 1 mL was prepared by adding 600 µL of urine to 400 µL of hydrogen peroxide.
- ***Group B6 (Drinking water group):*** 10 mL urine was divided equally into 2 subgroups:
  - ***Group B6L (Drinking water 20% group);*** 1 mL was prepared by adding 800 µL of urine to 200 µL of drinking water.
  - ***Group B6H (Drinking water 40% group);*** 1 mL was prepared by adding 600 µL of urine to 400 µL of drinking water.

Group C (Positive urine samples for tramadol): Each urine sample was equally subdivided into 6 groups:

- ***Group C1 (Tramadol-positive control group):*** 10 mL urine without adulteration.
- ***Group C2 (Carbonated water group):*** 10 mL urine was divided equally into 2 subgroups:
  - ***Group C2L (Carbonated water 20% group);*** 1 mL was prepared by adding 800 µL of urine to 200 µL of carbonated water.

- ***Group C2H (Carbonated water 40% group);*** 1 mL was prepared by adding 600 µL of urine to 400 µL of carbonated water.
- ***Group C3 (Laundry detergent gel):*** 10 mL urine was divided equally into 2 subgroups: ***Group C3L (Laundry detergent gel 20% group);*** 1 mL was prepared by adding 800 µL of urine to 200 µL of laundry detergent gel.
- ***Group C3H (Laundry detergent gel 40% group);*** 1 mL was prepared by adding 600 µL of urine to 400 µL of laundry detergent gel.
- ***Group C4 (Tea group):*** 10 mL urine was divided equally into 2 subgroups:
- ***Group C4L (Tea 20% group);*** 1 mL was prepared by adding 800 µL of urine to 200 µL of tea drink.
- ***Group C4H (Tea 40% group);*** 1 mL was prepared by adding 600 µL of urine to 400 µL of tea drink.
- ***Group C5 (Hydrogen peroxide group):*** 10 mL urine was divided equally into 2 subgroups:
- ***Group C5L (Hydrogen peroxide 20% group);*** 1 mL was prepared by adding 800 µL of urine to 200 µL of hydrogen peroxide.
- ***Group C5H (Hydrogen peroxide 40% group);*** 1 mL was prepared by adding 600 µL of urine to 400 µL of hydrogen peroxide).
- ***Group C6 (Drinking water group):*** 10 mL urine was divided equally into 2 subgroups:
- ***Group C6L (Drinking water 20% group);*** 1 mL was prepared by adding 800 µL of urine to 200 µL of drinking water.
- ***Group C6H (Drinking water 40% group);*** 1 mL was prepared by adding 600 µL of urine to 400 µL of drinking water.

Methods

After preparing the samples, they were examined for the following:

- **Physical properties** (odor, color, any changes in appearance as the presence of froth, sediments, turbidity).
- **Integrity strips and laboratory analysis:**

The pH, specific gravity (SG), and nitrites were checked using reagent strips for urinalysis, (model: URS-10H, Bioway Biological Technology CO., Ltd). The results from the integrity strips were visualized, recorded, and photographed in comparison to the color guide provided by the manufacturer. The pH level was verified using a pH meter (AD1030 model), and the SG was confirmed using a refractometer.

The determination of urine creatinine levels followed the Jaffe colorimetric method, which relies on the formation of a creatinine-picrate complex in an alkaline medium. In this method, creatinine in the sample reacts with picrate, and the rate of increase in absorbance at 500 nm is directly proportional to the creatinine concentration in the sample ***(Toora and Rajagopal, 2002)***.

The typical acceptable ranges for a standard urine sample are pH (4.5-8), SG (1005-1025), and creatinine: (20-100 mg/dL).

- **Drug screening immunoassay strips**

The rapid drug screening immunoassay (BioTina GmbH® One Step Diagnostic Strip, CAT NO.: R4018, Germany) was used according to manufacturer instructions to examine the presence of THC–COOH and tramadol. Two urine drops were carefully transferred into the sample well of the test strip. After a waiting period of 3 to 5 minutes, the results were assessed. The test strips are qualitative; therefore, a positive outcome indicates that the screened drug is probably present in the urine at a concentration exceeding the specified cut-off level. The cut-off level for THC–COOH is 50 ng/mL, while the cut-off level for tramadol is 100 ng/mL according to the manufacturer.

Principal of procedure

It is a rapid one-step chromatographic immunoassay utilizing chemically labeled drugs (drug-protein conjugates) to compete with drugs potentially present in urine for limited antibody binding sites. The test device comprises membrane strips pre-coated with drug-protein conjugates on the test band(s). The drug antibody-colloidal gold conjugate pad is placed at one end of each strip.

If there is no drug present in the urine, the colored antibody-colloidal gold conjugate solution moves chromatographically forming visible lines as the antibody complex interacts with the drug conjugates. If the drug is present in the urine, the drug/metabolite antigen competes with the drug-protein

conjugate on the test band region for the limited antibody and preventing the attachment of the colored antibody-colloidal gold conjugate to the drug-protein conjugate zone on the test band region ***(McBay, 1987)***.

- **DiaSystem automated immunoassay**

Auto-chemistry analyzer, (model, CDT240, SN: CA24-210800528), is a homogeneous enzyme immunoassay that utilizes a ready-to-use liquid reagent. The assay relies on the competition between antibodies for the drug in the sample and drug molecules labeled with glucose 6-phosphate dehydrogenase (G6PDH).

When antibodies bind to the drug, it leads to a reduction in enzyme activity, and the concentration of the drug in the sample is determined by measuring this enzyme activity. In instances where there is no drug present in the sample, a drug derivative labeled with G6PD conjugate binds to the antibodies, thereby inhibiting enzyme activity.

Conversely, when the sample contains the drug, the antibodies bind to the free drug, allowing the unbound drug derivative labeled with G6PD to exhibit its maximum enzyme activity. The active enzyme then converts nicotinamide adenine dinucleotide (NAD) to NADH, resulting in a change in absorbance that can be measured spectrophotometrically at a primary wavelength of 340 nm ***(Rainey and Baird, 2012)***.

Calibration procedures

- **THC–COOH analysis:**

Uni Cal THC–COOH negative calibrator L1 contains negative human urine with preservatives. Uni Cal THC–COOH calibrator Low 25: contains 25 ng/mL of THC–COOH. Uni Cal THC–COOH calibrator High: contains 50 ng/mL of THC–COOH. Uni Cal THC–COOH calibrator intermediate/ 100: contains 100 ng/mL of THC–COOH (**Figure 2**).

For qualitative analysis, the cut-off calibrator, which contains 50 ng/mL of THC–COOH, is used as a reference for distinguishing positive from negative samples according to the manufacturer.

- **Tramadol analysis:**

Uni Cal negative calibrator L1 contains negative human urine with preservatives. UniCal tramadol calibrator low 50: contains 50 ng/mL of tramadol. UniCal tramadol calibrator intermediate 250: contains 250ng/mL of tramadol. UniCal tramadol calibrator high 400: contains 400 ng/mL of tramadol (**Figure 3)**.

For qualitative analysis use the 200 ng/mL as the cut-off calibrator as a reference for distinguishing positive from negative samples according to the manufacturer.

- **GC-MS analysis**

Device: The analysis was done using a gas chromatograph (Model**:** GC-MS (ISQ™ LT trace 1300 coupled with an ISQ™ 7000 mass spectrometer), Thermo Fisher Scientific S.p.A. Milan, Italy (S.N. 420131158).

Principal of procedure

It is a combination of two distinct analytical techniques: gas chromatography (GC) and mass spectrometry (MS). GC operates on the principle that when a mixture is heated, its components separate. In this method, the sample was introduced into the GC inlet, where it vaporized and was carried into a chromatographic column by a carrier gas, typically helium (2mL/min). Within the column, the substances within the mixture were separated based on their interactions with both the column's coating (stationary phase) and the carrier gas (mobile phase).

As the sample continued through the column, it passed through a heated transfer line and reached the entrance to the ion source, where the compounds eluting from the column were transformed into ions. A stream of electrons ionized the sample molecules, resulting in the creation of molecular ions and smaller ions with distinct relative abundances. These relative abundances served as a unique 'fingerprint' for the molecular structure being analyzed. The mass analyzer was responsible for separating these ions, after which they were detected ***(Coskun, 2016)***.

The constituents of the extracts were thoroughly identified by matching their mass spectral fragmentation patterns with documented **NIST library** data bank spectral.

GC/MS operating parameters

- TR-DoA 35MS column: 15 meters in length, with an inner diameter of 0.25 mm, and coated with a 0.25-TG- stationary phase.
- Carrier gas: Helium, flowing at a rate of 1.5 mL/min, and Constant Flow mode
- Column temperature program: initial 70°C, held for 0.5 minute, followed by a ramp of 22°C/min to 250°C, and finally a ramp of 23°C/min to 320°C held for 2 minutes.
- Injection technique: Splitless with Surge mode. The splitless time: 1 min with split flow at a rate of 20 mL/min at 280 °C. The surge pressure: 172 kPa, flowing rate at 5 mL/min held for 1 min
- The retention time: 6.03 minutes.
- MS transfer line temperature at 250 °C, with Electron Ionization (EI) mode and the scan time :0.2 seconds.

Extraction procedure

- 30µL β-glucuronidase (Merck 5000 I.U.) were added to 3 mL of urine and incubated for 60 min at 56 °C.
- The sample was mixed with 3 mL of 2M acetate buffer, pH was checked and adjusted at 4.8.
- The sample was added to the HyperSep Verify CX cartridge (6 mL/200 mg, was conditioned with 3 mL MeOH then, 3 mL 0.1% formic acid) and a slight vacuum was applied to achieve elution rate.
- The elution was done with a mixture of 1 mL water + 0.1% formic acid, followed by a mixture of 1 mL methanol/water 50:50 + 0.1% formic acid, total volume 3 mL.
- The cartridge was dried after interference elution with strong vacuum.
- The sample was evaporated under nitrogen at 65 °C until dryness.
- Finally, the residue was dissolved in 0.1 mL of methanol and placed in 50 µL MS certified vials. The vials were subsequently centrifuged for precipitating the particles before putting them in the autosampler.

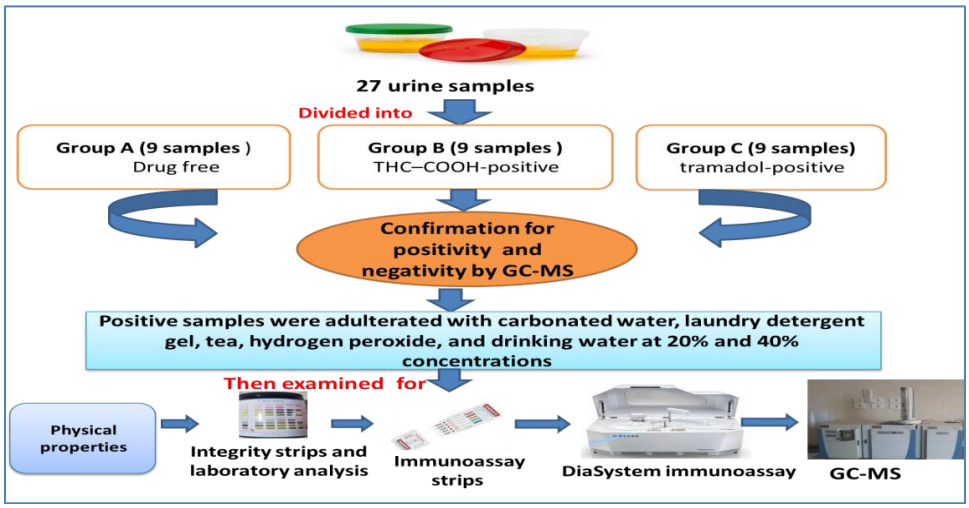

Figure (1): Experimental design

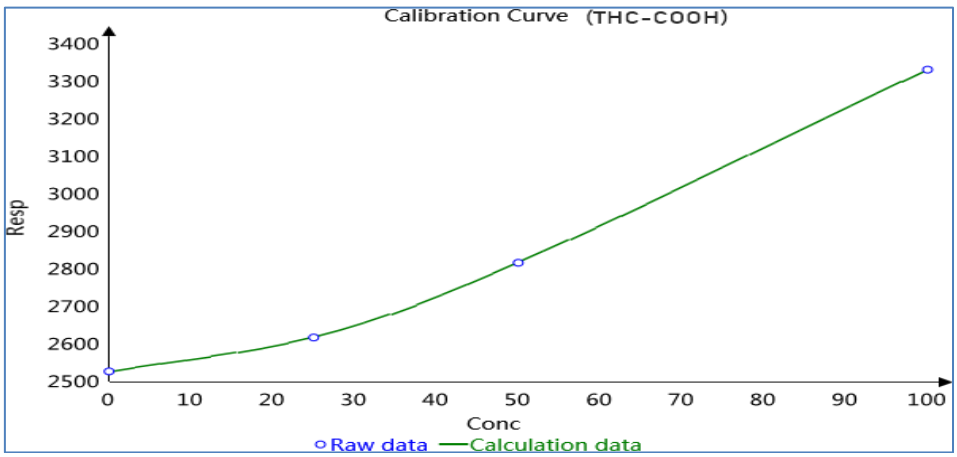

Figure (2): Calibration curve for THC-COOH on DiaSystem automated immunoassay.

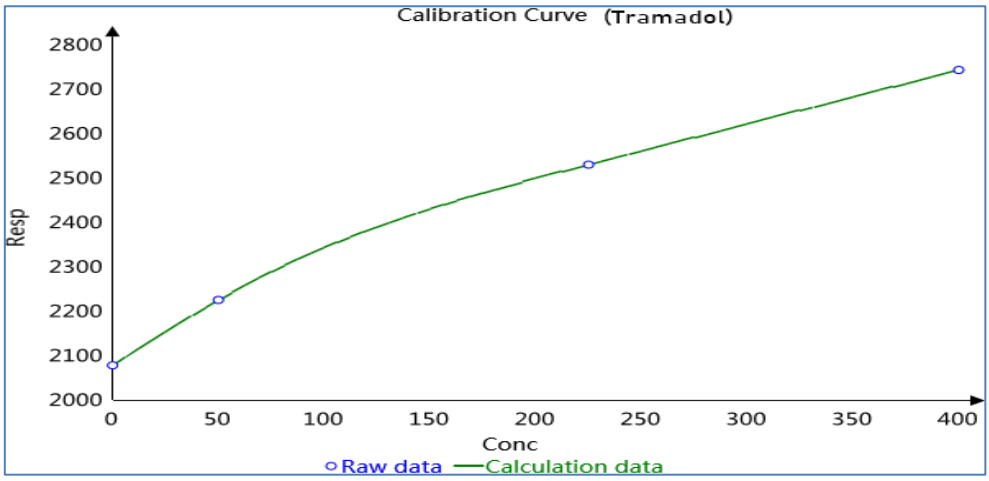

Figure (3): Calibration curve for tramadol on DiaSystem automated immunoassay.

STATISTICAL ANALYSIS:

SPSS (version 23.0), Graph Prism (version 9), and MedClac® statistical software (version 17.9.7) were used to collect, tabulate, and analyze the data. The data were expressed as mean±standard deviation. One-way ANOVA followed by a least significance test for comparison between different groups. Sensitivity and specificity were used to evaluate test performance, and accuracy was calculated to determine proximity to the true value. A p-value less than 0.05 was considered as a significant value.

RESULTS

Before preparing adulterated samples, urine samples were tested for positivity and negativity for THC–COOH and tramadol by GC-MS. **Figures (4, 5)** showed a chromatogram for urine samples positive for THC–COOH, with a retention time of 6.85 minute, and a chromatogram for urine samples positive for tramadol, with a retention time, of 8.76 minute respectively.

Physical properties

Negative and positive urine samples for THC–COOH and tramadol showed normal odor, with clear light to deep yellow color.

On examining positive urine samples for THC–COOH and tramadol after adulteration with 20% and 40% of carbonated water (**B2L, B2H, C2L, C2H**), it was observed a characteristic odor of the carbonated water with no change in color or appearance **(Figures 6, 7)**.

Cheating positive urine samples for THC–COOH and tramadol with 20% and 40% of laundry detergent gel (**B3L, B3H, C3L, C3H**). There was a noticeable change in the odor and color of urine associated with hazy and frothy appearance, these changes were more pronounced with 40% concentration **(Figures 6, 7)**.

Tea adulterated samples of 20% and 40% concentrations (**B4L, B4H, C4L, C4H**) showed alteration in the odor of urine with darkening in color (amber yellow) which was more pronounced with 40% concentration in comparison to control groups (**Figures 6, 7**).

Groups of **B5L, B5H, C5L, and C5H** displayed lightening in color (light yellow) after adding 20% and 40% of hydrogen peroxide compared with control groups without any change in odor or appearance **(Figures 6, 7)**.

By visual examination of the urine samples positive for THC–COOH and tramadol diluted with 20% and 40% of drinking water (**B6L, B6H, C6L, C6H**), there was observed lightening in color (light yellow) compared with control groups without any change in odor or appearance **(Figures 6, 7)**.

Integrity strip and laboratory analysis

Following the product instructions for integrity strip testing, which included assessment of pH, specific gravity, and nitrites, and through the laboratory analysis, the following results were observed:

Effect of carbonated water

The integrity strip results, the positive urine samples for THC–COOH and tramadol, adulterated with 20% and 40% of carbonated water (**B2L, B2H, C2L, C2H**) displayed no change in pH, specific gravity, and nitrites were negative **(Figures 8, 9)**.

The pH meter indicated a significant reduction in pH values within the **B2L, B2H, C2L, and C2H** groups when compared with the positive control groups **(B1, C1)** ($p<0.05$). However, the pH values remained within the normal reference range of (4.5-8). The specific gravity (SG) results by refractometer displayed significant elevation in SG in both concentrations of positive urine samples adulterated with carbonated water compared with positive control groups (**B1, C1**) ($p<0.05$) **(Tables 1, 2)**.

In terms of urine creatinine levels, both concentrations of positive urine samples adulterated with carbonated water exhibited lower creatinine levels compared with positive control groups ($p<0.05$). However, the urine creatinine levels remained within the normal reference range (20-100 mg/dL). Carbonated water 40% concentration groups (**B2H, C2H**) showed a significant reduction in urine creatinine levels compared with carbonated water 20% concentration groups (**B2L, C2L**) **(Tables 1, 2)**.

Effect of laundry detergent gel

The positive urine samples for THC–COOH and tramadol, adulterated with 20% and 40% of laundry detergent gel, (**B3L, B3H, C3L, C3H**) showed abnormal specific gravity (1030), normal pH, and negative for nitrites (**Figures 8, 9).**

The pH meter displayed non-significant pH changes in **B3L, B3H, C3L, and C3H** groups when compared with positive control groups ($p>0.05$).Refractometer showed significant elevation in SG at both concentrations of the positive THC–COOH and tramadol urine samples adulterated with laundry detergent gel compared with positive control groups (**B1, C1**) ($p<0.05$) **(Tables 1, 2)**.

Moreover, the urine creatinine levels showed significant decrements when compared with positive control groups ($p<0.05$), however, the creatinine levels remained within the normal reference range (20-100 mg/dL). Notably, the 40% concentration groups (**B3H, C3H**) showed a significant reduction in urine creatinine compared with the 20% groups (**B3L, C3L**) **(Tables 1, 2)**.

Effect of tea

The integrity strips indicated that positive urine samples for THC–COOH and tramadol adulterated with both 20% and 40% tea **(B4L, B4H, C4L, C4H)** displayed normal pH, specific gravity, and negative nitrites **(Figures 8, 9)**. Moreover, there were no significant pH changes, as tested by the pH meter, in the **B4L, B4H, C4L, and C4H** groups when compared with the positive control groups ($p>0.05$).

The refractometer showed non-significant changes in SG at 20% concentration of adulterated urine samples when compared with the positive control groups **(B1)** ($p>0.05$). However, the 40% concentration of tea **(B4H, C4H)** resulted in a significant elevation in SG when compared with the control group ($p<0.05$) **(Tables 1, 2)**.

In terms of urine creatinine, the adulterated urine samples at both concentrations **(B4L, B4H, C4L, C4H)** exhibited low creatinine levels compared with the positive control groups ($p<0.05$). Nevertheless, it's important to note that these creatinine levels remained within the normal reference range of 20-100 mg/dL. Notably, the 40% concentration of tea adulteration **(B4H, C4H)** showed a significant decrease in urine creatinine when compared with the 20% concentration of tea groups **(B4L, C4L) (Tables 1, 2)**.

Effect of hydrogen peroxide

Integrity strips showed normal pH, specific gravity, and negative nitrites in THC–COOH and tramadol urine samples adulterated with 20% and 40% hydrogen peroxide (**B5L, B5H, C5L, C5H**) **(Figures 7, 8)**. The pH meter showed non-significant changes in pH values in **B5L, B5H, C5L, and C5H** groups compared with the positive control groups ($p>0.05$) (**Tables 1, 2**).

The refractometer demonstrated a significant elevation in the SG of adulterated urine samples of both THC–COOH and tramadol at 20% and 40% hydrogen peroxide (**B5L, B5H, C5L, C5H**) compared with the positive control groups (**B1, C1**) ($p<0.05$) **(Tables 1, 2)**.

Regarding urine creatinine, the hydrogen peroxide-adulterated urine samples **(B5L, B5H, C5L, C5H)** displayed low creatinine compared with the control positive groups ($p<0.05$). However, it's important to note that the urine creatinine levels remained within the normal reference range of 20-100 mg/dL.

Hydrogen peroxide 40% groups (**B5H, C5H**) showed a significant decrease in urine creatinine compared with hydrogen peroxide 20% groups (**B5L, C5L**) **(Tables 1, 2)**.

Effect of drinking water

The urine samples tested positive for THC–COOH and tramadol and were diluted with 20% and 40% of drinking water (**B6L, B6H, C6L, C6H**), displayed normal pH, specific gravity, and negative nitrites **(Figures 8, 9).** The pH meter showed non-significant changes in pH values in **B6L, B6H, C6L, and C6H** groups compared with the positive control groups ($p>0.05$).

However, the refractometer showed a significant reduction of SG of both THC–COOH and tramadol samples diluted with 20% and 40% drinking water (**B6L, B6H, C6L, C6H**) compared with the positive control groups (**B1, C1**) ($p<0.05$). Regarding urine creatinine, the diluted urine samples displayed low creatinine compared with the positive control groups ($p<0.05$), furthermore, the drinking water at 40% concentration (**B6H, C6H**) showed a reduction of urine creatinine compared with 20% drinking water groups (**B6L, C6L**) ($p<0.05$) **(Tables 1, 2)**.

Table (1): Effect of cheating urine positive for THC-COOH by 20% and 40% of carbonated water, laundry detergent gel, tea, hydrogen peroxide, drinking water on nitrites, creatinine, pH and specific gravity.

Group / Parameter	pH by integrity strip Reference range (4.5-8)	pH by pH meter (Mean ±SD)	Specific gravity by integrity strip Reference range (1005-1025)	Specific gravity by refractometer (Mean ±SD)	Nitrites By integrity strip	Creatinine Normal range 20-100 mg/dl (Mean ±SD)
Negative control group (A)	Normal	5.72±0.61	Normal	1016.67±2.12	Negative	66.25±3.14
Positive control group (B1)	Normal	5.69±0.49	Normal	1017±3.16	Negative	68.50±4.70
Carbonated water 20% group (B2L)	Normal	4.88±0.31 a b	Normal	1026.22±4.12 a b	Negative	49.47±2.86 a b
Carbonated water 40% group (B2H)	Normal	4.88±0.45 a c	Normal	1027±2.92 a c	Negative	43.09±3.01 a c d
Laundry detergent gel 20% group (B3L)	Normal	5.52±1.78	Abnormal (1030)	1029.56±1.51 a b	Negative	49.42±1.43 a b
Laundry detergent gel 40% group (B3H)	Normal	6.08±0.36	Abnormal (1030)	1029.89±1.6۲ a c	Negative	41.67±3.99 a c e
Tea 20% group (B4L)	Normal	6.02±0.33	Normal	1019.44±3.97 b	Negative	43.63±1.09 a b
Tea 40% group (B4H)	Normal	6.03±0.10	Normal	1023.33±1.73 a c	Negative	34.22±3.18 a c f
Hydrogen peroxide 20% group (B5L)	Normal	5.48±1.69	Normal	1019.56±1.13 a b	Negative	45.40±1.51 a b
Hydrogen peroxide 40% group (B5H)	Normal	5.52±0.14	Normal	1022.22±3.80 a c	Negative	34.96±3.23 a c g
Drinking water 20% group (B6L)	Normal	5.97±0.28	Normal	1009.44±3.84 a	Negative	29.76±4.70 a
Drinking water 40% group (B6H)	Normal	5.75±0.26	Normal	1008.56±2.69 a	Negative	18.61±2.09 a h
F		2.446		53.19		182.4
P		0.0098		<0.0001		<0.0001

Number of samples in each group: 9, All results were expressed as mean ± SD. THC–COOH: 11-nor-9-carboxy-D9-tetrahydrocannabinol, ***a****: $p<0.05$ as compared with positive control group;* ***b****: $p<0.05$ as compared with drinking water 20%;* ***c****: $p<0.05$ as compared with drinking water 40%;* ***d****: $p<0.05$ as compared with Carbonated water 20%;* ***e****: $p<0.05$ as compared with laundry detergent gel 20%;* ***f:*** *$p<0.05$ as compared with Tea 20%;* ***g:*** *$p<0.05$ as compared with Hydrogen peroxide 20%;* ***h:*** *$p<0.05$ as compared with Drinking water 20%,* ***F:*** *analysis of variance test,* ***p****: p-value.*

Table (2): Effect of cheating urine positive for tramadol by 20% and 40% of carbonated water, laundry detergent gel, tea, hydrogen peroxide, drinking water on nitrites, creatinine, pH, and specific gravity.

Groups / Parameters	pH by integrity strip Reference range (4.5-8)	pH by pH meter (Mean ±SD)	Specific gravity by integrity strip Reference range (1005-1025)	Specific gravity by refractometer (Mean ±SD)	Nitrites by integrity strip	Creatinine Normal range 20-100 mg/dl (Mean ±SD)
Negative control group (A)	Normal	5.72±0.52	Normal	1016.67±2.12	Negative	66.25±3.14
Positive control group (C1)	Normal	5.88±0.34	Normal	1018.44±1.88	Negative	69.07±5.41
Carbonated water 20% group (C2L)	Normal	5.05±0.43 [a b]	Normal	1031.11±4.51 [a b]	Negative	53.26±2.29 [a b]
Carbonated water 40% group (C2H)	Normal	5.03±0.19 [a c]	normal	1030.78±1.56 [a c]	Negative	45.74±3.81 [a c d]
Laundry detergent gel 20% group (C3L)	Normal	5.72±0.23	Abnormal	1029.89±5.30 [a b]	Negative	53.97±4.74 [a b]
Laundry detergent gel 40% group (C3H)	Normal	5.77±0.42	Abnormal	1029±5.61 [a c]	Negative	42.86±3.64 [a c e]
Tea 20% group (C4L)	Normal	5.73±0.45	Normal	1019.44±1.51 [b]	Negative	49.95±5.23 [a b]
Tea 40% group (C4H)	Normal	5.82±0.44	Normal	1020.33±1.80 [a c]	Negative	39.69±2.28 [a c f]
Hydrogen peroxide 20% group (C5L)	Normal	5.82±0.37	Normal	1021±2.35 [a b]	Negative	50.71±3.03 [a b]
Hydrogen peroxide 40% group (C5H)	Normal	5.48±0.58	Normal	1021.67±2.06 [a c]	Negative	38.56±3.25 [a c g]
Drinking water 20% group (C6L)	Normal	5.73±0.32	Normal	1008.22±3.67 [a]	Negative	30.47±2.19 [a]
Drinking water 40% group (C6H)	Normal	5.61±0.46	Normal	1008.56±3.91 [a]	Negative	15.42±2.11 [a h]
F		4.359		50.16		149.2
P		<0.0001		<0.0001		<0.0001

Number of samples in each group: 9, All results were expressed as mean ± SD. ***a****: p<0.05 as compared with positive control group;* ***b****: p<0.05 as compared with drinking water 20%;* ***c****: p<0.05 as compared with drinking water 40%;* ***d****: p<0.05 as compared with Carbonated water 20%;* ***e****: p<0.05 as compared with laundry detergent gel 20%;* ***f:*** *p<0.05 as compared with Tea 20%;* ***g:*** *p<0.05 as compared with Hydrogen peroxide 20%;* ***h:*** *p<0.05 as compared with Drinking water 20%*
F: *analysis of variance test,* ***p****: p value.*

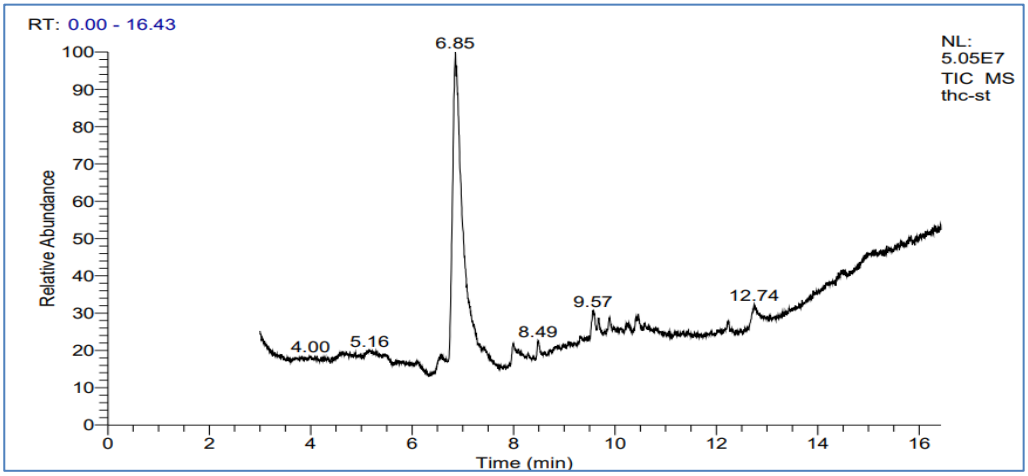

Figure (4): GC-MS chromatogram for THC-COOH analysis in the urine sample.

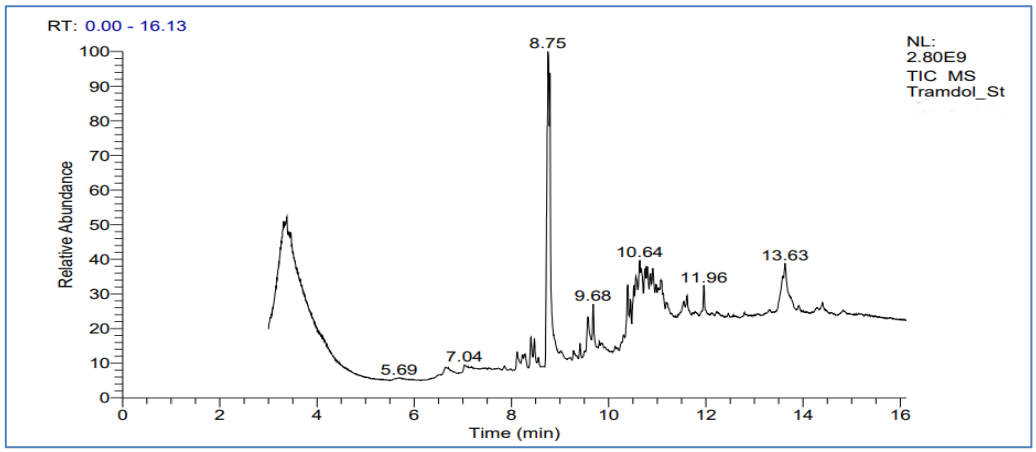

Figure (5): GC-MS chromatogram for tramadol analysis in the urine sample.

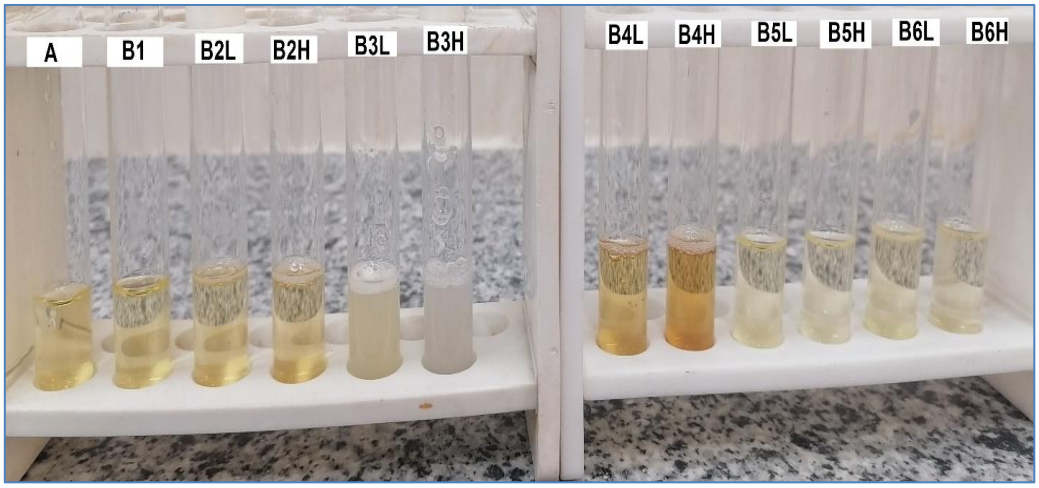

Figure (6): Color and appearance of cheated urine samples tested for THC-COOH. A: negative control group; B1: positive control group; B2L: Carbonated water 20% group; B2H: Carbonated water 40% group; B3L: Laundry detergent gel 20% group; B3H: Laundry detergent gel 40% group; B4L: Tea 20% group; B4H: Tea 40% group; B5L: Hydrogen peroxide 20% group; B5H: Hydrogen peroxide 40% group; B6L: Drinking water 20% group; B6H: Drinking water 40% group.

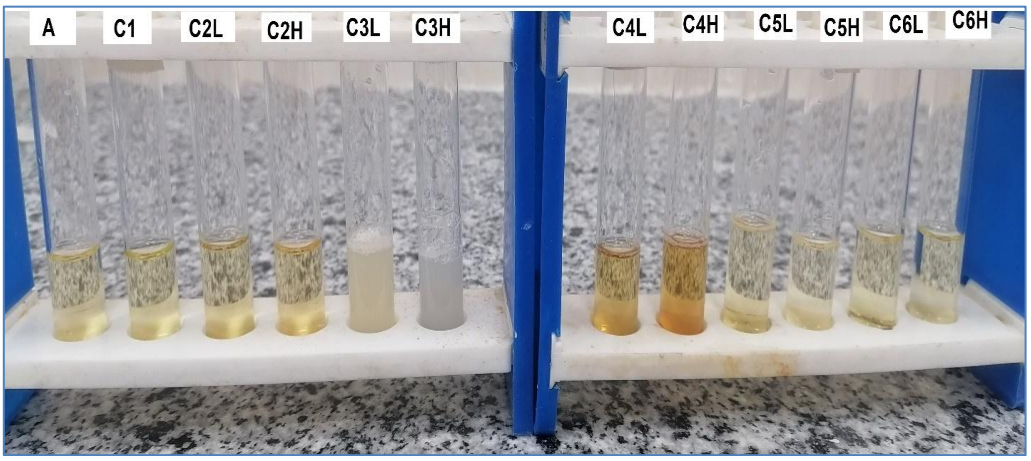

Figure (7): Color and appearance of cheated urine samples tested for tramadol. A: negative control group; C1: positive control group; C2L: Carbonated water 20% group; C2H: Carbonated water 40% group; C3L: Laundry detergent gel 20% group; C3H: Laundry detergent gel 40% group; C4L: Tea 20% group; C4H: Tea 40% group; C5L: Hydrogen peroxide 20% group; C5H: Hydrogen peroxide 40% group; C6L: Drinking water 20% group; C6H: Drinking water 40% group.

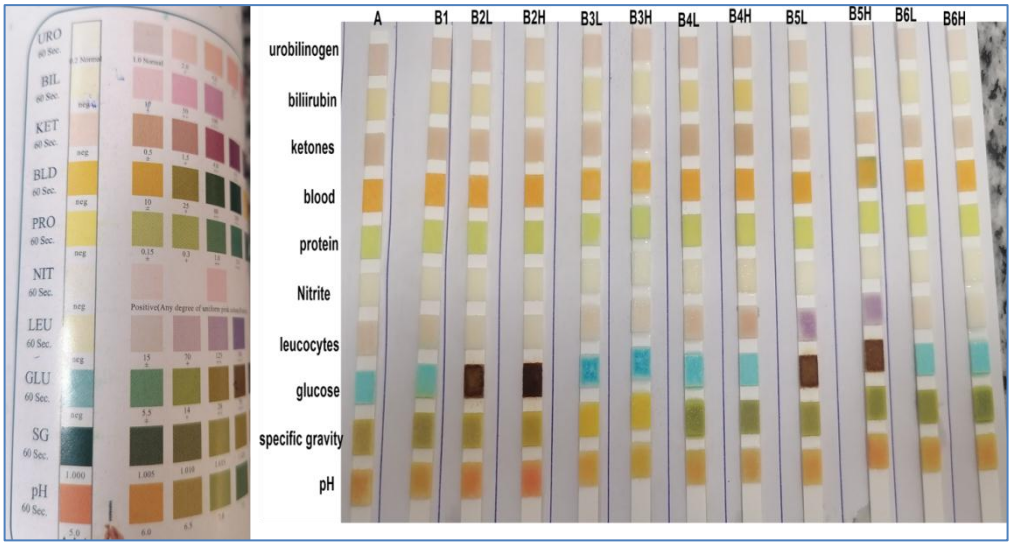

Figure (8): Effect of cheating of urine samples positive for THC-COOH by 20% and 40% of carbonated water, laundry detergent gel, tea, hydrogen peroxide, drinking water on pH, specific gravity, nitrites by integrity strips. A:negative control group; B1: positive control group; B2L: Carbonated water 20% group; B2H: Carbonated water 40% group; B3L: Laundry detergent gel 20% group; B3H: Laundry detergent gel 40% group; B4L: Tea 20% group; B4H: Tea 40% group; B5L: Hydrogen peroxide 20% group; B5H: Hydrogen peroxide 40% group; B6L: Drinking water 20% group; B6H: Drinking water 40% group.

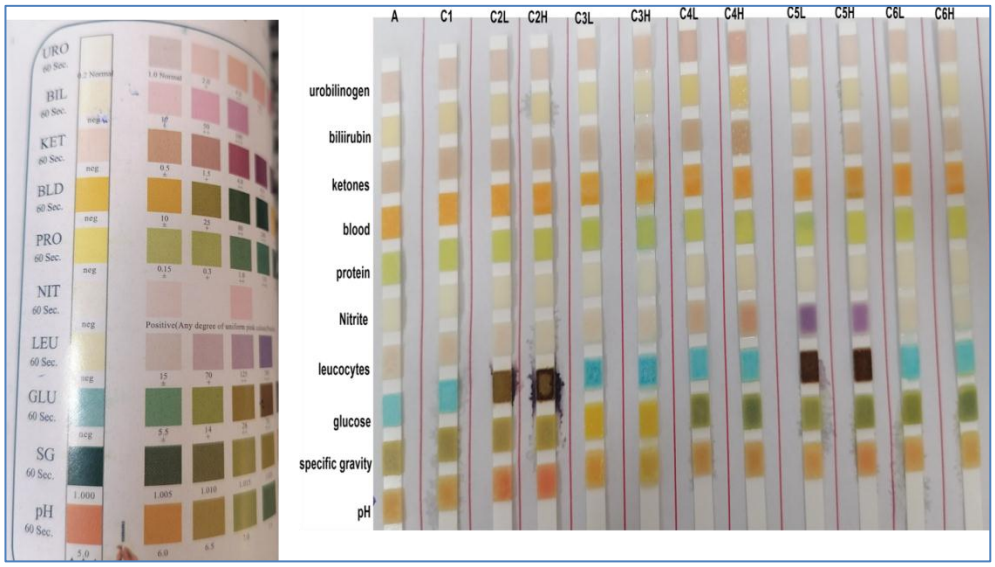

Figure (9): Effect of cheating of urine samples positive for tramadol by 20% and 40% of carbonated water, laundry detergent gel, tea, hydrogen peroxide, drinking water on pH, specific gravity, nitrites by integrity strips. A:negative control group; C1: positive control group; C2L: Carbonated water 20% group; C2H: Carbonated water 40% group; C3L: Laundry detergent gel 20% group; C3H: Laundry detergent gel 40% group; C4L: Tea 20% group; C4H: Tea 40% group; C5L: Hydrogen peroxide 20% group; C5H: Hydrogen peroxide 40% group; C6L: Drinking water 20% group; C6H: Drinking water 40% group.

Rapid screening immunoassay strip

The rapid screening strips displayed false-negative results for THC–COOH in a majority of the adulterated samples. At 20% and 40% concentration carbonated water showed false-negative results in 66.67% and 88.89% of samples, respectively. All samples containing 20% and 40% of laundry detergent gel exhibited false-negative results. Similarly, samples containing tea 20% and 40% showed false-negative results in 77.78% and 100% of samples, respectively. The hydrogen peroxide groups, both 20% and 40% conc., showed false-negative results in 66.67% and 55.56% of samples, respectively. Finally, the drinking water groups, both at 20% and 40%, showed false-negative results in 66.67% and 77.78% of cases, respectively (**Figure 10, Table 3)**.

The rapid screening strips employed to detect the presence of tramadol in adulterated samples yielded positive results in a majority of cases. However, carbonated water at 20% and 40% concentrations showed false-negative results in 22.22% and 33.33% of samples, respectively. Similarly, 11.11% of samples containing laundry detergent gel at 40% concentration showed false-negative results. Samples containing tea at 20% and 40% concentrations showed false-negative results in 33.33% and 77.78% of samples, respectively. The hydrogen peroxide groups, both 20% and 40%, showed false-negative results in 22.22% and 66.67% of samples, respectively. Finally, the drinking water groups, both 20% and 40%, showed false-negative results in 33.33% and 55.56% of cases, respectively (**Figure 11, Table 4)**.

DiaSystem automated immunoassay analysis

Immunoassay analysis for THC–COOH

Effect of carbonated water

The positive urine samples for THC–COOH adulterated with 20% and 40% conc. of carbonated water showed non-significant alterations (**B2L=86.3 ng/mL, B2H=86 ng/mL**) compared to the levels of the positive control group **(B1=86.7 ng/mL)** and the cut-off level = 50 ng/mL ($p>0.05$) **(Figure 12)**.

Effect of laundry detergent gel

Addition of 20% and 40% conc. of laundry detergent gel resulted in a significant reduction of THC–COOH levels **(B3L=16.8 ng/mL, B3H=3.7 ng/mL)** when compared to the positive control group **(B1=86.7 ng/mL)** ($p<0.05$) and non-significant differences with the negative control group **(A=17.7 ng/mL)** ($p>0.05$) **(Figure 12)**.

Effect of tea

The immunoassay screening of the urine samples adulterated with 20% and 40% conc. of tea showed significantly lower THC–COOH levels **(B4L=63.3 ng/mL, B4H=10.4 ng/mL)** when compared to the positive control group **(B1=86.7 ng/mL)** ($p<0.05$). Interestingly, the tea at 40% conc. group **(B4H=10.4 ng/mL)** displayed significant differences when compared with tea at 20% conc. group **(B4L=63.3 ng/mL)** ($p<0.05$) and its results were lower than the negative control group **(A=17.7 ng/mL)** and the cut-off level of THC–COOH **(Figure 12)**.

Effect of hydrogen peroxide

Non-significant changes in THC–COOH levels were observed in hydrogen peroxide at 20% and 40% conc. groups **(B5L=84.9 ng/mL, B5H=79 ng/mL)** when compared with the positive control group **(B1=86.7 ng/mL)** ($p>0.05$) **(Figure 12)**.

Effect of drinking water

Non-significant changes in THC–COOH levels were observed in both drinking water 20% and 40% conc. groups **(B6L=85.4 ng/mL, B6H=84.8 ng/mL)** when compared with the positive control group **(B1=86.7 ng/mL)** ($p>0.05$) **(Figure 12)**.

Immunoassay analysis for tramadol

Effect of carbonated water

Non-significant alterations in tramadol levels were detected with urine samples at 20% and 40% conc. of carbonated water (**C2L=328 ng/mL, C2H=326.3 ng/mL**) compared with the positive control group **(C1=342.7 ng/mL)** ($p>0.05$), where the tramadol the cut-off level=200 ng/mL **(Figure 13)**.

Effect of laundry detergent gel

Addition of 20% and 40% conc. of laundry detergent gel to tramadol urine samples resulted in a significant reduction of tramadol levels **(C3L=112.4 ng/mL, C3H=22.2 ng/mL)** when compared with the positive control group **(C1=342.7 ng/mL)** ($p<0.05$). Both concentrations displayed non-significant differences when compared with the negative

control group **(A= 61.5 ng/mL)** (p>0.05) **(Figure 13)**.

Effect of tea

The immunoassay screening of the urine samples adulterated with 20% tea showed non-significant differences in tramadol levels **(C4L=307.6 ng/mL)** compared to the positive control group **(C1=342.7 ng/mL)** (p<0.05). While the 40% conc. of tea groups **(C4H=149.1 ng/mL)**, which were below the cut-off level, displayed a significantly lower level of tramadol when compared with the positive control group **(C1=342.7 ng/mL)** (p<0.05)**.** The 40% conc. of tea group **(C4H=149.1 ng/mL**) showed a significant difference when compared to 20% conc. of tea group **(Figure 13)**.

Effect of hydrogen peroxide

Non-significant alterations in tramadol levels were noticed in both hydrogen peroxide 20% and 40% conc. groups **(C5L=334.6 ng/mL, C5H=337.5 ng/mL)** when compared with the positive control group **(C1=342.7 ng/mL)**, (p>0.05) **(Figure 13)**.

Effect of drinking water

Non-significant changes in tramadol levels were observed in both drinking water at 20% and 40% conc. groups **(C6L=334.7 ng/mL, C6H=327 ng/mL)** when compared with the positive control group **(C1=342.7 ng/mL)** (p>0.05) **(Figure 13)**.

Comparison of the diluted samples with adulterated samples

When comparing the pH results at 20% and 40% conc. of carbonated water (**B2L, C2L, B2H, C2H; respectively**), laundry detergent gel (**B3L, C3L, B3H, C3H; respectively**), tea (**B4L, C4L, B4H, C4H; respectively**), hydrogen peroxide (**B5L, C5L, B5H, C5H; respectively**) of both THC–COOH and tramadol adulterated groups with those of drinking water groups (**B6L, C6L, B6H, C6H; respectively**), no significant changes in pH values were observed (p>0.05) (**Tables 1, 2**).

Additionally, when comparing the SG and urine creatinine results at 20% and 40% conc. of drinking water groups (**B6L, C6L, B6H, C6H; respectively**) with those of carbonated water (**B2L, C2L, B2H, C2H; respectively**), laundry detergent gel groups (**B3L, C3L, B3H, C3H; respectively**), tea groups (**B4L, C4L, B4H, C4H; respectively**), and hydrogen peroxide groups (**B5L, C5, B5H, C5H; respectively**), significantly lower SG and creatinine values was observed in drinking water groups (p<0.05) **(Tables 1, 2)**. However, it's important to note that the urine creatinine levels remained within the normal reference range of 20-100 mg/dL.

Regarding DiaSystem automated immunoassay analysis, When comparing the results at 20% and 40% concentrations of carbonated water (**B2L, C2L, B2H, C2H; respectively**) and hydrogen peroxide (**B5L, C5L, B5H, C5H; respectively**) of both THC–COOH and tramadol adulterated groups with those of drinking water groups (**B6L, C6L, B6H, C6H; respectively**), no significant changes were observed (p>0.05) **(Figures 12, 13)**.

However the results of 20% and 40% conc. of laundry detergent gel (**B3L, C3L, B3H, C3H; respectively**) and tea (**B4L, C4L, B4H, C4H; respectively**) showed significantly lower levels of THC–COOH and tramadol when compared with those of drinking water groups (**B6L, C6L, B6H, C6H; respectively**) (p<0.05) **(Figures 12, 13)**.

Comparison of immunoassay systems results and GCMS

Tables (3) and **(4)** compared the results of GC-MS detection of adulterated positive samples for THC–COOH and tramadol with those of rapid immunoassay screening strip and DiaSystem automated immunoassay. The rapid immunoassay screening strip displayed the percentage of positive and negative results for THC–COOH and tramadol in adulterated and diluted urine samples. The GC-MS results confirmed the results obtained from the DiaSystem automated immunoassay, which were the positive results for THC–COOH and tramadol in most adulterated and diluted samples at both concentrations.

Furthermore, the GC-MS results confirmed positive results for THC–COOH in only 44.4% of laundry detergent gel and 20% conc. samples, 22.2% of laundry detergent gel 40% conc. samples, and 66.7% of tea 40% conc. samples. While the GC-MS results confirmed positive results for tramadol only 55.6% of laundry detergent gel 20% conc. samples,

33.3% of laundry detergent gel 40% conc. samples, and 77.8% of tea 40% conc. samples.

Validity and accuracy analysis of immunoassay systems and GCMS

Tables (5) and **(6)** compared the validity and accuracy results of rapid immunoassay screening strips and DiaSystem automated immunoassay with the results of GC-MS. We observed that rapid immunoassay strips exhibited a low sensitivity of 27.27% and an accuracy rate of 33.33% for detecting THC–COOH, along with 67.68% and 70.37%, respectively, for tramadol detection after adulterating and diluting urine samples.

On the other hand, the DiaSystem automated immunoassay demonstrated a higher sensitivity of 72.73% and an accuracy rate of 75% for detecting THC–COOH, as well as 75.76% and 77.78%, respectively, for tramadol detection after adulterating urine samples. Meanwhile, GC-MS showed a sensitivity of 84.85% and an accuracy of 86.11% for detecting THC–COOH and 87.88% and 88.89% respectively for tramadol detection after adulterating and diluting urine samples.

Table (3): Effect of cheating urine positive for THC-COOH by 20% and 40% of carbonated water, laundry detergent gel, tea, hydrogen peroxide, and drinking water on results of rapid immunoassay screening dipstick strip, DiaSystem automated immunoassay, and GC-MS.

Groups / Method	Rapid immunoassay screening strip results				DiaSystem automated immunoassay results				GC-MS results			
	Negative		Positive		Negative		Positive		Negative		Positive	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Negative control group (A)	9	100	0	0	9	100	0	0	9	100	0	0
Positive control group (B1)	0	0	9	100	0	0	9	100	0	0	9	100
Carbonated water 20% group (B2L)	6	66.67	3	33.33	0	0	9	100	0	0	9	100
Carbonated water 40% group (B2H)	8	88.89	1	11.11	0	0	9	100	0	0	9	100
Laundry detergent gel 20% group (B3L)	9	100	0	0	9	100	0	0	5	55.56	4	44.44
Laundry detergent gel 40% group (B3H)	9	100	0	0	9	100	0	0	7	77.78	2	22.22
Tea 20% group (B4L)	7	77.78	2	22.22	0	0	9	100	0	0	9	100
Tea 40% group (B4H)	9	100	0	0	9	100	0	0	3	33.33	6	66.67
Hydrogen peroxide 20% group (B5L)	6	66.67	3	33.33	0	0	9	100	0	0	9	100
Hydrogen peroxide 40% group (B5H)	5	55.56	4	44.44	0	0	9	100	0	0	9	100
Drinking water 20% group (B6L)	6	66.67	3	33.33	0	0	9	100	0	0	9	100
Drinking water 40% group (B6H)	7	77.78	2	22.22	0	0	9	100	0	0	9	100

N: number. % percent. GC-MS: gas chromatograph mass spectrometry. THC–COOH: 11-nor-9-carboxy-D9-tetrahydrocannabinol

Table (4): Effect of cheating urine positive for tramadol by 20% and 40% of carbonated water, laundry detergent gel, tea, hydrogen peroxide, and drinking water on results of rapid immunoassay screening dipstick strip, DiaSystem automated immunoassay, and GC-MS.

Groups / Method	Rapid immunoassay screening strip results				DiaSystem automated immunoassay results				GC-MS results			
	Negative		Positive		Negative		Positive		Negative		Positive	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Negative control group (A)	9	100	0	0	9	100	0	0	9	100	0	0
Positive control group (C1)	0	0	9	100	0	0	9	100	0	0	9	100
Carbonated water 20% group (C2L)	2	22.22	7	77.78	0	0	9	100	0	0	9	100
Carbonated water 40% group (C2H)	3	33.33	6	66.67	0	0	9	100	0	0	9	100
Laundry detergent gel 20% group (C3L)	0	0	9	100	8	88.89	1	11.11	4	44.44	5	55.56
Laundry detergent gel 40% group (C3H)	1	11.11	8	88.89	9	100	0	0	6	66.67	3	33.33
Tea 20% group (C4L)	3	33.33	6	66.67	0	0	9	100	0	0	9	100
Tea 40% group (C4H)	7	77.78	2	22.22	7	77.78	2	22.22	2	22.22	7	77.78
Hydrogen peroxide 20% group (C5L)	2	22.22	7	77.78	0	0	9	100	0	0	9	100
Hydrogen peroxide 40% group (C5H)	6	66.67	3	33.33	0	0	9	100	0	0	9	100
Drinking water 20% group (C6L)	3	33.33	6	66.67	0	0	9	100	0	0	9	100
Drinking water 40% group (C6H)	5	55.56	4	44.44	0	0	9	100	0	0	9	100

N: number. % percent. GC-MS: gas chromatograph mass spectrometry.

Table (5): Validity and accuracy of rapid immunoassay screening dipstick strip, DiaSystem automated immunoassay, and GC-MS in detecting THC-COOH after adulterating urine samples.

	Sensitivity	Specificity	PPV	NPV	Accuracy
Rapid immunoassay screening strip	27.27%	100.00%	100.00%	11.11%	33.33%
DiaSystem automated immunoassay	72.73%	100.00%	100.00%	25.00%	75.00%
GC-MS	84.85%	100.00%	100.00%	37.50%	86.11%

PPV: positive predictive value, NPV: negative predictive value. GC-MS: gas chromatograph mass spectrometry. THC–COOH: 11-nor-9-carboxy-D9-tetrahydrocannabinol

Table (6): Validity and accuracy of rapid immunoassay screening dipstick strip, DiaSystem automated immunoassay, and GC-MS in detecting tramadol after adulterating urine samples.

	Sensitivity	Specificity	PPV	NPV	Accuracy
Rapid immunoassay screening strip	67.68%	100.00%	100.00%	21.95%	70.37%
DiaSystem automated immunoassay	75.76%	100.00%	100.00%	27.27%	77.78%
GC-MS	87.88%	100.00%	100.00%	42.86%	88.89%

PPV: positive predictive value, NPV: negative predictive value. GC-MS: gas chromatograph mass spectrometry.

Figure (10): Effect of cheating of urine samples positive for THC-COOH by 20% and 40% of carbonated water, laundry detergent gel, tea, hydrogen peroxide, and drinking water on drug screening immunoassay strips. A:Negative control group; B1: Positive control group; B2L: Carbonated water 20% group; B2H: Carbonated water 40% group; B3L: Laundry detergent gel 20% group; B3H: Laundry detergent gel 40% group; B4L: Tea 20% group; B4H: Tea 40% group; B5L: Hydrogen peroxide 20% group; B5H: Hydrogen peroxide 40% group; B6L: Drinking water 20% group; B6H: Drinking water 40% group.

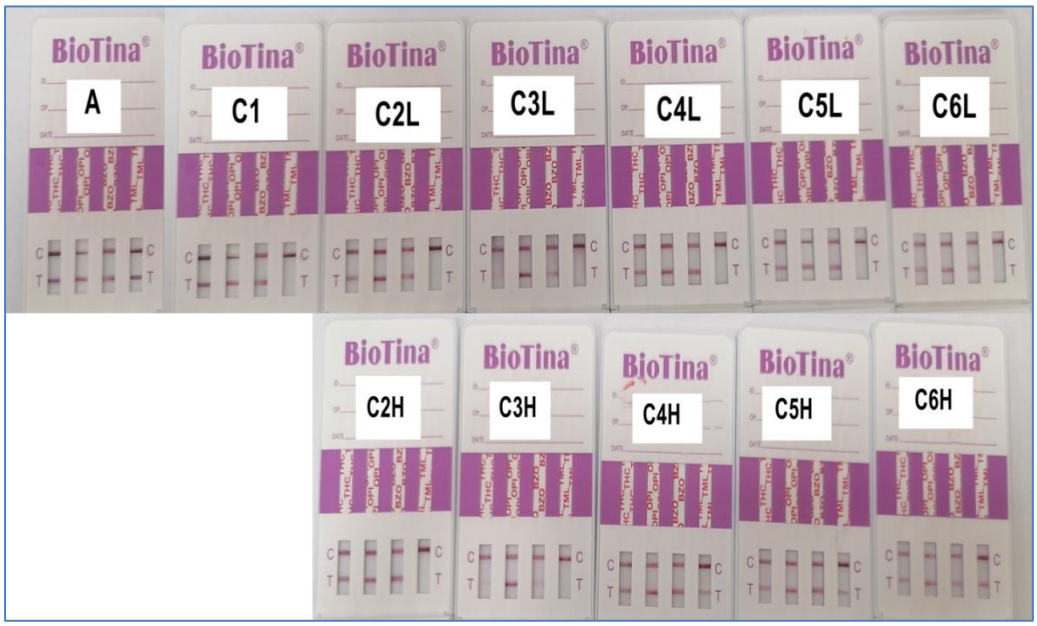

Figure (11): Effect of cheating of urine samples positive for tramadol by 20% and 40% of carbonated water, laundry detergent gel, tea, hydrogen peroxide, and drinking water on drug screening immunoassay strips. A: Negative control group; C1: Positive control group; C2L: Carbonated water 20% group; C2H: Carbonated water 40% group; C3L: Laundry detergent gel 20% group; C3H: Laundry detergent gel 40% group; C4L: Tea 20% group; C4H: Tea 40% group; C5L: Hydrogen peroxide 20% group; C5H: Hydrogen peroxide 40% group; C6L: Drinking water 20% group; C6H: Drinking water 40% group.

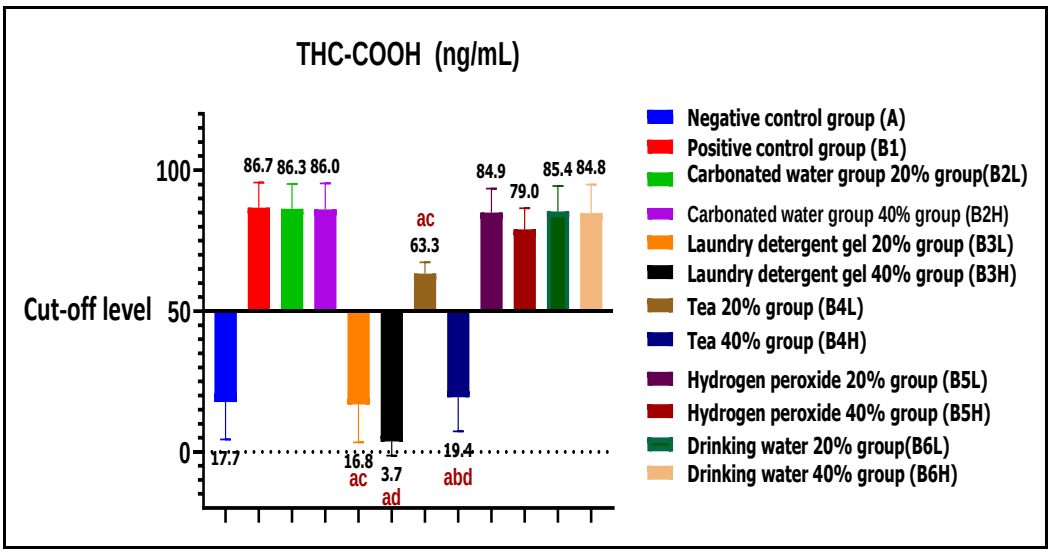

Figure (12): Effect of cheating urine positive for THC-COOH by 20% and 40% of carbonated water, laundry detergent gel, tea, hydrogen peroxide, and drinking water on results of DiaSystem automated immunoassay. a: p<0.05 as compared to the positive control group; b: p<0.05 as compared to tea 20% group; c: p<0.05 as compared to drinking water 20% group; d: p<0.05 as compared to drinking water 40% group.

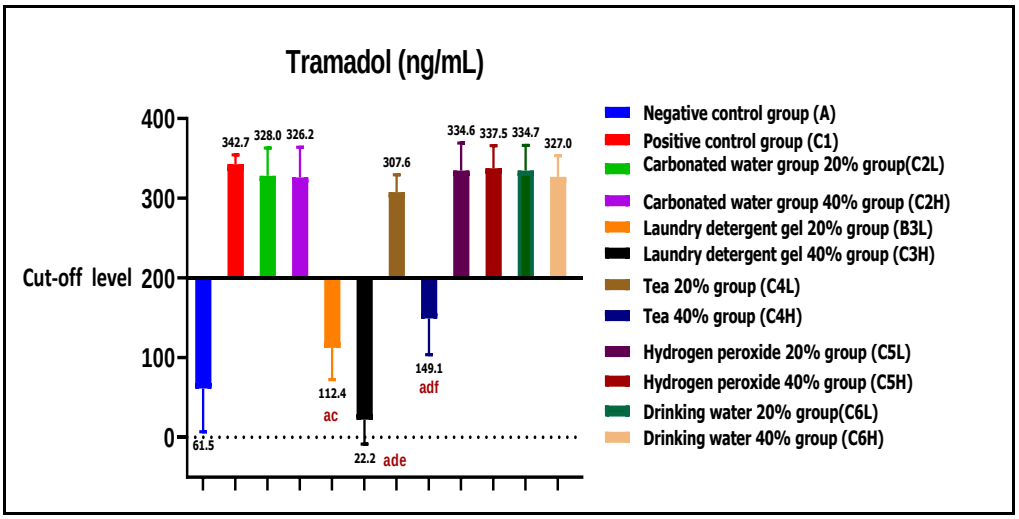

Figure (13): Effect of cheating urine positive for tramadol by 20% and 40% of carbonated water, laundry detergent gel, tea, hydrogen peroxide, and drinking water on results of DiaSystem automated immunoassay. a: p<0.05 as compared to the positive control group; c: p<0.05 as compared to drinking water 20% group; d: p<0.05 as compared to drinking water 40% group; e: as compared to laundry detergent gel 20% group; f: p<0.05 as compared to tea 20% group.

DISCUSSION

Drug testing plays a crucial role in various aspects of healthcare. Urine drug testing is a commonly employed method for monitoring medication adherence and identifying illicit drug use ***(Jenkins and Goldberger, 2002)***. However, cheating of urine samples has become prevalent among drug users, particularly, to evade routine workplace screenings. The products used for urine adulteration by drug abusers are various, depending on their availability and their degree of deception. The products used in this study were from easily obtained market and household products.

The current study examined two methods of cheating on positive urine samples. The first is the adulteration method with (carbonated water, tea, and laundry detergent gel, hydrogen peroxide) in low concentration 20% and higher concentration 40% and the second is the dilution method with drinking water at both tempering concentrations. Both concentrations of other adulterants, such as vinegar, bleach, Visine eye drops, and liquid Drano, have sparked considerable debate in previous studies due to their potential to yield false negative or positive results ***(Abdel Ati et al., 2020; Elsayed et al., 2021)***. Therefore, we selected both concentrations for further investigation.

The current study employed different methods for the detection of cheating-positive urine samples, including urine examination of physical property changes. The urine samples were assessed for integrity using integrity strips, and the results were compared to those obtained from a pH meter and refractometer. The chemical analysis was utilized to detect creatinine levels in urine samples. Finally, the urine samples were examined by DiaSystem automated immunoassay, which is considered a screening and semiquantitative method and were further confirmed through GC-MS.

Regarding the physical results of the current study, there was a change in the odor of urine samples adulterated with carbonated water, tea, and laundry detergent gel in both concentrations, and no change was observed in urine samples adulterated with hydrogen peroxide, and diluted with drinking water. The urine samples adulterated with laundry detergent gel, tea, and hydrogen peroxide and diluted with drinking water showed color changes, particularly at 40% concentration of both THC–COOH and tramadol-positive urine samples. Meanwhile, no color change was observed in carbonated water. Laundry detergent gel showed a frothy appearance at 40% concentration.

Our results are consistent with previous studies concerning cheating of urine samples among drug abusers ***(Abdel Ati et al., 2020; Aydoğdu and Akgür, 2021)***, who reported alteration of color and appearance of urine samples adulterated with different household products such as vinegar, bleach, drain opener, eye drop, and liquid hand soap. ***Mikkelsen and Ash (1988)*** had reported the darkening of urine samples adulterated with Golden-seal tea. The color changes in urine samples adulterated with hydrogen peroxide were attributed to heptavalent chromium reduction ***(Wong, 2002)***.

The current study, using integrity strips, revealed that the studied-adulterated and diluted urine samples at both 20% and 40% concentrations showed normal pH, specific gravity (SG), and negative nitrites when compared to the positive control groups. The exceptions were laundry detergent gel, which exhibited higher SG, and carbonated water, which reduced the pH and elevated SG.

These findings were corroborated by pH meter and refractometer measurements, which indicated no significant alterations in the pH and SG values of the studied-adulterated and diluted samples, except for carbonated water (which showed reduced pH and elevated SG), tea (which showed an elevated SG at a 40% concentration), laundry detergent gel, hydrogen peroxide (both resulting in an elevated SG) and drinking water (showed lower SG). Notably, the pH values remained within the normal range.

Despite the urine creatinine levels in the studied-adulterated and diluted urine samples at both concentrations remaining within the normal reference range, they exhibited a significant reduction when compared with the positive control group. Moreover, the 40% concentration of the studied-adulterated and diluted samples exhibited a significant reduction of creatinine compared with the

20% concentration. Interestingly, the urine samples adulterated with 40% tea displayed a significant reduction in creatinine compared with the 20% tea-adulterated samples.

To the best of our knowledge, this study represents the initial investigation of the impact of carbonated water and tea adulteration on urine samples that had tested positive for THC–COOH and tramadol. Carbonated water (fayrouz) was chosen for its color characteristics, resembling those of a urine sample. The slightly acidic nature of carbonated water might be the cause of the pH reduction in adulterated urine samples. A previous article reported that carbonated water is water pressurized with carbon dioxide gas and typically has a pH in the range of 3–4 ***(Spritzler, 2019)***.

Furthermore, the primary chemical constituents found in tea include eight catechins, caffeine, theaflavin, gallic acid, chlorogenic acid, ellagic acid, and kaempferol-3-G ***(Zhao et al., 2019)***. These compounds can exhibit varying levels of tea acidity despite the results showing no change in pH values.

Previous studies on products containing acids, such as vinegar, have reported decreased pH levels, which can affect the binding, reaction times, and drug solubility ***(Olivieri et al., 2018)***. Another study noted that the addition of 40% vinegar to urine samples led to a reduction in both pH and creatinine levels below their typical range, while specific gravity increased. When 20% vinegar was added, pH levels decreased and specific gravity increased ***(Abdel Ati et al., 2020)***. These findings are consistent with the results obtained from the products used in our study.

Our results are consistent with those of ***Abdel Ati et al. (2020)***, who found that the addition of 20% liquid hand soap to tramadol-positive urine samples increased SG, pH, and reduced creatinine levels. However, the elevated pH levels are inconsistent with our results.

Furthermore, ***Aydoğdu and Akgür (2021)***, documented lower sensitivity of integrity strips when compared with a pH meter. The contrasting findings of integrity strips and the refractometer can be explained by the fact that the refractometer quantifies the refractive index, which is associated with the overall quantity of dissolved substances in urine. Substances with high molecular weight, such as glucose, protein, or radiographic contrast agents, will exert a more pronounced influence on SG. On the other hand, reagent strips gauge ionic strength and remain unaffected by the presence of protein, glucose, or contrast agents ***(Mina et al., 2021)***. Utilizing integrity strips, particularly in comprehensive drug urine screening, presents challenges associated with timeliness, individual perspectives, and costs ***(Olivieri et al., 2018)***.

A number of oxidizing adulterants have effectively been employed to manipulate the physical and chemical characteristics of urine samples, leading to inaccurate negative test results ***(Thevis et al., 2008)***.

Dilution involves mixing regular water with urine in an attempt to reduce the concentration of drugs, allowing the sample to meet the drug test's cut-off criteria ***(Cone et al., 1998)***. To identify the potential dilution of urine, the most effective tests to conduct are for creatinine levels and specific gravity ***(Riahi-Zanjani, 2014)***. Despite dilution is likely one of the most commonly used techniques for manipulating urine, however, the presence of any notable departure from the anticipated results in specimen integrity or creatinine tests could suggest potential tampering with the urine ***(Fu, 2016)***.

In the context of the rapid screening immunoassay strips, the current study yielded false-negative results for THC–COOH in the majority of higher concentration (40%) of adulterated and diluted urine samples. The percentage of positive results was relatively low, ranging between 11.11% to 44.44% in all adulterated and diluted samples. Interestingly, the urine sample adulterated with laundry detergent gel at both concentrations (20% and 40%) displayed false-negative results.

Nevertheless, positive results for tramadol were evident in the majority of both adulterated and diluted samples at both concentrations. The highest incidence of false-negative results occurred at the 40% concentration for tea, hydrogen peroxide, and drinking water. Notably, laundry detergent gel at 20% and 40% concentrations exhibited

positive results, with percentages of 100% and 88.9%, respectively and only 11.11% showed false-negative results.

Our results are consistent with ***Rajsic et al. (2020)***, who reported that the cannabinoid displayed higher vulnerability to sample adulteration compared to other drugs. They suggested that the acids proved to be a strong adulterant in the test strip system under investigation, and frequently resulting in negative screening outcomes.

In this study, the majority of higher concentrations (40%) of acid-containing tea and hydrogen peroxide were effective adulterants with tramadol. As well as the dilution with 40% conc. of drinking water also showed this effective potential. ***Mizrak (2019)*** suggested that peroxides in adulterated urine effectively masked opiate presence on screening or confirmatory assay.

Regarding the DiaSystem automated immunoassay method, the current study yielded false-negative results for both THC–COOH and tramadol at concentrations of 20% and 40% of adulterated urine samples with laundry detergent gel when compared to positive and negative control groups. Even though their levels were lower than the cut-off levels of THC–COOH and tramadol.

Similarly, the 40% conc. of tea adulterated urine samples of THC–COOH and tramadol yielded false-negative results when compared to only the positive control group and their levels were lower than the cut-off level of THC–COOH and tramadol. Furthermore, the 40% conc. of laundry detergent gel and tea showed more potential for adulteration.

In the current study, THC–COOH negative results obtained using the rapid immunoassay strip turned positive when tested with the automated immunoassay. Notably, both methods had the same cut-off value of 50 ng/mL. Conversely, positive results for tramadol using the automated immunoassay were obtained at a higher cut-off value (200 ng/mL) compared to the rapid immunoassay strip (100 ng/mL), which yielded negative results for tramadol.

Concerning the false negative results detected by the automated immunoassay method, our results align with studies conducted on urine samples adulterated with hand soap and dish detergents. ***Wu (2003); Huppertz et al. (2018)*** and ***Elsayed et al. (2021)*** reported false negative results of urine samples that tested positive for multiple drugs including THC–COOH and tramadol even in low concentrations by using screening radio-immunoassay or ELISA methods.

It was suggested that these detergents interfered with urine samples during preparation ***(Huppertz et al., 2018)***.

Dasgupta (2010) reported that soap can potentially modify the pH levels in urine samples and disrupt the binding of drugs in immunoassays. Laundry detergent gel is composed of surfactants and alkaline builders that lead to false negative results for multiple different drugs ***(Schwarzhoff and Cody, 1993)***.

No review in the literature discussing the effects of tea on any immunoassay technique. Thus, the effect of tea adulteration on urine samples couldn't be explained clearly. However, the acidic composition of tea as reported earlier ***(Zhao et al., 2019)*** might reduce the drug level in urine samples. It was reported that elevated acidity caused drug level reduction in urine samples ***(Thabet et al., 2016)*** or altering the specified drugs to form distinct chemical compounds, resulting in the interference of immunoassays ***(Wu et al., 1999)***.

The current study showed that the DiaSystem automated immunoassay method yielded positive results for THC–COOH and tramadol in carbonated water and hydrogen peroxide adulterated urine samples as well as the diluted urine samples at both 20% and 40% concentrations.

Inconsistent with our results ***Fu (2016)*** recorded that the false negative results for THC–COOH and opiates could be generated in hydrogen peroxide adulterated urine samples using immunoassay at levels ranging from 125% to 150% of the cut-off values. Our study recorded the positive results of hydrogen peroxide adulterated urine samples nearly equivalent to 158% and 167% of the cut-off levels of THC–COOH and tramadol; respectively. It may be attributed to the fact that, in order to minimize the occurrence of false-negative results, screening methods typically employ higher detection cut-off

levels compared to testing methods ***(Jaffee et al., 2007)***.

It was reported that the tampering of urine samples with water may potentially diminish the quantity of sample antigens accessible for competitive binding. This leads to a reduction of metabolite concentration below the designated cut-off thresholds. Simple dilution has also been demonstrated to produce false negative responses on enzyme immunoassay instruments ***(George and Braithwaite, 1995; Pham et al., 2013)***.

However, our study reported positive results for THC–COOH and tramadol in diluted urine samples at lower and higher concentrations nearly equivalent to 170% and 163.5 % of the cut-off levels of THC–COOH and tramadol; respectively.

No review in the literature discussing the effects of carbonated water on any immunoassay technique. However, our study reported positive results for THC–COOH and tramadol in adulterated urine samples with carbonated water at both lower and higher concentrations nearly equivalent to 172% and 164 of the cut-off levels of THC–COOH and tramadol; respectively. Although, the significant fluctuations in the pH of urine media and ionic strength can be detrimental to the interaction between antigens and antibodies causing false negative results ***(Riahi-Zanjani, 2014)***. However, it is reversed by these new results.

The capability of drug screening methods is variable in the detection of false negative and positive results, it depends on multiple factors such as the used kits and programming provided, high drug test cut-off level ***(Alwaeel et al., 2022)***, the carry-over needle contamination of the previous sample, and very low cut-off *values* ***(Kirschbaum et al., 2011)***. For example, cloned enzyme donor immunoassays (CEDIA) were used for the identification of chemical adulterants such as acids, alkalis, oxidizing agents, and detergents. This immunoassay effectively identified acids and alkalis. However, while the CEDIA test successfully detected higher concentrations of adulterants, it exhibited some limitations for samples with low adulterant concentrations ***(Matriciani et al., 2018)***.

Other studies have demonstrated that adulterants, even at low concentrations, can lead to false-negative ELISA results ***(Olivieri et al., 2018)***.

In the current study, when comparing the integrity results of THC–COOH and tramadol positive samples diluted with drinking water at both conc. with those of adulterant containing positive samples, we observed non-significant changes in pH values and lower levels of SG and creatinine levels. Furthermore, there were non-significant changes in the DiaSystem automated immunoassay outcomes when comparing the results THC–COOH and tramadol positive samples diluted with drinking water at both conc. with those of adulterants containing positive samples. This indicates that manipulating positive urine samples for THC–COOH and tramadol, either through household product adulteration or dilution via drinking water, yields similar results.

The exception was noted with adulterants that produced false negative results (20% and 40% concentrations of laundry detergent gel and 40% concentration of tea samples), where the levels of THC–COOH and tramadol were lower than those observed in diluted samples.

Regarding the confirmatory results of GC-MS, our results revealed that the GC-MS detected the positive results for THC–COOH and tramadol in most of adulterated and diluted samples. Interestingly, the false negative results for THC–COOH and tramadol by GC-MS were detected with 20% and 40% conc. of laundry detergent gel and 40% conc. of tea samples.

Upon comparing number and percentage between immunoassay systems with GC-MS, our results demonstrated the effectiveness of the DiaSystem automated immunoassay in detecting of the majority of positive urine samples that were adulterated and diluted with the specified products in our study. Regarding sensitivity and accuracy, DiaSystem automated immunoassay showed its efficiency in detecting positive abused samples in conditions of adulteration and dilution better than rapid immunoassay, but still less than GC-MS.

The ability of GC-MS to produce false negative results was detected earlier and was

attributed to various causes. Such as elevated concentrations of interfering drug, competition of interfering drug with targeted drug for the derivatization reagent, co-elution of interfering drug with targeted drug, and lack of ionization efficiency of the target agent ***(Wu, 1995)***.

The efforts to tamper with positive urine samples can impact the screening results, the confirmation analysis, or both ***(Fu, 2016)***. Many approaches to manipulate or adulterate samples were discussed, one approach involves subverting the limits of detection or cut-offs set for the screening or confirmation test. Achieving this objective may involve diluting the urine sample that can result in true negative outcomes. Another approach involves interfering with the analysis of the specimens by obstructing the analytical methods. For instance, the addition of detergents to the urine sample can affect both extractions and immunoassay detection through disruption of the chromatographic system used in confirmation analysis. These approaches can lead to false-negative screening results ***(Wissenbach et al., 2023)***.

CONCLUSION

The current study identified several issues. Firstly, the DiaSystem automated immunoassay provided acceptable results for drug screening of adulterated and diluted urine samples that tested positive for THC–COOH and tramadol without requiring the precautions, specimen preparation, and costs typically associated with GC-MS.

Secondly, among the adulterants used in our study, both lower and higher concentrations of laundry detergent gel, as well as the higher concentration of the tea, can produce false negative results using both DiaSystem automated immunoassay and GC-MS.

Additionally, the adulterated positive urine samples for THC–COOH are more susceptible to producing false negative results than tramadol using rapid screening immunoassay strips. Finally, the integrity strips are inadequate for detecting adulteration with the used products.

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Authors' Contributions

All authors contributed equally to the collection of data, data analysis, and writing of the present study. In addition, the article was revised by the corresponding author. All authors contributed to and have approved the final manuscript.

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