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Papain: a Novel Urine Adulterant.

David Burrows
East Tennessee State University

Papain, A Novel Urine Adulterant

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by
David Lewis Burrows
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Kenneth E. Ferslew, Ph.D., Chair
Peter J. Rice, Ph.D.
Michelle M. Duffourc, Ph.D.
Brunhilde Tober-Meyer, D.V.M., M.S.
Richard M. Kostrzewa, Ph.D.
Brian P. Rowe, Ph.D.

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ABSTRACT

Papain, A Novel Urine Adulterant

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The estimated number of employees in the United Stated screened annually for illicit drugs is approximately 20 million, with marijuana being the most frequently abused drug. Urine adulterants provide an opportunity for illicit drug users to obtain a false negative result on commonly used primary drug screening methods such as the Fluorescence Polarized Immunoassay (FPIA) technique. Typical chemical adulterants such as nitrites are easily detected or render the urine specimen invalid as defined in the proposed federal guidelines for specimen validity testing based on creatinine, specific gravity, and pH. Papain is a cysteine protease with intrinsic ester hydrolysis capability. The primary metabolite of the psychoactive chemical in marijuana, 11-norcarboxy-delta-9-tetrahydrocannibinol (THC-COOH), was assayed by FPIA in concentrations ranging from 25 to 500 ng/mL, at pH values ranging from 4.5 to 8, over the course of 3 days with papain concentrations ranging from 0 to 10 mg/mL. FPIA analysis of other frequently abused drugs: amphetamines, barbiturates, benzodiazepines, cocaine, opiates, and phencyclidine, along with gas chromatography/mass spectrometry (GC/MS) of THC-COOH and high pressure liquid chromatography/ultraviolet detection (HPLC/UV) of nordiazepam was performed in order to determine if the mechanism of urine adulteration by papain was analyte specific. Control and adulterated urine specimens (n=30) were assayed for creatinine, specific gravity, and pH to determine if papain rendered the specimens invalid based on the proposed federal guidelines. There was a direct pH, temperature, and time dependent correlate between the increase in papain concentration and the decrease in THC-COOH concentration from the untreated control groups ($p<0.01$). The average 72 hour THC-COOH concentration decrease at pH 6.2 with a papain concentration of 10 mg/mL was 50%. Papain did not significantly decrease the concentration of the other drugs analyzed with the exception of nordiazepam. GC/MS of THC-COOH and HPLC/UV of nordiazepam revealed a 66% and 24% decrease in concentration of the respective analyte with 10 mg/mL papain after 24 hours at room

temperature (~23 °C). No adulterated specimens were rendered invalid based on the SAMHSA guidelines. Immediate FPIA analysis is suggested to minimize the interfering effects of papain with regards to primary drug screening.

DEDICATION

I dedicate this manuscript to my wife, Kimberly, and to my beloved son, Zachary. Science delineates our universe and the intricacies of human life. Art, beauty, and romance are what we live for.

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CHAPTER 1
INTRODUCTION

A Brief History of Drug Testing

Circa 1950, drug abuse treatment facilities were one of the first institutions to implement drug testing (Coombs and West 1991). In 1967, the International Olympic Committee outlined the definition of doping and the concept of banned drugs, however testing was met with resistance because it would be potentially damaging to the public and commercial image of athletes (Wolmar 1992). Concurrently, the military began screening for heroin use among those returning from Vietnam in the late 1960s and early 1970s, and drug screening was subsequently extended to include all soldiers reporting for active duty. In 1981, an investigation into an aircraft crash aboard an aircraft carrier revealed that cannabinoids were present in over 50% of the total fatalities. This finding accelerated the Navy's decision to implement across-the-board drug screening (Zwerling 1993).

Federal Statute CFR49R mandated that all federally regulated industries, shipping, railway, and airline employees be drug tested due to their responsibilities that pertain to the safety of large numbers of individuals (SAMHSA 2004). National sports leagues (i.e., NFL and NHL) began drug testing during the mid-1980s (NCDFS 2003). The Drug Free Workplace Act (DFWA) of 1988 stated that businesses and institutions receiving federal funds ensure that their employees were drug free (Holtorf 1998). The DFWA led to a multitude of private company policy changes to include pre-employment and/or random drug testing. The estimated number of employees screened annually is approximately 20 million, at a cost of several hundred million dollars to industry.

Drug Testing Procedures

Drug testing consists of two levels: the primary drug screen to efficiently detect multiple drugs or classes of drugs and the confirmation of any positive results obtained from the primary drug screen by a more specific and sensitive method. Primary drugs screens are commonly performed by an immunoassay technique due to efficiency and automaticity. Immunoassays involve the binding of a specific antibody with a labeled drug or with an enzyme-drug complex.

The overall principle involved with immunoassays is the competitive binding of the analytes of interest that may be present in the specimen and displacement of the labeled complex from the antibody. The degree by which the labeled complex is displaced is proportional to the concentration of analytes in the specimen. Depending on the specific type of immunoassay, a secondary phenomenon occurs after the labeled complex is displaced that allows the investigator to calculate the analyte concentration. Secondary phenomena include, but are not limited to, absorption of ultraviolet light from an enzymatic substrate conversion, reflection of polarized light from polymerized tracers, and emission of radioactivity from radio-labeled tracers.

The ability to differentiate between a positive and negative result lies in the practice of using a "cutoff" concentration. A cutoff concentration is a concentration of drug below which all specimens are to be considered negative and at or above which all specimens are to be considered positive. Oversight agencies dictate the specific cutoff concentration for each drug or drug class of interest. The practice of using cutoffs normalizes interlaboratory results and standardizes the interpretation of results that can differentiate active and passive drug usage. Table 1 lists the cutoff concentrations for screening and confirmation procedures for the most commonly abused/highest potential for abuse illicit drugs.

Table 1 Cutoff concentrations for screening and confirmation procedures

Analyte	Screening (ng/mL)	Confirmation (ng/mL)
Amphetamines	1000	
Amphetamine		500
Methamphetamine		500+200 amphetamine
Barbiturates	200	200
Benzodiazepines	200	200
Cannabinoids	50	15
Cocaine	300	150
Opiates	300	
Codeine		300
Morphine		300
Phencyclidine	25	25

Source: Adapted from Liu R, Goldberger B. 1996. Handbook of Workplace Drug Testing. Washington DC. AACC Press. 390p.

Assays, Specimens and Analytes of Interest

Enzyme Multiplied Immunoassay Technique (EMIT)

The EMIT assay is based on competitive binding between drug in the specimen and drug labeled with the enzyme glucose-6-phosphate dehydrogenase (G6PD) for antibody binding sites. In the cannabinoid assay, the antibody is biomanufactured to preferentially bind with the THC-COOH metabolite. G6PD activity is stereo-chemically hindered upon binding to the antibody. Unobstructed G6PD converts nicotinamide adenine dinucleotide (NAD) to the protonated form, NADH resulting in an absorbance change that is measured spectrophotometrically at 340 nm (Levine 2003). Assay reagents and specimen are added to a heated (37 °C) reaction vessel and absorbance is measured for a period of 30 to 60 seconds after an incubation period (if applicable, depending on the analyte being measured). The rate of the absorbance change (delta-mabs/t) is proportional to the amount of free drug in the specimen. Figure 1 illustrates the EMIT assay mechanism. A negative, cutoff, and high control are assayed daily to set the rate of absorbance change that correlates to the cutoff concentration for a particular analyte. The EMIT immunoassay is a qualitative assay and is limited to providing categorical data, i.e., a specimen is either positive or negative. Dose-dependent effects of adulterating substances are difficult to observe and analyze statistically. Therefore, we reserved the use of the EMIT assay for the purpose obtaining preliminary data.

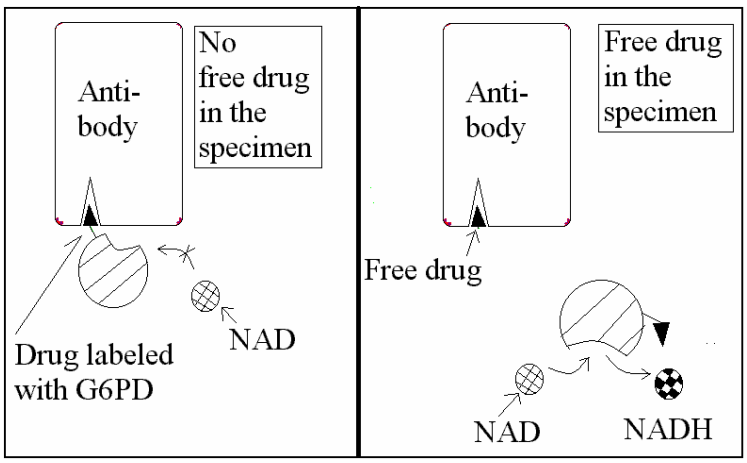

Figure 1 Depiction of the EMIT reaction

Fluoresence Polarized Immunoassay (FPIA)

The FPIA immunoassay is based on competitive binding between drug in the specimen and a drug-fluorophore complex for the antibody (Figure 2). Energy absorbed from polarized light focused on the specimen rotates the molecules in the solution. Larger molecules (i.e., the antibody:tracer complex) will rotate slower than the smaller molecules (i.e., tracer molecules alone). Slower rotating molecules reflect the polarity of the incident beam. Therefore, a high quantity of reflected polarized light is indicative of a specimen with a relatively low concentration of free drug. Conversely, a low quantity of reflected polarized light is indicative of a specimen with a relatively high concentration of free drug. The FPIA and radiological immunoassay (RIA) do not tend to yield false negative results due to the inverse proportionality of the measured immunoassay indicator and free drug in the specimen. Specimens and reagents are added to a reaction vessel with an automated probe. The reaction vessel is allowed to incubate for 10-12 minutes depending on the assay before the quantity of polarized light is quantitated. The FPIA is a quantitative immunoassay that requires each lot of reagents to be calibrated with a six point standard curve that is confirmed daily with bi-level controls.

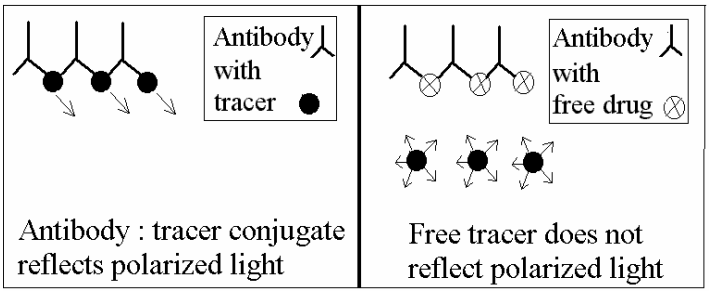

Figure 2 Depiction of the FPIA reaction
Source: Adapted from Levine B. 2003. Principles of forensic toxicology. Washington DC: AACC Press. 385 p.

Confirmation of Positive Drug Screen Results

Confirmation of positive primary drug screen results is performed by a more specific and a more sensitive method of detection of drugs of interest. If a particular immunoassay is established as a primary drug screening method, gas chromatography or high performance liquid chromatography with mass spectrometry (GC/MS, HPLC/MS) or high performance liquid chromatography/ultraviolet detection (HPLC/UV) are usually employed for confirmation of

positive drug screen results. Compared to the methods performed for drug screening procedures, GC, GC/MS, LC/MS, and HPLC/UV methodologies are more resistant to urine specimens that have been adulterated in an attempt to obtain a false negative result from a drug test.

Gas Chromatography/Mass Spectroscopy. GC/MS is a powerful tool in quantitative chemical analysis. Gas chromatography involves the volatilization of a specimen such that all of the analytes exist in the gas phase. The volatilized specimen transverses a column under pressure of an inert carrier gas such as nitrogen or helium. In a capillary column, the analytes are separated due to chemical interactions with the stationary phase on the capillary walls as they transverse the column, these interactions cause the analytes to elute from the column with individual retention times. The chemical interactions within the stationary phase are a factor of: the McReynolds constants that define the stationary phase, column temperature, carrier gas flow rate, and the chemistry of the analyte itself. Electron ionization mass spectroscopy involves a focused electron beam that fractionates a molecule into discrete molecular weights or mass fragments for detection by a mass selective ion detector. The elution time, presence, and ratios of specific molecular weights are unique to every molecule and become a "fingerprint" to identify and quantitate analytes within a specimen.

High Performance Liquid Chromatography/Ultraviolet Detection. HPLC/UV is another vital tool in quantitative chemical analysis. A liquid specimen dissolved in an aqueous mobile phase is forced through a radial compression or stainless steel column by a precision tooled piston pump. The analytes are separated due to chemical interactions with column packing material, these interactions cause the analytes to elute from the column with individual retention times. The chemical interactions within the column are a function of: the phase of the packing material, the flow rate of the specimen through the column, the polarity of the mobile phase, and the chemistry of the analyte itself. Analytes such as benzodiazepines will absorb ultraviolet light of a specific wavelength due to the configuration of the electron orbitals within the molecule. Thus, the degree of ultraviolet absorption is proportional to the concentration of the analyte.

Specimens

The presence of parent drugs and/or their metabolites can be detected in many biological specimens. Urine is typically the specimen of choice for drug testing for several reasons. The collection of urine is non-invasive and has less potential to accidentally transmit pathogens such as those associated with a blood collection. Urine typically has a higher concentration of the analytes of interest due to the physiology of the kidney which collects and concentrates hydrophilic drug metabolites. The matrix effect of a urine specimen is typically less than that of a blood specimen due to the decreased number of lipophilic constituents. Alternative matrices including salvia and hair are available for drug testing. The pharmacodynamics and interpretation of the results obtained from alternative matrix testing are not established (Dolan and others 2004). Urine is therefore the specimen most frequently assayed for drugs and/or drug metabolites.

Primary Analyte of Interest

Cannabinoids are a class of compounds that encompass the psychoactive parent drug found in marijuana, delta-9-tetrahydrocannabinol (THC), four primary metabolites: 11-hydroxy-THC, 8,11-dihydroxy-THC, 11-oxo-THC, and 11-norcarboxy-delta-9-THC (THC-COOH), and over 60 natural and synthetic related structures (Burstein 1979). Marijuana is the most frequently administered illicit drug in the United States (SAMHSA 2002). The EMIT and FPIA immunoassays are optimized to measure THC-COOH because it is the principle urine metabolite that has a decreased lipid solubility relative to the parent compound. The hydrophilicity of THC-COOH allows it to partition into urine in far greater quantities than the lipophilic parent compound. The chemical structure of THC-COOH is illustrated in Figure 3.

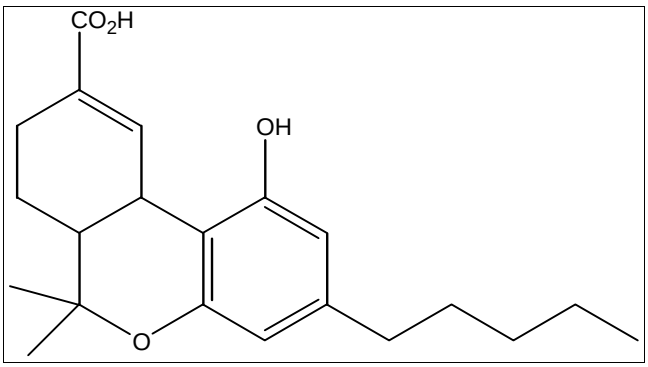

Figure 3 The chemical structure of THC-COOH

Other analytes of interest include: amphetamine, secobarbital, nordiazepam, benzoylecgonine, morphine, and phencyclidine. These parent compounds or metabolites are the substances that are assayed in drug screens for amphetamines, barbiturates, benzodiazepines, opiates, and phencyclidine; respectively. Each analyte presents structurally different chemical moieties and functional groups as illustrated in Figure 4. Each chemical moiety may dictate the susceptibility of the analyte to the effects of a urine adulterant. Previous literature has demonstrated that only specific analytes may be susceptible to the effects of certain urine adulterants (Cody and Valtier 2001).

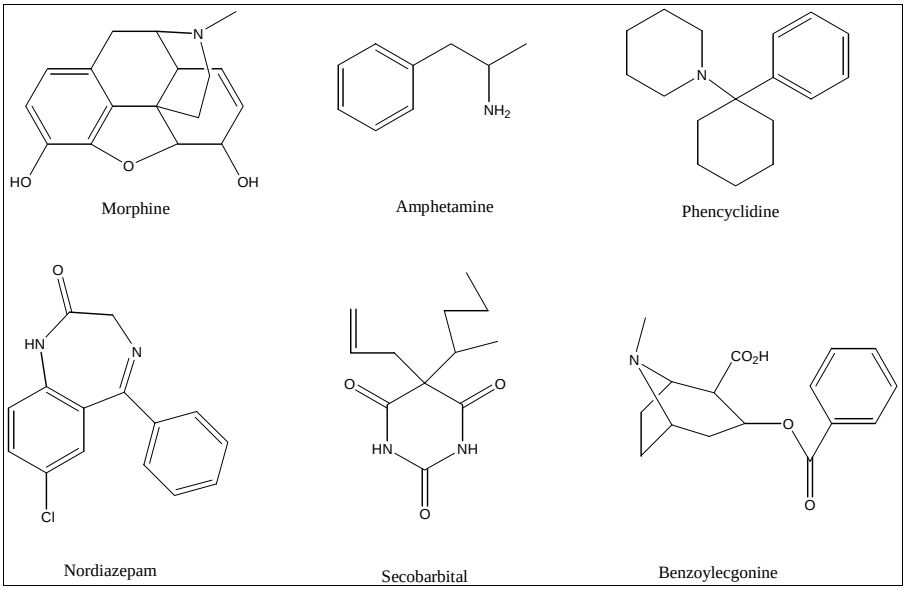

Figure 4 The chemical structures of morphine, amphetamine, phencyclidine, nordiazepam, secobarbital, and benzoylecgonine

Methods Currently Used to Adulterate a Urine Specimen

Illicit drug users who wish to mask the detection of drugs in a urine specimen have several *in vivo* and *in vitro* methods currently available to them. Each method differs with respect to the target of the adulterating agent (i.e., the analyte in the specimen, the matrix, or reagents within the assay) and the complexity of the mechanism of interference associated with the adulterating agent. The target of the adulterating agent is of concern to forensic toxicologists because it dictates the protocol that will be followed to resolve this issue caused by the particular urine adulterant. Urine adulterants that target the reagents within the immunoassay generally require the reformulation of the immunoassay reagents by the manufacturer. If a urine adulterant targets individual analytes, the drug testing laboratories will focus on methodologies to detect the adulterant. In attempt to suppress information that could be used by individuals who intend to adulterate their urine specimen, there are relatively few scientific publications describing urine adulterants. The scientific literature cited herein comprises a vast majority of the total published scientific literature related to urine adulterant. Much of the information pertaining to novel urine adulterants and adulteration methodologies is obtained by “counter-culture” and “pro-drug” media.

Pharmaceutical and Chemical Adulteration of Urine

In vivo methods of urine adulteration include sodium bicarbonate ingestion that can increase urine pH and modify the excretion profile of parent drugs or metabolites. Diuretics will produce a more dilute urine by a variety of mechanisms dependent on their class (Winek and others 1993; Cone and others 1998). Salicylate containing drugs in the urine will decrease the absorbance at 340 nm.

In vitro methods of urine adulteration include diluting the specimen with tap water to bring the analytes of interest below their cutoff concentrations. Substitution is also an effective means of obtaining a false negative drug screen result in which an individual can adhere a plastic container that is filled with a "clean" specimen close to the body and add the clean specimen to the urine cup at the time of collection. Detergents added to urine specimens can encapsulate analytes of interest in micelles rendering them unattainable to the antibody in immunoassays. Urine adulteration is also achieved by the addition of acidic and basic

chemicals that cleave analytes of interest that contain susceptible moieties. Bleach, nitrates, and nitrites will oxidize and modify the chemical structure of the analytes or immunoassay reagents when added to a urine specimen. Many commercially available oxidant based urine adulterants are easily detected in urine via osmolality or capillary ion electrophoresis (Ferslew, and others 2003).

Adulteration by Biologically Active Substances

In addition to the chemical means of adulteration, the adulteration of urine has been accomplished by the addition of biologically active substances such as enzymes. The kinetics of oxidizing materials such as nitrate and nitrites alone may be insufficient in those urine specimens that contain a high concentration of the analyte to effectively produce a false negative drug screen result. Some commercial adulterants consist of an oxidant containing solution that is fortified with a peroxidase. Peroxidases are a family of enzymes that catalyze the oxidation of a substrate. The oxidizing adulterant reacts more quickly and efficiently with the analytes in the urine specimen which decreases the possibility of detecting an oxidizing adulterant while simultaneously yielding a lower concentration of the analyte of interest. Those peroxidases that have been thoroughly investigated have molecular weights ranging from 45-150 kDa, and have Michaelis-Menton constants (Km) ranging from 0.004-181.3 mM, depending on the substrate (Kariya and others 1987).

Proteases are enzymes that target proteins and cleave them at specific locations based on a particular amino acid, amino acid sequences, or any number of physical properties or functional groups. Papain is a cysteine protease that cleaves peptide bonds of basic amino acids and can hydrolyze esters and amides (Townes-Anderson and others 1985). Papain is obtained from the latex of the papaya plant, Carica Papaya, and is the active ingredient in Adolph's® Meat Tenderizer and other consumer products. Scientific investigation into the fermentative properties of papain began circa the 1870s (Wittmack 1878). The application of papain in wound debridement has been described by Mekkes and others (1997). Kinetic data for papain is listed in Table 2 and papain's quaternary structure that reveals 6 alpha helices and 8 beta sheets is illustrated in Figure 5. In addition to the variety of substrates that are enzymatic substrates to papain, it has demonstrated nonspecific binding to a variety of substances (An and others 2004).

The proliferative quantities of papaya latex worldwide yields gross quantities of papain to be sold in a variety of preparations with purities ranging from crude latex to refined-recrystallized enzyme. Each preparation has an associated quantity of papain enzyme with innate enzymatic activity. Quantitation of the effects of a particular preparation of papain must be interpreted within the context of that preparation. For example, the crude latex preparation of papain contains other proteins, both proteolytic and inert, including globulin, albumin, and peptones (Hwang and Ivey 1951).

Currently, there are no published scientific literature sources that implicate papain as a potential or currently used urine adulterant. Counter-culture internet web sites have cited papain as a potential urine adulterant (Erowid 1998). Papain has the potential to be employed as a urine adulterant due to it being readily available, concealable, and relatively inexpensive.

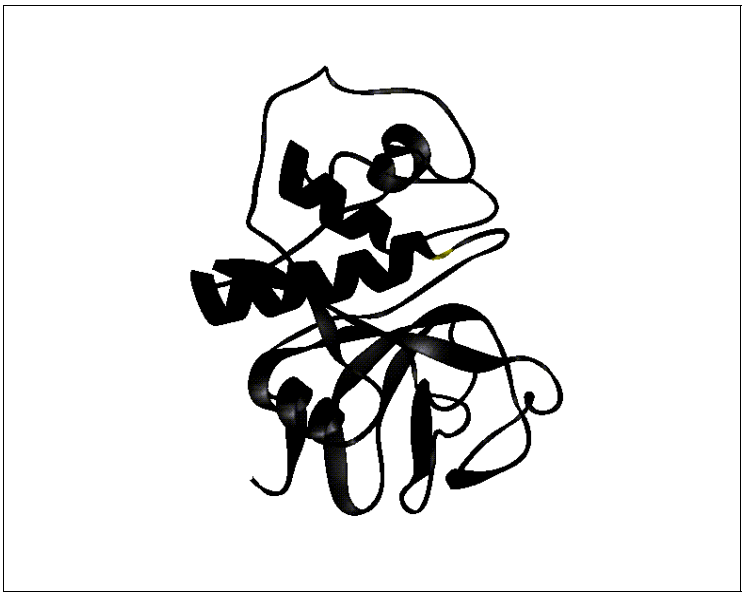

Figure 5 The quarternary structure of papain

In general, commercially available urine adulterants are formulated with sufficient quantities of the active ingredient to mask the detection of drugs and/or metabolites in a chronic "heavy" user. The product will then be effective for all types of illicit drug users. The excessive

quantities of active ingredient(s) in currently available urine adulterants are easily detected by a variety of screening methods including colorimetric test strips such as MASK® and confirmation procedures such as capillary ion electrophoresis (Ferslew and others 2003). The implementation of a chemical or biologically active substance as a novel method to effectively adulterate a urine specimen requires that the chemical or biologically active substance avoid detection. The urine adulterant should therefore be stabile and easily transported at room temperature, able to be discretely added to the urine specimen, and not produce a gross abnormal appearance to the urine specimen.

Table 2 Kinetic parameters of papain

Parameter	Value
Molecular weight	20 - 24 kDa[1,2]
Km	0.008 - 320 mM,[1]
Turnover number	3.84 - 2 x 10^5 per sec[3,4]
Optimum pH	6 - 9.5[5]
Optimum temperature	40 - 85 °C[6]
pI	9.6[7]

Sources: [1] Glazer A, Smith E. 1971. Papain and other plant sulfhydryl proteolytic enzymes. The Enzymes, 3rd. Ed., 3:501-546; [2] Brocklehurst K, Baines B, Kierstan M. 1981. Papain and other constituents of Carica papaya L, Top. Enzyme Ferment. Biotechnol., 5:262-335; [3] Khouri H, and others. 1991. Engineering of papain, Biochemistry, 30:8929-8936; [4]Storer A, Carey P. 1985. Comparison of the kinetics and mechanism of the papain-catalyzed hydrolysis of esters and thiono esters. Biochemistry. 24:6808-6818; [5] Skelton G. 1968. Papaya proteinases. I. Temperature-and pH-stability curves. Enzymologia 35:270-274; [6]Chiou R, Beuchat L. 1986. Characteristics and application of immobilized papain in a continuous-flow reactor. Biotechnol. Appl. Biochem.8:529-536; [7]Balls A, Lineweaver J. 1939. Isolation and properties of crystalline papain. J. Biol. Chem. 130: 669.

Adulteration Detection

The Department of Health and Human Services has developed guidelines in which they propose the implementation of adulterant testing by drug testing laboratories to determine if a given specimen is diluted, substituted, or adulterated (NLCP Program Document #35 1998). The guidelines define the ranges of specific gravity (SG), pH, nitrite concentration, and creatinine values that label the urine specimens as normal, substituted, diluted, or adulterated. Table 3 lists the normal values of SG, pH, and creatinine for random urine specimens (n=40).

A specimen is considered diluted if the creatinine is < 20 mg/dL and the specific gravity is < 1.003. Substituted urine specimens have creatinine concentrations < 5 mg/dL and specific gravities < 1.001 or > 1.020. The urine is adulterated if the pH is < 3 or > 11 or a nitrite concentration >500 μg/mL (NLCP Program Document #35 1998). Dual qualifying parameters (i.e., creatinine *and* specific gravity) were necessary to differentiate a urine specimen from a patient with polydipsia or renal disease from a diluted or substituted urine specimen. In an evaluation of over 100 polydipsia case studies, there were no instances in which the patient's urine was classified as substituted, diluted, or adulterated based on the Department of Health and Human Services guidelines (Cook and others 2000).

Table 3 Creatinine, specific gravity, and pH values of normal urine

Parameter	Value
Creatinine	37 – 300 mg/dL (female) 44 – 250 mg/dL (male)
Specific gravity	1.002-1.030
pH	4.5-8

Adapted from: Tietz N. 2001. Fundamentals of clinical chemistry, 5rd ed. Philadelphia: WB Saunders. 1010 p.

Point of care (POC) adulterant testing kits such as Multiple Adulterant Strip Kemistry (MASK) allow the rapid detection/semi-quantitation of multiple urine parameters and adulterants including specific gravity, creatinine, pH, nitrite concentration, and the presence of oxidants and glutaraldehyde. The MASK test strip contains multiple 1 cm x 1 cm absorbent pads infused with an adulterant chromaphor substrate that produces a color reaction in the presence of a particular adulterant or urine analyte. The absorbent pads are then compared with a reference chart that illustrates examples of absorbent pads which indicate positive or negative results, or an approximate concentration of an analyte.

Specific Aims of this Study

Hypothesis: The concentration of THC-COOH in urine specimens, as measured by primary immunoassay drug screen analyses and confirmation analyses, will be reduced by the

adulteration of the urine specimen with papain such that the urine specimen will not be rendered invalid based on the current federal specimen validity guidelines.

The hypothesis will be addressed by the following specific aims:

1. To determine if papain yields a false negative result by qualitative EMIT analysis on a specimen that contains THC-COOH.
2. To determine the quantities of papain required to obtain a false negative result on synthetic urine specimens that contain various amounts of THC-COOH, with respect to pH, papain concentration, and time.
3. To determine if the addition of papain to urine specimens will yield a false negative result by FPIA analysis on specimens that contain other commonly abused drugs.
4. To determine if the observed effects of papain are obtained with purified protein and with purified protein that has its enzymatic site is irreversibly inhibited.
5. To use a more selective and sensitive assay to determine if the mechanism of the effects of papain are due to manipulation of the analyte.
6. To determine if the maximum concentration of papain involved in adulterating the specimen renders the specimen invalid based on the proposed guidelines for substituted, adulterated, or diluted urine specimens, or due to qualitative analysis based on rapid chromaphore adulterant testing.

CHAPTER 2
MATERIALS AND METHODS

Volumetric and Gravimetric Equipment

The analytical balance (Mettler H33AR, Mettler-Toledo, Columbus OH) used to obtain reagent masses in our research was calibrated with the National Institute of Standards and Technology (NIST) mass standards. The following pipettes were calibrated by volumetric analysis with 18 MOhm deionized water from Nanopure water systems (Barnstead Company, Boston, MA.): Wheaton Calibra® 20-200 microliter adjustable pipette (Fisher Scientific, Pittsburgh, PA.); Wheaton Calibra® 2-20 microliter adjustable pipette (Fisher); Finnpipette® 100-1000 microliter adjustable pipette (Fisher); Eppendorf Repeater® repeating single channel pipette (Brinkmann Instruments, Westbury, NY). Eppendorf Research® 10, 50, and 100 µL pipettes (Fisher). All graduated cylinders and ground glass volumetric flasks with penny stoppers used in our research were Pyrex™ brand obtained from Fisher Scientific. All volumetric pipettes used in our research were Kimax™ brand obtained from Fisher Scientific.

Laboratory Instruments

Specimen and buffer pH was measured on a model 4500 digital pH meter (Beckmann, Norcross, GA.) with a calomel glass combination electrode that was calibrated with a pH 7.0 standard (Fisher) and a pH 4.0 or pH 10 standard (Fisher) as necessitated by the pH range measured. Specimens were vortexed on a Barnstead/Thermolyne M16715 mixer (Barnstead International, Dubuque, IA.). Liquid/liquid specimen extraction was assisted by a test tube rocker (American Dade, Miami, Fl.). EMIT analyses were performed on a ETS™ analyzer (Syva-Dade Behring, San Jose, CA.) calibrated daily with negative, cutoff, and high calibrators as described below. FPIA analyses were performed on an Abbott Axsym™ (Abbott Diagnostics, Abbott Park, IL.) FPIA analyzer. Each lot of reagent kits was calibrated once with a 6 point standard curve in duplicate and two standard controls were assayed every 8 hour shift as described below. Operation, maintenance, and proper handling of the reagents, controls, and calibrators were followed as instructed by the respective ETS™ and Axsym™ operation manuals. UV/Vis Spectrophotometry was performed on a Cary 300 Bio UV/VIS split beam

spectrophotometer (Varian, Palto Alto, CA.) integrated to a Dell Optiplex® PC (Dell Inc., Round Rock, TX.). Microplate spectrophotometry was performed on a Elx808 microplate reader (Bio-Tek Instruments, Winooski, VT.) integrated to a Dell Optiplex PC (Dell Inc.). Gas chroma-tography/mass spectroscopy was performed on a model 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA.) with a model 18596C autosampler (Hewlett Packard), a model 18593B autoinjector (Hewlett Packard), and a 30 m x 0.25 mm DB-5 capillary column (Supelco, St. Louis, MO.) coupled to a model 5972 electronic ionization mass selective ion detector (Hewlett Packard) integrated into a Vectra PC (Hewlett Packard). High performance liquid chromatography/ultraviolet detection was performed on a model M-45 solvent delivery system (Waters, Millford, MA.) with a model 717 plus autosampler (Waters) and a 8 mm x 100 mm NOVA-PAK C18-4 micron radial compression column (Waters) coupled to a Lambda-Max 480 ultraviolet detector (Waters) integrated into a model 746 data module (Waters). Specific gravity was measured with a model 300026 temperature compensated digital refractometer (Sper Scientific, Scottsdale, AZ.). Osmolality was measured with a model 302 Advanced Digimatic® osmometer (Advanced Instruments, Needham Heights, MA.) or on an Osmette S automatic osmometer (Precision Systems Inc., Natick, MA.).

Enzymes and Pharmaceutical Standards

Papain (Sigma Chemical, St. Louis, MO. Catalog # P3375, Lot # 51K1540), 1.5-3.5 U/mg, was purchased in the form of crude latex granules. The crude latex granules were pulverized with a pestle and mortar to a fine powder and refrigerated at 4 °C. Unless otherwise noted, all assays were performed with the crude latex powder form of papain. A 26.4 mg/mL stock suspension of twice recrystallized papain (RP) with a standardized enzyme activity of 21 U/mg (Sigma, Catalog # P3125, Lot # 30H8030) was donated (Johnson 2002) and refrigerated at 4 °C (Dubois and others 1988). A 200 mM dithiothreitol (DTT) stock standard solution was prepared by placing 309 mg of dithiothreitol (Sigma, Catalog # D-9163, Lot # unknown) in a 10 mL volumetric flask with 5 drops of dimethyl sulfoxide (DMSO, Sigma) and diluting to volume with 18 MOhm deionized water. N-alpha-Benzoyl-DL-Arg-p-Nitroanalide, BANI, (Sigma Chemical, Catalog # B4875, Lot # 63F-0436) was donated (Johnson 2002) and refrigerated at 4 °C.

Working standards of THC-COOH (Cerilliant, Round Rock, TX, Catalog #01422, Lot #'s 44,45) were purchased in the form of a 100 mcg/mL stock methanol standards in a sealed 1.5 mL glass vial. Deuterated 11-norcarboxy-delta-9-tetrahydrocannibinol (d_3 -THC-COOH) (Cerilliant, Catalog # T-015, Lot # 35002-42C) was purchased in the form of a 10 mcg/mL stock methanol standard in a sealed 1.5 mL glass vial. A 1 mg/mL nordiazepam stock methanol standard was prepared by placing 10 mg of nordiazepam free base (Sigma, Catalog # D7282, Lot # 17F-4024) in a 10 mL volumetric flask and diluting to volume with methanol (Optima®, Fisher). A 1 mg/mL benzoylecgonine stock methanol standard was prepared by placing 10 mg of benzoylecgonine free base (Sigma, Catalog # B4147, Lot #68F-4001) in a 10 mL volumetric flask and diluting to volume with methanol (Optima®, Fisher). A 1 mg/mL prazepam stock methanol standard was prepared by placing 10 mg of prazepam free base (Sigma, Catalog #P3654, Lot #56F-0684) in a 10 mL volumetric flask and diluting to volume with methanol (Optima®, Fisher). Phencyclidine (Cerilliant, Catalog# P-007, Lot # 65004-06D) was purchased in a 10 mcg/mL stock methanol standard in a sealed 1.5 mL glass vial. A 1 mg/mL morphine stock methanol standard was prepared by placing 10.4 mg of morphine sulfate (Mallinckrodt, Hazelwood, MO, Catalog # unknown, Lot # E633-102)) in a 10 mL volumetric flask and diluting to volume with methanol (Optima®, Fisher). A 1 mg/mL amphetamine stock methanol solution was prepared by placing 13.6 mg of amphetamine sulfate (Smith-Kline, Research Triangle Park, NC., Catalog # Unknown, Lot # Unknown) in a 10 mL volumetric flask and diluting to volume with methanol (Optima®, Fisher). A 1 mg/mL secobarbital stock methanol solution was prepared by placing 10 mg of secobarbital free acid (Sigma, Catalog # S1503, Lot # 030H0288) in a 10 mL volumetric flask and diluting to volume with methanol (Optima®, Fisher).

Papain Standardization

The proteolytic activity of papain was quantitated by spectrophotometric observance of the hydrolysis of N-alpha-Benzoyl-DL-Arg-p-Nitroanalide (BANI) adapted from a previously published method (Erlanger, Kokowsky, and Cohen 1961). The activity of cysteine proteases are augmented by a reducing agent such as sodium sulfide or DTT. A 20 mM DTT standard solution was prepared by pipetting 1 mL of the 200 mM stock DTT solution into a 10 mL

volumetric flask and diluting to volume with 18 MOhm deionized water and vortexing for 1 minute. A 20 mM stock solution of BANI was prepared by placing 87.0 mg of BANI into a 10 mL volumetric flask with 1 mL of DMSO and diluting to volume with 18 MOhm deionized water. The solution was stoppered and vortexed for 1 minute. A 2 mM BANI/DTT working solution was prepared by pipetting 1 mL of the 20 mM BANI stock solution and 1 mL of the 20 mM standard solution into a 10 mL flask and diluting to volume with 18 MOhm deionized water to yield a working solution of 2 mM BANI/DTT. The 2 mM BANI/DTT working solution was stoppered and mixed thoroughly by repeated inversion of the volumetric flask. A working solution of 0.13 mg/mL (2.73 U/mL) twice recrystallized papain (RP) was prepared by pipetting 49.0 μL of the 26.4 mg/mL RP stock standard into a 10 mL volumetric flask and diluting to volume with 18 MOhm water. A 0.065 mg/mL (1.37 U/mL) RP working standard was prepared by pipetting 50 μL of 0.13 mg/mL RP standard in a 1.5 mL flat top microcentrifuge tube (Fisher) followed by 50 μL of 18 MOhm deionized water. A 0.0325 mg/mL (0.68 U/mL) RP working standard was prepared by pipetting 50 μL of 0.065 mg/mL RP standard in a 1.5 mL flat top microcentrifuge tube (Fisher) followed by 50 μL of 18 MOhm deionized water. A blank 2 mM DTT working solution was prepared by pipetting 10 μL of the 200 mM DTT into a 1 mL volumetric flask and diluting to volume with 18 MOhm deionized water. A 10 mg/mL crude latex papain (CLP) solution was prepared by placing 10.0 mg of CLP in a 10 mL volumetric flask and diluting to volume with 18 MOhm deionized water. A 5 mg/mL CLP solution was prepared by pipetting 200 μL of the 10 mg/mL papain solution into a 1.5 mL flat top microcentrifuge tube (Fisher) followed by 200 μL of 18 MOhm deionized water. Repeated serial dilutions with equal volumes of 18 MOhm deionized water were performed to obtain 2.5 and 1.25 mg/mL CLP solutions.

The Cary UV/Vis spectrophotometer was allowed to stabilize for 1 hour. The accompanied software program “Kinetics” was opened and wavelength was adjusted to 410 nm. The reference cuvette consisted of 1.0 mL of the 2 mM BANI/DTT solution and 67 μL of 18 MOhm deionized water pipetted into a 1-cm matched quartz cuvette (Fisher). The sample cell contained 1.0 mL of the 2 mM BANI/DTT solution. Immediately prior to recording absorbance data, 67 μL of the 0.13 mg/mL RP standard was pipetted into the sample cuvette and mixed by repeated pumping of the pipette plunger. The sample cuvette was placed in the

spectrophotometer and the absorbance was measured for 20 minutes. The initial rate of absorbance change (delta-mabs/min) was obtained in the first 45 seconds of the reaction. The analysis was repeated with 67 µL of 0.065 mg/mL, 0.0325 mg/mL RP working standards, and blank 2 mM DTT working solution. The BANI proteolytic activity assay was repeated with CLP solutions in concentrations of 10, 5, 2.5, and 1.25 mg/mL.

Synthetic Urine

Enzyme activity is dependent on several parameters such as pH, temperature, co-factors and ionic strength. The effect of papain on THC-COOH and other drugs of abuse was monitored in a matrix of synthetic urine. The synthetic urine provided a matrix with discrete constituents that defined the environment in which the enzyme was active. The synthetic urine also provided a matrix that was completely devoid of over-the-counter pharmaceuticals or other potentially adulterating substances that may be present in a pooled urine matrix. Two-4 liter batches of synthetic urine were prepared and homogenized as described by Kark and others (1964). Each batch of synthetic urine was prepared by placing the quantities, in grams, of the following constituents (Table 4) into a 5.5 L polypropylene beaker (Nalgene®, Fisher): urea (Fisher), 64; sodium chloride (Fisher), 9.3; potassium chloride (Fisher), 13.7; creatinine (Fisher), 4.4; sodium sulfate (Fisher), 17.25; ammonium chloride (Fisher), 4.25; citric acid (Fisher), 2.2; magnesium sulfate (Fisher), 1.85; sodium phosphate dibasic monohydrate (Fisher), 12.55; calcium chloride dihydrate (Fisher), 3.75; sodium oxalate (Fisher), 0.119; lactic acid (Fisher), 320 µL of 85% solution; glucose (Fisher), 1.95; sodium silicate pentahydrate (Fisher), 0.212; pepsin (Fisher), 0.1; sodium fluoride (Fisher), 1.0. Two-2000 mL aliquots of deionized water were added to the 5.5 liter beaker by a 2000 mL graduated cylinder (Fisher). Both 4 liter batches of synthetic urine were mixed for 1 hour by a 3 inch magnetic stir bar on a Fisherbrand® variable speed mixer (Fisher). The synthetic urine was titrated to pH 6.2 with concentrated HCl (Optima®, Fisher). The two batches of synthetic urine were then homogenized with each other and stored at 4 °C until needed. The osmolality of the synthetic urine was measured on an Osmette S automatic osmometer (Precision Systems Inc.). The osmometer was turned on and allowed to stabilize for 1 hour. The instrument was calibrated with a 500 mOsm/L calibration standard (Advanced Instruments). A 200 µL aliquot of the calibrator and

synthetic urine was pipetted into disposable plastic osmometer sample cups. The sample cup was placed into the sample cup reservoir and the probe was lowered into the sample cup. Osmolality was measured directly by depressing the "seed" switch.

Table 4. Constituents of synthetic urine

Constituent	Quantity (g/4 kg)
Urea	64
Sodium Chloride	9.3
Potassium Chloride	13.7
Creatinine	4.4
Sodium Sulfate	17.25
Ammonium Chloride	4.25
Citric Acid	2.2
Magnesium Sulfate	1.85
Sodium Phosphate Dibasic Monohydrate	12.55
Calcium Chloride Dihydrate	3.75
Sodium Oxalate	0.119
Lactic Acid	320 µL of 85% solution
Glucose	1.95
Sodium Silicate Pentahydrate	0.212
Pepsin	0.1
NaF	1

Source: Adapted from Kark RM, Lawrence JR, Pollack VE, Pirani CL, Muehrcke RC, Silva H. 1964. A primer of urinalysis (2nd ed.). New York: Hoeber Medical Division, Harper & Row. 74 p.

Enzyme Multiplied Immunoassay Technique

A preliminary investigation into papain's effects on synthetic urine specimens containing THC-COOH was performed by enzyme multiplied immunoassay technique. A 125 ng/mL THC-COOH solution was prepared by pipetting 125 µL of 100 mg/mL THC-COOH stock methanol standard into a 100 mL volumetric flask with 1 mL of 200 mM DTT and diluting to volume with pH 6.2 synthetic urine. Six replicate adulterated specimens containing 10 mg/mL papain were prepared by placing 10 mg of papain in a 1.5 mL flat top microcentrifuge tube (Fisher) and delivering 1 mL of the 125 ng/mL THC-COOH solution with an Eppendorf Repeater®. Six positive control specimens were prepared by delivering 1 mL of the 125 ng/mL THC-COOH solution in a 1.5 mL flat top microcentrifuge tube (Fisher) with an Eppendorf Repeater®. The specimens incubated at room temperature (~23 ^{0}C) for 5 hours.

The ETS™ analyzer was allowed to stabilize for 1 hour. The initialization and calibration procedure as defined in the operator's manual was followed to calibrate the instrument. Negative (0 ng/mL THC-COOH), cutoff (100 ng/mL THC-COOH), and high (200 ng/mL THC-COOH) calibrators were removed from the refrigerator and allowed to attain room temperature for 1 hour. Calibrators were pipetted into ETS™ sample cups with a 200 µL disposable glass pasteur pipette. The ETS™ sample cups were placed on an ETS™ sample cup carousel that was placed in and ETS™ reagent rack position on the instrument. The instrument calibration, involving duplicate assays of the cutoff calibrator and a single assays of the negative and high calibrators, was performed. The delta-mabs/t separations between the negative and the cutoff calibrator values, and between the cutoff and the high calibrator values exceed the minimum values for the particular reagent lot used. A 200 µL aliquot of the prepared unadulterated and adulterated synthetic urine specimen was pipetted into an ETS™ sample cup with a 200 µL disposable glass pasteur pipette. The ETS™ sample cups were placed on an ETS™ sample cup carousel that was placed in and ETS™ reagent rack position on the instrument. "Cannabinoid 100" assay was selected for each specimen and the assay sequence was started.

Fluorescence Polarized Immunoassay

The following FPIA analyses provided information on the dose dependent effects of papain on synthetic urine with respect to the analyte of interest, the pH of the urine matrix, the temperature of the urine matrix, and the time of analyte exposure to papain. The experiments were constructed to emulate specimen analysis performed in laboratories within 8 hours, 24 hours , and 3 days of after the time of specimen collection . A typical FPIA cannabinoid assay calibration curve is illustrated in Figure 6. The quantity of polarized light (mP) of THC-COOH calibrator was plotted against its respective THC-COOH calibrator concentration (ng/mL), and the mathematical fit, mP= A+(B/(C+[THC-COOH]^D)), as defined by the Axsym™ operator's manual was performed to yield a standard curve with a calculated correlation coefficient (r^2). All daily calibrators were within the allowable range as noted by the Axsym™ operator's manual guidelines.

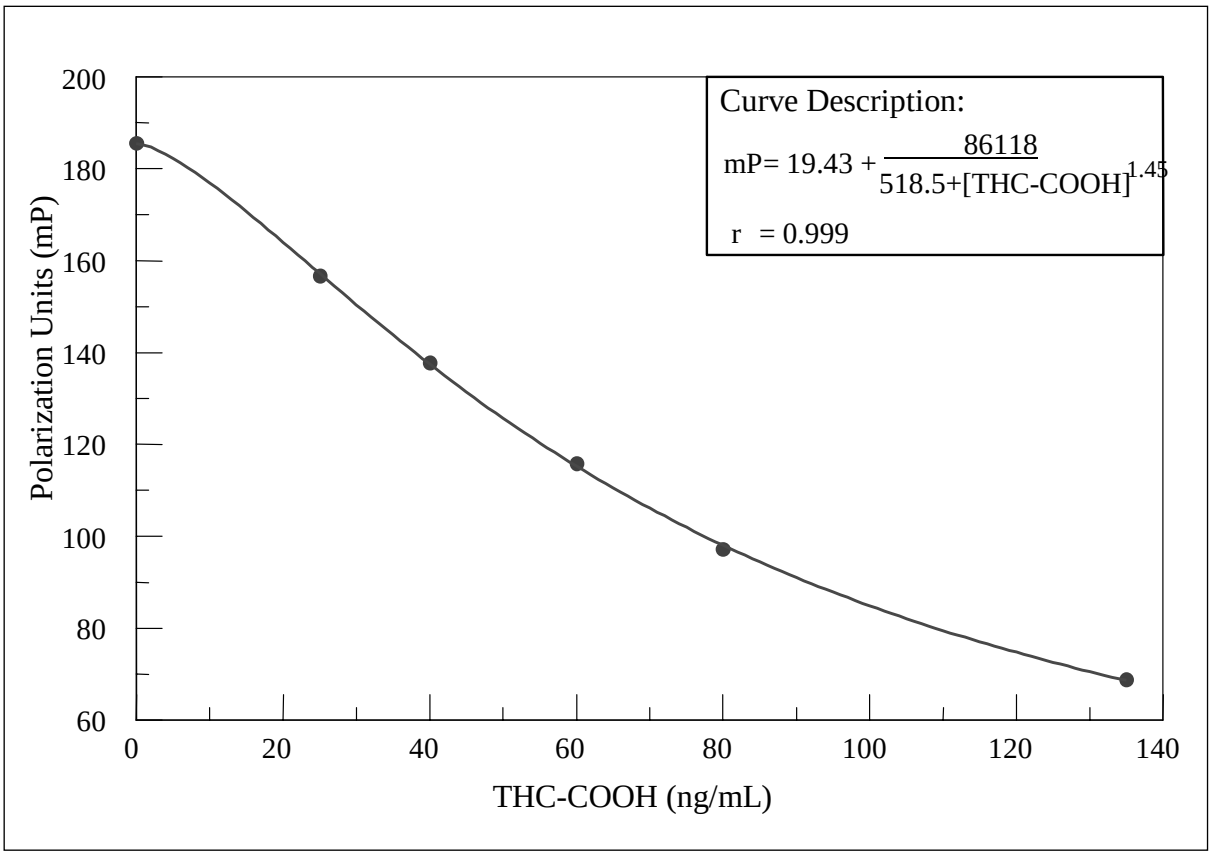

Figure 6 Typical FPIA cannabinoid assay calibration curve

THC-COOH Assays

A 25 ng/mL THC-COOH solution was prepared by pipetting 50 µL of the 100 mcg/mL THC-COOH stock methanol standard into a 200 mL volumetric flask with 2 mL of 200 mM DTT. The flask was diluted to volume with pH 6.2 synthetic urine. Six replicate specimens of pH 6.2 synthetic urine containing 0, 0.5, 1, 2, 5, and 10 mg/mL papain (36 total specimens) were prepared by placing 0, 2.5, 5, 10, 25, and 50 mg of papain in 13 x 100 mm glass screw top test tubes (Fisher) and delivering 5 mL of pH 6.2 synthetic urine with an Eppendorf Repeater® to every tube.

The Axsym™ analyzer was allowed to stabilize for 1 hour. The initialization calibration procedure as defined in the operator's manual was followed to calibrate the instrument. Each cannabinoid reagent lot was calibrated with a 6 point control. A 200 µL aliquot of 0, 25, 40, 60, 80, 135 ng/mL THC-COOH calibrators (Abbott) were pipetted into Axsym™ sample cups with a 100-1000 mL Eppendorf™ pipette. The sample cups were placed in a sample cup rack

and placed on the sample cup rack carousel. The calibrators were assayed to calibrate the reagent lot. A 200 µL aliquot of the 36 prepared specimens and 2 cannabinoid controls (Abbott) were pipetted into the Axsym™ sample cups with a 100-1000 mL Eppendorf™ pipette. The sample cups were placed in a sample cup rack and placed on the sample cup rack carousel. Specimen identifiers were entered on the Axsym™ touch screen and assayed for cannabinoids. The specimens were capped with a teflon screw cap and allowed to remain at room temperature (~23 °C) before being re-assayed for cannabinoids 4 and 6 hours after the specimens were initially prepared. The specimens were then refrigerated at 4 °C before being re-assayed for cannabinoids at 24 and 72 hours after the initial preparation of the specimens.

Standard solutions of pH 6.2 synthetic urine containing 75, 100, 250, and 500 ng/mL THC-COOH were prepared by pipetting 150, 200, 500, and 1000 µL of 100 mcg/mL THC-COOH stock methanol standard into separate 200 mL volumetric flasks with 2 mL of 200 mM DTT and diluting to volume with pH 6.2 synthetic urine. The set of FPIA assays as described above for 25 ng/mL THC-COOH were performed with the synthetic urine containing 75 - 500 ng/mL THC-COOH. The upper limit of the calibration curve for the cannabinoid assay was 135 ng/mL. Prepared specimens that had reported concentrations >135 ng/mL were diluted accordingly with blank synthetic urine and re-assayed such that the reported concentration was <135 ng/mL.

Standard solutions of pH 8.0 and 4.5 synthetic urine were prepared by placing 1.2 liters of pH 6.2 synthetic urine in a 2 L Kimax beaker (Fisher) with a magnetic stir bar. The beaker was then placed on an automatic stir plate and allowed to stir with the pH electrode protruding into the liquid. Adjustment to the pH 4.5 or 8.0 was accomplished with the titration of concentrated 12 M hydrochloric acid (Reagent Grade®, Fisher) or 10 M sodium hydroxide (Fisher). The solutions were placed in a 1 L brown glass screw top bottle, capped, and placed in the refrigerator at 4 °C until needed.

Standards consisting of 25, 75, 100, 250, and 500 ng/mL THC-COOH in pH 4.5 and 8.0 synthetic urine were prepared as described for 6.2 synthetic urine. Six replicate specimens containing 0, 0.5, 1, 2, 5, and 10 mg/mL papain (36 total specimens per set) were prepared as described above for each THC-COOH standard at pH 4.5 and 8.0 (10 total sets). The specimens

were assayed for cannabinoids under the same time-temperature treatments as described above for pH 6.2 synthetic urine.

FPIA Assays for Other Drugs of Abuse

To determine if papain had an effect on the reported concentrations of other drugs of abuse, FPIA analyses were performed for amphetamine, barbiturates, benzodiazepines, cocaine, opiates, and phencyclidine in pH 6.2 synthetic urine adulterated with 10 mg/mL papain. Two standard solutions, a positive and a negative, were prepared for each analyte in pH 6.2 synthetic urine. The positive solution contained 150% of a respective analytes' cutoff concentration, and the negative solution contained 50% of the a respective analytes' cutoff concentration. The 1500 ng/mL and 500 ng/mL amphetamine solutions were prepared by pipetting 150 and 50 µL of 1 mg/mL amphetamine stock methanol standard into two 100 mL volumetric flask with 1 mL 200 mM DTT and diluting to volume with pH 6.2 synthetic urine. The 300 ng/mL and 100 ng/mL secobarbital solutions were prepared by pipetting 30 and 10 µL of 1 mg/mL secobarbital stock methanol standard into two 100 mL volumetric flask with 1 mL 200 mM DTT and diluting to volume with pH 6.2 synthetic urine. The 300 ng/mL and 100 ng/mL nordiazepam solutions were prepared by pipetting 30 and 10 µL of 1 mg/mL nordiazepam stock methanol standard into two 100 mL volumetric flask with 1 mL 200 mM DTT and diluting to volume with pH 6.2 synthetic urine. The 450 ng/mL and 150 ng/mL benzoylecgonine solutions were prepared by pipetting 45 and 15 µL of 1 mg/mL benzoylecgonine stock methanol standard into two 100 mL volumetric flask with 1 mL 200 mM DTT and diluting to volume with pH 6.2 synthetic urine. The 450 ng/mL and the 150 ng/mL morphine solutions were prepared by pipetting 45 and 15 µL of 1 mg/mL morphine stock methanol standard into two 100 mL volumetric flask with 1 mL 200 mM DTT and diluting to volume with pH 6.2 synthetic urine. The 37.5 ng/mL and the 12.5 ng/mL phencyclidine solutions were prepared by pipetting 37.5 and 12.5 µL of 0.1 mg/mL phency-clidine stock methanol standard into two 100 mL volumetric flask with 1 mL 200 mM DTT and diluting to volume with pH 6.2 synthetic urine.

Six unadulterated specimens and 6 specimens adulterated with 10 mg/mL papain were prepared for both the negative and the positive solutions for each analyte (144 total specimens). The adulterated specimens were prepared by placing 50 mg of papain into 13 x 100 screw top

test tubes and delivering 5 mL of the respective solution to every tube with an Eppendorf Repeater®. This process was repeated for each analyte's negative and positive solution. The unadulterated specimens were prepared by delivering 5 mL of the respective solution to an empty 13 x 100 screw top test tube with an Eppendorf Repeater®. This process was repeated for each analyte's negative and positive solution. The reagent lot for the amphetamine, barbiturates, benzodiazepines, cocaine, opiate, and phencyclidine assays were calibrated by their respective assay calibrators (Abbott) as described for the cannabinoid assay. A 200 µL aliquot of the prepared specimens and 2 assay controls (Abbott) for each assay were pipetted into the Axsym™ sample cups with a 100-1000 mL Eppendorf™ pipette. The sample cups were placed in a sample cup rack and placed on the sample cup rack carousel. Sample identifiers were entered on the Axsym™ touch screen and assayed for their respective analyte. The prepared specimens were capped with a teflon screw cap and allowed to remain at room temperature (~ 23 °C) for 6 hours after the initial preparation before being re-assayed on the Axsym™ for their respective analyte. The specimens were then capped with a teflon screw cap and refrigerated (4 °C) and re-assayed on the Axsym™ for their respective analyte at 72 hours after preparation.

Recrystallized Papain and Inhibition by E-64

Based on results obtained by FPIA analysis for cannabinoids and other drugs of abuse in specimens adulterated with papain, the following FPIA analyses were performed with twice recrystallized papain (RP) with a standardized activity of 21 U/mg. Inhibition of RP enzymatic activity with trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) was necessary to determine if the observed effects of papain on specimens containing THC-COOH and nordiazepam were due to its enzymatic activity. E-64 covalently binds to the cysteine-25 active site on papain preventing enzymatic activity.

BANI Assay of Inhibited RP Enzymatic Activity. To determine the quantity of E-64 required to inhibit a 1 mg/mL solution of RP, a BANI enzyme activity assay was performed. The microplate reader was allowed to stabilize during the preparation of the specimens. The software program "KC Jr." was opened and the wavelength was set to 405 nm. The kinetics program instructed the microplate reader to read the specimen wells every 20 seconds for 4

minutes. A working solution of 1 mg/mL RP was prepared by pipetting 76 µL of 26.4 mg/mL RP stock suspension into a 2 mL volumetric flask with 20 µL of 200 mM DTT and diluting to volume with 18 MOhm deionized water. A working solution of deactivated recrystallized papain (DRP) was prepared by placing 1 mg of E-64 into a 1 mL volumetric flask and diluting to volume with the 1 mg/mL RP working solution, yielding a 58.7 molar excess of E-64 to papain. The DRP solution incubated for 2 hours at room temperature to allow the E-64 to covalently bind to papain. A solution of 2 mM DTT was prepared by pipetting 10 µL of 200 mM DTT stock standard solution into a 1 mL volumetric flask and diluting to volume with 18 MOhm deionized water. A stock solution of 20 mM BANI was prepared by placing 43.5 mg into a 5 mL volumetric flask with 1 mL DMSO (Sigma) and diluting to volume with 18 MOhm deionized water. A working solution of 4 mM BANI was prepared by pipetting 1 mL of the 20 mM BANI stock solution into a 5 mL volumetric flask with 50 mL of 200 mM DTT and diluting to volume with 18 MOhm deionized water. A blank specimen was prepared by pipetting 150 µL of 4 mM BANI into a well of a 96 well acrylic microplate (Neogen) and adding 150 µL of 2 mM DTT. Control specimens of RP were prepared in triplicate by pipetting 150 µL of 4 mM BANI into 3 wells of the 96 well microplate with 150 mL of 1 mg/mL RP solution. Triplicate specimens of DRP were prepared by pipetting 150 µL of 4 mM BANI into 3 wells of the 96 well microplate with 150 mL of 1 mg/mL DRP solution. The absorbance in every specimen well was sequentially measured at 405 nm every 5 seconds for 4 minutes.

FPIA Assays with RP and DRP. A 2 mg/mL RP working solution was prepared by pipetting 152 µL of 26.4 mg/mL RP stock suspension into a 2 mL volumetric flask with 20 µL of 200 mM DTT and diluting to volume with 18 MOhm deionized water. A 2 mg/mL DRP working solution was prepared by placing 2 mg of E-64 in a 1 mL volumetric flask and diluting to volume with the 2 mg/mL RP working solution and allowing the DRP solution to incubate for 2 hours at room temperature. A 2 mM DTT solution was prepared by pipetting 100 µL of 200 mM DTT into a 10 mL volumetric flask and diluting to volume with 18 MOhm deionized water. A 120 ng/mL THC-COOH working solution was prepared by pipetting 120 µL of 100 mg/mL THC-COOH stock methanol standard into a 10 mL volumetric flask with 100 µL of 200 mM DTT and diluting to volume with 18 MOhm deionized water. Six replicate blank specimens

were prepared by pipetting 100 µL of the 120 ng/mL THC-COOH working solution into 6 Axsym™ sample cups with 100 µL of 2mM DTT solution. Six replicate RP adulterated specimens were prepared by pipetting 100 µL of the 120 ng/mL THC-COOH working solution into 6 Axsym™ sample cup with 100 µL of the 2 mg/mL RP working solution. Six replicate DRP adulterated specimens were prepared by pipetting 100 µL of the 120 ng/mL THC-COOH working solution into 6 Axsym™ sample cups with 100 µL of the 2 mg/mL DRP working solution. The specimens were allowed to incubate at room temperature for 2 hours before being assayed for cannabinoids on the calibrated Axsym™ analyzer as described in the previous section.

A 500 ng/mL working solution of nordiazepam was prepared by pipetting 5 µL of the 1 mg/mL nordiazepam stock methanol standard into a 10 mL volumetric flask with 100 µL of 2 mM DTT. The 2 mg/mL RP and DRP working solutions were prepared as described as above for the THC-COOH assay. Six replicate blank specimens were prepared by pipetting 100 µL of the 500 ng/mL nordiazepam working solution into 6 Axsym™ sample cups with 100 µL of 2mM DTT solution. Six replicate RP adulterated specimens were prepared by pipetting 100 µL of the 500 ng/mL nordiazepam working solution into 6 Axsym™ sample cups with 100 µL of the 2 mg/mL RP working solution. Six replicate DRP adulterated specimens were prepared by pipetting 100 µL of the 500 ng/mL nordiazepam working solution into 6 Axsym™ sample cups with 100 µL of the 2 mg/mL DRP working solution. The specimens were allowed to incubate at room temperature for 2 hours before being assayed for benzodiazepines on the calibrated Axsym™ analyzer as described in the previous section.

Gas Chromatography/ Mass Spectroscopy of THC-COOH

Preparation of Buffers/Reagents

A solution of 100 mM hydrochloric acid (HCl) was prepared by placing 4.2 mL concentrated HCl (Optima®, Fisher) into a 500 mL volumetric flask and diluting to volume with 18 MOhm deionized water. A solution of 100 mM HCl/acetonitrile (70/30) was prepared by placing 70 mL of 100 mM HCl in a 100 mL volumetric flask and diluting to volume with acetonitrile (Optima®, Fisher). A solution of hexane/ethyl acetate (50/50) was prepared by

placing 25 mL of hexane (Optima®, Fisher) into a 50 mL volumetric flask with a volumetric pipette and diluting to volume with ethyl acetate (Optima®, Fisher). The derivatizing reagent N,O-bis[Trimethylsilyl]trifluoroacetamide (BSTFA, Sigma) was purchased in 1 mL aliquots sealed in a 1.5 mL glass vial.

Preparation of Standards and Specimens

A 500 ng/mL THC-COOH working standard was prepared by pipetting 250 µL of the 100 mcg/mL THC-COOH stock methanol standard into a 50 mL volumetric flask with 500 µL of 200 mM DTT. The flask was diluted to volume with pH 6.2 synthetic urine. A solution of 2 mM DTT was prepared in blank synthetic urine by pipetting 25 µL of the 200 mM DTT stock solution into a 25 mL volumetric flask and diluting to volume with pH 6.2 synthetic urine. A standard curve was prepared by pipetting 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the 500 ng/mL THC-COOH working standard into six 13 x 100 mm glass screw top test tubes that contained 5.0, 4.0, 3.0, 2.0, 1.0, and 0.0 mL of 2 mM DTT in blank pH 6.2 synthetic urine to yield concentrations of 0, 100, 200, 300, 400, and 500 ng/mL THC-COOH. Six replicate adulterated specimens that contained 500 ng/mL THC-COOH and 10 mg/mL papain were prepared by pipetting 5.0 mL of the 500 ng/mL THC-COOH working standard into six 13 x 100 mm glass screw top test tubes that each contained 50 mg of papain. A blank adulterated specimen was also prepared by pipetting 5.0 mL of blank pH 6.2 synthetic urine in a 13 x 100 mm glass screw top test tube that contained 50 mg of papain. All standards and specimens were capped with a teflon screw cap and allowed to remain at room temperature for 24 hours before analysis.

Solid Phase Extraction and Derivatization

All standards and specimens were extracted by solid phase extraction (SPE) according to a method adapted from the United Chemical Technologies (United Chemical Technologies, Bristol, PA) Applications Manual. Just prior to beginning the extraction procedure, 10 µL of the 10 mcg/mL d_3-THC-COOH stock methanol standard was pipetted to every specimen tube with an Eppendorf Repeater® as an internal standard and vortexed. The pH of the standards and specimens was assessed by pHydron™ papers, 4.5-7.5 range (Micro Essential Laboratories, Brooklyn, N.Y.) and were adjusted to pH 6.0 +/- 0.5 with 100 mM (mono-, di-) basic sodium

phosphate as needed. Thirteen SPE cartridges, model ZSDAU020 (United Chemical Technologies) were labeled and placed on a 29 cm x 7.5 cm x 10 cm vacuum apparatus connected to a model DOL-101-AA vacuum pump (Gast, Benton Harbor, MI.). The vacuum pump was adjusted to apply a 25 mmHg negative pressure to the vacuum apparatus. The SPE cartridges were conditioned with 3 mL of methanol (Optima®, Fisher), followed by 3 mL of deionized water, then 1 mL of 100 mM HCl. Standards and specimens were applied to the column at 1 to 2 mL/min. The column was washed with 2 mL of deionized water, followed by 2 mL of 100 mM HCl/acetonitrile (70/30) before being allowed to dry. A 200 mL aliquot of hexane was pipetted into the cartridges before shutting off the vacuum pump and eluting the THC-COOH. The specimens were eluted by gravity into 5 mL conical glass screw top derivatization vials (Sigma) with 3 mL of hexane/ethyl acetate (50/50). The eluate was evaporated to dryness overnight in a laboratory hood.

A 50 µL aliquot of BSTFA was added to every derivatization vial with an Eppendorf Repeater® before being capped with a teflon top and plastic screw cap. The derivatization vials were incubated at room temperature (~ 23 °C) overnight. The derivatized standards and specimens were then transferred with a disposable glass pasteur pipette (Fisher) to 12 x 32 mm glass autosampler vials (National Scientific, Duluth, GA.) containing a glass microsample inserts (National Scientific) supported by an insert compression spring (National Scientific). The derivatized specimens and standards were then capped with a teflon lined screw cap (National Scientific) and placed on the gas chromatograph autosampler for subsequent assay.

GC/MS Parameters

The GC/MS software method “northcsm” (Table 5) was initialized with the following instrument parameters: injection volume, 3 µL; injector temperature, 250 °C; helium flow, 1 mL/min; column temperature program, 200 °C for 0.5 min, then raised at 30 °C/min to 300 °C for 6.5 minutes; GC/MS interface temperature, 250 °C; mass selective ion detector temperature, 250 °C; monitored ions (m/z), THC-COOH: 371, 473, 488, d_3-THC-COOH: 374, 476, 491.

Table 5 Gas chromatography/mass spectroscopy parameters for "northcsm"

Instrument	Hewlett-Packard 5890 gas chromatograph coupled to a 5972 mass selective ion detector
Injection volume	3 μL
Injector temperature	250 °C
Column	30 m x 0.25 mm DB-5 capillary column
Column conditions	200 °C for 0.5 min, then raised at 30 °C/min to 300 °C for 6.5 minutes
Mass selective ion detector	Electronic ionization with 70 eV at 250 °C, and an electron multiplier voltage of 1753mV.
Monitored ions (quantitation)	THC-COOH: (371), 473, 488; d_3-THC-COOH: (374), 476, 491

Figure 7 illustrates the high degree of linearity ($r^2 = 0.998$) of the PAR (371:374) versus THC-COOH (ng/mL) standard curve.

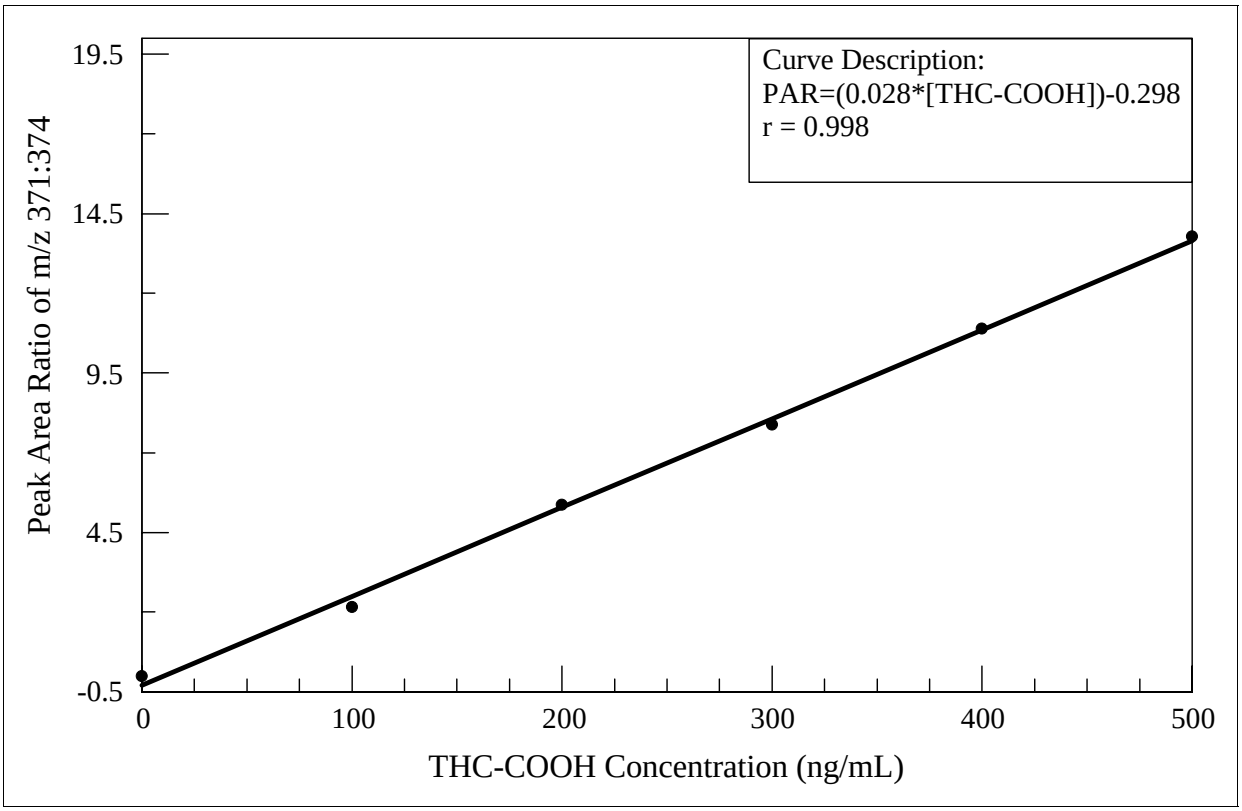

Figure 7 The standard curve of peak area ratio (PAR) versus THC-COOH concentration (ng/mL)

High Pressure Liquid Chromatography/Ultraviolet Detection of Nordiazepam

Preparation of Buffers/Reagents

An ammonium chloride/ammonium hydroxide (NH_4Cl/NH_4OH) buffer, pH 9.2, was prepared by saturating a 50 mL aliquot of 18 MOhm deionized water with ammonium chloride (Fisher), followed by titration to pH 9.2 with concentrated ammonium hydroxide (reagent grade, Fisher) with a digital pH meter. A 100 mM monobasic potassium phosphate (KH_2PO_4) solution was prepared by dissolving 13.61 g of KH_2PO_4 in a one liter volumetric flask that contained 500 mL 18 MOhm deionized water and 5.0 mL of methanol. The flask was then diluted to volume with 18 MOhm deionized water. A 100 mM phosphoric acid solution (H_3PO_4) was prepared by pipetting 6.7 mL of 85% H_3PO_4 (reagent grade, Fisher) into a 1 L volumetric flask and diluting to volume with 18 MOhm deionized water. A 15 mM phosphate buffer, pH 3.3, was prepared by pouring 300 mL of the 100 mM KH_2PO_4 buffer, pH 9.2, into a 500 mL graduated cylinder and adding the contents to a 2 L volumetric flask, then diluting to volume with 18 MOhm deionized water. The 15 mM phosphate buffer was poured into a 5.5 L polypropylene bucket (Nalgene, Fisher) and titrated to pH 3.3 with 16 mL of 100mM H_3PO_4 solution with a digital pH meter. A 107 mL aliquot of 18 MOhm deionized water was added with a 500 mL graduated cylinder to the titrated phosphate buffer obtain a 15 mM phosphate concentration. The HPLC/UV mobile phase was prepared by adding 1.4 L of acetonitrile (Optima®, Fisher) with a 2 L graduated cylinder. The mobile phase was then filtered and de-gassed under vacuum with a 0.45 micron glass microfiber filter (Whatman, Middlesex, U.K.) and placed in a 4-liter brown glass screw top container. A methanol/water (50/50) solution was prepared by placing 500 mL of methanol (Optima®, Fisher) in a 1 L volumetric flask with a graduated cylinder and diluting to volume with 18 MOhm deionized water. The methanol/water (50/50) solution was degassed and filtered under vacuum with a 0.45 micron glass microfiber filter (Whatman) and placed in a 1 L brown glass screw top container. Methanol (Optima®, Fisher) was degassed and filtered under vacuum with a 0.45 micron glass microfiber filter (Whatman) and placed in a 1 L brown glass screw top container.

Preparation of Standards and Specimens

A 500 ng/mL nordiazepam working standard was prepared by pipetting 25 µL of the 1 mg/mL nordiazepam stock methanol standard into a 50 mL volumetric flask that contained 500 µL of 200 mM DTT. The flask was to volume with pH 6.2 synthetic urine. A solution of 2 mM DTT was prepared in blank synthetic urine by pipetting 25 µL of the 200 mM DTT stock solution into a 25 mL volumetric flask and diluting to volume with pH 6.2 synthetic urine. A standard curve was prepared by pipetting 0.0, 2.0, 2.5, 3.0, and 4.0 mL of the 500 ng/mL nordiazepam working standard into five 13 x 100 mm glass screw top test tubes that contained 5.0, 3.0, 2.5, 2.0, and 1.0 mL of 2 mM DTT in pH 6.2 synthetic urine to yield standards of 0, 200, 250, 300, and 400 ng/mL nordiazepam. Six replicate adulterated specimens that contained 300 ng/mL nordiazepam and 10 mg/mL papain were prepared by pipetting 3.0 mL of the 500 ng/mL nordiazepam working standard into six 13 x 100 mm glass screw top test tubes that each contained 50 mg of papain and 2.0 mL of 2mM DTT in pH 6.2 synthetic urine. An adulterated blank specimen that contained 10 mg/mL papain was prepared by placing 50 mg of papain into a 13 x 100 mm glass screw top test tube that contained 5.0 mL of 2 mM DTT in pH 6.2 synthetic urine. All standards and specimens were capped with a teflon screw cap and allowed to incubate at room temperature for 24 hours.

Liquid/Liquid Extraction

A 2 mL aliquot of each specimen and standard was pipetted into new 13 x 100 mm glass screw top test tubes and 10 µL of the 1 mg/mL prazepam internal standard was added to every tube with and Eppendorf Repeater® and vortexed. A 1 mL aliquot of pH 9.2 NH_4Cl/NH_4OH buffer was pipetted into every specimen and vortexed. A 4mL aliquot of butyl chloride (HPLC grade, Fisher) was added to every specimen with an adjustable volume solvent dispenser (Wheaton, Fisher), capped with a teflon screw cap, and placed on a test tube rocker for 15 minutes. The organic phase was transferred with a disposable glass pasteur pipettes (Fisher) to 25 mL conical glass concentration vials (Supelco). The contents of the concentration vials were allowed to evaporate overnight at room temperature under a laboratory hood. The specimens and standards were reconstituted with 50 µL of mobile phase and vortexed for 30 seconds. The reconstituted specimens and standards were then transferred with a disposable glass pasteur

pipette (Fisher) to 2 cm x 12 cm glass autosampler vials (Waters) containing a 0.25 microcentrifuge tube (Fisher) supported by an insert compression spring (Waters). The specimens and standards were then capped with a PTFE lined H-style screw cap (Waters) and placed on the HPLC autosampler for subsequent assay.

HPLC/UV Operation and Assay Parameters

The radial compression column was conditioned with methanol (Optima®, Fisher) at 2 mL/min for 10 minutes, followed by a solution of methanol/water (50/50) at 2 mL/min for 10 minutes, followed by the prepared mobile phase at 2 mL/min for 10 minutes. The autosampler was programmed to inject 20 µL of sample with a run time of 25 minutes and a 1 minute purge between samples. The flow rate of the mobile phase was adjusted to 2.5 mL/minute. The ultraviolet detector was adjusted to 254 nm. The data module was programmed as follows: attenuation, 32; chart speed, 0.5; TFN=PM=1; PT EVAL, <250; Dialog: enable, baseline drawing-N; storage menu-N; function number-O; file name, benzos; TT, 25; TF, ER; TV, 1; <return><return> (Table 6).

Table 6 High performance liquid chromatography/ultraviolet detection parameters

Instrument	Waters M45 solvent delivery system coupled to a Lambda-Max UV detector and a 717 autosampler
Injection volume	20 µL every 26 minutes
Flow rate	2.5 mL/min
Column	8 mm x 100 mm NOVA-PAK C18-4 micron radial compression
Ultraviolet detector	254 nm
Data module	Attenuation = 32 chart speed = 0.5 TFN = PM = 1 PT EVAL < 250 dialog: enable baseline drawing = N storage menu = N function number = O file name = benzos TT = 25 TF = ER TV = 1 <return><return>

Figure 8 illustrates the high degree of linearity ($r^2 = 0.997$) of the PAR (nordiazepam:prazepam) versus nordiazepam (ng/mL) standard curve.

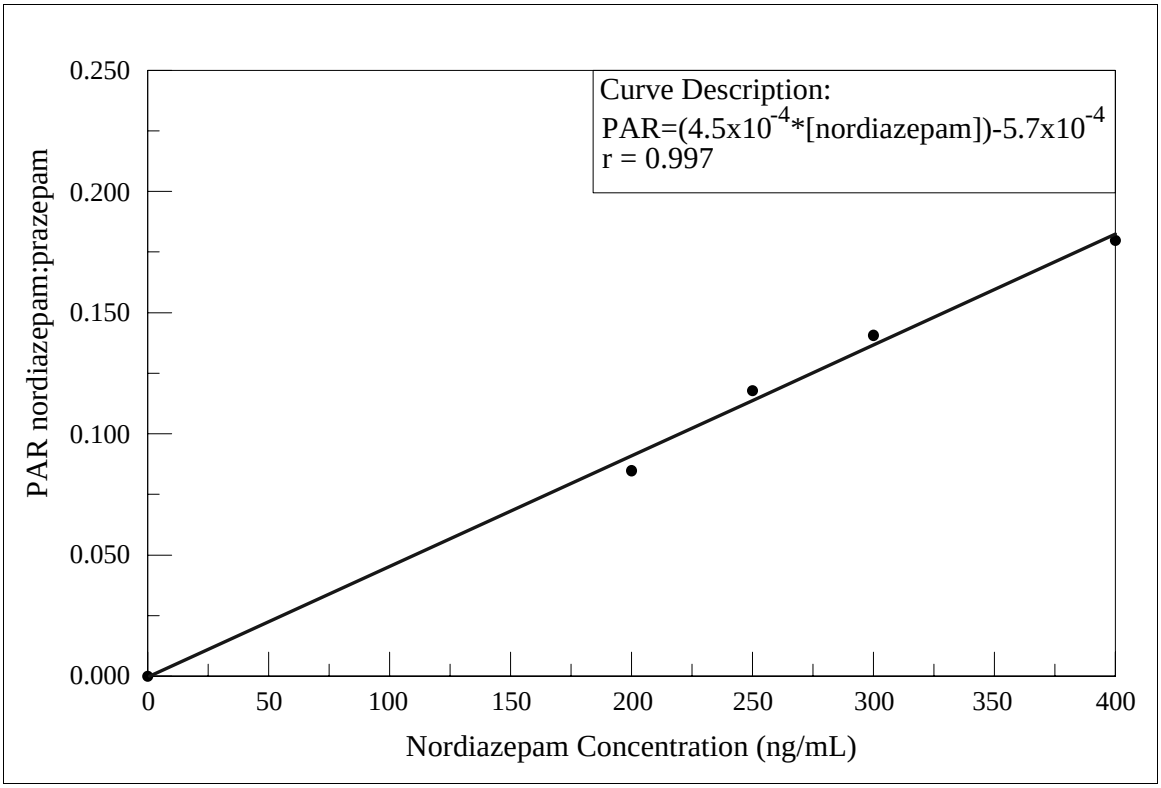

Figure 8 The standard curve of peak area ratio (PAR) versus nordiazepam concentration (ng/mL)

Specimen Validity Testing

An Institutional Review Board (IRB) from East Tennessee State University and the Veterans' Affair Medical Center approved the following research protocol for the investigation of papain's effects on parameters of specimen validity testing. To examine the effect of papain on a population of urine specimens with respect to creatinine, pH, osmolality, and specific gravity, 30 urine specimens were randomly selected from our specimen refrigerator. The specimens were residual quantities that remained after other unrelated clinical assays were performed. All patient identifiers were removed and the specimens were arbitrarily numbered from 1 to 30. For osmolality, pH, creatinine, and specific gravity assays, specimens 1-30 were

pipetted into thirty 1.5 mL flat top microcentrifuge tubes. Paired adulterated specimens that contained 10 mg/mL papain were prepared by pipetting 1 mL of the unadulterated specimens into thirty 1.5 mL flat top microcentrifuge tubes that contained 10 mg of papain. All 60 specimens were assayed immediately for creatinine, specific gravity, pH and osmolality as described below. Six urine specimens that encompassed the range of creatinine, specific gravity, pH and osmolality values observed out of the 30 random urine specimens measured, were selected to undergo time course experiments. The six specimens, unadulterated and adulterated with 10 mg/mL papain, were prepared by pipetting 1 mL of each specimen into a 1.5 mL flat top microcentrifuge tube and a 1.5 mL flat top microcentrifuge tube that contained 10 mg of papain. The twelve specimens were assayed for creatinine, osmolality, pH, and specific gravity, then capped and allowed to remain at room temperature (~23 °C) for 6 hours before being re-assayed for each parameter. The specimens were then refrigerated (4 °C) and re-assayed for creatinine, osmolality, pH, and specific gravity 72 hours after their initial preparation. The effect of 10 mg/mL papain on urine specimens with respect to nitrite concentration and the presence of glutaraldehyde and oxidants was measured in a subset of 6 urine specimens that encompassed the range of observed specific gravity values out of the original 30 random urine specimen measured.

Creatinine

The following method was adapted from a previously published method to quantitate urine creatinine (Kroll and others, 1986). The software program "Kinetics" was opened and the wavelength adjusted to 520nm. A 151 mM sodium hydroxide (NaOH) stock solution was prepared by placing 302 mg of sodium hydroxide (Fisher) in a 50 mL volumetric flask and diluting to volume with 18 MOhm deionized water. A 50 mL alkaline picric acid working solution was prepared by pipetting 9.2 mL of saturated (1.3% w/v) picric acid solution (Sigma) into a 50 mL volumetric flask that contained 20 mL of the 151 mM NaOH stock solution and diluting to volume with 18 MOhm deionized water. The Cary UV/Vis spectrophotometer was turned on and allowed to stabilize for 1 hour. The reference cuvette consisted of 360 µL of the alkaline picric acid working solution and 9 µL of 18 MOhm deionized water pipetted into a 1-cm matched quartz cuvette (Fisher).

A standard curve was constructed with 0, 3, 7, and 23 mg/dL creatinine standards (Sciteck Diagnostics, Raleigh, NC.) and two quality control specimens were prepared with 5 and 12 mg/dL creatinine standards (Scitek). The sample cell contained of 360 µL of the alkaline picric acid working solution. Just prior to recording absorbance data, 9 µL of standard, quality control, or urine specimen was pipetted into the sample cuvette and mixed by repeated pumping of the pipette plunger. The absorbance of the picric acid chromaphor being evolved was measured for 30 seconds and the rate of absorbance change (delta-mabs/min) was obtained. Specimens that produced an absorbance change greater than the rate of absorbance change of the 23 mg/dL creatinine standard were diluted accordingly with 18 MOhm deionized water and re-assayed. Figure 9 depicts a typical standard curve of creatinine analysis by UV/Vis spectroscopy and illustrates the high degree of linearity of the creatinine standard curve with a correlation coefficient(r^2) of 0.999. The 5.0 and 12.0 mg/dL creatinine quality control specimens yielded values of 4.45 and 11.4 mg/dL creatinine.

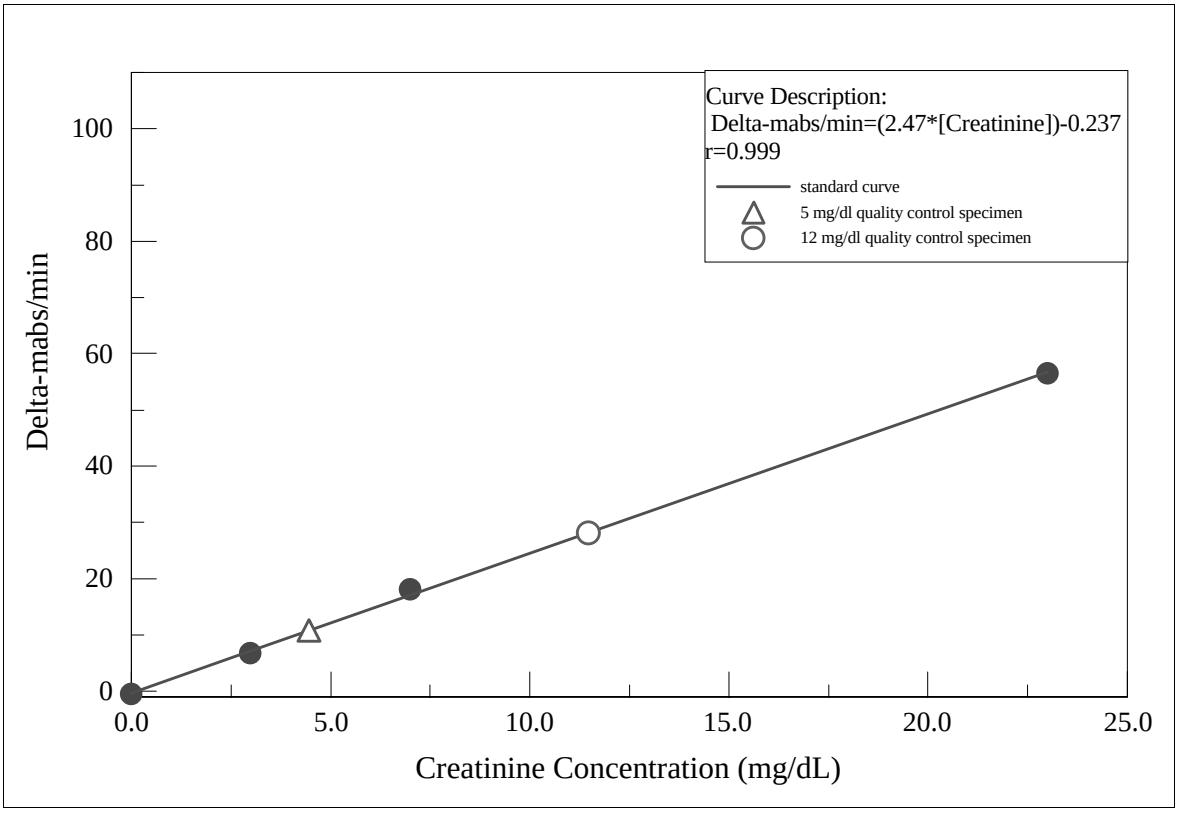

Figure 9 A typical standard curve of urine creatinine analysis by UV/Vis spectroscopy

Specific Gravity

The Sper scientific model 300026 digital refractometer was turned on and calibrated with distilled water (Sper). A quality control specimens consisted of 1.002, 1.005, and 1.030 specific gravity standards (Scitek). Specific gravity of the standards and the prepared urine specimens was measured directly by placing 3 drops of standards or specimens on the optical sample plate with a polypropylene disposable pipette and pressing the “mes” button.

pH

The Beckmann model 4500 digital pH meter was calibrated with pH 7.0 and 4.0 calibrators (Fisher) and the temperature knob was adjusted to the current room temperature. Specimen pH was measured directly by placing the pH electrode into the 1.5 mL flat top microcentrifuge tube that contained the aliquoted unadulterated and adulterated specimens.

Osmolality

The Advanced Digmatic® osmometer was calibrated with 100 and 900 mOsm/L calibration standards (Advanced Instruments) and the calibration was confirmed with a 200 mOsm/L quality control standard (Advanced Instruments). The osmolality of the calibrators, quality control and prepared urine specimens were measured directly by pipetting 200 µL of the calibrator, quality control or prepared urine specimens into the disposable plastic osmometer sample cup that was placed in the osmometer and pressing the “read” button.

Nitrates, Glutaraldehyde, and Oxidants

MASK tri-level controls (Kacey Incorporated, Asheville, NC.) were placed on 3 individual MASK test strips and their absorbent pads were compared to the reference chart printed on the test strip container. Six urine specimens that encompassed the range of specific gravity values observed out of the 30 random urine specimens measured, were selected. The 6 specimens, unadulterated and adulterated with 10 mg/mL papain, were prepared by pipetting 1 mL of each specimen into a 1.5 mL flat top microcentrifuge tube and a 1.5 mL flat top microcentrifuge tube that contained 10 mg of papain. The 12 specimens and control solutions were placed on individual MASK test strips with a disposable polypropylene pipettes

immediately after preparation. The MASK test strips were then compared to the reference chart for the presence of nitrite, oxidants, and glutaraldehyde.

CHAPTER 3
CALCULATIONS AND STATISTICS

Papain Standardization

The rate of absorbance change (delta-mabs/min) of each RP standard was plotted against its respective RP standard concentration (U/mL), and a linear regression was performed to yield a standard curve with a calculated slope (m), intercept (b), and correlation coefficient (r). The CLP concentrations ((C), U/mL) were obtained from interpolation (C=((rate of absorbance change)-b)/m) of the standard curve. The CLP activity (U/mg) was obtained by dividing the interpolated CLP concentration (U/mL) by the concentration of the prepared CLP solutions (mg/mL). An average (arithmetic mean) and standard deviation (SD) were calculated from the 4 prepared CLP solutions.

Enzyme Multiplied Immunoassay Technique

The ETS™ analyzer was calibrated daily in accordance with the standard operating procedure published by Syva Dade-Behring. A chi squared analysis was performed to compare the data obtained between the unadulterated control group and the group adulterated with 10 mg/mL papain. Groups were denoted as being significantly different if the comparison yielded a p-value <0.01.

Fluorescence Polarized Immunoassay Technique

THC-COOH Assays

For each concentration of THC-COOH in pH 4.5, 6.2, and 8.0 synthetic urine, an analysis of variance (ANOVA) was performed to compare the mean values obtained between the unadulterated control group and the groups containing 0.5, 1, 2, 5, and 10 mg/mL papain within each time point. These were designated as the “intra-temporal” ANOVAs. Paired sample ANOVAs were also performed to compare the mean values obtained between the groups containing 0, 0.5, 1, 2, 5, and 10 mg/mL papain at baseline (T=0 hours) and their respective group containing 0, 0.5, 1, 2, 5, and 10 mg/mL papain at the latter time points (T= 4, 6, 24, and

72 hours). These were designated as the "inter-temporal" ANOVAs. A significant effect was attributed to papain only if both the inter-temporal and intra-temporal ANOVAs yielded a p-value <0.01. The minimum percent decrease, less the decrease in the unadulterated control, was calculated by subtracting the adulterated group that exhibited the least effect from its own baseline value, then subtracting the value obtained from the difference of the unadulterated control at the same time point and its baseline value. The overall difference was divided by the initial THC-COOH concentration and multiplied by 100 to obtain a percentage difference. In addition, the adulterated groups that yielded THC-COOH concentration values < 50 ng/mL were noted to summarize the results of each set of FPIA assays for the purposes of discussion. The effects of papain concentration, time, THC-COOH concentration, and pH were compared were calculated as described below only with the experimental groups that had a significant effect attributed to papain.

The Effect of Papain Concentration, Time, Initial THC-COOH Concentration, and pH on the Percent Decrease of THC-COOH. The percent decrease in THC-COOH concentration after 72 hours, less the THC-COOH concentration decrease in the unadulterated control, in specimens adulterated with 0.5 to 10 mg/mL papain in pH 6.2 synthetic urine were plotted versus their respective initial THC-COOH concentration. ANOVAs were performed to compare the mean values between the papain concentration groups within each initial THC-COOH concentration group. The percent decrease in THC-COOH concentration after 4, 24, and 72 hours, less the THC-COOH concentration decrease in the unadulterated control, in specimens adulterated with 10 mg/mL papain in pH 6.2 synthetic urine were plotted versus their respective initial THC-COOH concentrations. Paired sample ANOVAs were performed to compare the mean values between incubation times within a particular THC-COOH concentration group. The percent decrease in THC-COOH concentration after 72 hours, less the THC-COOH concentration decrease in the unadulterated control, in specimens adulterated with 10 mg/mL papain in pH 6.2 synthetic urine were plotted versus their respective initial THC-COOH concentrations. ANOVAs were performed compare the mean values between the initial THC-COOH concentration groups. The percent decrease in THC-COOH concentration after 72 hours, less the THC-COOH concentration decrease in the unadulterated control, in specimens

adulterated with 10 mg/mL papain in pH 4.5, 6.2, and 8.0 synthetic urine were plotted versus their respective initial THC-COOH concentration. ANOVAs were performed compare the mean values between the papain concentration groups within each initial THC-COOH concentration group. In every comparison chart, significant differences between mean values were established with $p<0.01$.

FPIA Assays for Other Drugs of Abuse

For each set of amphetamine, barbiturate, benzodiazepine, cocaine, opiate, and phencyclidine assays an "intra-temporal" ANOVA was performed to compare the values obtained between the unadulterated control group and the group adulterated with 10 mg/mL for each time point. A paired sample "inter-temporal" ANOVA was also performed between the groups containing 0 and 10 mg/mL papain and their respective group containing 0 and 10 mg/mL papain at the latter time points (T= 6 and 72 hours). A significant effect was attributed to papain only if both the inter-temporal and intra-temporal ANOVAs yielded a p-value <0.01.

BANI Assay of Inhibited RP Enzymatic Activity

The absorbance change (delta-mabs) in 4 minutes of each RP and DRP specimen was averaged and compared with the absorbance change of the blank specimen. The absorbance change (delta-mabs) in 4 minutes of each RP specimens was compared with the limit of detection (1 delta-mabs/min) for enzyme activity.

FPIA Assays with RP and DRP

For each set of cannabinoid and benzodiazepine assays an ANOVA was performed to compare the values between the unadulterated control group and each group adulterated with 1 mg/mL RP and with 1 mg/mL DRP. An ANOVA was also performed to compare the values between the group adulterated with 1 mg/mL RP and the group adulterated with 1 mg/mL DRP. Groups were denoted as being significantly different, if the ANOVA yielded a p-value <0.01.

Gas Chromatography/Mass Spectroscopy of THC-COOH

Quantitation ions for THC-COOH (m/z 371) and d_3-THC-COOH (m/z 374) co-eluted at 6.44 minutes and were integrated by the RTE integrator to yield peak areas of each ion. The quotients of the peak areas were calculated into peak area ratios (PAR) of ions 371 : 374. The PAR of each standard was plotted against its respective concentration (ng/mL THC-COOH) and a linear regression was performed to yield a standard curve with a given slope (m), intercept (b), and correlation coefficient (r). Concentrations (C) of the adulterated specimens were obtained from interpolation (C=(PAR-b)/m) of the standard curve.

High Pressure Liquid Chromatography/Ultraviolet Detection of Nordiazepam

Nordiazepam eluted at 5.01 minutes, 0.2343 relative retention time to the internal standard prazepam that eluted at 21.4 minutes. Peak areas of each peak were obtained by the integration on the 746 data module. The peak areas were then quotiented into a PAR of nordiazepam : prazepam. The PAR of each standard was plotted against its respective concentration (ng/mL nordiazepam) and a linear regression was performed to yield a standard curve with a given slope (m), intercept (b), and correlation coefficient (r). Concentrations (C) of the adulterated specimens were obtained from interpolation (C=(PAR-b)/m) of the standard curve.

THC-COOH Binding Plots

The quantities of bound THC-COOH (ng/mg of papain), B, were plotted versus the concentration of free THC-COOH, $THC\text{-}COOH_{free}$ in an attempt to elucidate a potential mechanism of the interference of papain on the concentration of THC-COOH as measured by FPIA. Bound THC-COOH was defined as the difference between the mean concentration of THC-COOH in the control group less the mean concentration of THC-COOH in the adulterated group after 24 hours. The free concentration of THC-COOH was defined as the reported concentration of THC-COOH at 24 hours. A linear regression was performed to yield a line with a calculated slope of k_{obs}.

Creatinine

The rate of absorbance change (delta-mabs/min) of each creatinine standard was plotted against its respective creatinine standard concentration (mg/dL), and a linear regression was performed to yield a standard curve with a given slope (m), intercept (b), and correlation coefficient (r). The creatinine concentrations (mg/dL) of the prepared urine and quality control specimens were obtained from interpolation of the standard curve. A paired t-test was performed to compare the creatinine values obtained from unadulterated group and the group adulterated with 10 mg/mL papain in the population of 30 urine specimens. Groups were denoted as being significantly different, if the t-test yielded a p-value <0.05. A paired t-test was performed to compare the creatinine values obtained from the unadulterated group and the group adulterated with 10 mg/mL papain in the subpopulation of 6 urine specimens at each time point (T=0, 6, and 72 hours). Groups were denoted as being significantly different if the t-test yielded a p-value <0.05.

Specific Gravity

A paired t-test was performed to compare the specific gravity values obtained from unadulterated group and the group adulterated with 10 mg/mL papain in the population of 30 urine specimens. Groups were denoted as being significantly different if the t-test yielded a p-value <0.05. A paired t-test was performed to compare the specific gravity values obtained from the unadulterated group and the group adulterated with 10 mg/mL papain in the subpopulation of 6 urine specimens at each time point (T=0, 6, and 72 hours). Groups were denoted as being significantly different if the t-test yielded a p-value <0.05.

pH

A paired t-test was performed to compare the pH values obtained from unadulterated group and the group adulterated with 10 mg/mL papain in the population of 30 urine specimens. Groups were denoted as being significantly different, if the t-test yielded a p-value <0.05. A paired t-test was performed to compare the pH values obtained from the unadulterated group and the group adulterated with 10 mg/mL papain in the sub-population of 6 urine specimens at each

time point (T=0, 6, and 72 hours). Groups were denoted as being significantly different if the t-test yielded a p-value <0.05.

Osmolality

A paired t-test was performed to compare the osmolality values obtained from unadulterated group and the group adulterated with 10 mg/mL papain in the population of 30 urine specimens. Groups were denoted as being significantly different if the t-test yielded a p-value <0.05. A paired t-test was performed to compare the osmolality values obtained from the unadulterated group and the group adulterated with 10 mg/mL papain in the sub-population of 6 urine specimens at each time point (T=0, 6, and 72 hours). Groups were denoted as being significantly different if the t-test yielded a p-value <0.05.

Nitrates, Glutaraldehyde, and Oxidants

Chi square analyses were performed to compare the data obtained between the unadulterated group and the group adulterated with 10 mg/mL papain in the population of 6 urine specimens. Groups were denoted as being significantly different if the chi square analyses yielded a p-value <0.05.

CHAPTER 4

RESULTS

Papain Standardization

Figure 10 illustrates a recrystallized papain (RP) enzyme activity standard curve with a correlation coefficient of 0.998. The rate of absorbance change was a maximum of 5 mabs/min in an absorbance range of approximately 0.100 abs. The linearity of the BANI assay for RP activity suggests the limitations of the assay were not exceeded in terms of a maximum UV absorbance of 2000 mabs or depletion of the BANI substrate.

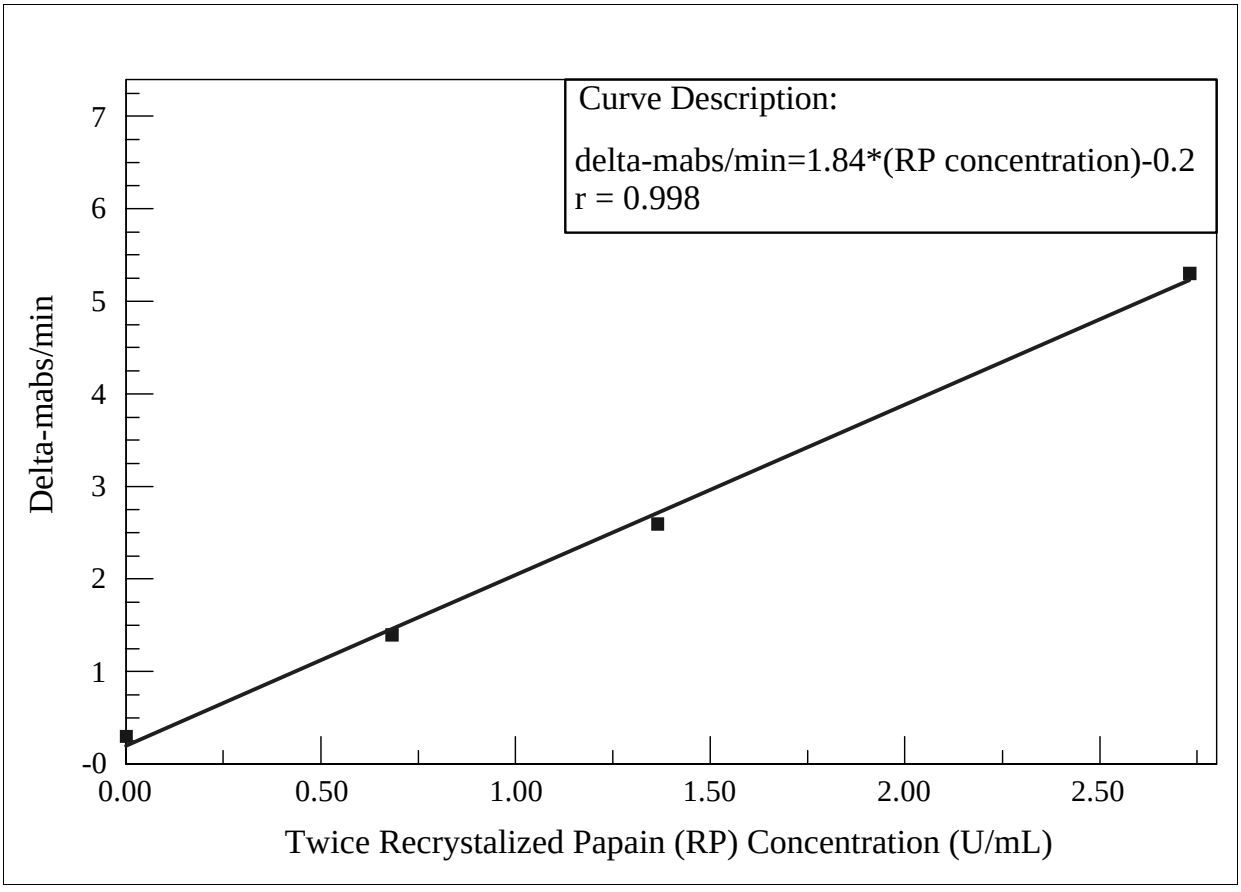

Figure 10 Standard curve of the rate of absorbance change (delta-mabs/min) versus the concentration (U/mL) of twice recrystallized papain

The third column in Table 7 lists the average activity of CLP interpolated from the standard curve in Figure 10 was 0.912 (+/- 0.050) U/mg. The coefficient of variation of the results from the CLP standardization assays was 5.4%.

Table 7 The activity of crude latex papain

Concentration of prepared CLP (mg/mL)	Activity interpolated from the standard curve (U/mL)	Activity interpolated from the standard curve (U/mg)	
1.25	1.08		0.869
2.5	2.17		0.869
5.0	4.72		0.945
10.0	9.66		0.966
		Average	0.912
		SD	0.050

Synthetic Urine

The osmolality of the synthetic urine was 627 (+/- 1) mOsm/kg and within the range of normal urine osmolality of 500-800 mOsm (Tietz 2001). The particular preparation for synthetic urine adapted for our research is currently used by Pacific Northwest Laboratories for method validation and is cited by the United States Department of Commerce as a valid matrix to conduct laboratory research (PNL-6490 1988).

Enzyme Multiplied Immunoassay Technique

The results given in Table 8 of the EMIT cannabinoid assays indicate a significant difference, $p<0.01$, between the control group of unadulterated specimens and specimens adulterated with 10 mg/mL papain.

Table 8 Results of EMIT analysis of specimens adulterated with papain

Group	Results	
	Negative	Positive
Unadulterated, n=6		6
Adulterated, n=6, 10 mg/mL papain*	6	

* Significant difference, $p<0.01$

Fluorescence Polarized Immunoassay Technique

THC-COOH Assays

Figures 11-15 and Tables 9-13 depict the effect of papain (0-10 mg/mL) on various concentrations of THC-COOH (25-500 ng/mL) over time (0-72 hours) in pH 6.2 synthetic urine as measured by FPIA analyses.

The overall data in Figure 11 and Table 9 illustrate a minimum 24% decrease, less the decrease in the unadulterated control, in THC-COOH concentration in almost every group of adulterated specimens with various papain concentrations at 4, 6, 24, and 72 hours. The cannabinoid assays at 72 hours revealed an apparent 31% increase in THC-COOH concentration for specimens adulterated with 0.5 and 1.0 mg/mL papain. There were no adulterated groups that yielded a THC-COOH concentration > 50 ng/mL, indicating a false positive result over 72 hours.

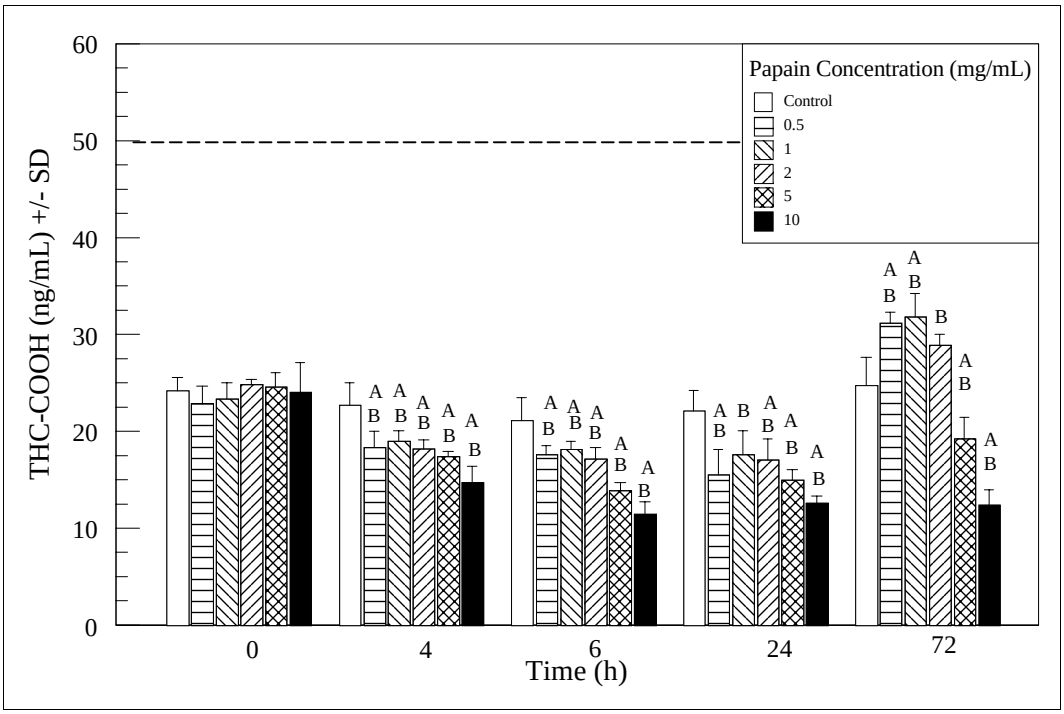

Figure 11 Effects of papain on measured THC-COOH (25 ng/mL) over time in pH 6.2 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 9 The average concentration of THC-COOH in pH 6.2 synthetic urine containing 25 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL), n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	24.15	1.41		
	0.5	22.84	1.85		
	1.0	23.36	1.65		
	2.0	24.81	0.54		
	5.0	24.61	1.42		
	10.0	24.00	3.10		
4	0.0	22.71	2.30		
	0.5	18.34	1.66	X	X
	1.0	18.96	1.11	X	X
	2.0	18.23	0.85	X	X
	5.0	17.37	0.53	X	X
	10.0	14.73	1.63	X	X
6	0.0	21.10	2.39		
	0.5	17.59	0.98	X	X
	1.0	18.16	0.78	X	X
	2.0	17.18	1.14	X	X
	5.0	13.92	0.78	X	X
	10.0	11.47	1.26	X	X
24	0.0	22.06	2.14		
	0.5	15.45	2.64	X	X
	1.0	17.56	2.48	X	X
	2.0	17.00	2.22	X	X
	5.0	14.96	1.07	X	X
	10.0	12.58	0.75	X	X
72	0.0	24.69	2.92		
	0.5	31.16	1.11	X	X
	1.0	31.80	2.43	X	X
	2.0	28.87	1.14	X	X
	5.0	19.25	2.24	X	X
	10.0	12.35	1.59	X	X

The overall data in Figure 12 and Table 10 illustrated a minimum 16% decrease, less the decrease in the unadulterated control, in THC-COOH concentration in every group of adulterated specimens with various papain concentrations at 4, 24 and 72 hours. Groups adulterated with 1.0, 5.0, and 10 mg/mL papain after 4 hours; and all adulterated groups after 24 and 72 hours yielded an average THC-COOH concentration < 50 ng/mL, indicating false negative results.

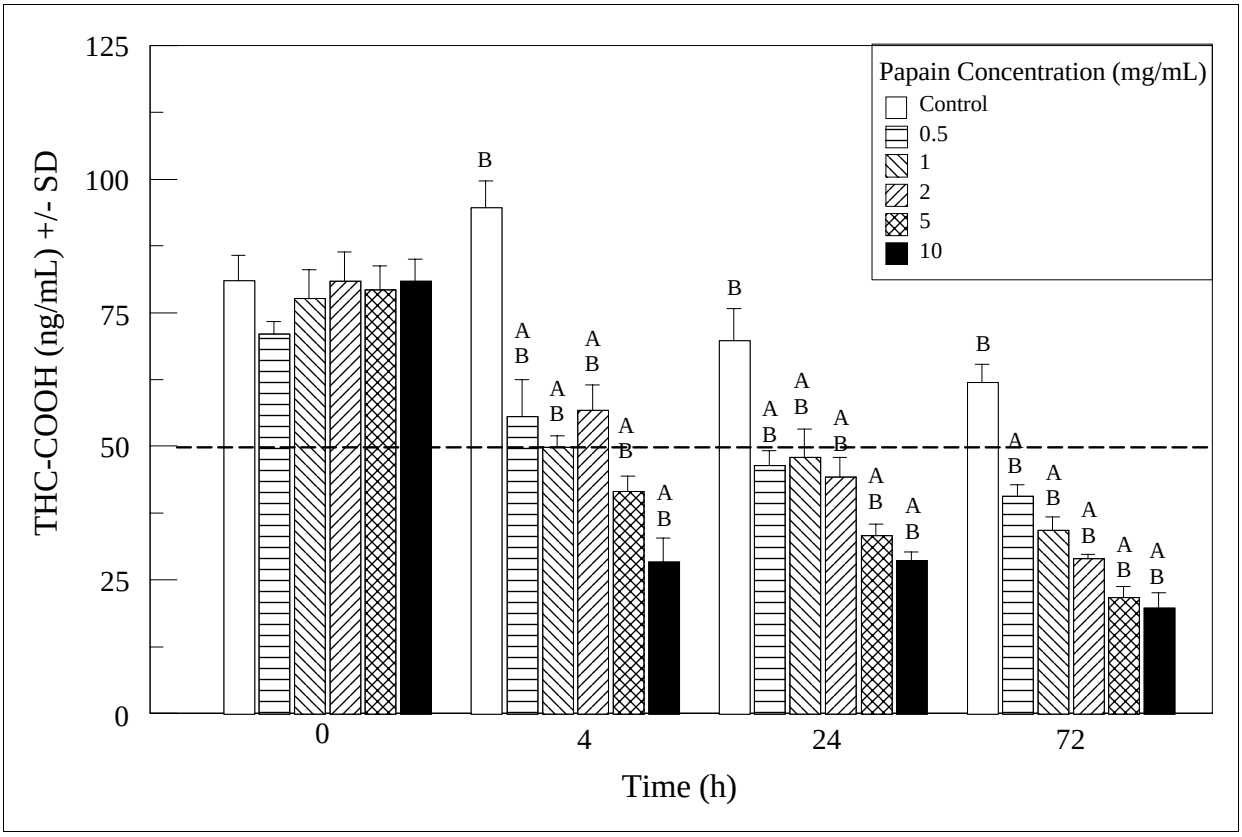

Figure 12 Effects of papain on measured THC-COOH (75 ng/mL) over time in pH 6.2 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------

Table 10 The average concentration of THC-COOH in pH 6.2 synthetic urine containing 75 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	80.99	4.86		
	0.5	71.10	2.23		
	1.0	77.76	5.27		
	2.0	80.87	5.54		
	5.0	79.31	4.38		
	10.0	80.87	4.21		
4	0.0	94.58	5.10		X
	0.5	55.61	6.92	X	X
	1.0	49.73	2.24	X	X
	2.0	56.68	4.79	X	X
	5.0	41.48	3.00	X	X
	10.0	28.35	4.52	X	X
24	0.0	69.80	6.01		X
	0.5	46.41	2.82	X	X
	1.0	47.87	5.39	X	X
	2.0	44.28	3.57	X	X
	5.0	33.31	2.13	X	X
	10.0	28.69	1.57	X	X
72	0.0	61.92	3.52		X
	0.5	40.61	2.20	X	X
	1.0	34.32	2.48	X	X
	2.0	28.89	0.86	X	X
	5.0	21.70	2.03	X	X
	10.0	19.72	2.81	X	X

The overall data in Figure 13 and Table 11 indicate that papain contributed to a significant effect on 100 ng/mL THC-COOH in pH 6.2 synthetic urine over time. There was a minimum 7% decrease, less the decrease in the unadulterated control, in THC-COOH concentration in every group of adulterated specimens with various papain concentrations at 4, 24 and 72 hours. Groups adulterated with 5.0 and 10 mg/mL papain after 4 hours; 2.0, 5.0, and 10 mg/mL papain after 24 hours; and all adulterated groups after 72 hours yielded an average THC-COOH concentration < 50 ng/mL, indicating false negative results.

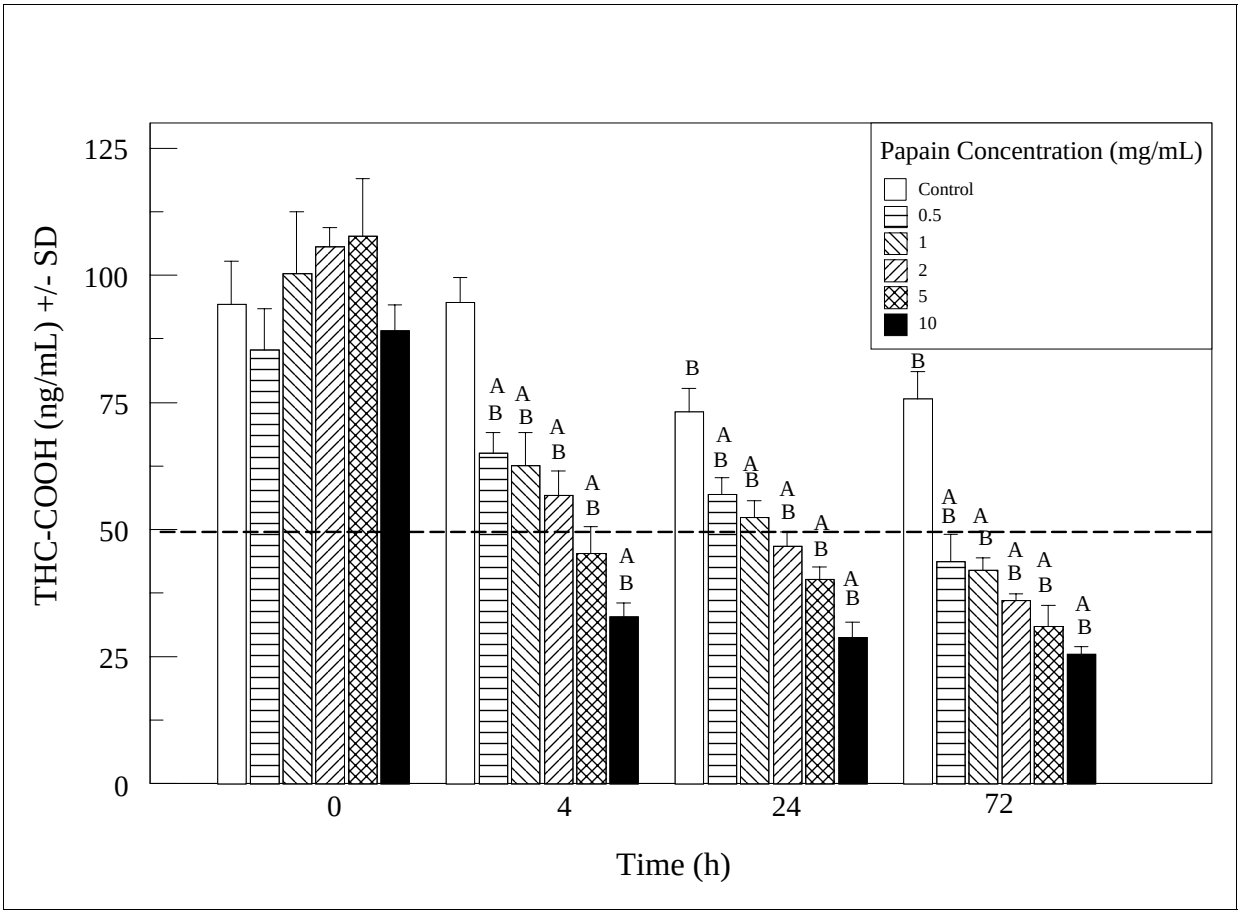

Figure 13 Effects of papain on measured THC-COOH (100 ng/mL) over time in pH 6.2 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 11 The average concentration of THC-COOH in pH 6.2 synthetic urine containing 100 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	94.41	8.39		
	0.5	85.27	8.15		
	1.0	100.43	12.14		
	2.0	105.57	3.89		
	5.0	107.73	11.23		
	10.0	89.12	5.03		
4	0.0	94.58	5.10		
	0.5	65.05	3.99	X	X
	1.0	62.57	6.44	X	X
	2.0	56.68	4.79	X	X
	5.0	45.35	5.21	X	X
	10.0	32.74	2.72	X	X
24	0.0	73.13	4.69		X
	0.5	56.83	3.28	X	X
	1.0	52.40	3.22	X	X
	2.0	46.72	2.86	X	X
	5.0	40.24	2.36	X	X
	10.0	28.78	3.04	X	X
72	0.0	75.62	5.42		X
	0.5	43.58	5.51	X	X
	1.0	42.00	2.40	X	X
	2.0	35.97	1.33	X	X
	5.0	30.78	4.24	X	X
	10.0	25.48	1.52	X	X

The overall data in Figure 14 and Table 12 indicate that papain contributed to a significant effect on 250 ng/mL THC-COOH in pH 6.2 synthetic urine over time. There was a minimum 23% decrease, less the decrease in the unadulterated control, in THC-COOH concentration in almost every group of adulterated specimens with various papain concentrations at 4, 6, 24 and 72 hours. Only the group adulterated with 10 mg/mL papain after 72 hours yielded an average THC-COOH concentration < 50 ng/mL, indicating a false negative result.

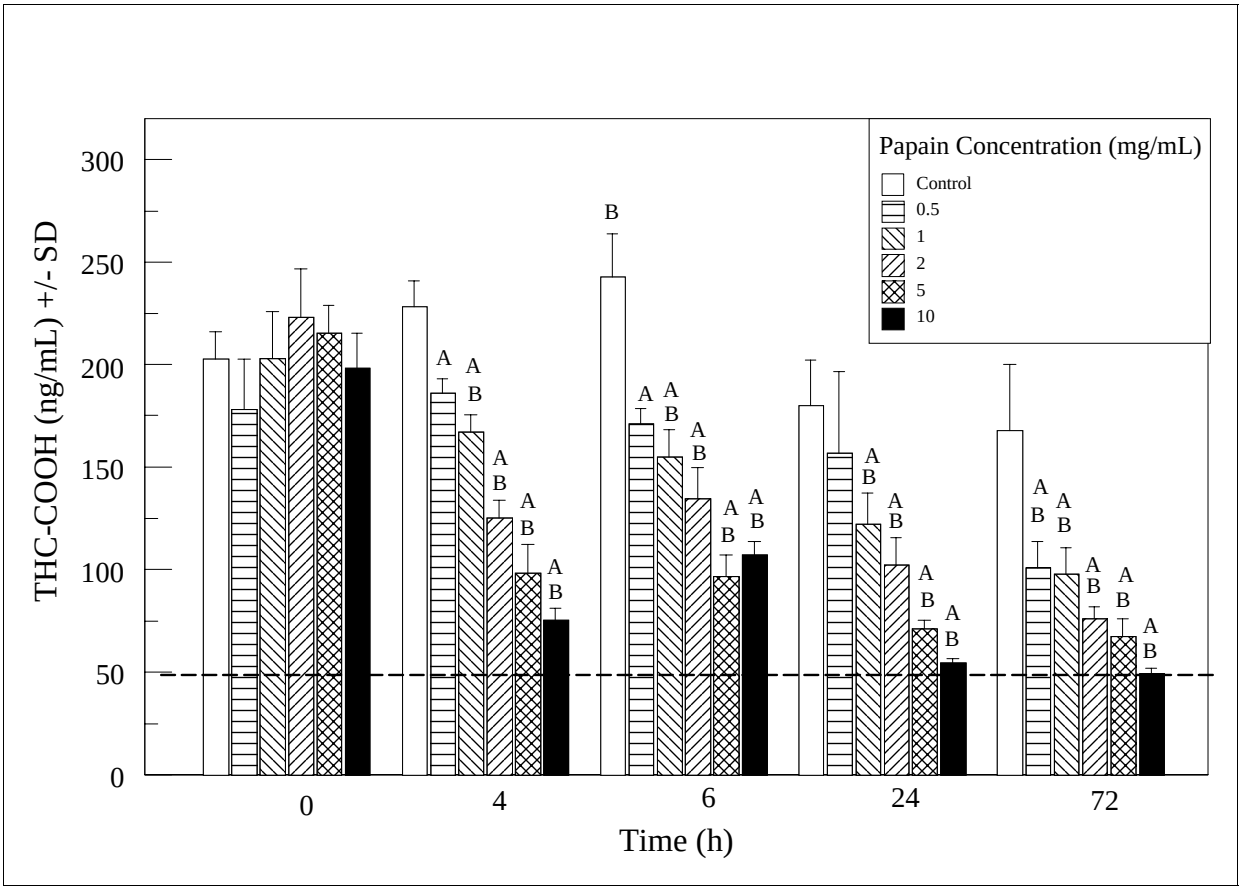

Figure 14 Effects of papain on measured THC-COOH (250 ng/mL) over time in pH 6.2 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 12 The average concentration of THC-COOH in pH 6.2 synthetic urine containing 250 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	202.60	13.47		
	0.5	178.23	24.36		
	1.0	203.09	22.91		
	2.0	222.78	23.97		
	5.0	215.19	13.79		
	10.0	198.03	16.99		
4	0.0	228.01	12.57		
	0.5	186.03	7.11	X	X
	1.0	167.18	8.43	X	X
	2.0	125.08	8.79	X	X
	5.0	98.11	14.29	X	X
	10.0	75.12	5.81	X	X
6	0.0	242.43	21.33		X
	0.5	171.00	7.64	X	X
	1.0	154.65	13.58	X	X
	2.0	134.45	15.22	X	X
	5.0	96.64	10.50	X	X
	10.0	106.93	6.61	X	X
24	0.0	179.92	22.22		
	0.5	156.51	40.34		X
	1.0	122.26	15.22	X	X
	2.0	102.09	13.43	X	X
	5.0	71.23	4.07	X	X
	10.0	54.38	2.41	X	X
72	0.0	167.89	32.21		
	0.5	100.76	13.05	X	X
	1.0	97.89	12.71	X	X
	2.0	75.78	6.05	X	X
	5.0	67.55	8.44	X	X
	10.0	49.31	2.39	X	X

The overall data in Figure 15 and Table 13 indicate that papain contributed to a significant effect on 500 ng/mL THC-COOH in pH 6.2 synthetic urine over time. There was a minimum 16% decrease, less the decrease in the unadulterated control, in THC-COOH concentration in almost every group of adulterated specimens with various papain concentrations at 4, 6, 24, and 72 hours. There were no adulterated groups that yielded an average THC-COOH concentration < 50 ng/mL over 72 hours.

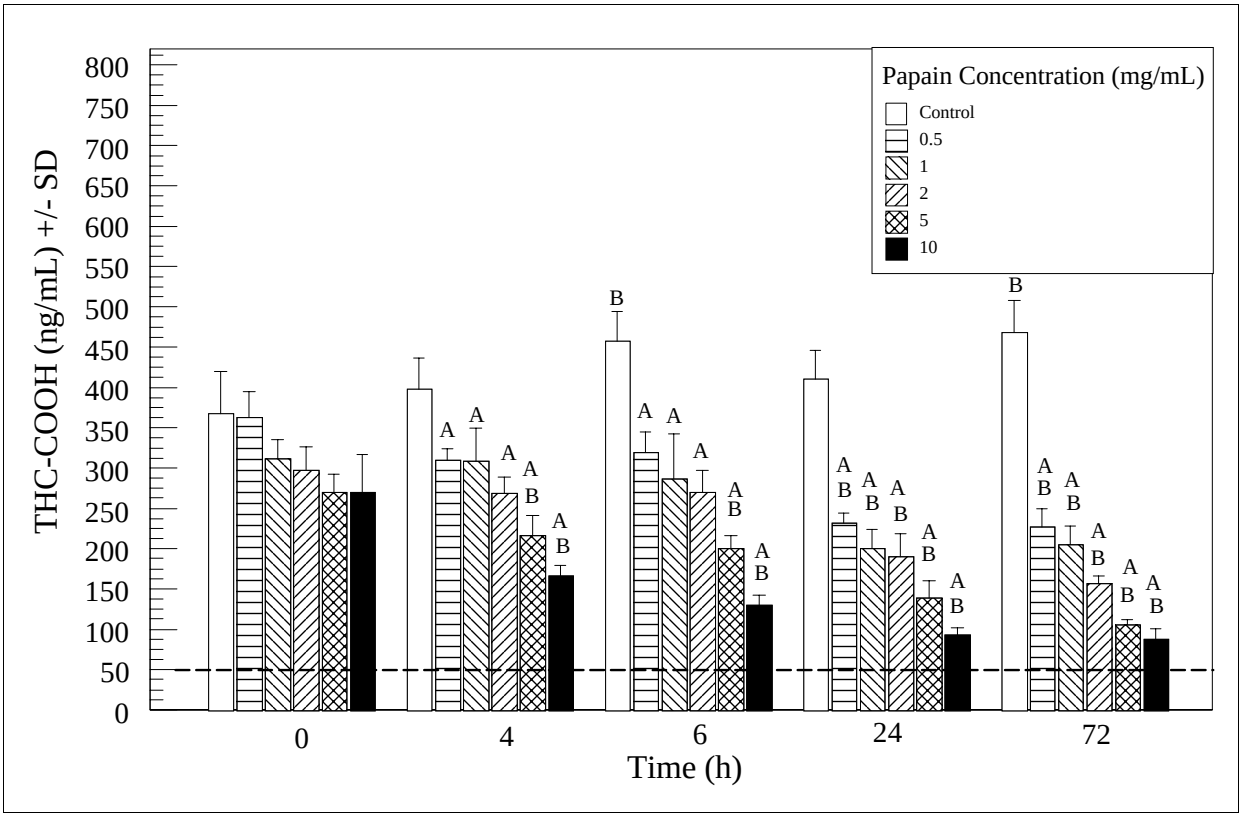

Figure 15 Effects of papain on measured THC-COOH (500 ng/mL) over time in pH 6.2 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 13 The average concentration of THC-COOH in pH 6.2 synthetic urine containing 500 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	367.49	52.33		
	0.5	362.22	32.38		
	1.0	311.41	23.94		
	2.0	296.83	30.05		
	5.0	269.28	23.25		
	10.0	269.56	46.98		
4	0.0	397.33	39.54		
	0.5	309.16	14.81	X	
	1.0	308.13	40.92	X	
	2.0	268.91	19.30	X	
	5.0	216.05	25.42	X	X
	10.0	166.18	13.62	X	X
6	0.0	457.23	37.05		X
	0.5	318.86	25.46	X	
	1.0	286.54	55.65	X	
	2.0	269.93	26.78	X	
	5.0	200.34	16.04	X	X
	10.0	130.42	11.49	X	X
24	0.0	410.35	35.91		
	0.5	231.61	12.13		X
	1.0	200.10	23.95	X	X
	2.0	190.06	28.17	X	X
	5.0	138.17	21.88	X	X
	10.0	92.54	9.32	X	X
72	0.0	467.83	39.70		X
	0.5	227.11	22.63	X	X
	1.0	204.86	23.23	X	X
	2.0	156.82	9.61	X	X
	5.0	105.53	5.98	X	X
	10.0	87.42	12.80	X	X

Figures 16-20 and Tables 14-18 depict the effect of papain (0-10 mg/mL) on various concentrations of THC-COOH (25-500 ng/mL) over time in pH 4.5 synthetic urine as measured by FPIA analyses.

The overall data in Figure 16 and Table 14 indicate that papain did not contribute to a significant effect on 25 ng/mL THC-COOH in pH 4.5 synthetic urine over time. Almost every group of adulterated specimens was not significantly different from its intra-temporal unadulterated control. A minimum 5% decrease, less the decrease in the unadulterated control, in THC-COOH concentration was noted for the group adulterated with 2 mg/mL papain at 4 hours and all adulterated groups at 72 hours. There were no adulterated groups that yielded a THC-COOH concentration > 50 ng/mL, indicating a false positive result, over 72 hours.

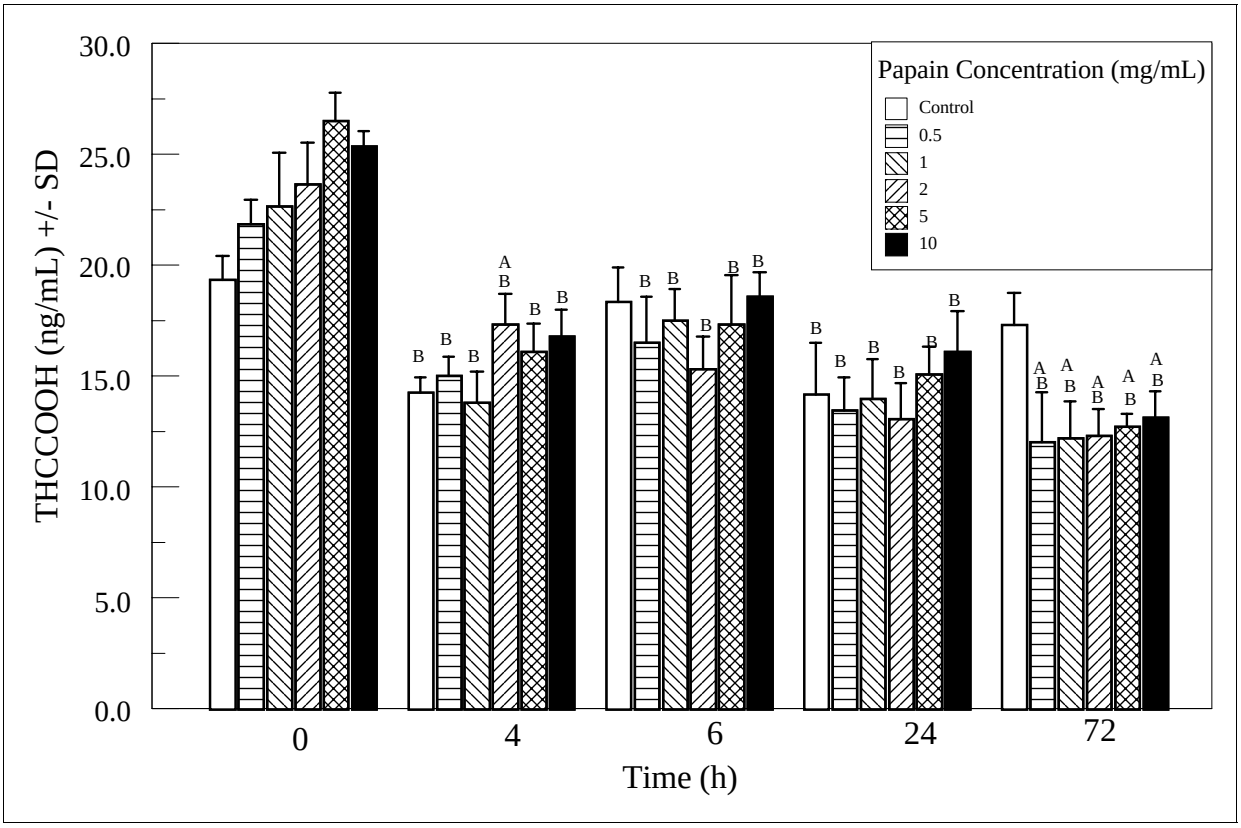

Figure 16 Effects of papain on measured THC-COOH (25 ng/mL) over time in pH 4.5 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Table 14 The average concentration of THC-COOH in pH 4.5 synthetic urine containing 25 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	19.34	1.10		
	0.5	21.84	1.11		
	1.0	22.63	2.46		
	2.0	23.63	1.88		
	5.0	26.50	1.26		
	10.0	25.33	0.70		
4	0.0	14.24	0.68		X
	0.5	15.00	0.83		X
	1.0	13.77	1.44		X
	2.0	17.33	1.34	X	X
	5.0	16.06	1.30		X
	10.0	16.76	1.21		X
6	0.0	18.31	1.56		X
	0.5	16.51	2.07		X
	1.0	17.50	1.44		X
	2.0	15.33	1.46		X
	5.0	17.34	2.23		X
	10.0	18.58	1.11		X
24	0.0	14.18	2.32		X
	0.5	13.43	1.50		X
	1.0	13.96	1.82		X
	2.0	13.04	1.64		X
	5.0	15.06	1.27		X
	10.0	16.09	1.83		X
72	0.0	17.28	1.48	X	X
	0.5	12.03	2.24	X	X
	1.0	12.20	1.65	X	X
	2.0	12.29	1.21	X	X
	5.0	12.70	0.61	X	X
	10.0	13.11	1.21	X	X

The overall data in Figure 17 and Table 15 indicate that papain sporadically affected 75 ng/mL THC-COOH in pH 4.5 synthetic urine over time. There was a minimum 15% decrease, less the decrease in the unadulterated control, in THC-COOH concentration for adulterated groups in which papain had a significant effect at 4, 6, 24, and 72 hours. All unadulterated and adulterated groups yielded average THC-COOH concentrations < 50 ng/mL, indicating false negative results. Although papain may have augmented the decrease in THC-COOH in pH 4.5 synthetic urine, a majority of this effect is attributable to the pH of the matrix.

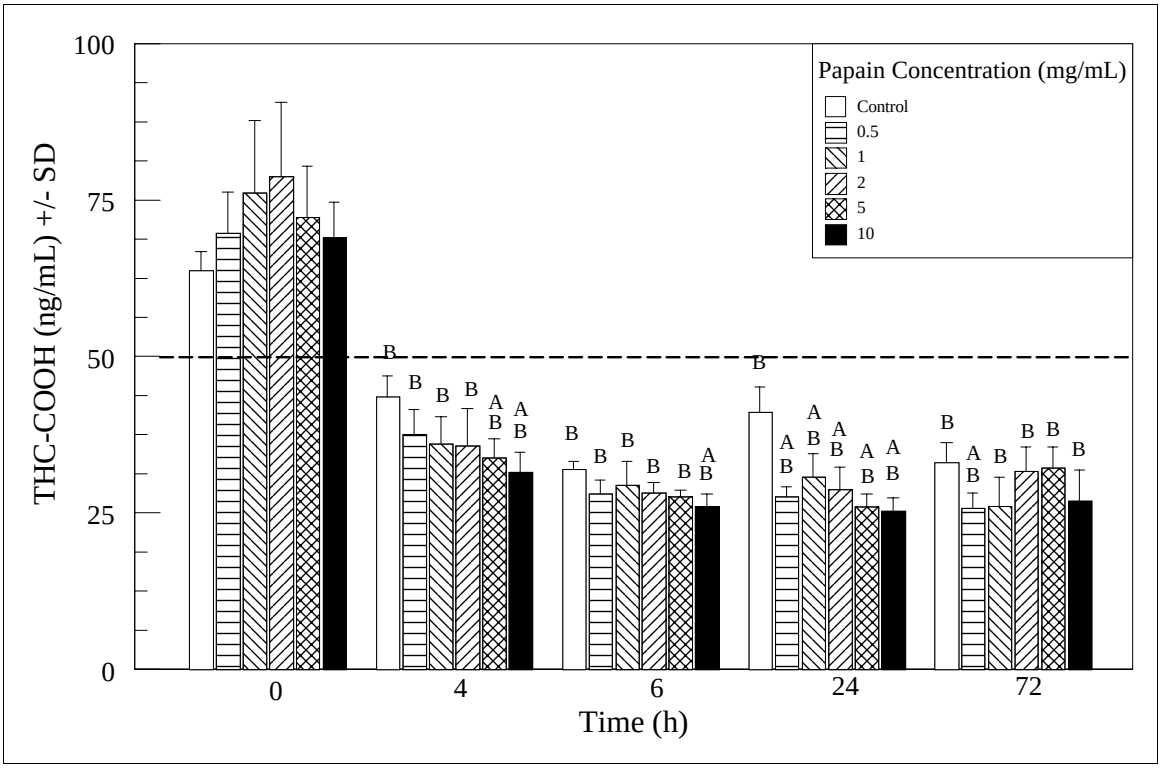

Figure 17 Effects of papain on measured THC-COOH (75 ng/mL) over time in pH 4.5 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 15 The average concentration of THC-COOH in pH 4.5 synthetic urine containing 75 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	63.69	3.07		
	0.5	69.68	6.55		
	1.0	76.18	11.51		
	2.0	78.65	11.94		
	5.0	72.26	8.13		
	10.0	69.00	5.67		
4	0.0	43.56	3.37		X
	0.5	37.48	4.07		X
	1.0	35.96	4.40		X
	2.0	35.65	6.03		X
	5.0	33.84	2.97	X	X
	10.0	31.50	3.26	X	X
6	0.0	31.96	1.23		X
	0.5	28.06	2.13		X
	1.0	29.42	3.76		X
	2.0	28.14	1.73		X
	5.0	27.59	1.00		X
	10.0	26.05	1.95	X	X
24	0.0	41.09	4.05		X
	0.5	27.54	1.58	X	X
	1.0	30.65	3.86	X	X
	2.0	28.76	3.56	X	X
	5.0	25.94	2.12	X	X
	10.0	25.22	2.25	X	X
72	0.0	33.01	3.24		X
	0.5	25.70	2.47	X	X
	1.0	26.06	4.56		X
	2.0	31.65	3.92		X
	5.0	32.19	3.30		X
	10.0	26.84	4.94		X

The overall data in Figure 18 and Table 16 indicate papain sporadically affected 100 ng/mL THC-COOH in pH 4.5 synthetic urine over time. There was a minimum 15% decrease, less the decrease in the unadulterated control, in THC-COOH concentration for adulterated groups in which papain had a significant effect at 4, 6, 24, and 72 hours. Almost every unadulterated and adulterated groups yielded average THC-COOH concentrations < 50 ng/mL, indicating false negative results. Although papain may have augmented the decrease in THC-COOH in pH 4.5 synthetic urine, a majority of this effect is attributable to the pH of the matrix.

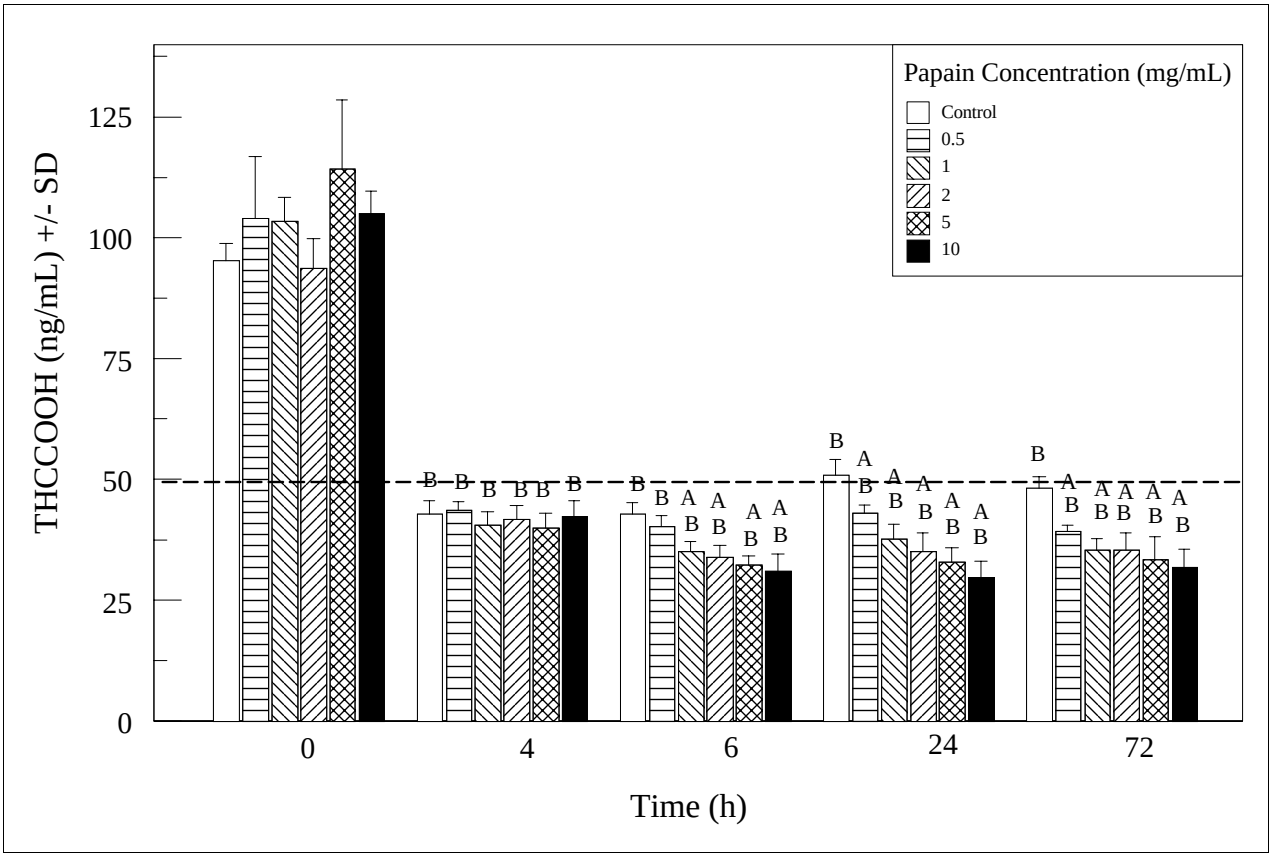

Figure 18 Effects of papain on measured THC-COOH (100 ng/mL) over time in pH 4.5 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 16 The average concentration of THC-COOH in pH 4.5 synthetic urine containing 100 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	95.21	3.58		
	0.5	104.04	12.81		
	1.0	103.32	5.13		
	2.0	93.59	6.31		
	5.0	114.16	14.33		
	10.0	104.97	4.72		
4	0.0	42.80	2.76		X
	0.5	43.54	1.81		X
	1.0	40.46	2.82		X
	2.0	41.61	2.98		X
	5.0	39.81	3.09		X
	10.0	42.31	3.30		X
6	0.0	42.75	2.43		X
	0.5	40.17	2.24		X
	1.0	35.02	2.08	X	X
	2.0	33.89	2.39	X	X
	5.0	32.31	1.86	X	X
	10.0	30.92	3.60	X	X
24	0.0	50.85	3.27		X
	0.5	42.92	1.80	X	X
	1.0	37.58	3.05	X	X
	2.0	34.94	3.94	X	X
	5.0	32.90	2.98	X	X
	10.0	29.64	3.44	X	X
72	0.0	48.08	2.50		X
	0.5	39.25	1.21	X	X
	1.0	35.31	2.45	X	X
	2.0	35.37	3.49	X	X
	5.0	33.45	4.66	X	X
	10.0	31.71	3.84	X	X

The overall data in Figure 19 and Table 17 indicate that papain contributed to a significant effect on 250 ng/mL THC-COOH in pH 4.5 synthetic urine over time. There was a minimum 4% decrease, less the decrease in the unadulterated control, in THC-COOH concentration for adulterated groups in which papain had a significant effect at 4, 6, 24, and 72 hours. Groups adulterated with 5.0 and 10 mg/mL papain after 6 hours and the group adulterated groups after 72 hours yielded an average THC-COOH concentration < 50 ng/mL, indicating false negative results. Although papain may have augmented the decrease in THC-COOH in pH 4.5 synthetic urine, a majority of this effect is attributable to the pH of the matrix.

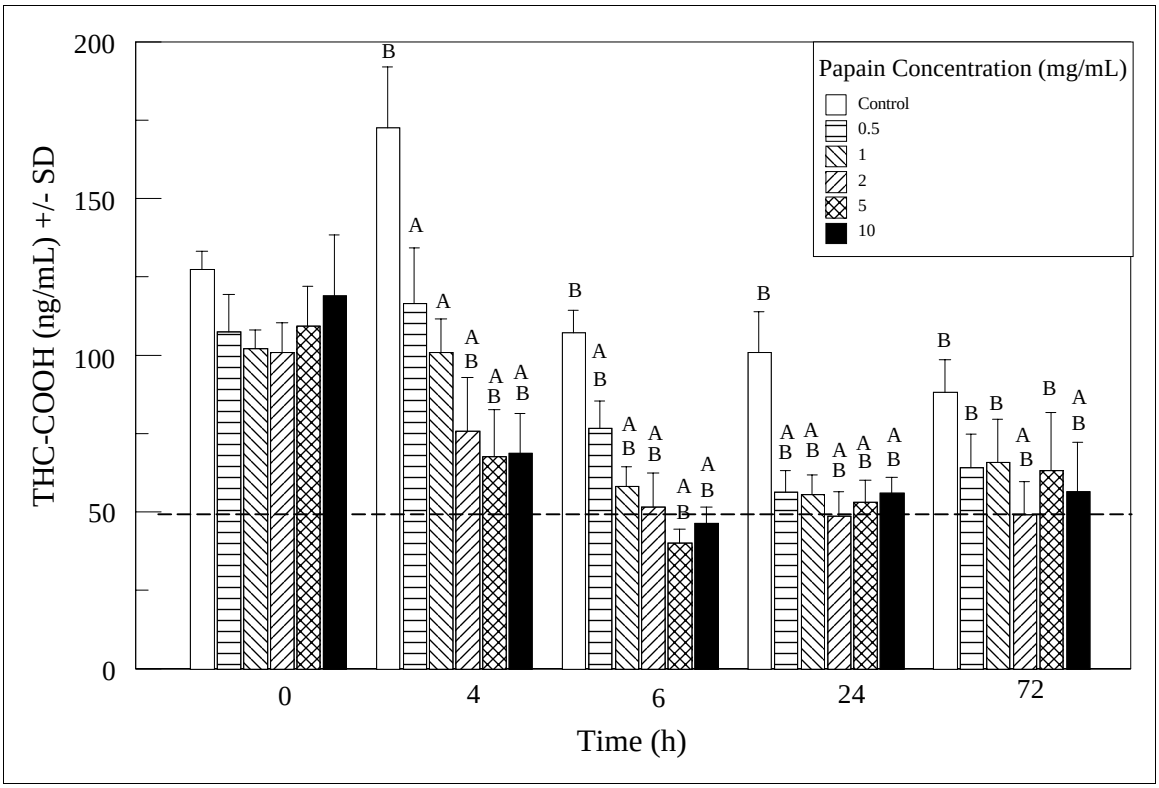

Figure 19 Effects of papain on measured THC-COOH (250 ng/mL) over time in pH 4.5 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 17 The average concentration of THC-COOH in pH 4.5 synthetic urine containing 250 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	127.26	5.80		
	0.5	107.40	12.13		
	1.0	102.12	5.92		
	2.0	100.85	9.63		
	5.0	109.25	12.70		
	10.0	118.93	19.43		
4	0.0	172.43	19.50		X
	0.5	116.43	17.83	X	
	1.0	100.90	10.63	X	
	2.0	75.64	17.11	X	X
	5.0	67.68	14.88	X	X
	10.0	68.65	12.73	X	X
6	0.0	107.18	7.16		X
	0.5	76.63	8.89	X	X
	1.0	58.03	6.23	X	X
	2.0	51.66	10.94	X	X
	5.0	40.13	4.38	X	X
	10.0	46.40	5.31	X	X
24	0.0	100.93	12.97		X
	0.5	56.15	6.94	X	X
	1.0	55.51	6.23	X	X
	2.0	48.67	7.86	X	X
	5.0	53.00	7.24	X	X
	10.0	56.00	5.14	X	X
72	0.0	88.08	10.64		X
	0.5	64.18	10.67		X
	1.0	65.76	13.88		X
	2.0	49.12	10.68	X	X
	5.0	63.25	18.56		X
	10.0	56.59	15.58	X	X

The overall data in Figure 20 and Table 18 indicate that papain sporadically affected 500 ng/mL THC-COOH in pH 4.5 synthetic urine over time. There was a minimum 11% decrease, less the decrease in the unadulterated control, in THC-COOH concentration for adulterated groups in which papain had a significant effect at 4, 6, 24, and 72 hours. No adulterated groups yielded at THC-COOH concentration < 50 ng/mL. Although papain may have augmented the decrease in THC-COOH in pH 4.5 synthetic urine, a majority of this effect is attributable to the pH of the matrix.

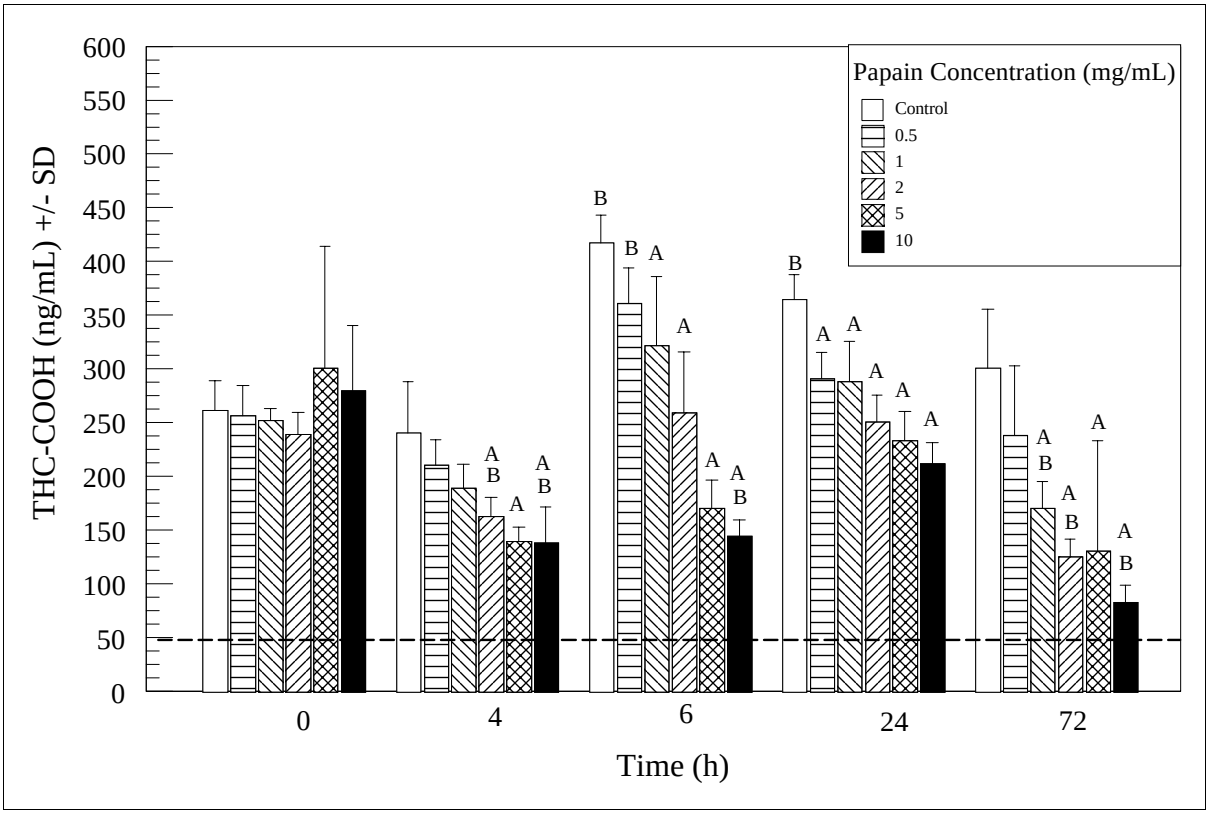

Figure 20 Effects of papain on measured THC-COOH (500 ng/mL) over time in pH 4.5 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 18 The average concentration of THC-COOH in pH 4.5 synthetic urine containing 500 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	261.07	27.71		
	0.5	256.16	28.70		
	1.0	252.15	11.11		
	2.0	239.13	20.69		
	5.0	301.02	112.55		
	10.0	279.72	60.53		
4	0.0	239.97	48.23		
	0.5	210.45	23.25		
	1.0	188.65	22.30		
	2.0	162.22	18.16	X	X
	5.0	139.31	13.60	X	
	10.0	138.39	33.19	X	X
6	0.0	417.27	26.04		X
	0.5	360.84	32.65		X
	1.0	321.56	64.47	X	
	2.0	258.75	56.96	X	
	5.0	170.46	25.91	X	
	10.0	144.35	15.49	X	X
24	0.0	364.44	22.73		X
	0.5	291.03	24.51	X	
	1.0	288.07	37.92	X	
	2.0	250.35	25.46	X	
	5.0	233.56	27.09	X	
	10.0	211.50	19.80	X	
72	0.0	300.62	55.17		
	0.5	238.23	64.32		
	1.0	170.38	24.97	X	X
	2.0	125.31	16.69	X	X
	5.0	130.45	102.61	X	
	10.0	82.91	15.39	X	X

Figures 21-25 and Tables 19-23 depict the effect of papain (0-10 mg/mL) on various concentrations of THC-COOH (25-500 ng/mL) over time in pH 8.0 synthetic urine as measured by FPIA analyses.

The overall data in Figure 21 and Table 19 indicate that papain sporadically affected 25 ng/mL THC-COOH in pH 8.0 synthetic urine over time. There was a minimum 21% decrease, less the decrease in the unadulterated control, in THC-COOH concentration for adulterated groups in which papain had a significant effect at 4, 6, 24, and 72 hours. There were no adulterated groups that yielded at THC-COOH concentration > 50 ng/mL , indicating a false positive result, over 72 hours.

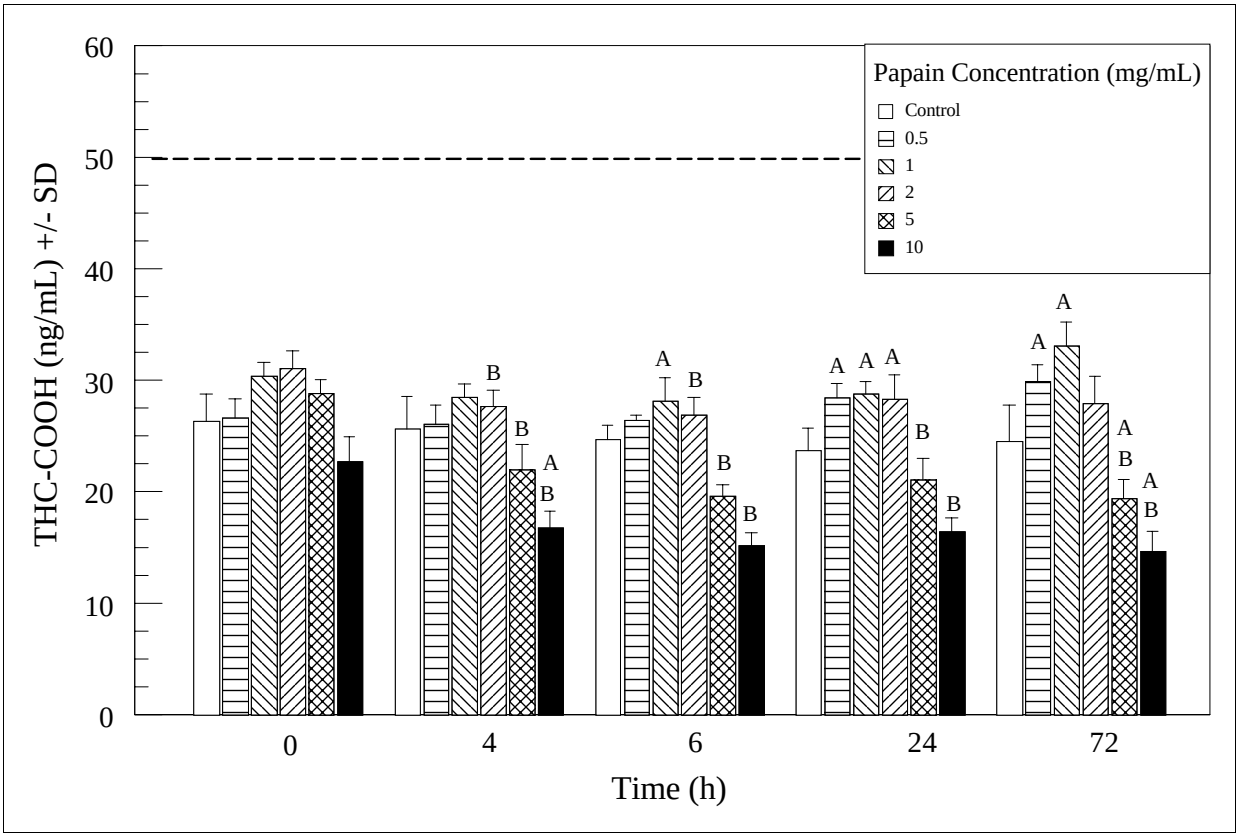

Figure 21 Effects of papain on measured THC-COOH (25 ng/mL) over time in pH 8.0 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 19 The average concentration of THC-COOH in pH 8.0 synthetic urine containing 25 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	26.32	2.40		
	0.5	26.58	1.75		
	1.0	30.34	1.24		
	2.0	31.07	1.56		
	5.0	28.84	1.23		
	10.0	22.69	2.23		
4	0.0	25.62	2.91		
	0.5	26.07	1.69		
	1.0	28.47	1.20		
	2.0	27.65	1.43		X
	5.0	21.94	2.27		X
	10.0	16.72	1.52	X	X
6	0.0	24.68	1.27		
	0.5	26.37	0.50		
	1.0	28.11	2.10	X	
	2.0	26.85	1.61		X
	5.0	19.56	1.10	X	X
	10.0	15.14	1.22	X	X
24	0.0	23.65	2.05		
	0.5	28.40	1.34	X	
	1.0	28.76	1.08	X	
	2.0	28.26	2.19	X	
	5.0	21.05	1.95		X
	10.0	16.38	1.23	X	X
72	0.0	24.52	3.28		
	0.5	29.87	1.48	X	
	1.0	33.04	2.14	X	
	2.0	27.95	2.41		
	5.0	19.36	1.76	X	X
	10.0	14.62	1.86	X	X

The overall data in Figure 22 and Table 20 indicate that papain contributed to a significant effect on 75 ng/mL THC-COOH in pH 8.0 synthetic urine over time. There was a minimum 17% decrease, less the decrease in the unadulterated control, in THC-COOH concentration in almost every group of adulterated specimens with various papain concentrations at 4, 6, 24, and 72 hours. Groups adulterated with 10 mg/mL papain after 4 and 6hours; 5.0 and 10 mg/mL papain after 24 and 72 hours yielded an average THC-COOH concentration < 50 ng/mL, indicating false negative results.

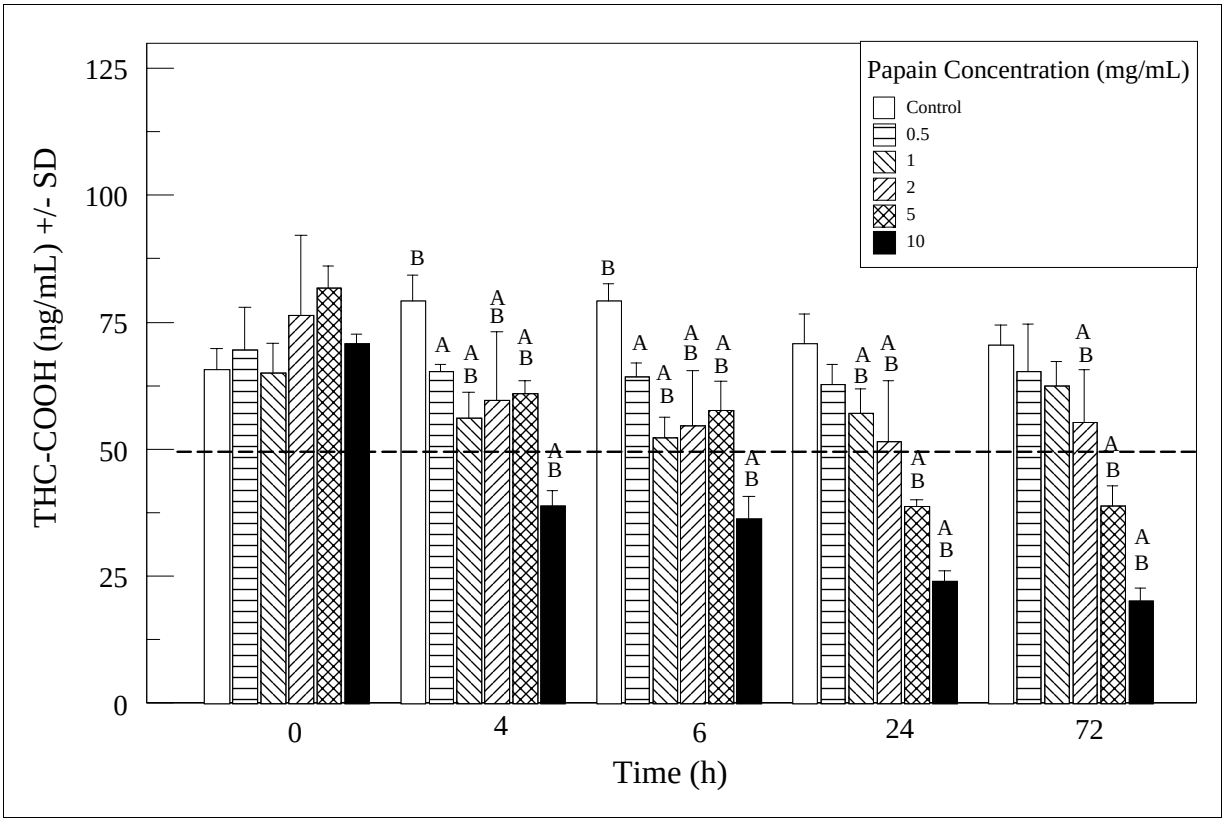

Figure 22 Effects of papain on measured THC-COOH (75 ng/mL) over time in pH 8.0 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 20 The average concentration of THC-COOH in pH 8.0 synthetic urine containing 75 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	65.63	4.13		
	0.5	69.48	8.50		
	1.0	64.93	5.93		
	2.0	76.22	15.88		
	5.0	81.77	4.34		
	10.0	70.67	2.02		
4	0.0	79.18	5.04		X
	0.5	65.26	1.39	X	
	1.0	56.10	5.18	X	X
	2.0	59.60	13.47	X	X
	5.0	60.95	2.60	X	X
	10.0	38.75	3.04	X	X
6	0.0	79.13	3.51		X
	0.5	64.18	2.81	X	
	1.0	52.22	4.10	X	X
	2.0	54.63	10.77	X	X
	5.0	57.65	5.69	X	X
	10.0	36.22	4.39	X	X
24	0.0	70.75	5.82		
	0.5	62.79	3.86		
	1.0	57.04	4.78	X	X
	2.0	51.45	12.01	X	X
	5.0	38.68	1.30	X	X
	10.0	23.97	2.03	X	X
72	0.0	70.47	4.02		
	0.5	65.23	9.34		
	1.0	62.43	4.75		
	2.0	55.22	10.38	X	X
	5.0	38.76	3.95	X	X
	10.0	20.05	2.53	X	X

The overall data in Figure 23 and Table 21 indicate that papain contributed to a significant bi-modal effect on 100 ng/mL THC-COOH in pH 8.0 synthetic urine over time. There was a minimum 24% decrease, the decrease in the unadulterated control, in the specimens adulterated with 5.0 and 10 mg/mL papain over 4, 6, 24, and 72 hours. There was an apparent maximum 35% increase, less the decrease in the unadulterated control, in the specimens adulterated with 0.5 and 1.0 mg/mL papain over 6, 24, and 72 hours. Groups adulterated with 10 mg/mL papain after 6 and 24 hours; 5.0 and 10 mg/mL papain after 72 hours yielded an average THC-COOH concentration < 50 ng/mL, indicating false negative results.

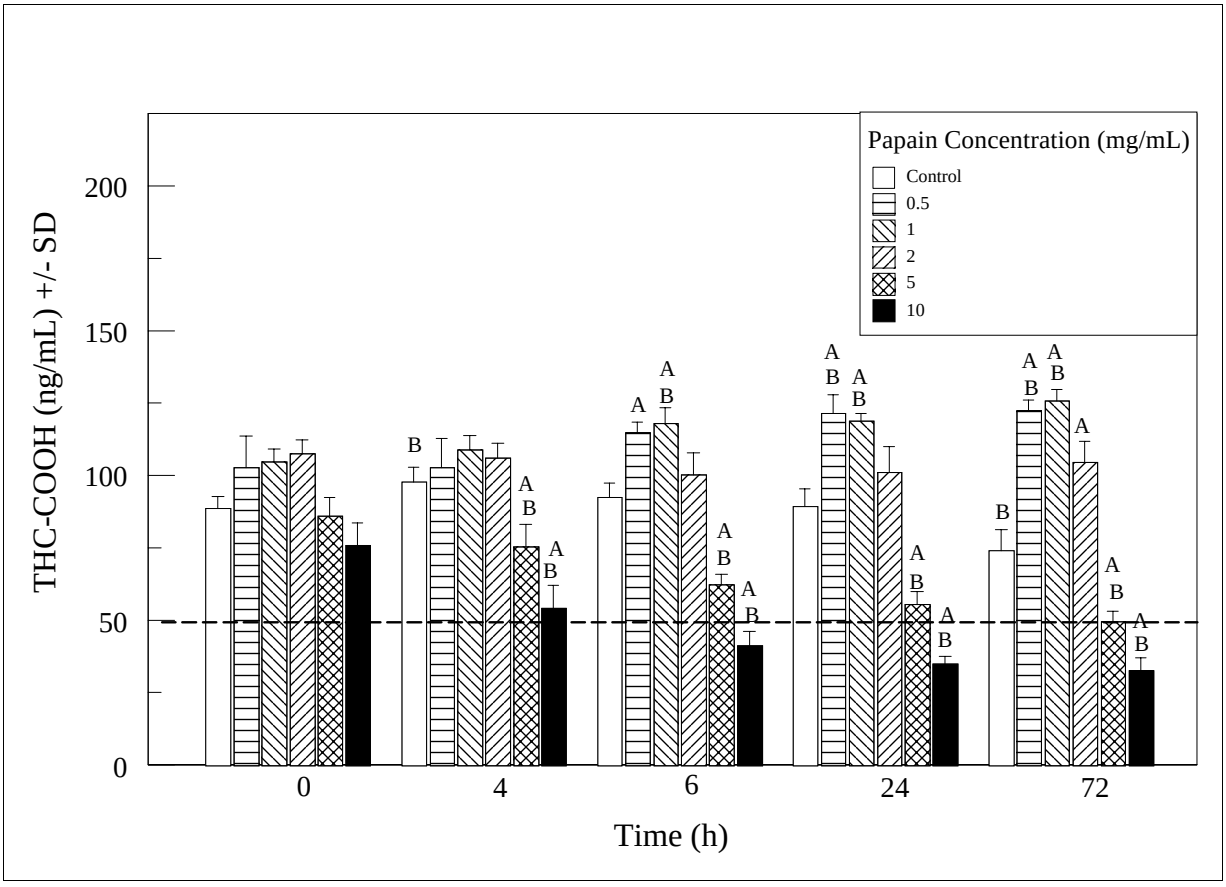

Figure 23 Effects of papain on measured THC-COOH (100 ng/mL) over time in pH 8.0 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 21 The average concentration of THC-COOH in pH 8.0 synthetic urine containing 100 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	88.56	4.18		
	0.5	102.73	10.78		
	1.0	104.70	4.35		
	2.0	107.53	4.64		
	5.0	86.02	6.51		
	10.0	75.79	7.93		
4	0.0	97.72	5.05		X
	0.5	102.69	9.98		
	1.0	108.98	4.92		
	2.0	106.04	5.13		
	5.0	75.14	8.05	X	X
	10.0	54.17	7.87	X	X
6	0.0	92.41	4.94		
	0.5	114.90	3.51	X	
	1.0	117.93	5.39	X	X
	2.0	100.27	7.53		
	5.0	62.22	3.60	X	X
	10.0	41.15	4.91	X	X
24	0.0	89.33	5.97		
	0.5	121.47	6.30	X	X
	1.0	118.71	2.52	X	X
	2.0	100.92	9.05		
	5.0	55.58	4.44	X	X
	10.0	34.98	2.45	X	X
72	0.0	73.90	7.46		X
	0.5	122.40	3.66	X	X
	1.0	125.82	3.78	X	X
	2.0	104.33	7.27	X	
	5.0	49.41	3.84	X	X
	10.0	32.63	4.32	X	X

The overall data in Figure 24 and Table 22 indicate that papain sporadically affected 250 ng/mL THC-COOH in pH 8.0 synthetic urine over time. There was a minimum 18% decrease, less the decrease in the unadulterated control, in THC-COOH concentration for groups in which papain had a significant effect at 4, 6, 24, and 72 hours. There were no adulterated groups that yielded a concentration < 50 ng/mL over 72 hours.

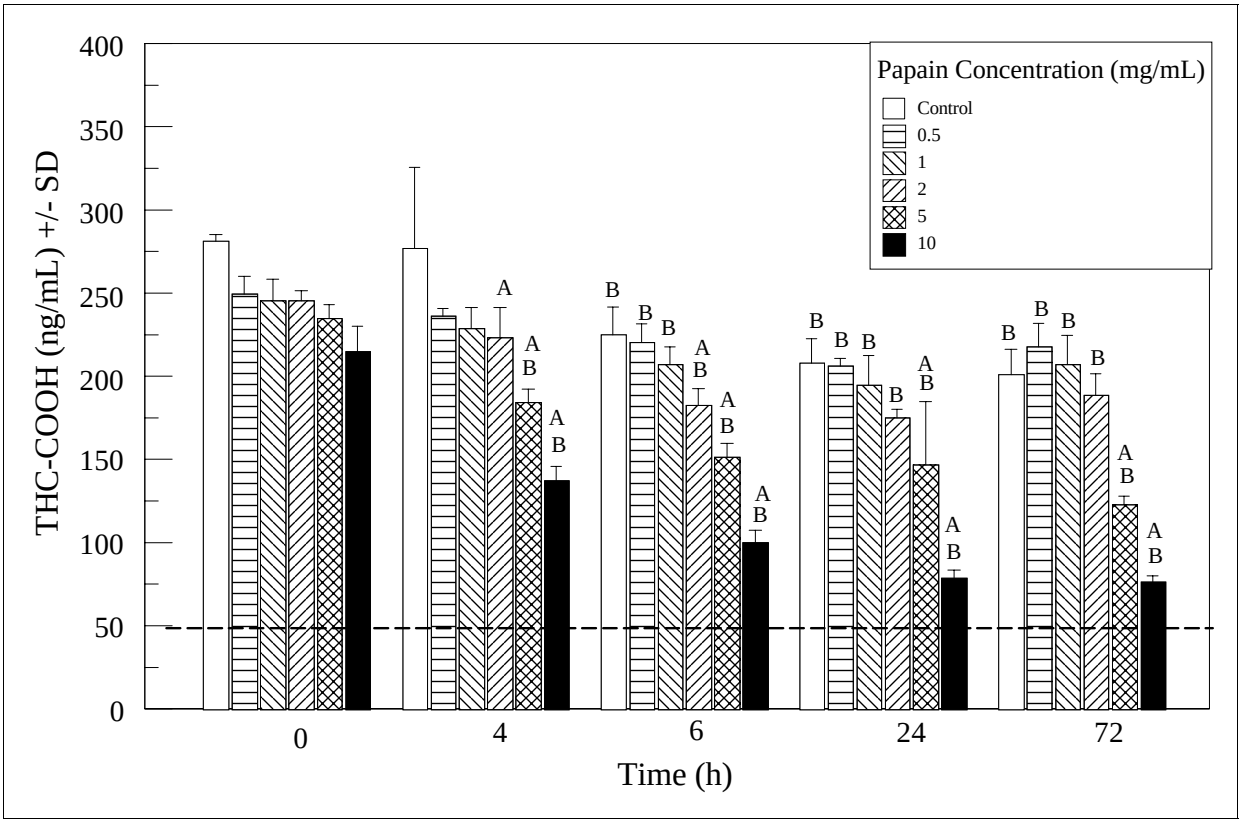

Figure 24 Effects of papain on measured THC-COOH (250 ng/mL) over time in pH 8.0 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 22 The average concentration of THC-COOH in pH 8.0 synthetic urine containing 250 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	281.00	4.27		
	0.5	249.55	10.52		
	1.0	245.47	12.95		
	2.0	245.24	6.17		
	5.0	234.53	8.33		
	10.0	214.59	15.62		
4	0.0	276.86	48.53		
	0.5	235.91	4.96		
	1.0	228.60	12.40		
	2.0	223.33	17.70	X	
	5.0	184.44	7.81	X	X
	10.0	137.01	8.91	X	X
6	0.0	224.98	16.48		X
	0.5	220.34	10.94		X
	1.0	206.77	10.60		X
	2.0	182.29	10.47	X	X
	5.0	151.42	8.27	X	X
	10.0	99.88	7.60	X	X
24	0.0	208.07	14.59		X
	0.5	205.92	4.70		X
	1.0	194.40	18.28		X
	2.0	174.96	4.97		X
	5.0	146.72	38.04	X	X
	10.0	78.51	4.83	X	X
72	0.0	200.98	15.15		X
	0.5	217.52	14.60		X
	1.0	207.07	17.55		X
	2.0	188.34	13.02		X
	5.0	122.65	5.16	X	X
	10.0	76.40	3.80	X	X

The overall data in Figure 25 and Table 23 indicate that papain sporadically affected 500 ng/mL THC-COOH in pH 8.0 synthetic urine over time. There was a minimum 13% decrease, less the decrease in the unadulterated control, in THC-COOH concentration for groups in which papain had a significant effect at 4, 6, 24, and 72 hours. There were no adulterated groups that yielded a concentration < 50 ng/mL over 72 hours.

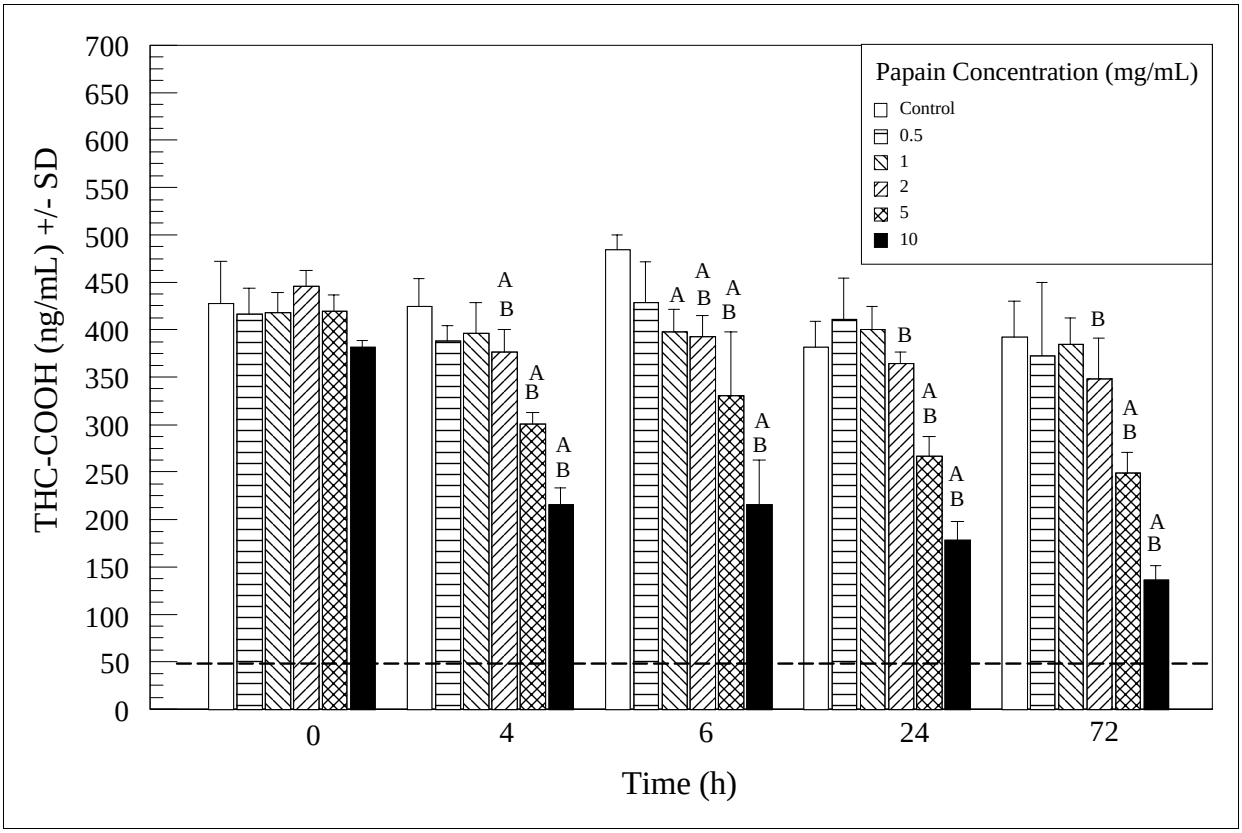

Figure 25 Effects of papain on measured THC-COOH (500 ng/mL) over time in pH 8.0 synthetic urine, n=6

[A] Significant "intra-temporal" difference, $p<0.01$

[B] Significant "inter-temporal" difference, $p<0.01$

Cutoff concentration, 50 ng/mL, ------.

Table 23 The average concentration of THC-COOH in pH 8.0 synthetic urine containing 500 ng/mL THC-COOH with various concentrations of papain over time

Time(h)	Papain concentration (mg/mL)	Average THC-COOH concentration (ng/mL) n=6	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
0	0.0	428.09	44.53		
	0.5	416.70	27.30		
	1.0	417.80	21.28		
	2.0	445.54	17.22		
	5.0	419.44	17.59		
	10.0	381.73	7.37		
4	0.0	424.89	28.68		
	0.5	388.06	16.16		
	1.0	396.29	32.33		
	2.0	376.37	23.67	X	X
	5.0	300.63	11.77	X	X
	10.0	215.35	18.27	X	X
6	0.0	484.10	15.41		
	0.5	428.99	42.47		
	1.0	398.04	22.96	X	
	2.0	393.11	21.66	X	X
	5.0	330.46	67.30	X	X
	10.0	215.44	46.97	X	X
24	0.0	381.62	27.18		
	0.5	410.84	43.56		
	1.0	400.24	24.50		
	2.0	364.79	11.70		X
	5.0	266.39	21.03	X	X
	10.0	178.18	19.11	X	X
72	0.0	392.16	38.23		
	0.5	372.80	76.97		
	1.0	384.67	28.08		
	2.0	348.78	42.75		X
	5.0	249.02	21.58	X	X
	10.0	135.72	15.85	X	X

The Effect of Papain Concentration, Time, THC-COOH Concentration, and pH on the Percent Decrease of THC-COOH. Figure 26 illustrates and Table 24 lists the percent decrease

in THC-COOH concentration after 72 hours, less the THC-COOH concentration decrease in the unadulterated control, in specimens adulterated with 0.5 to 10 mg/mL papain in pH 6.2 synthetic urine plotted versus their respective initial THC-COOH concentration. Data indicate the percent decrease in THC-COOH in pH 6.2 synthetic urine with 0.5 to 10 mg/mL papain after 72 hours was statistically less than or equal to 2.0 mg/mL papain over all concentrations of THC-COOH. There was an average (SD) 22% (15%) decrease in THC-COOH for specimens adulterated with 1 mg/mL papain over 25 to 500 ng/mL initial THC-COOH concentrations in pH 6.2 synthetic urine. There was an average (SD) 50% (15%) decrease in THC-COOH for specimens adulterated with 10 mg/mL papain over 25 to 500 ng/mL initial THC-COOH concentrations in pH 6.2 synthetic urine. Although the most demonstrative effects were observed in specimens adulterated with 10 mg/mL papain, they were not statistically different from specimens adulterated with 2.0 or 5.0 mg/mL papain. The lack of statistical difference between 2.0, 5.0, and 10.0 mg/mL papain concentrations was due, in part, to the large standard deviations that resulted from the propagation of the 4 standard deviations required for the comparison. The overall data suggest a direct correlate between the percent decrease in THC-COOH and the concentration of papain

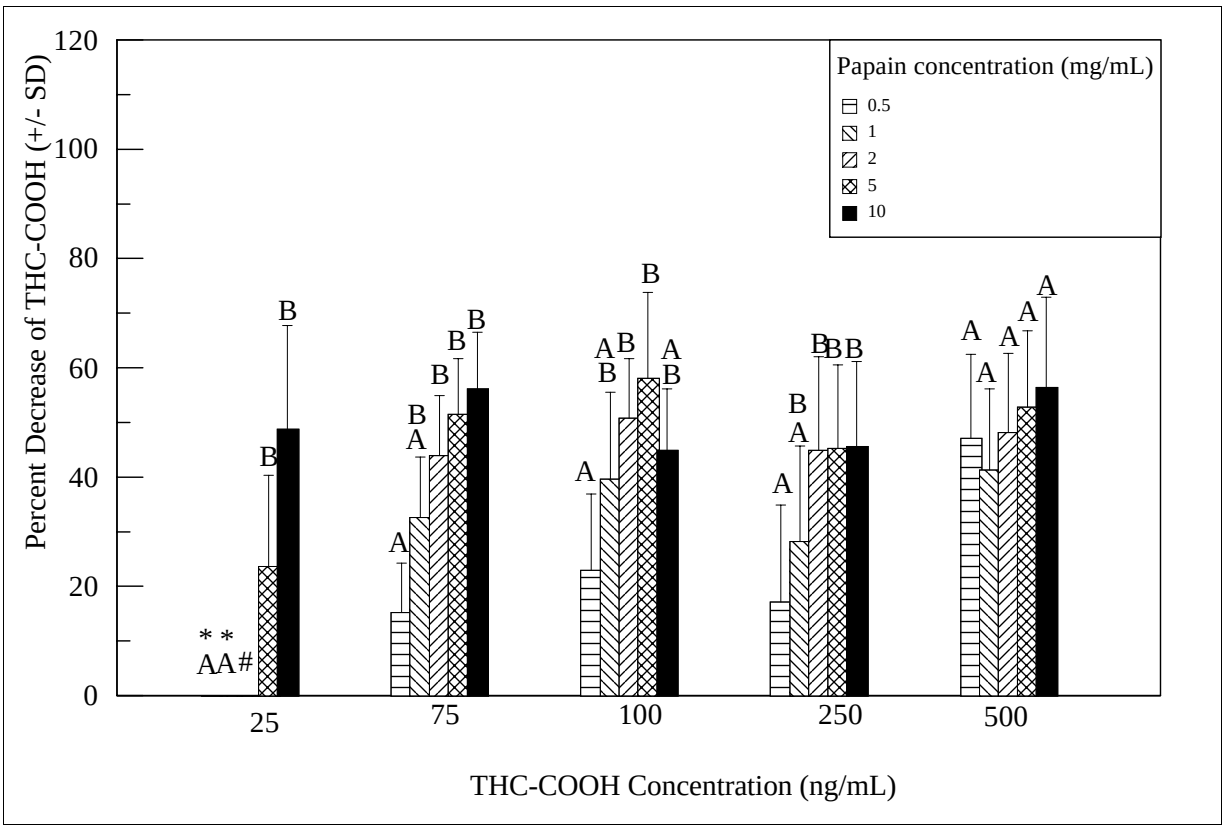

Figure 26 The percent decrease of THC-COOH after 72 hours versus specimens adulterated with 0.5-10 mg/mL papain containing various initial THC-COOH concentrations in pH 6.2 synthetic urine

[A,B,C] Groups denoted with the same letter are not significantly different, $p<0.01$

[*] Papain concentrations of 0.5 and 1.0 mg/mL yielded a 31% increase

[#] A significant effect was not attributed to the 2 mg/mL papain at this concentration of THC-COOH

Table 24 The percent decrease of THC-COOH over 72 hours in specimens adulterated with 0.5-10 mg/mL papain in specimens containing various concentrations of THC-COOH

Initial THC-COOH concentration (ng/mL)	Papain concentration (mg/mL)	Percent decrease in THC-COOH	S.D.	Statistical differences*
25	0.5	-31.13	15.57	A
	1	-31.59	17.51	A
	2#			
	5	23.61	16.76	B
	10	48.74	19.02	B
75	0.5	15.23	9.03	A
	1	32.50	11.16	AB
	2	43.89	10.95	B
	5	51.40	10.27	B
	10	56.11	10.47	B
100	0.5	22.90	14.02	A
	1	39.65	15.90	AB
	2	50.82	10.80	B
	5	58.16	15.62	B
	10	44.85	11.29	AB
250	0.5	17.10	17.81	A
	1	28.19	17.46	AB
	2	44.92	17.11	B
	5	45.17	15.39	B
	10	45.60	15.56	B
500	0.5	47.09	15.33	A
	1	41.38	14.73	A
	2	48.07	14.57	A
	5	52.82	13.99	A
	10	56.50	16.35	A

* Groups denoted with the same letter are not statistically different within a THC-COOH concentration group, $p<0.01$

A significant effect was not attributed to the 2 mg/mL papain at this concentration of THC-COOH

Figure 27 illustrates and Table 25 lists the percent decrease in THC-COOH concentration after 4, 24, and 72 hours, less the THC-COOH concentration decrease in the unadulterated control, in specimens adulterated with 10 mg/mL papain in pH 6.2 synthetic urine plotted versus their respective initial THC-COOH concentrations. Data indicate the percent decreases in THC-COOH in pH 6.2 synthetic urine with 10 mg/mL papain at 24 and 72 hours, were statistically less than or equal to the percent decrease at 4 hours for initial THC-COOH concentrations of 25 to 250 ng/mL. Within the first 4 hours, there was an average (SD) 52% (14%) decrease in THC-COOH for specimens adulterated with 10 mg/mL papain over 25 to 500 ng/mL initial THC-COOH concentrations. After 72 hours, there was an average (SD) 50% (15%) decrease in THC-COOH for specimens adulterated with 10 mg/mL papain over 25 to 500 ng/mL initial THC-COOH concentrations. The data suggest the reduction of THC-COOH in synthetic urine is not enzymatic, as the percent reduction in THC-COOH was not greater after 24 or 72 hours than after 4 hours except for THC-COOH concentrations of 500 ng/mL.

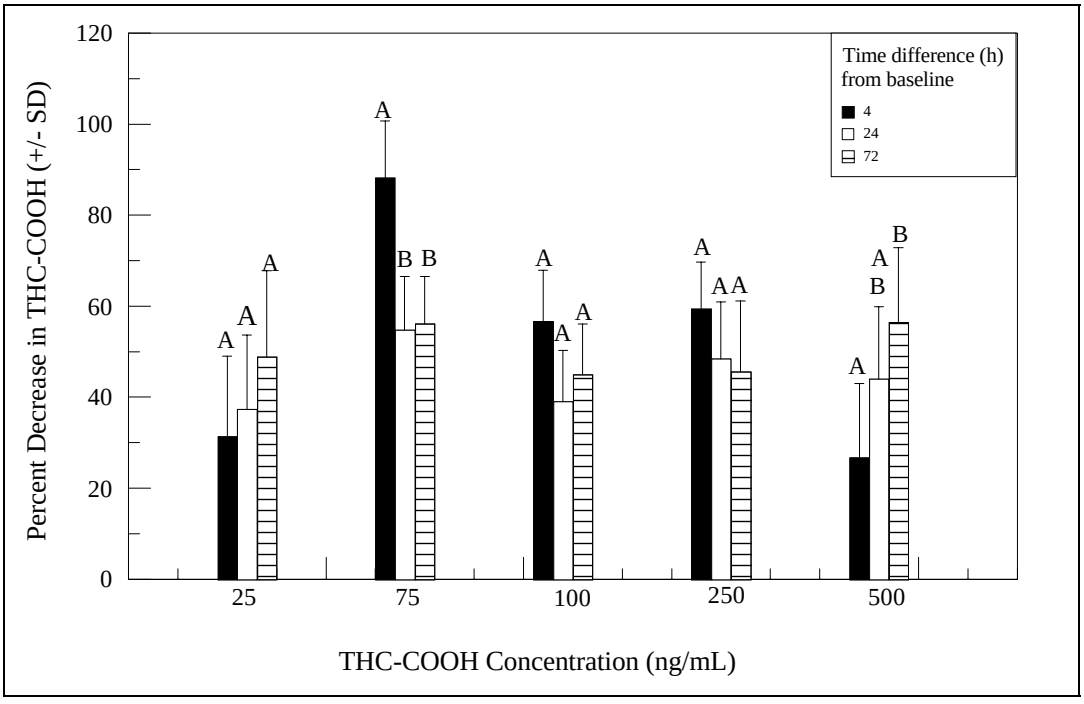

Figure 27 The percent decrease of THC-COOH over 4, 24, and 72 hours versus specimens containing various initial THC-COOH concentrations in pH 6.2 synthetic urine adulterated with 10 mg/mL papain

[A,B] Groups denoted with the same letter are not significantly different within a THC-COOH concentration group, $p<0.01$

Table 25 The percent decrease of THC-COOH concentration over 4, 24, and 72 hours in specimens containing various initial THC-COOH concentrations in pH 6.2 synthetic urine adulterated with 10 mg/mL papain

Initial THC-COOH concentration (ng/mL)	Time (h) from baseline	Percent decrease in THC-COOH, n=6	S.D.	Statistical differences*
25	4	31.30	17.68	A
	24	37.29	16.36	A
	72	48.74	19.02	A
75	4	88.16	12.49	A
	24	54.66	11.92	B
	72	56.11	10.47	B
100	4	56.55	11.36	A
	24	39.05	11.27	A
	72	44.85	11.29	A
250	4	59.33	10.29	A
	24	48.39	12.46	A
	72	45.60	15.56	A
500	4	26.65	16.36	A
	24	43.98	15.90	AB
	72	56.50	16.35	B

* Groups with the same letter are not statistically different within a THC-COOH concentration group.

Figure 28 illustrates and Table 26 lists the percent decrease in THC-COOH concentration after 72 hours, less the THC-COOH concentration decrease in the unadulterated control, in specimens adulterated with 10 mg/mL papain in pH 6.2 synthetic urine plotted versus their respective initial THC-COOH concentrations. Data indicate the percent decreases in THC-COOH in pH 6.2 synthetic urine containing 25 to 500 ng/mL THC-COOH were not statistically different across the concentration range of THC-COOH. There was an average (SD) 50% (15%) decrease in THC-COOH for specimens adulterated with 10 mg/mL papain over 25 to 500 ng/mL initial THC-COOH concentrations. An equal percent decrease in THC-COOH in specimens

containing higher concentrations of THC-COOH translates into a greater absolute decrease in THC-COOH that directly correlates with the initial THC-COOH concentration.

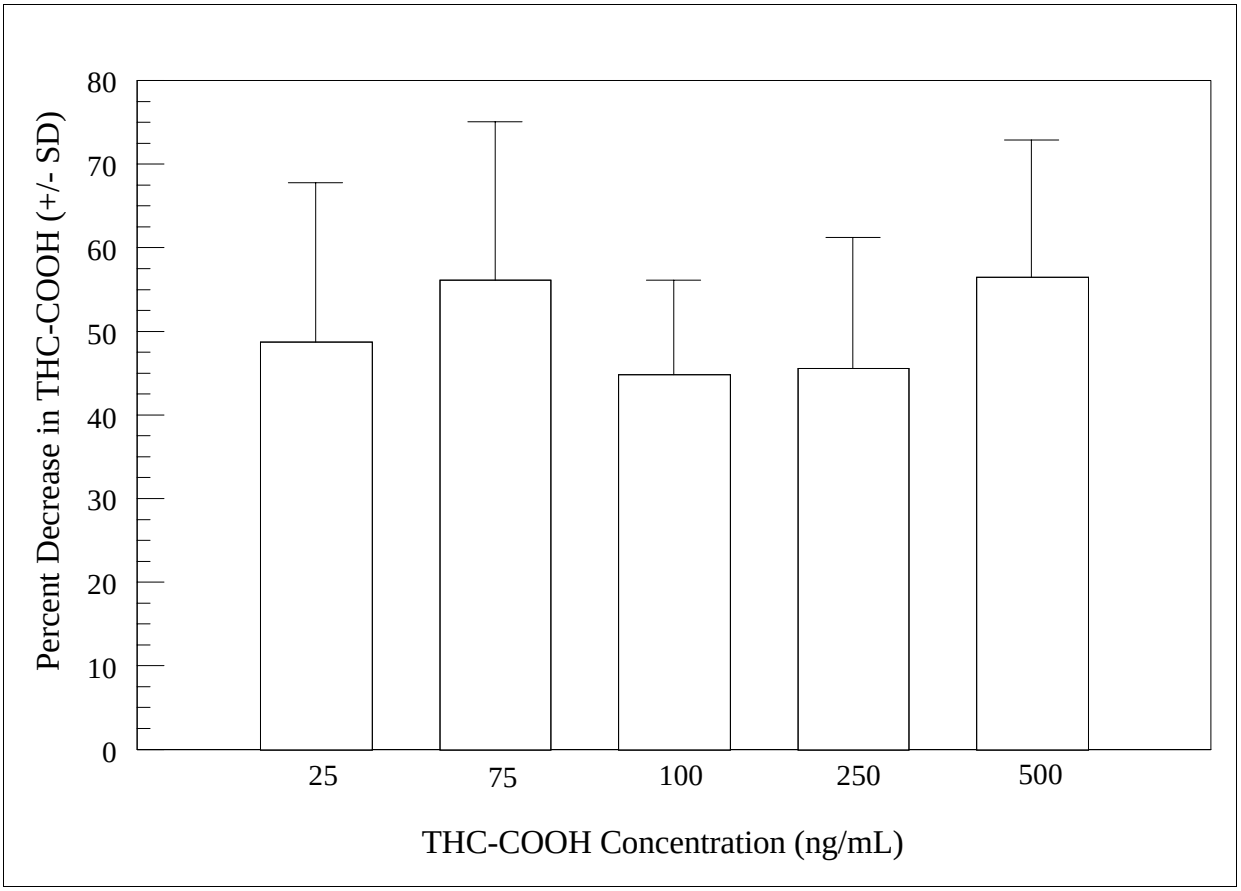

Figure 28 The percent decrease of THC-COOH over 72 hours versus specimens containing various initial THC-COOH concentrations in pH 6.2 synthetic urine adulterated with 10 mg/mL papain.

Table 26 The percent decrease in THC-COOH concentration over 72 hours in specimens containing various initial THC-COOH concentrations in pH 6.2 synthetic urine adulterated with 10 mg/mL papain

Initial THC-COOH concentration (ng/mL)	Percent decrease in THC-COOH, n=6	S.D.	Statistical difference, p<0.01
25	48.74	19.02	
75	56.11	10.47	
100	44.85	11.29	
250	45.60	15.56	
500	56.50	16.35	

Figure 29 illustrates and Table 27 lists the percent decrease in THC-COOH concentration after 72 hours, less the THC-COOH concentration decrease in the unadulterated control, in specimens adulterated with 10 mg/mL papain in pH 4.5, 6.2, and 8.0 synthetic urine plotted versus their respective initial THC-COOH concentration. Data indicate the percent decreases in THC-COOH in pH 4.5, 6.2, and 8.0 synthetic urine were most demonstrative at pH 6.2. For pH 4.5, 6.2, and 8.0 synthetic urine, there was an average (+/-SD) decrease of 31% (11%), 50% (15%), and 39% (13%) in THC-COOH concentrations for specimens adulterated with 10 mg/mL papain over 25 to 500 ng/mL initial THC-COOH concentrations. The percent decrease in THC-COOH in pH 6.2 synthetic urine was not statistically different from the percent decrease in THC-COOH in pH 8.0 synthetic urine, and in some instances pH 4.5 synthetic urine. However, the lack of significant difference is attributed, in part, to the large standard deviations that resulted from the propagation of the 4 standard deviations required for the comparison. The data in Figure 29 and Table 27 also indicate that a matrix with an acidic pH attenuated papain's effect on the reduction of THC-COOH concentration. The distribution of pH values in normal urine is greatest circa 6.2, while the distribution at the extrema (pH 4.5 and 8.0) are minimal as defined by the Gaussian distribution. Therefore, the results observed in pH 6.2 synthetic urine would correlate to the effects observed in the greatest percentage of the population.

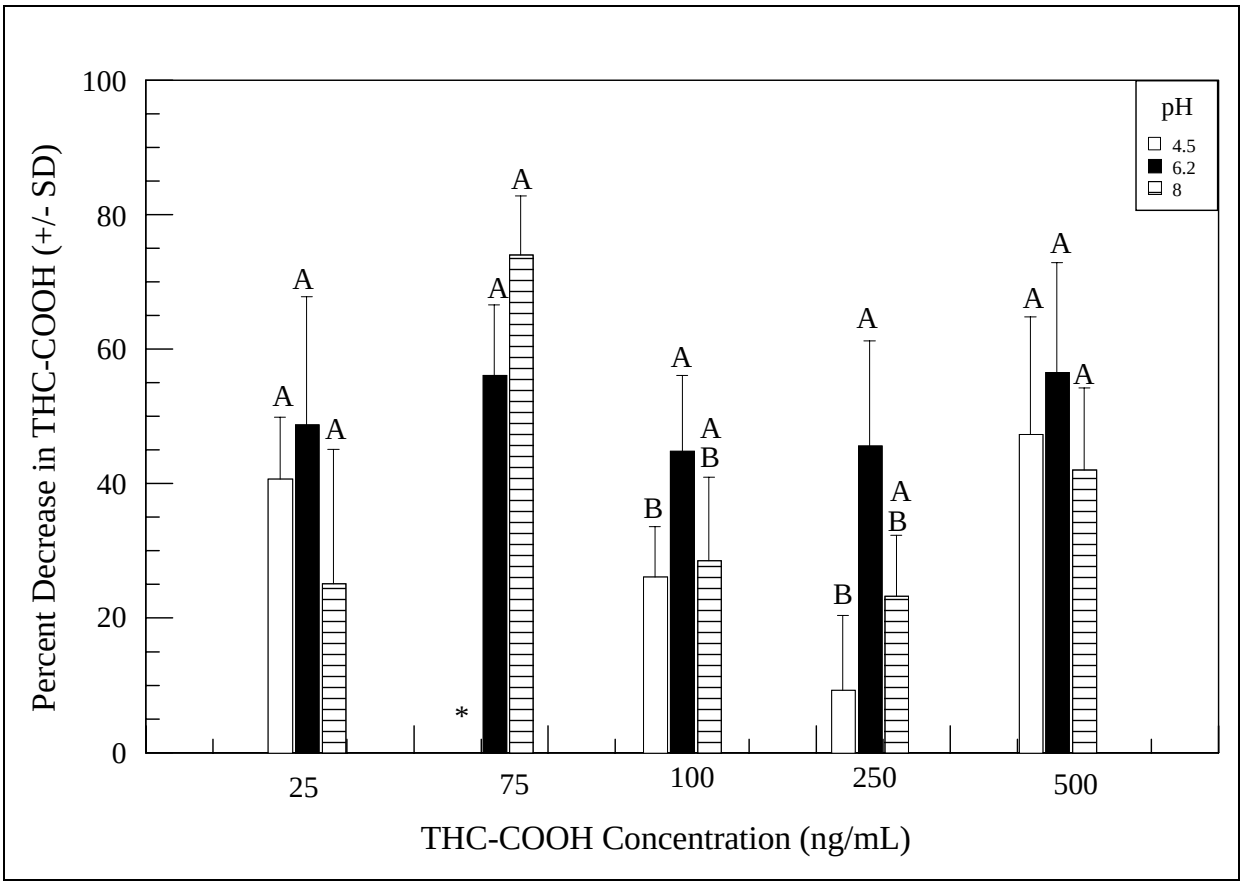

Figure 29 The percent decrease of THC-COOH over 72 hours versus specimens adulterated with 10 mg/mL papain containing various initial THC-COOH concentrations in pH 4.5, 6.2, and 8.0 synthetic urine

[A,B] Groups denoted with the same letter are not significantly different, $p<0.01$

[*] A significant effect was not attributed to papain at 75 ng/mL THC-COOH in pH 4.5 synthetic urine

Table 27 The percent decrease of THC-COOH concentration over 72 hours in specimens containing various initial THC-COOH concentrations in pH 4.5, 6.2, and 8.0 synthetic urine adulterated with 10 mg/mL papain

Initial THC-COOH concentration (ng/mL)	pH	Percent decrease in THC-COOH	S.D.	Statistical differences*
25	4.5	40.65	9.23	A
	6.2	48.74	19.02	A
	8.0	25.09	20.00	A
75	4.5#			
	6.2	56.11	10.47	A
	8.0	73.96	8.81	A
100	4.5	26.12	7.49	B
	6.2	44.85	11.29	A
	8.0	28.51	12.44	AB
250	4.5	9.26	11.08	B
	6.2	45.60	15.56	A
	8.0	23.27	9.00	AB
500	4.5	47.27	17.56	A
	6.2	56.50	16.35	A
	8.0	42.02	12.25	A

* Groups denoted with the same letter are not statistically different within a THC-COOH concentration group

A significant effect was not attributed to papain at 75 ng/mL THC-COOH in pH 4.5 synthetic urine

FPIA Assays for Other Drugs of Abuse

The Figures 30-35 and Tables 28-33 illustrate the effects of 10 mg/mL papain in pH 6.2 synthetic urine specimens that contained amphetamine, secobarbital, nordiazepam, phencyclidine, morphine, and benzoylecgonine at +/- 50% of their respective cutoff concentrations. Figures 30-34 and Tables 28-32 indicate that 10 mg/mL papain did not have a significant effect on the FPIA analyses of either concentration of the amphetamine, secobarbital,

phencyclidine, morphine, or benzoylecgonine solutions assayed over time. Figure 35 and Table 33 indicate that papain had a significant effect in both concentrations of the nordiazepam solutions assayed over time by FPIA. There was a maximum 12% decrease, less the decrease in the unadulterated control, among all of the nordiazepam assays.

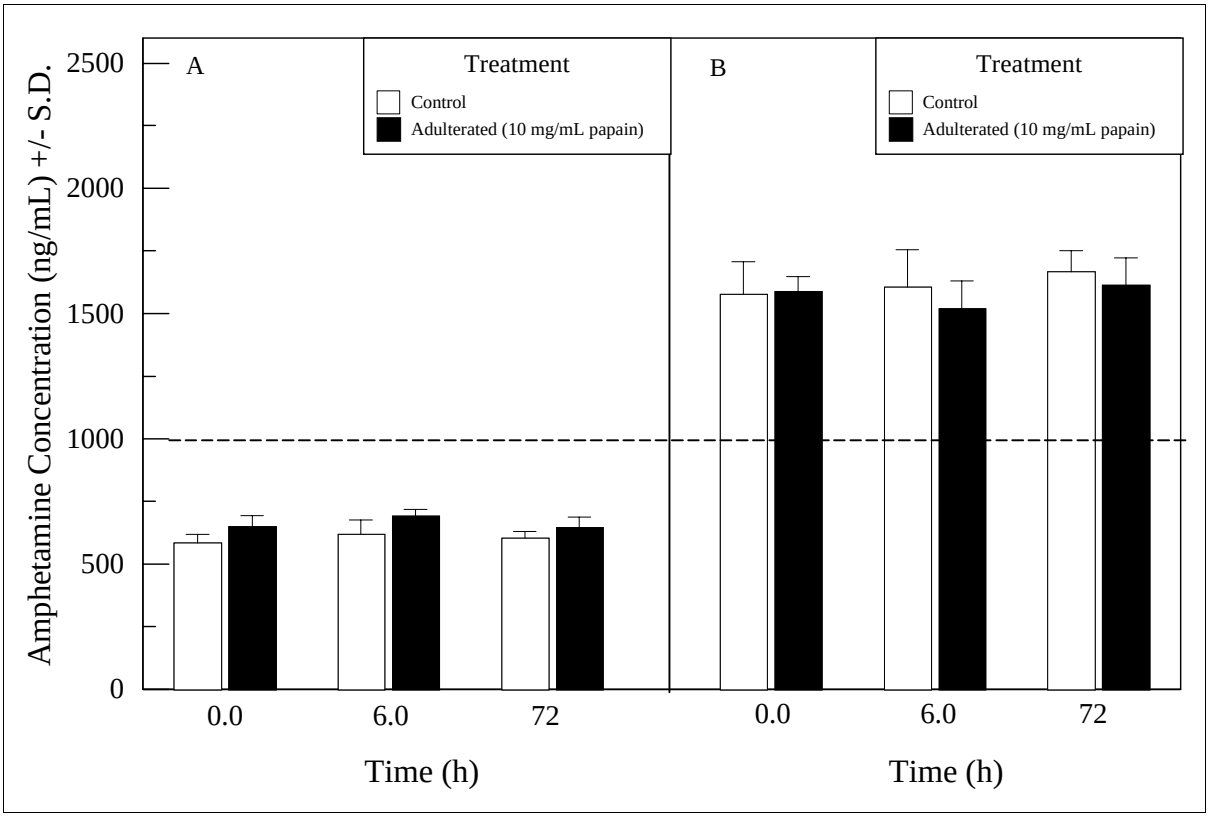

Figure 30 Effects of papain (10 mg/mL) on amphetamine concentrations over time in pH 6.2 synthetic urine, n=6
Panel A- 500 ng/mL amphetamine solution
Panel B- 1500 ng/mL amphetamine solution
Cutoff concentration, 1000 ng/mL, ------.

Table 28 The average amphetamine concentration over time in pH 6.2 synthetic urine containing 0 and 10 mg/mL papain

Initial amphetamine concentration (ng/mL)	Time (h)	Papain concentration	Average amphetamine concentration (ng/mL)	S.D.	Intra-temporal, p<0.01	Inter-temporal, p<0.01
500	0	0	584.41	33.31		
	0	10	650.44	43.78		
	6	0	618.16	58.44		
	6	10	689.90	27.61		
	72	0	604.46	26.79		
	72	10	645.65	42.77		
1500	0	0	1576.8	129.3		
	0	10	1589.7	56.27		
	6	0	1604.8	151.2		
	6	10	1520.0	110.6		
	72	0	1662.6	83.30		
	72	10	1612.4	109.5		

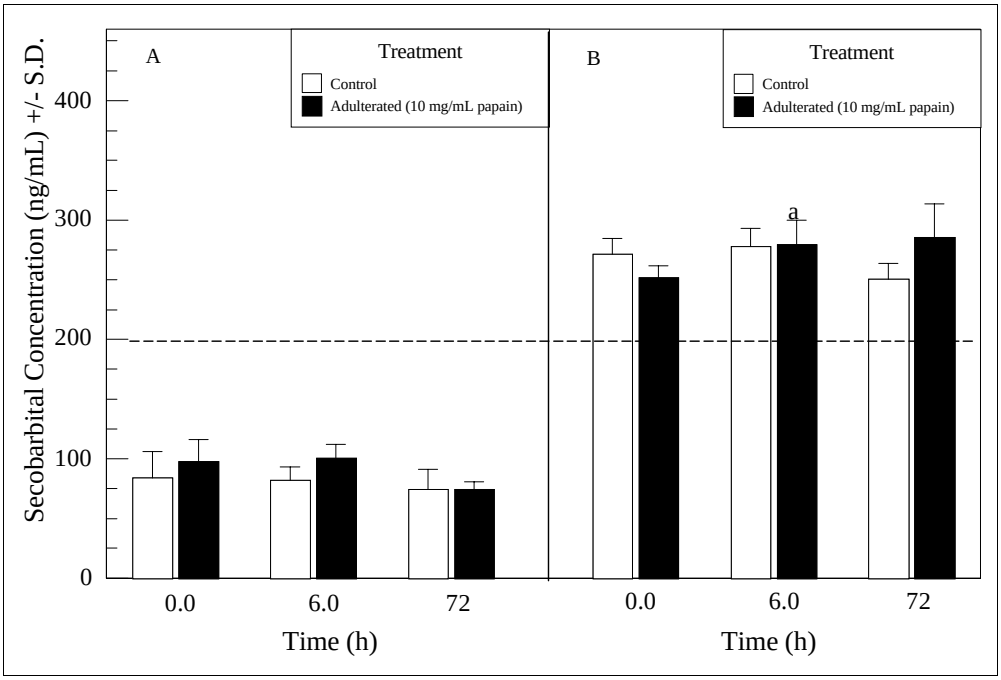

Figure 31 Effects of papain (10 mg/mL) on secobarbital concentrations over time in pH 6.2 synthetic urine, n=6

Panel A- 100 ng/mL secobarbital solution

Panel B- 300 ng/mL secobarbital solution

[a] Significant "inter-temporal" difference, p<0.01

Cutoff concentration, 200 ng/mL, ------.

Table 29 The average secobarbital concentration over time in pH 6.2 synthetic urine containing 0 and 10 mg/mL papain

Initial amphetamine concentration (ng/mL)	Time (h)	Papain concentration	Average secobarbital concentration (ng/mL)	SD	Intra-temporal, p<0.01	Inter-temporal, p<0.01
100	0	0	83.99	21.91		
	0	10	97.37	19.09		
	6	0	81.94	11.49		
	6	10	100.45	11.76		
	72	0	74.24	16.77		
	72	10	74.09	6.99		
300	0	0	271.29	13.36		
	0	10	251.93	9.79		
	6	0	277.76	15.22		
	6	10	279.46	20.29		X
	72	0	250.50	12.97		
	72	10	285.27	28.06		

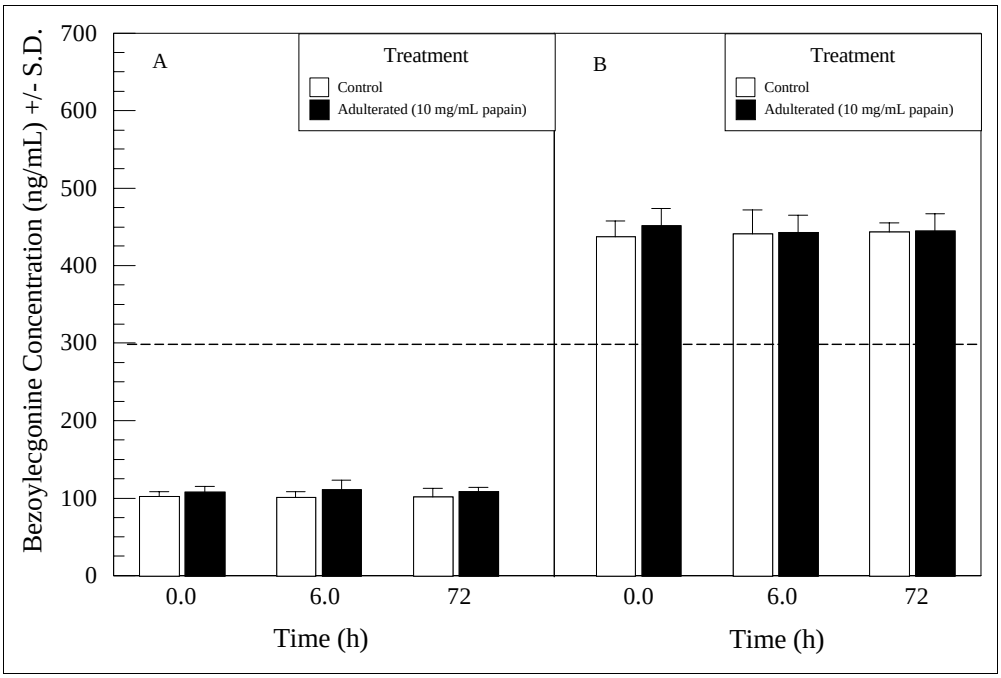

Figure 32 Effects of papain (10 mg/mL) on benzoylecgonine concentrations over time in pH 6.2 synthetic urine, n=6
Panel A- 450 ng/mL benzoylecgonine solution
Panel B- 150 ng/mL benzoylecgonine solution
Cutoff concentration, 300 ng/mL, ------.

Table 30 The average benzoylecgonine concentration over time in pH 6.2 synthetic urine containing 0 and 10 mg/mL papain

Initial amphetamine concentration (ng/mL)	Time (h)	Papain concentration	Average benzoylecgo-nine concentration (ng/mL)	SD	Intra-temporal, p<0.01	Inter-temporal, p<0.01
150	0	0	102.30	5.89		
	0	10	107.75	7.65		
	6	0	101.13	6.86		
	6	10	110.95	12.45		
	72	0	101.89	11.08		
	72	10	108.54	5.64		
450	0	0	436.54	20.76		
	0	10	450.97	22.78		
	6	0	440.69	31.21		
	6	10	442.53	22.16		
	72	0	443.35	11.64		
	72	10	444.33	22.44		

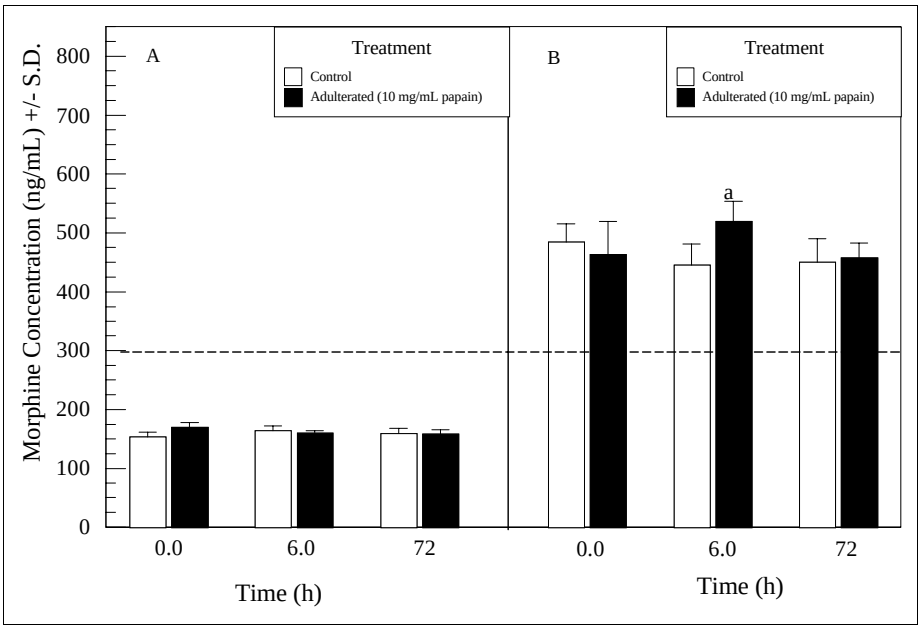

Figure 33 Effects of papain (10 mg/mL) on morphine concentrations over time in pH 6.2 synthetic urine, n=6
Panel A- 450 ng/mL morphine solution
Panel B- 150 ng/mL morphine solution
[a] Significant "intra-temporal" difference, p<0.01
Cutoff concentration, 300 ng/mL, ------.

Table 31 The average morphine concentration over time in pH 6.2 synthetic urine containing 0 and 10 mg/mL papain

Initial amphetamine concentration (ng/mL)	Time (h)	Papain concentration	Average morphine concentration (ng/mL)	SD	Intra-temporal, p<0.01	Inter-temporal, p<0.01
150	0	0	153.20	8.21		
	0	10	170.13	7.56		
	6	0	163.81	8.72		
	6	10	159.95	3.94		
	72	0	159.20	9.53		
	72	10	157.86	7.72		
450	0	0	484.25	30.88		
	0	10	463.19	56.35		
	6	0	445.15	35.71		
	6	10	519.50	33.99	X	
	72	0	449.99	40.41		
	72	10	457.21	25.07		

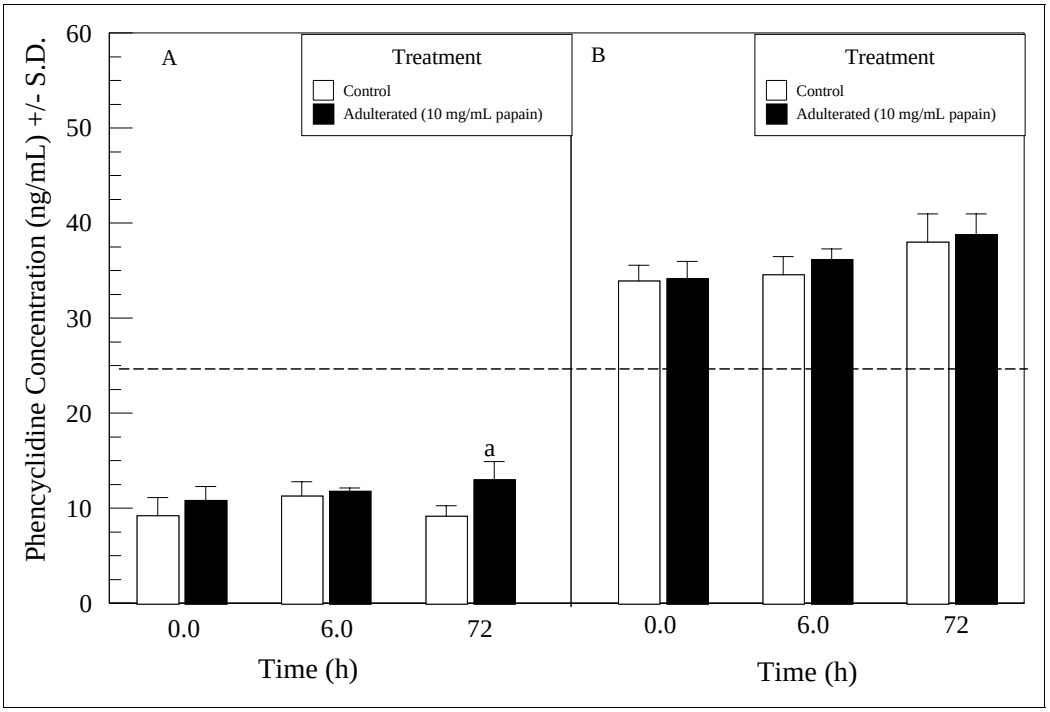

Figure 34 Effects of papain (10 mg/mL) on phencyclidine concentrations over time in pH 6.2 synthetic urine, n=6

Panel A- 37.5 ng/mL phencyclidine solution

Panel B- 12.5 ng/mL phencyclidine solution

[a] Significant "intra-temporal" difference, p<0.01

Table 32 The average phencyclidine concentration over time in pH 6.2 synthetic urine containing 0 and 10 mg/mL papain

Initial amphetamine concentration (ng/mL)	Time (h)	Papain concentration	Average phencyclidine concentration (ng/mL)	SD	Intra-temporal, p<0.01	Inter-temporal, p<0.01
12.5	0	0	9.22	1.88		
	0	10	10.85	1.41		
	6	0	11.27	1.50		
	6	10	11.75	0.41		
	72	0	9.15	1.15		
	72	10	13.02	1.91	X	
37.5	0	0	33.89	1.65		
	0	10	34.18	1.80		
	6	0	34.56	1.96		
	6	10	36.17	1.11		
	72	0	37.99	3.03		
	72	10	38.84	2.17		

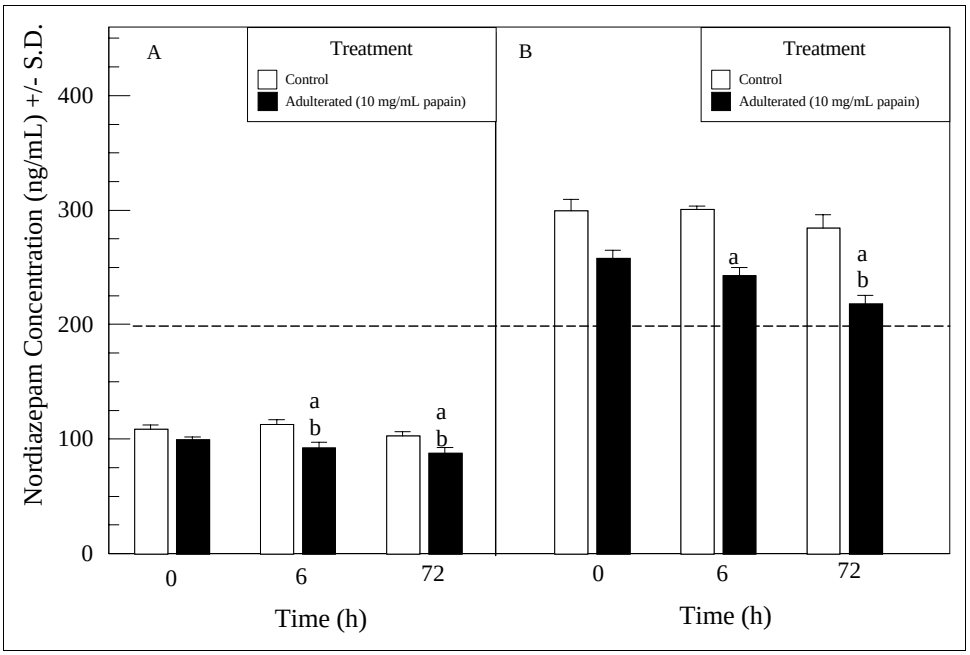

Figure 35 Effects of papain (10 mg/mL) on nordiazepam concentrations over time in pH 6.2 synthetic urine, n=6

Panel A- 300 ng/mL nordiazepam solution

Panel B- 100 ng/mL nordiazepam solution

[a] Significant "intra-temporal" difference, p<0.01

[b] Significant "inter-temporal" difference, p<0.01

Table 33 The average nordiazepam concentration over time in pH 6.2 synthetic urine containing 0 and 10 mg/mL papain

Initial amphetamine concentration (ng/mL)	Time (h)	Papain concentration	Average nordiazepam concentration (ng/mL)	SD	Intra-temporal, p<0.01	Inter-temporal, p<0.01
100	0	0	108.60	4.04		
	0	10	99.40	2.52		
	6	0	113.03	3.64		
	6	10	91.84	5.22	X	X
	72	0	102.47	3.87		
	72	10	87.64	4.67	X	X
300	0	0	299.44	9.63		
	0	10	257.64	7.46		
	6	0	300.86	2.79		
	6	10	242.95	6.98	X	
	72	0	284.15	11.9		
	72	10	218.06	7.43	X	X

BANI Assay of Inhibited RP Enzymatic Activity

The average absorbance changes (delta-mabs) in 4 minutes of the blank, RP, and DRP specimens are given in Table 34. Absorbance changes less than that of the blank specimen indicate abolishment of enzymatic activity. Table 34 indicates the rate of absorbance change of the DRP preparations was less than the rate of absorbance change for the blank. A milligram ratio of 1:1 of E-64 to papain is therefore a sufficient quantity to render the papain enzymatically inactive. The RP preparation yielded an absorbance change of 36 mabs/min, thus verifying the enzymatic activity of the recrystallized papain.

Table 34 The average absorbance rate change for blank, RP, and DRP specimens, n=3

Specimen	Average delta-mabs in 4 minutes	S.D.
Blank	1	0
RP	36	1
DRP	0	0

FPIA Assays with RP and DRP

The effect of 1 mg/mL RP and 1 mg/mL deactivated DRP on THC-COOH and nordiazepam concentrations are illustrated in Figures 36 and 37 and Tables 35 and 36. Figure 36 and Table 35 indicate that both the 1 mg/mL RP and DRP solutions had a significant effect on 60 ng/mL THC-COOH after 2 hours. The groups adulterated with RP and DRP were not statistically different. There was an average (SD) 37% (6%) difference between the control group and the groups adulterated with 1 mg/mL RP and DRP. Data suggest the reductions in THC-COOH concentrations observed with the crude latex powder were, at the very least, partly attributable to papain itself versus an effect from the latex matrix. They also suggest that the reduction in THC-COOH is not enzymatic, and may be due to nonspecific binding.

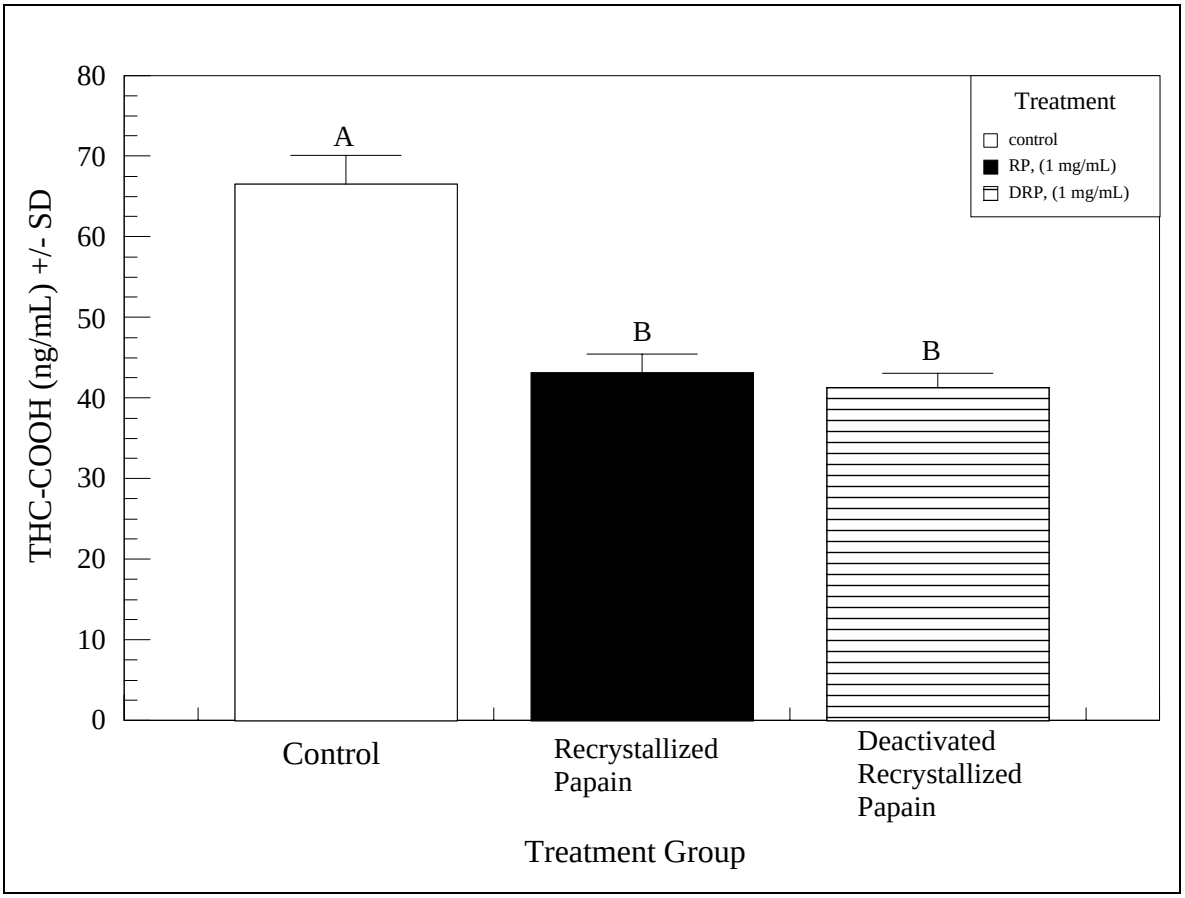

Figure 36 The effect of recrystallized papain (RP) and deactivated recrystallized papain (DRP) on THC-COOH concentrations, n=6

[A,B] Groups denoted with the same letter are not significantly different, $p<0.01$

Table 35 The average concentration of THC-COOH in pH 6.2 synthetic urine containing 60 ng/mL THC-COOH with 1 mg/mL recrystallized and deactivated recrystallized papain

Group	Average THC-COOH concentration (ng/mL)	SD	Significant difference, P<0.01
Control	66.52	3.56	A
Recrystallized papain (1 mg/mL)	43.08	2.32	B
Deactivated recrystallized papain (1 mg/mL)	41.29	1.73	B

[A,B] Groups denoted with the same letter are not significantly different, p<0.01

Figure 37 and Table 36 indicate that both the 1 mg/mL RP and DRP solutions did not have a significant effect on 250 ng/mL nordiazepam after 2 hours. The groups adulterated with RP and DRP were not statistically different from the unadulterated control group. Data indicate reductions the in nordiazepam concentrations observed with the crude latex powder were entirely attributable to the latex matrix.

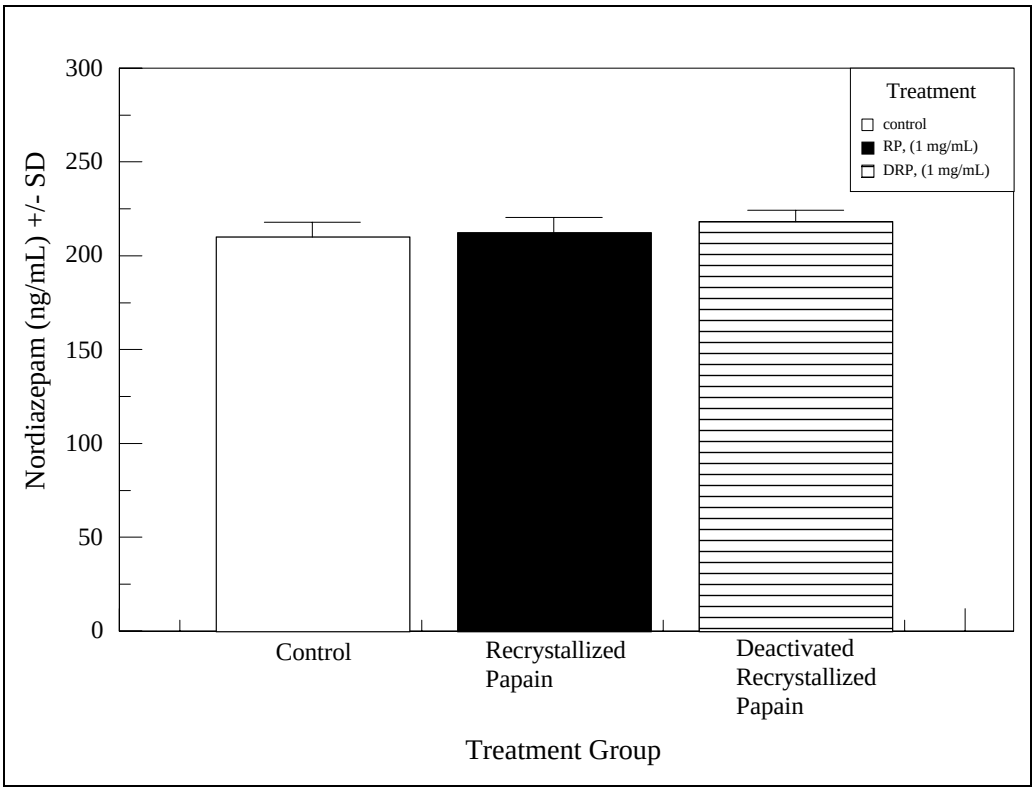

Figure 37 The effect of recrystallized papain (RP) and deactivated recrystallized papain (DRP) on nordiazepam concentrations, n=6

Table 36 The average concentration of nordiazepam in pH 6.2 synthetic urine containing 250 ng/mL nordiazepam with 1 mg/mL recrystallized and deactivated recrystallized papain

Group	Average nordiazepam concentration (ng/mL)	SD	Significant difference, P<0.01
Control	209.97	7.79	
Recrystallized papain (1 mg/mL)	212.31	8.28	
Deactivated recrystallized papain (1 mg/mL)	218.09	6.19	

Gas Chromatography/Mass Spectroscopy of THC-COOH

The THC-COOH concentrations of adulterated specimens initially containing 500 ng/mL THC-COOH in pH 6.2 synthetic urine with 10 mg/mL papain are listed in Table 37. Data indicate the average (SD) concentration of the specimens adulterated with 10 mg/mL papain was 168.36 (+/- 30.34) ng/mL THC-COOH, a 66% decrease from the initial THC-COOH concentration of 500 ng/mL. Data suggest the reported decrease in THC-COOH concentrations observed in the FPIA assays involves an interaction between papain and THC-COOH versus an interference with the FPIA assay. Furthermore, the chromatogram and mass spectra of the adulterated specimens did not reveal mass fragments that could be attributed to the degradation of THC-COOH by papain (data not shown).

Table 37 THC-COOH concentrations of adulterated specimens that contained 500 ng/mL THC-COOH and 10 mg/mL papain.

Replicate number	THC-COOH concentration (ng/mL)
1	208
2	145
3	141
4	141
5	173
6	199
Average	168
SD	30.3

High Pressure Liquid Chromatography/Ultraviolet Detection of Nordiazepam

The nordiazepam concentrations of adulterated specimens initially containing 300 ng/mL nordiazepam in pH 6.2 synthetic urine with 10 mg/mL papain are listed in Table 38. Data

indicate the average (SD) concentration of the specimens adulterated with 10 mg/mL papain was 228.18 (+/- 12.14) ng/mL nordiazepam, a 24% decrease from the initial nordiazepam concentration of 300 ng/mL. Data suggest the reported decrease in nordiazepam concentrations observed in the FPIA assays involves an interaction between papain and nordiazepam versus an interference with the FPIA assay.

Table 38 Nordiazepam concentrations of adulterated specimens that contained 300 ng/mL nordiazepam and 10 mg/mL papain

Replicate number	Nordiazepam concentration (ng/mL)
1	249
2	226
3	232
4	220
5	224
6	214
Average	228
SD	12.1

THC-COOH Binding Plots

Figures 38-40 illustrate the binding (B) of THC-COOH (ng/mg papain) versus free THC-COOH (THC-$COOH_{free}$) as measured by FPIA analyses in synthetic urine at pH 4.5, 6.2, and 8.0. Data that yielded "negative" binding of THC-COOH, representing the few instances in which the concentration of THC-COOH increased, were not included in the model. The linear regression performed on the data from pH 6.2 and 8.0 analyses yielded a lined defined by the equation: $B= k_{obs} * [THC\text{-}COOH_{free}]$, $k_{obs}= \Sigma k_n$, where k_n represents the binding constant of a particular constituent of the CLP preparation. The k_{obs} for the binding of THC-COOH in pH 6.2 synthetic urine was 0.7685. The k_{obs} for the binding of THC-COOH in pH 8.0 synthetic urine was 0.0784. The linearity of the binding plots from pH 6.2 and 8.0 are indicative of nonspecific binding of THC-COOH to papain and further corroborates with the data obtained from the experiments performed with RP and DRP preparations of papain, and the data obtained from the GC/MS experiments. The data from pH 4.5 analyses were unable to be fit by a mathematical model that describes either specific (hyperbolic) or nonspecific binding (linear), Figure 40. A

mechanism of interference is therefore more difficult to postulate as other factors, i.e. a matrix effect, have yielded data that is not able to be interpreted at this time.

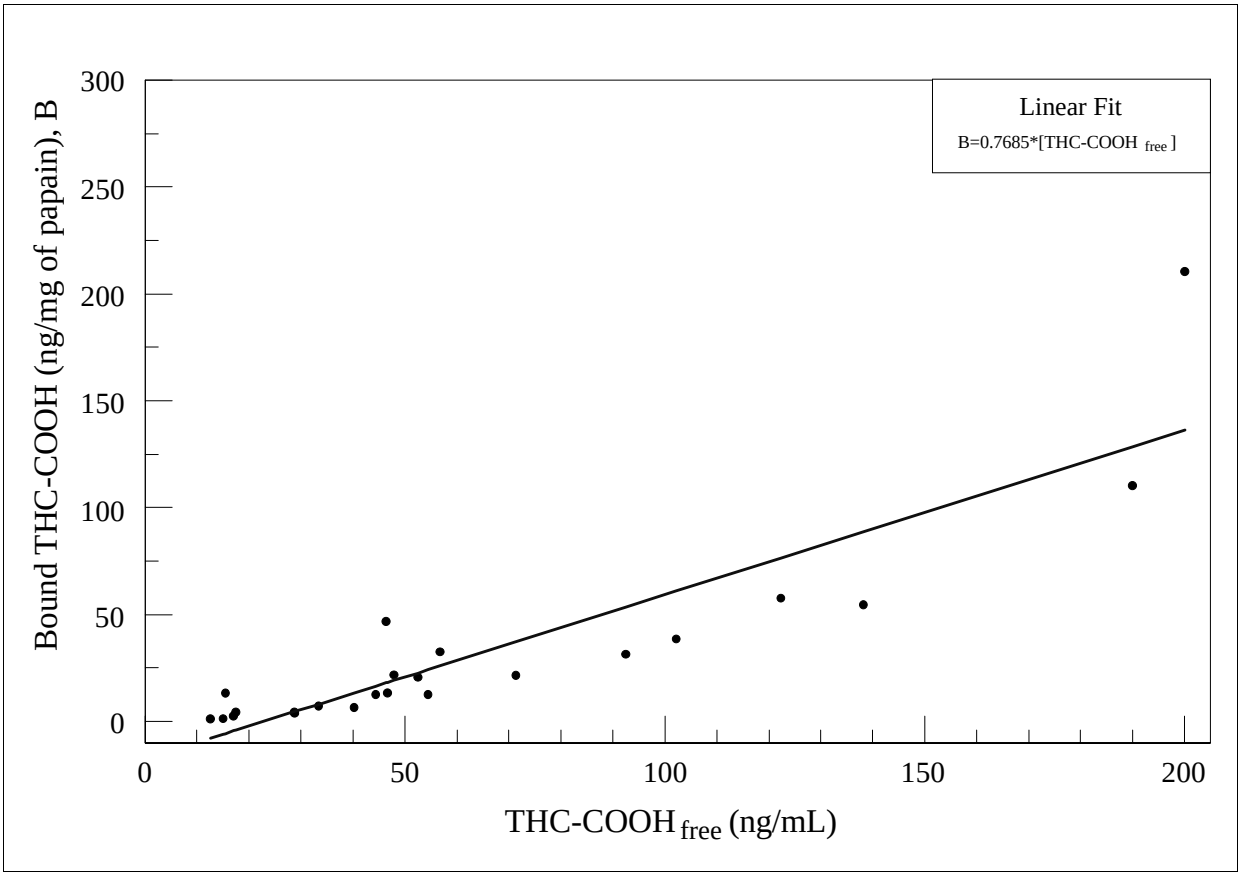

Figure 38 The binding of THC-COOH (ng/mg papain) versus free THC-COOH, pH 6.2

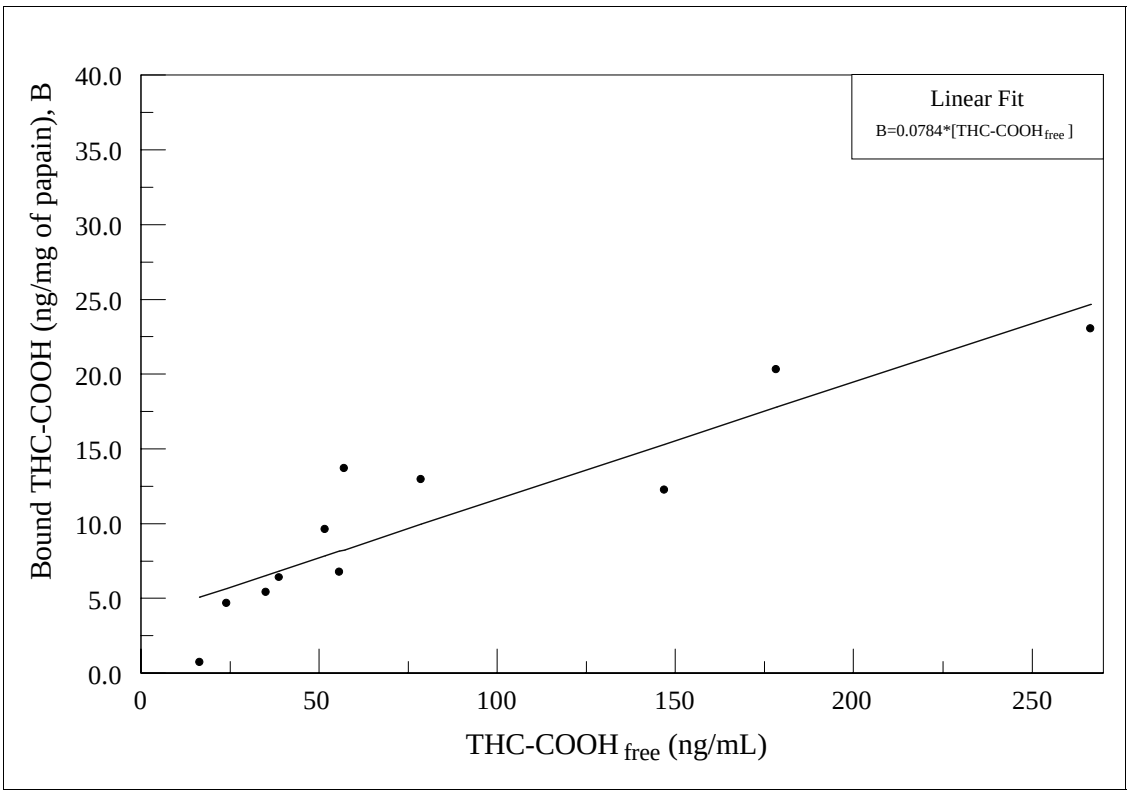

Figure 39 The binding of THC-COOH (ng/mg papain) versus free THC-COOH, pH 8.0

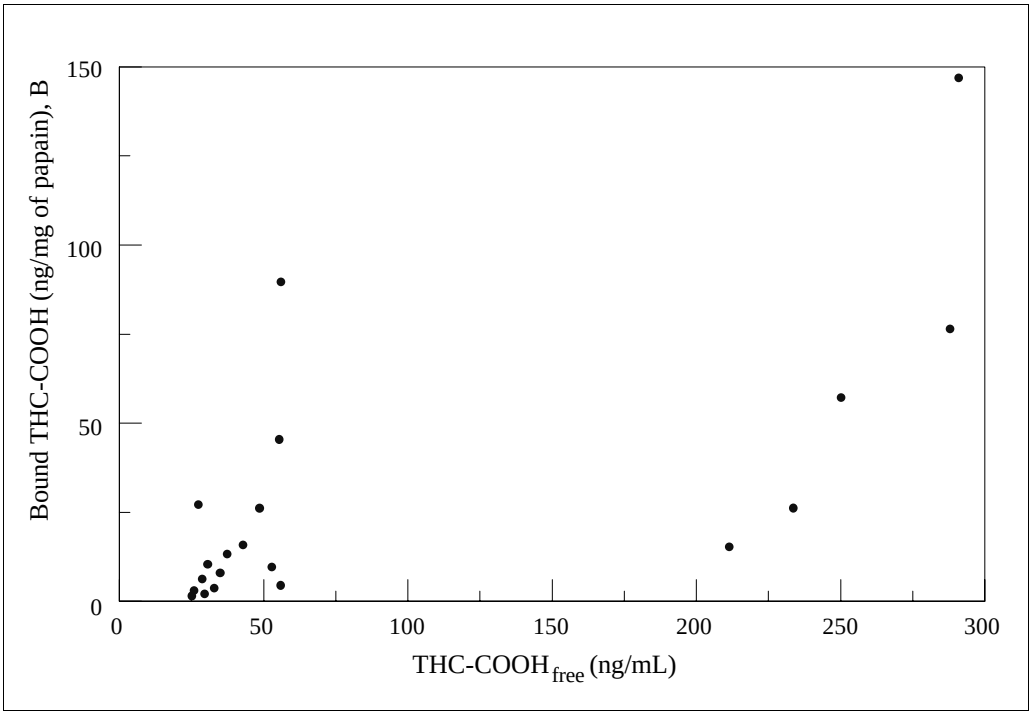

Figure 40 The binding of THC-COOH (ng/mg papain) versus free THC-COOH, pH 4.5

Creatinine

Figure 41 and Table 39 describe the immediate effect of 10 mg/mL papain on creatinine in 30 random urine specimens. The median creatinine concentration, skewness, and kurtosis values of the control population were 87.8, 1.025, and 0.682. The median creatinine concentration, skewness, and kurtosis values of the population adulterated with 10 mg/mL papain were 88.9, 1.29, and 1.74. Figure 42 and Table 40 describe the effect of 10 mg/mL papain on creatinine over time in 6 random urine specimens. Figures 41 and 42 and Tables 39 and 40 indicate that 10 mg/mL papain did not have a significant effect on the distribution of urine creatinine values in 30 random urine specimens, nor did 10 mg/mL papain have a significant effect on the urine creatinine values over time in 6 random urine specimens. Individuals adulterating their urine with up to 10 mg/mL papain would therefore not alter their urine creatinine concentration.

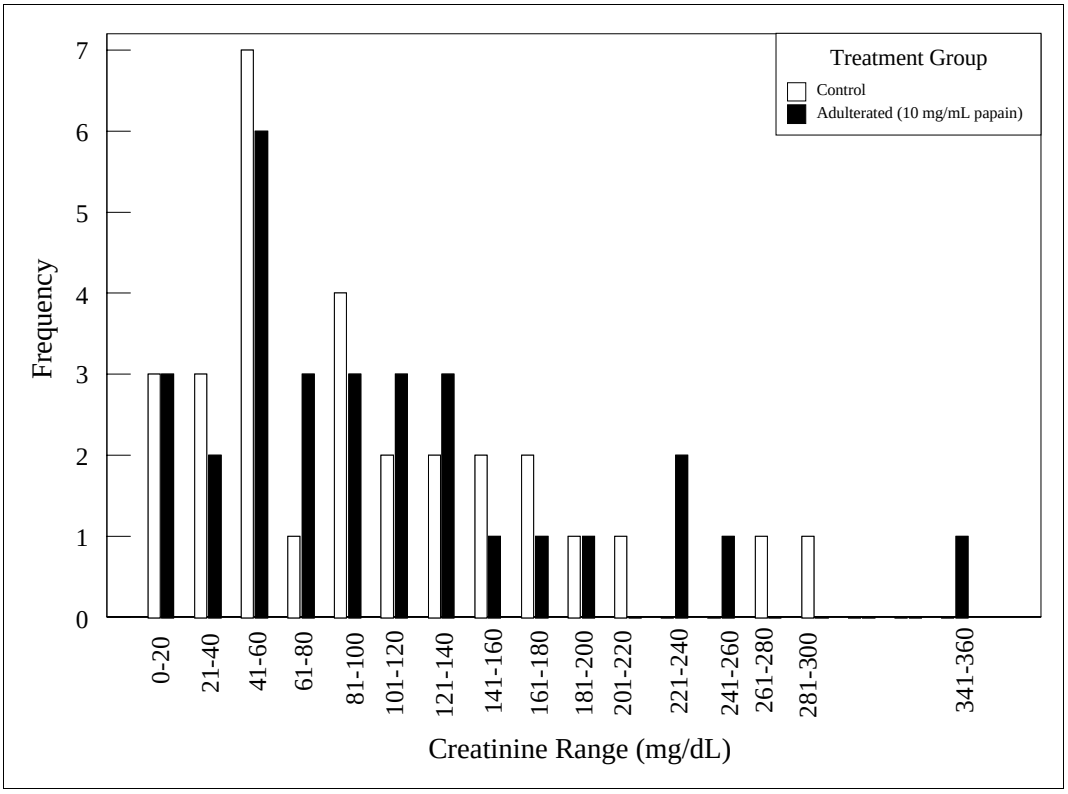

Figure 41 The effect of 10 mg/mL papain on the distribution of urine creatinine concentrations in a population (n=30) of random urine specimens

Table 39 The distribution of urine creatinine concentrations in a population (n=30) of unadulterated random urine specimens and specimens adulterated with 10 mg/mL papain

Creatinine concentration range (mg/dL)	Frequency of unadulterated specimens (n=30)	Frequency of specimens (n=30) adulterated with 10 mg/mL papain
0-20	3	3
21-40	3	2
41-60	7	6
61-80	1	3
81-100	4	3
101-120	2	3
121-140	2	3
141-160	2	1
161-180	2	1
181-200	1	1
201-220	1	
221-240	0	2
241-260	0	1
261-280	1	
281-300	1	
341-360		1

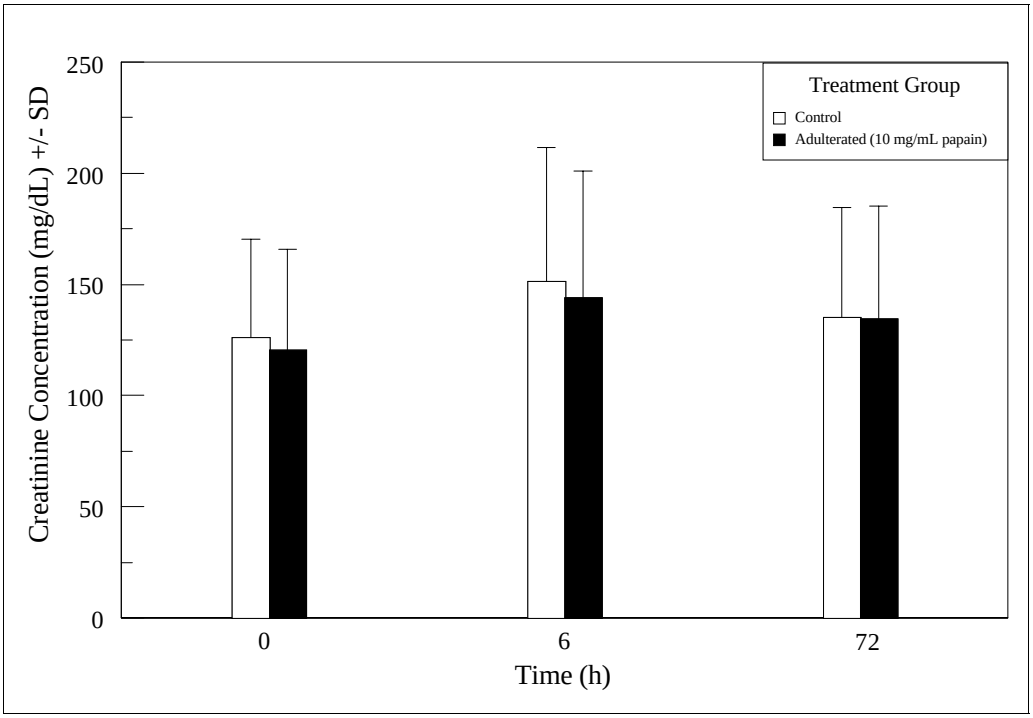

Figure 42 The effect of 10 mg/mL papain on the average urine creatinine concentration over time in a population (n=6) of random urine specimens

Table 40 The average urine creatinine concentration in a population (n=6) of unadulterated random urine specimens and specimens adulterated with 10 mg/mL papain

Time (h)	Average creatinine concentration (mg/dL) in the unadulterated specimens (n=6)	Average creatinine concentration (mg/dL) in the specimens (n=6) adulterated with 10 mg/mL papain
0	126.1	120.3
6	151.2	144.0
72	135.2	134.7

Specific Gravity

Table 41 lists the typical reported values of the 1.002, 1.005, and 1.030 specific gravity quality control standards. Table 41 illustrates that the refractometer reported specific gravity values with a maximum 0.3% error. Data indicate the method employed for obtaining the specific gravity in urine is valid up to the limit (1.05) of the refractometer.

Table 41 Specific gravity values of the quality control specimens

Quality control specimen	Reported value
1.002	1.002
1.005	1.005
1.030	1.033

Figure 43 and Table 42 describe the immediate effect of 10 mg/mL papain on urine specific gravity in 30 random urine specimens. The median specific gravity, skewness, and kurtosis values of the control population were 1.014, 0.415, and -0.337. The median specific gravity, skewness, and kurtosis values of the population adulterated with 10 mg/mL papain were 1.019, 0.545, and -0.070. Figure 44 and Table 43 describe the effect of 10 mg/mL papain on urine specific gravity over time in 6 random urine specimens. Figures 43 and 44 and Tables 42 and 43 illustrate that 10 mg/mL had a significant effect on the distribution of urine specific gravity values in 30 random urine specimens, and 10 mg/mL papain had a significant effect on the urine specific gravity values over time in 6 random urine specimens. The maximum increase

in urine specific gravity was < 0.008 (0.7%) and there were no specimens in which the specific gravity was less than 1.001. Overall, 10 mg/mL papain in 30 random urine specimens gave the appearance of a greater urine concentration. Individuals adulterating their urine with up to 10 mg/mL papain would not increase the urine specific gravity above 0.7%.

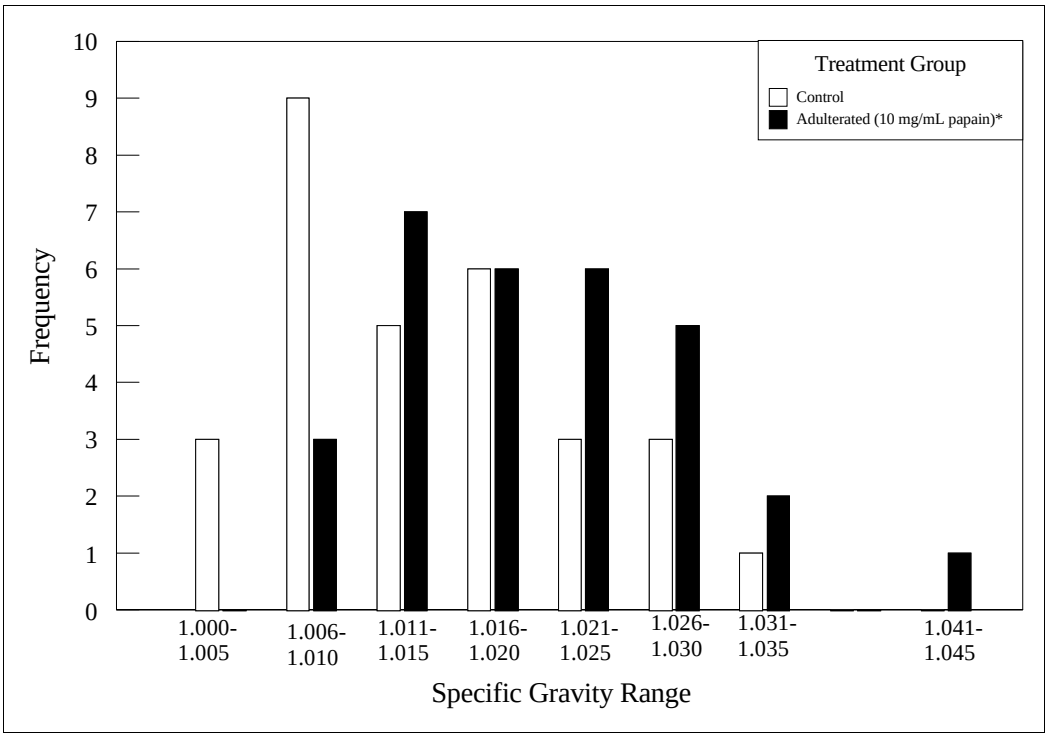

Figure 43 The effect of 10 mg/mL papain on the distribution of urine specific gravity in a population (n=30) of random urine specimens

* Significant difference, $p<0.05$

Table 42 The distribution of urine specific gravity in a population (n=30) of unadulterated random urine specimens and specimens adulterated with 10 mg/mL papain

Specific gravity range	Frequency of unadulterated specimens (n=30)	Frequency of specimens (n=30) adulterated with 10 mg/mL papain
1.001-1.005	3	0
1.006-1.010	9	3
1.011-1.015	5	7
1.016-1.020	6	6
1.021-1.025	3	6
1.026-1.030	3	5
1.031-1.035	1	2
1.041-1.045		1

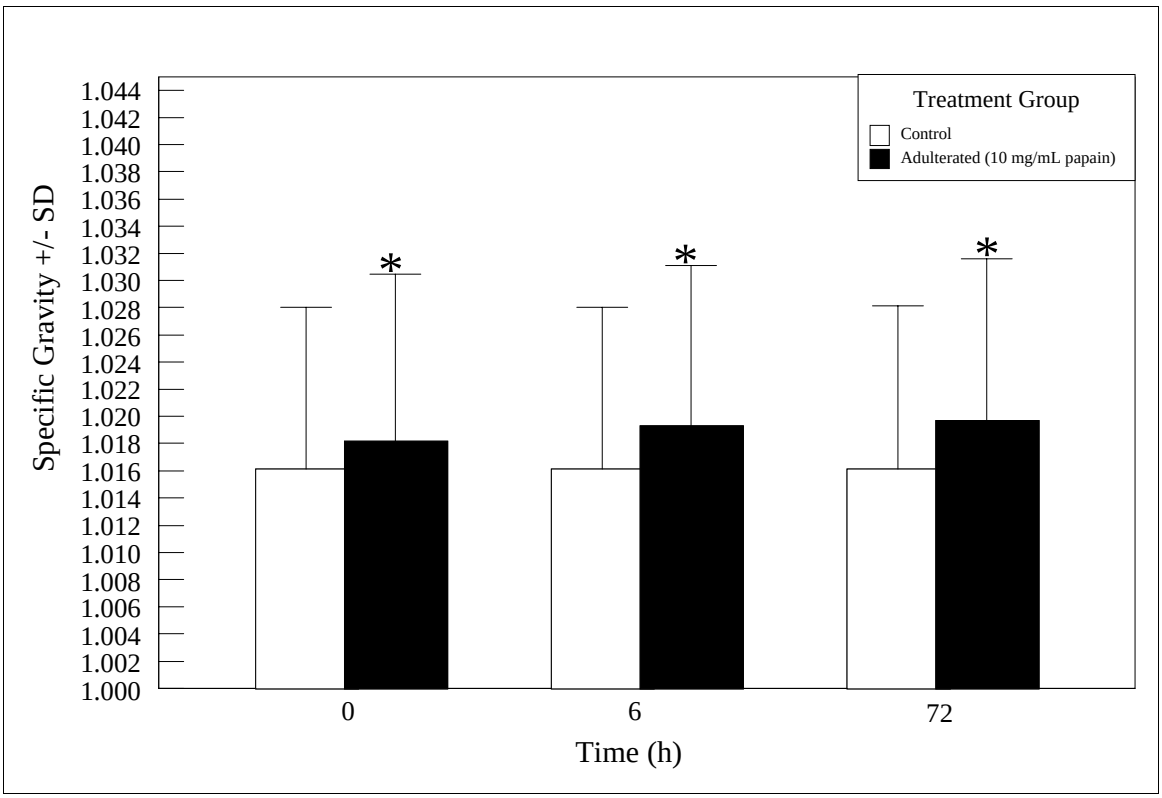

Figure 44 The effect of 10 mg/mL papain on the average urine specific gravity over time in a population (n=6) of random urine specimens

* Significant difference, $p<0.05$

Table 43 The average urine specific gravity in a population (n=6) of unadulterated random urine specimens and specimens adulterated with 10 mg/mL papain

Time (h)	Average specific gravity in the unadulterated specimens (n=6)	Average specific gravity in the specimens (n=6) adulterated with 10 mg/mL papain
0	1.016	1.018
6	1.016	1.019
72	1.016	1.020

pH

Figure 45 and Table 44 describe the immediate effect of 10 mg/mL papain on urine pH in 30 random urine specimens. The median pH, skewness, and kurtosis values of the control population were 6.06, 0.485, and -0.616. The median pH, skewness, and kurtosis values of the population adulterated with 10 mg/mL papain were 5.85, 1.06, and 1.67. Figure 46 and Table 45 describe the effect of 10 mg/mL papain on urine pH over time in 6 random urine specimens. Figure 45 and Table 44 indicate that 10 mg/mL papain had a significant effect on the decrease in pH of thirty random urine specimens. The median pH range of the 30 unadulterated

specimens was 6.0 to 6.2 while the median pH range of the 30 specimens adulterated with 10 mg/mL papain was 5.7 to 5.9. Figure 46 and Table 45 indicate that 10 mg/mL papain did not have a significant effect on the pH of the 30 random urine specimens. Although there was a minor decrease in the pH in the adulterated specimens over time, it was not statistically different than the unadulterated control. There were no unadulterated or adulterated specimens with a pH >11 or <3. Individuals adulterating their urine specimens with up to 10 mg/mL papain would not increase the pH of their urine to a pH >11 or decrease their urine to a pH <3, thus rendering the urine specimen invalid.

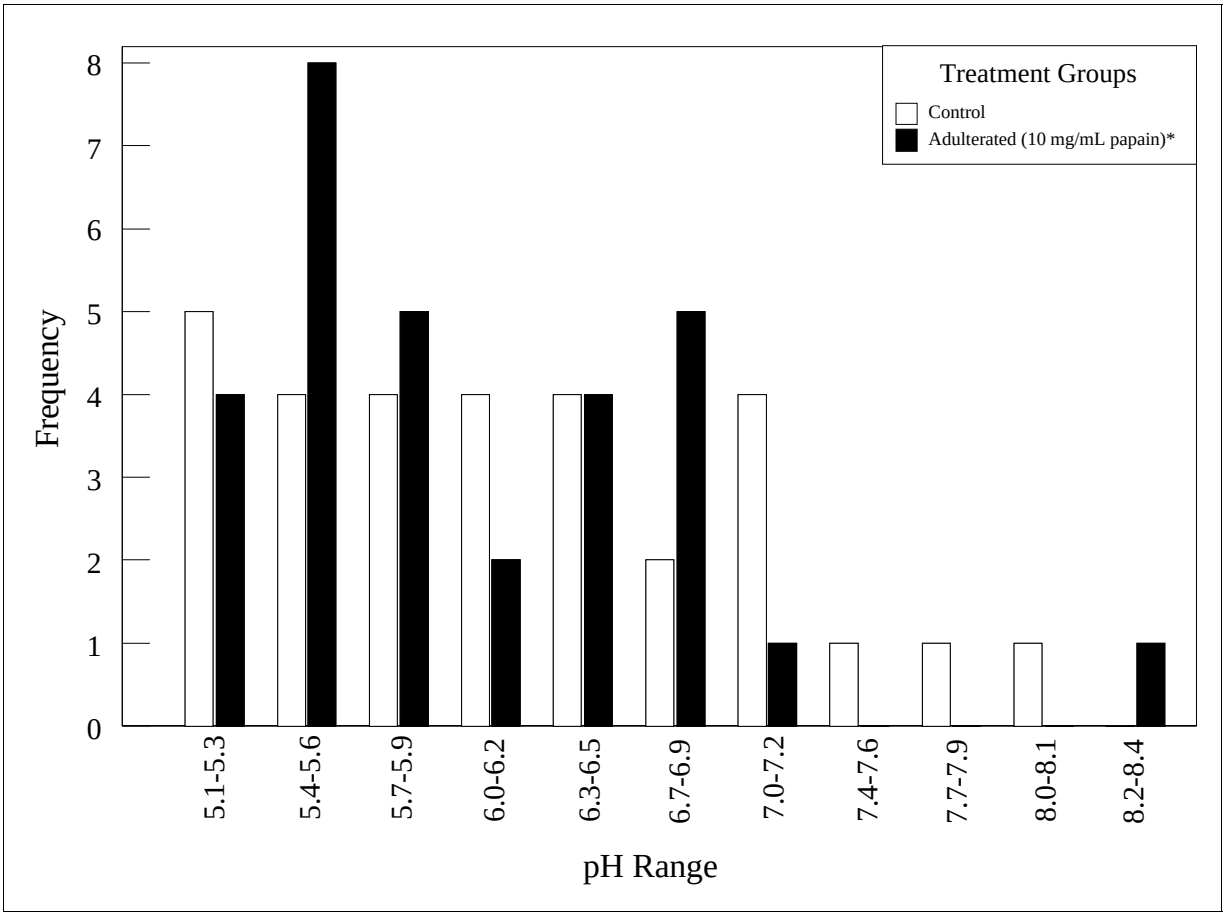

Figure 45 The effect of 10 mg/mL papain on the distribution of urine pH in a population (n=30) of random urine specimens

* Significant difference, $p<0.05$

Table 44 The distribution of urine pH in a population (n=30) of unadulterated random urine specimens and specimens adulterated with 10 mg/mL papain

pH range	Frequency of unadulterated specimens (n=30)	Frequency of specimens (n=30) adulterated with 10 mg/mL papain
5.1-5.3	5	4
5.4-5.6	4	8
5.7-5.9	4	5
6.0-6.2	4	2
6.3-6.5	4	4
6.6-6.8	2	5
6.9-7.1	4	1
7.2-7.3	1	
7.4-7.6	1	
7.7-7.9	1	
8.0-8.2		1

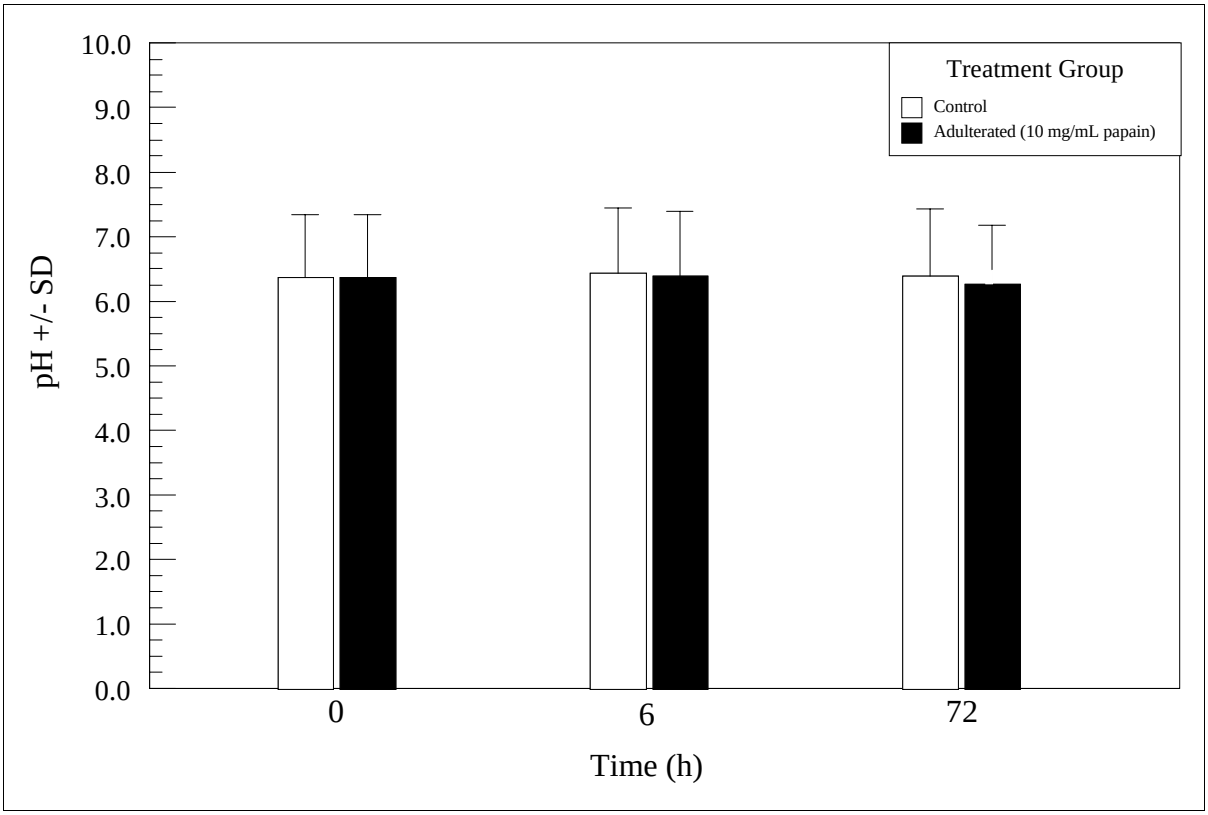

Figure 46 The effect of 10 mg/mL papain on the average urine pH over time in a population (n=6) of random urine specimens

Table 45 The average urine pH in a population (n=6) of unadulterated random urine specimens and specimens adulterated with 10 mg/mL papain

Time (h)	Average pH values in the unadulterated specimens (n=6)	Average pH values in the specimens (n=6) adulterated with 10 mg/mL papain
0	6.4	6.4
6	6.4	6.4
72	6.4	6.3

Osmolality

Figure 47 and Table 46 describe the immediate effect of 10 mg/mL papain on urine osmolality in 30 random urine specimens. The median osmolality, skewness, and kurtosis values of the control population were 509, 0.304, and -0.039. The median osmolality, skewness, and kurtosis values of the population adulterated with 10 mg/mL papain were 535, 0.261, and -0.095. Figure 48 and Table 47 describe the effect of 10 mg/mL papain on urine osmolality over time in 6 random urine specimens. Figures 47 and 48 and Tables 46 and 47 indicate that 10 mg/mL had a significant effect on the distribution of urine osmolality in 30 random urine specimens, and 10 mg/mL papain had a significant effect on the urine osmolality over time in 6 random urine specimens. The maximum increase in the average urine osmolality was less than 5.3% over time and there were no specimens in which the urine osmolality < 59 mOsm/kg. Individuals adulterating their urine with up to 10 mg/mL papain would not increase their urine osmolality by more than 5.3%. In comparison to the maximum percent increase in specific gravity (0.7%), the maximum percent increase in osmolality (5.3%) represents a method of quantifying urine concentration that is more sensitive to the addition of adulterating substances.

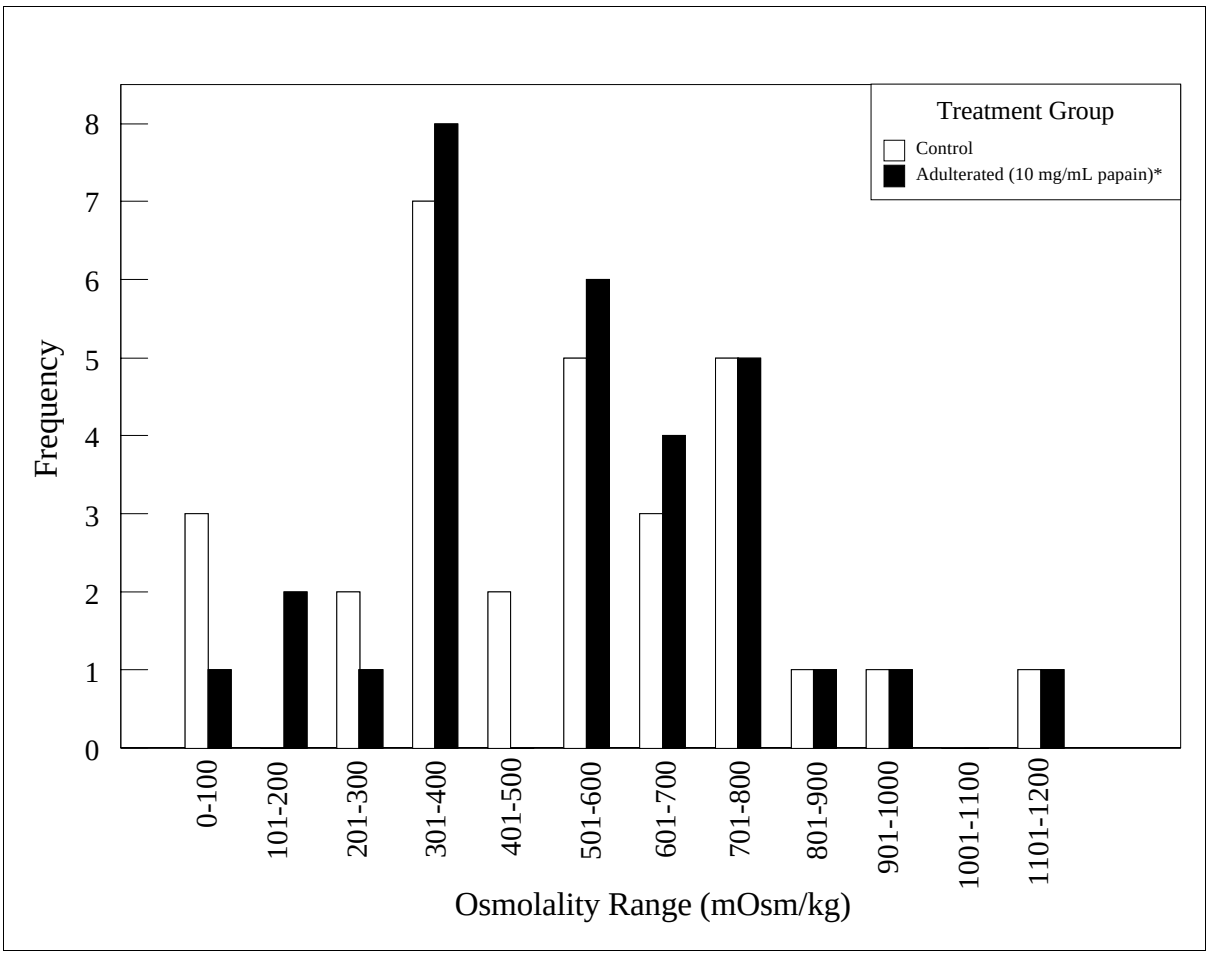

Figure 47 The effect of 10 mg/mL papain on the distribution of urine osmolality in a population (n=30) of random urine specimens

* Significant difference, $p<0.05$

Table 46 The distribution of urine osmolality in a population (n=30) of unadulterated random urine specimens and specimens adulterated with 10 mg/mL papain

Osmolality range (mOsm/kg)	Frequency of unadulterated specimens (n=30)	Frequency of specimens (n=30) adulterated with 10 mg/mL papain
0-100	3	1
101-200		2
201-300	2	1
301-400	7	8
401-500	2	
501-600	5	6
601-700	3	4
701-800	5	5
801-900	1	1
901-1000	1	1
1101-1200	1	1

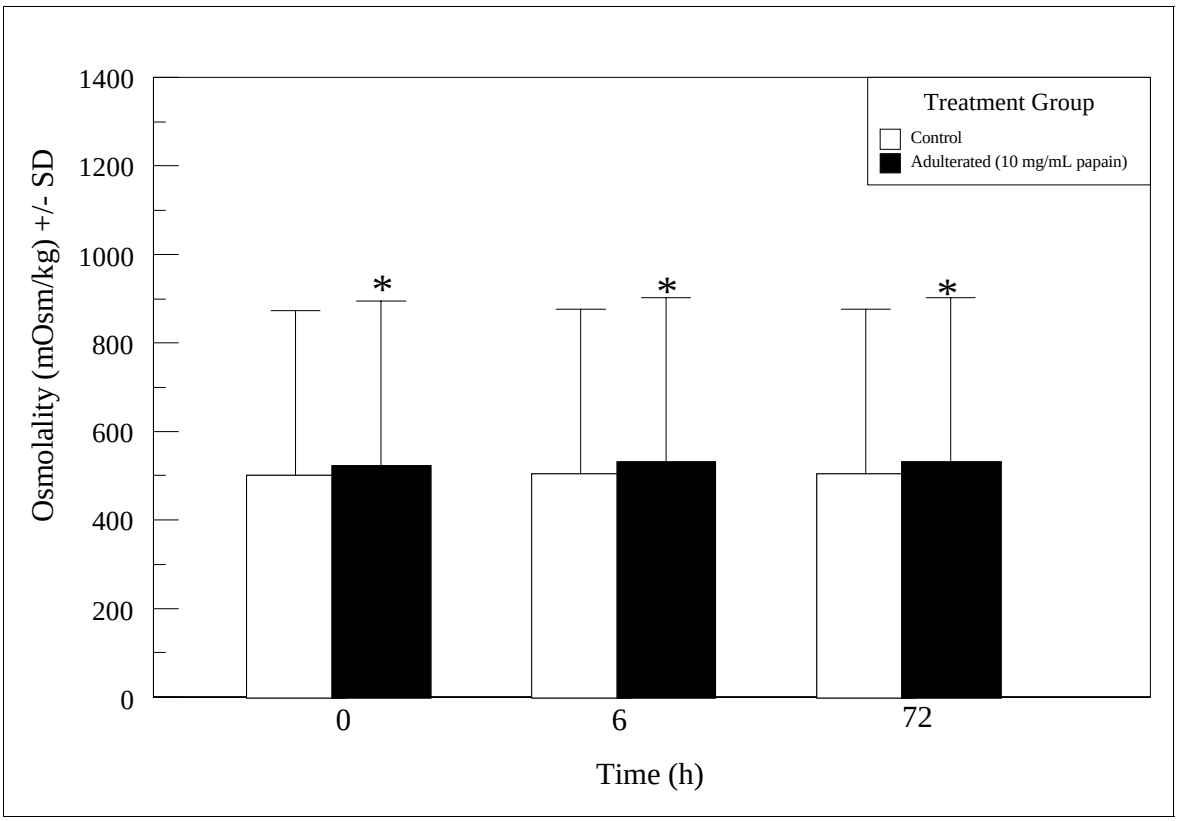

Figure 48 The effect of 10 mg/mL papain on the average urine osmolality over time in a population (n=6) of random urine specimens

* Significant difference, $p<0.05$

Table 47 The average urine osmolality in a population (n=6) of unadulterated random urine specimens and specimens adulterated with 10 mg/mL papain

Time (h)	Average osmolality in the unadulterated specimens (n=6)	Average osmolality in the specimens (n=6) adulterated with papain (10 mg/mL)
0	501	524
6	502	525
72	504	531

Nitrates, Glutaraldehyde, and Oxidants

Table 48 illustrates the effect of 10 mg/mL papain on POC chromaphore assays for nitrites, glutaraldehyde, and oxidants on a population of random urine specimens (n=6). Table 48 illustrates that 10 mg/mL papain did not have a significant effect on the presence of nitrates, glutaraldehyde or oxidants in 30 random urine specimens. The nitrite concentration revealed in the unadulterated specimen and paired specimen adulterated with 10 mg/mL papain was < 500 µg/mL, and therefore below the cutoff level that denotes an adulterated specimen. The

adulterated urine of individuals who add up to 10 mg/mL papain to their urine would not test positive for the presence of glutaraldehyde or oxidants, nor would the concentration of nitrites increase due to the presence of papain.

Table 48 The presence of nitrite, glutaraldehyde, and oxidants in a population (n=6) with 10 mg/mL papain

Group	Nitrite		Glutaraldehyde		Oxidants	
	Positive	Negative	Positive	Negative	Positive	Negative
Unadulterated, n=6	1[a]	5	0	6	0	6
Adulterated, 10 mg/mL papain, n=6	1[a]	5	0	6	0	6

[a] The nitrite concentration was <500 μg/mL

CHAPTER 5
DISCUSSION

Papain Standardization

The choice of the crude latex preparation of papain (CLP) as the subject of our research involved several economic and practical issues. The commercial manufacture of the crude latex preparation of papain as a urine adulterant would be a more economical than a purified preparation and is therefore a more marketable adulteration product. The effects of CLP on the measured concentrations of THC-COOH would also represent the minimal effects observed if a commercial urine adulterant contained a more refined preparation of papain as the active ingredient. An individual intending to adulterate a urine specimen would be able to transport sufficient quantities of a crude powder preparation at room temperature in a clandestine 0.5 cubic centimeter plastic pouch.

In contrast to the recrystallized papain, the CLP preparation had an enzyme activity of 4.32% per milligram the enzyme activity of recrystallized papain. As research project revealed the enzymatic activity was not an essential component of the mechanism of adulteration, the significance of the enzymatic activity of CLP preparation is therefore somewhat diminished.

Enzyme Multiplied Immunoassay Technique

The preliminary data suggested that further research was required by an alternative method to delineate the dose dependent effects of papain with respect to pH, THC-COOH concentration, and time. One hundred percent of the adulterated positive specimens assayed yielded a false-negative result. The further investigation into papain's effect on the measured concentration of THC-COOH by another "class" of immunoassay not only delineated the dose dependent effects of papain but revealed more information about the possible mechanism of interference and the range of immunoassays that papain's effects might be observed. As previously stated, the target of the adulterant is of concern, be it the analytes or the immunoassay reagents. A reduction in the measured concentration of THC-COOH by two different classes of immunoassays would indicate the mechanism of interference may involve the analyte versus the immunoassay's reagents.

Fluorescence Polarized Immunoassay Technique

THC-COOH Assays

A majority (94%) of all adulterated specimens, over time and a physiological urine pH range, reported a decreased concentration of THC-COOH relative to the unadulterated control and the individual baseline value (T=0). Employing the conservative p-value of 0.01 in the "intra-temporal" and "inter-temporal" ANOVAs ensured that the differences between the control and the adulterated urine specimens were indeed genuine. In Figures 12,14,19, and 20 an apparent increase of THC-COOH was reported in the control group at selected time points. Every analytical toxicology method has an associated degree of variability in which the control specimens for a particular assay must lye. The established FPIA method for the quantitation of drugs of abuse allows for a 10 to 32% variation in the reported value of the daily calibrators. These variations are established by the U.S. Food and Drug Administration based on the research validating the FPIA methodology. In most instances, the apparent increase in the THC-COOH concentration of the control group were within the allowed variations of the assay. In the few instances in which the THC-COOH concentration of the control group increased from the baseline value, the adulterated specimens were consistent in reporting THC-COOH concentrations less than the control group. Figures 11 and 23 exhibited an apparent increase in the reported concentration of THC-COOH in the specimens adulterated with less than 5.0 mg/mL papain. A possible explanation of the apparent increase in THC-COOH concentration is an interference with the FPIA assay. Interference with immunoassays such as the FPIA have previously been observed with other adulterants, as the quantitation of the analyte is inversely proportional to the amount of polarized light emitted from the specimen (Cody and Schwartzhoff 1989). It should be noted however, that the apparent increase in THC-COOH concentration occurred in less than 6% of all cannabinoid assays performed and were not consistent across THC-COOH concentrations or within a specific pH of synthetic urine. Furthermore, the apparent increase was not observed in the FPIA assays for other drugs of abuse, Figures 30-35. These observations are also not consistent with the degradation of papain, as a trend for increased analyte concentration over time was not observed among all papain

concentration, or FPIA assays. The lack of consistency across THC-COOH concentrations, pH, or among FPIA assays for other drugs of abuse and the infrequency of the apparent increase in THC-COOH concentration occurrence is evidence that the apparent increase was artifactual.

The Effect of Papain Concentration, Time, THC-COOH Concentration, and pH on the Percent Decrease of THC-COOH. Although the statistical significance of papain's effects on THC-COOH concentrations between papain concentrations of 2.0, 5.0 and 10 mg/mL were occluded by the large variations generated by the analysis, a direct correlation between the percent decrease of THC-COOH concentration and the papain concentration was observed. Data does not indicate the decrease in THC-COOH concentration involves an enzymatic mechanism as gross significant reductions in THC-COOH concentrations were not observed after 4 to 6 hours. A proportional decrease in the absolute THC-COOH concentration should be expected relative to the initial THC-COOH concentration as illustrated by Figure 28.

Generally, 10 mg/mL papain had the most demonstrative effects in pH 6.2 synthetic urine in terms of percent difference of THC-COOH from the unadulterated control. The largest absolute decrease in THC-COOH was observed in pH 4.5 synthetic urine; however, the decrease in THC-COOH concentration was partially attributed to the pH of the matrix. If an individual urine specimen contains < 50 ng/mL THC-COOH, a false positive FPIA result would not be expected if an individual adulterated his urine with 10 mg/mL papain.

FPIA Assays for Other Drugs of Abuse

The average percent decrease in THC-COOH (~50%) over 72 hours is in stark contrast to the maximum percent decrease in nordiazepam (~12%) in specimens adulterated with 10 mg/mL papain in pH 6.2 synthetic urine. The difference in percent decreases between the two analytes is suggestive of an alternative mechanism of adulteration. Nevertheless, the potential for papain to yield a false negative result by FPIA analyses for the 7 analytes involved in our research was limited to cannabinoids and benzodiazepines. To reiterate, the lack of papain's effect on the reported concentrations of other drugs of abuse by the FPIA immunoassay suggests that the mechanism of interference involves an interaction with specific analytes versus the FPIA reagents. Figure 4 illustrates that each analyte is structurally foreign to one another and each

analyte would therefore have a unique affinity for potentially binding macromolecules such as papain.

FPIA Assays with RP and DRP

The ability for the RP to elicit a 37% reduction in the reported THC-COOH concentration after only 2 hours not only implicates papain (versus the latex matrix) as an entity participating in the adulteration of a urine specimen by a CLP preparation but illustrates the potential for an RP preparation of papain to be profoundly efficacious as a urine adulterant. Data also indicate a reformulation of immunoassay reagents to include protease inhibitors would not prevent adulteration of urine specimens. The inability for the RP preparation to reduce the nordiazepam concentration implicates the latex matrix in the CLP preparation as the sole entity adulterating these urine specimens. The differences in the percent reduction between THC-COOH (~50%) and nordiazepam (~12%) by the CLP preparations can be explained by the different entities involved in adulterating the urine specimen.

Gas Chromatography/Mass Spectroscopy of THC-COOH

The reduction in the THC-COOH concentration as reported by GC/MS verifies, by an alternative and more sensitive assay, that the mechanism of interference involves an interaction with the analyte. The quantitation of THC-COOH by GC/MS also verifies the adulterant has the potential to interfere with the results obtained from a confirmatory assay. Therefore screening of all specimens, both negative and positive, for THC-COOH by GC/MS would be both economically and practically ineffective in revealing specimens that have been adulterated with papain.

High Pressure Liquid Chromatography/Ultraviolet Detection of Nordiazepam

The reduction in the nordiazepam concentration as reported by HPLC/UV verifies, by an alternative and more sensitive assay, that the mechanism of interference involves an interaction with the analyte and the adulterant has the potential to interfere with the results obtained from a confirmatory assay. Therefore screening of all specimens, both negative and positive, for

nordiazepam by HPLC/UV would be both economically and practically ineffective in revealing specimens that have been adulterated with papain.

Nonspecific Binding, A Putative Mechanism of Interference

It is imperative to note that the binding of THC-COOH (ng/mg of papain) was performed with data from the crude latex preparation of papain. The data are therefore not interpreted as the binding to one specific entity, as the CLP preparation contains multiple constituents. The binding constant is therefore denoted as an observed binding constant, k_{obs}. The experiments with RP and DRP revealed that the papain enzyme itself was at least partially responsible for the decrease in THC-COOH with the CLP preparations; however, it did not eliminate the contribution of the other constituents.

The summation of the data obtained from the effects of CLP, RP, and DRP on THC-COOH as measured by immunoassay and GC/MS indicate that mechanism of interference is nonspecific binding of THC-COOH to papain. A linear plot of the binding of THC-COOH (ng/mg papain) versus free THC-COOH as observed from data obtained from pH 6.2 and 8.0 is further indication of this mechanism. As the pH of the synthetic urine approached the pI of papain (9.6), it would be expected that a greater quantity of THC-COOH would participate in nonspecific binding. However, the k_{obs} of the binding in pH 8.0 synthetic urine was approximately one-tenth that of the binding in pH 6.2 synthetic urine. This observation may be due to either the other constituents in the CLP preparation of papain exerting a more significant effect at pH 6.2 than at 8.0, or a matrix effect on the FPIA assay could also be implicated.

Specimen Validity Testing

Specimen validity testing relies on dual parameters (i.e., creatinine and specific gravity) to differentiate an adulterated, substituted, or diluted specimen from a specimen obtained from an individual with renal pathologies. Although papain had a significant effect on urine osmolality, pH, and specific gravity, the urine creatinine and nitrite concentrations were not affected by papain. An individual with a “normal” urine specimen before adulteration with 10mg/mL papain would not have his urine specimen rendered invalid after adulteration with papain based on the dual qualifying parameters of specimen validity testing or the presence of

oxidants or glutaraldehyde. Conversely, if an unadulterated urine specimen were to be considered diluted or substituted based on a specific gravity < 1.001 before adulteration with papain, the urine has the potential to be considered “normal” after adulteration with 10 mg/mL papain due to the increase in specific gravity.

CHAPTER 6
SUMMARY AND CONCLUSIONS

As the proprietary active ingredient(s) in commercially available urine adulterants become public knowledge, the manufacturers of the urine adulterants are required to modify the constituents of the urine adulterant to avoid detection by drug testing laboratories. The detection of substances currently employed to adulterate urine specimens and modification of guidelines for specimen valid testing have forced manufacturers of urine adulterants to reformulate their products with novel ingredients that adulterate urine specimens in a manner that does not render the specimen invalid. The investigation of the effects of papain in a crude latex matrix on the FPIA analysis for primary drug screens provides information on the potential for papain to become a novel commercially available urine adulterant.

The synthetic urine matrix used in our research delineates the conditions in which papain exerted its effects with respect to the discrete concentrations of the constituents and pH. The data revealed by EMIT analyses of specimens containing THC-COOH not only provided justification for further investigation into the effects of papain but demonstrated that papain is an effective urine adulterant by an alternative immunoassay. The overall FPIA analyses of specimens containing THC-COOH revealed that papain exerted a dose dependent significant effect on the concentration THC-COOH with respect to pH, THC-COOH concentration, and time. False negative urine results attributed solely to papain were observed most frequently in pH 6.2 synthetic urine after 72 hours in specimens containing < 100 ng/mL THC-COOH.

The effects observed from specimens adulterated with papain suggest an interaction with the analytes versus an interference with the assay as demonstrated by a lack of observed effects in FPIA analyses of other drugs of abuse, and was also demonstrated by the confirmation (GC/MS, HPLC/UV) analyses of THC-COOH and nordiazepam that revealed a decrease in analyte concentration by a direct measurement of the analytes. The interactions of papain with THC-COOH and nordiazepam suggest that the interaction was not enzymatic as demonstrated by the “plateau” of the THC-COOH concentrations after 4 to 6 hours, and was also demonstrated by FPIA assays of THC-COOH and nordiazepam that involved papain deactivated

by E-64. The latex matrix in which CLP resides can, in itself, act as an adulterant by reducing the concentrations of analytes in the specimen as demonstrated by an effect observed in the FPIA analyses of nordiazepam with CLP versus a lack of observed effects in the FPIA analyses of nordiazepam with RP.

An attempt by an individual (with an average urine pH ~6.2) using CLP to obtain a false negative FPIA urine drug screen result for cannabinoids within 4 hours would be most successful if the individual first minimized the concentration of THC-COOH in the urine by the consumption of a large quantity of water followed by the in vitro addition of 10 mg/mL papain. If the diuresis induced by the consumption of a large quantity of water yields an initial urine THC-COOH concentration <100 ng/mL, then the average 50% decrease of THC-COOH concentration by 10 mg/mL papain would render the urine THC-COOH concentration < 50 ng/mL. An attempt by an individual using CLP to obtain a false negative FPIA urine drug screen result for benzodiazepines may or may not be as successful. The effects of the CLP on the reduction of nordiazepam concentration are entirely due to the latex matrix and would therefore be difficult to assay for a standard effect.

An individual who attempts to adulterate his urine with 10 mg/mL papain would not have their urine specimen rendered invalid based on the parameters of specimen validity testing or the presence of glutaraldehyde or oxidants. If the papain was enzymatically active, a microplate BANI enzyme activity assay would readily detect a general class of proteases in the urine specimen. As proteases are not a constituent of normal urine, the specimen could be identified as adulterated. However, enzyme activity is not necessary for a reduction in THC-COOH concentration, and the sinister placement of inactive papain in a commercially available urine adulterant would be likely to avoid detection by an enzyme activity assay. Papain could specifically be identified in a urine specimen by a Western blot. However, performing a Western blot on every specimen in the laboratory is impractical due to the cost and time involved in performing the Western blot. Furthermore, depending on the specificity of the probe, the Western blot would only detect papain and not other classes of proteins that could potentially interact with THC-COOH. Although urine typically does not contain proteins > 60 µg/mL or proteins > 20kDa, a concentration of proteins > 60 µg/mL as determined by a nonspecific assay such as coomassie staining, does not differentiate an adulterated specimen

from a specimen attained from an individual with renal pathologies. Only the dual qualifying parameters of specimen validity testing will differentiate an adulterated, diluted, or substituted urine from an individual with renal pathologies. Issues of patient privacy (HIPAA) arise in regards to federal guidelines that have not implemented a protocol for an observed urine collection for drug testing. A closely observed urine collection, although not guaranteed to prevent urine adulteration, is the best measure to prevent adulteration of urine specimens by foreign substances.

The overall goal of our research, as defined by the specific aims of this study, was attained by delineating the effects of papain as they apply to the practical application of a urine adulterant with respect to the target of papain's interaction, the concentration of THC-COOH, pH of the matrix, time of interaction, and the parameters of specimen validity testing. Based on our results, papain has the potential to be employed as the active ingredient in a commercially available urine adulterant and represents the minimal effects that could be observed in a novel class of urine adulterants. The mechanism of interaction of papain with THC-COOH, as suggested by our data, is putatively the nonspecific binding of THC-COOH to papain. The observed binding constant was greatest at pH 6.2, a reasonable median value for typical urine pH. The nonspecific binding of THC-COOH would render the THC-COOH unidentifiable to the antibody complex in the immunoassays and would not allow solid phase extraction of THC-COOH in preparation for gas chromatography/mass spectroscopy.

The information contained in this research has the potential to be exploited by the manufacturers of urine adulterant to include papain as the active ingredient in their products. This research can also pioneer investigations into novel classes of urine adulterants in which the mechanism of adulteration is the binding and steric hindrance of chemical moieties on the analyte. A scenario involving the adulteration of a urine specimen by microgram quantities of polyclonal antibodies, not unlike those used in the immunoassays themselves, has the potential to be marketed by commercial manufacturers of urine adulterants. The polyclonal antibodies would be more costly to produce and market than papain but would certainly be more efficacious in adulterating a urine specimen.

The information contained in this research also has the potential to persuade federal agencies in drafting a standard protocol for an observed urine collection for drug screening. The

most effective practice in thwarting an attempted *in vitro* urine adulteration attempt is to perform a closely observed urine collection. To circumvent the issue of infringed patient privacy, federal agencies are expending tremendous capital to enact guidelines that will allow the interpretation of the results from alternative matrix drug screens. A standard protocol for urine collection that is compliant with current HIPAA guidelines would be a more practical approach than alternative matrice drug testing.

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APPENDICES

APPENDIX A

List of Abbreviations

Abbreviation	Description
CLP	Crude Latex Papain
DRP	Deactivated Recrystallized Papain
EMIT	Enzyme Multiplied ImmunoAssay Technique
FPIA	Fluorescence Polarized ImmunoAssay
GC/MS	Gas Chromatography/Mass Spectrometry
HIPAA	Health Insurance Portability and Accountability Act
HPLC/UV	High Performance Liquid Chromatography/Ultraviolet Detection
RP	Recrystallized Papain
SAMHSA	Substance Abuse & Mental Health Administration
THC	Delta-9-tetrahydrocannabinol
THC-COOH	11-nor-delta-9-tetrahydrocannabinol

APPENDIX B

Papain Standardizaiton

Preparation	Concentration (mg/mL)	delta-mabs
Twice recrystallized	0.0	-0.1
	0.0325	1
	0.065	2.2
	0.13	4.9
Crude latex	1.25	1.8
	2.5	3.8
	5	8.5
	10	17.9

APPENDIX C

EMIT

Specimen (125 ng/mL THC-COOH)	Replicate #	Result
Control (0 mg/mL papain)	1	positive
	2	positive
	3	positive
	4	positive
	5	positive
	6	positive
Adulterated (10 mg/mL papain)	1	negative
	2	negative
	3	negative
	4	negative
	5	negative
	6	negative

APPENDIX D

FPIA Assays

THC-COOH Assays-25 ng/mL in pH 4.5 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	17.2	22.67	20.77	25.46	28.12	26.14
	2	20.36	23.21	23.66	26.45	25.7	26.24
	3	19.67	20.12	26.81	22.55	27.04	25.08
	4	19.64	21.25	20.32	21.81	24.85	25.09
	5	19.77	21.56	23.17	23.21	27.56	24.88
	6	19.37	22.25	21.02	22.31	25.75	24.55
4	1	14.76	14.63	13.37	15.99	16.22	17.9
	2	13.77	13.94	12.2	19.64	13.87	16.91
	3	13.55	14.54	15.89	17.89	15.28	15.3
	4	15.33	16.46	13.3	16.87	17.49	18.3
	5	13.89	14.89	12.71	17.68	16.73	15.53
	6	14.15	15.53	15.15	15.91	16.81	16.65
6	1	16.57	15.49	15.19	15.62	13.88	19.62
	2	18.63	14.06	17.09	16.69	17.18	20.05
	3	17.1	18.08	17.58	14.74	16.35	17.49
	4	20.13	19.02	19.7	13.1	18.3	18.95
	5	17.34	17.85	17.66	17.08	17.68	17.73
	6	20.14	14.55	17.74	14.72	20.62	17.65
24	1	10.73	15.06	13.38	14.91	14.05	18.84
	2	13.73	11.06	11.92	10.77	14.35	17.4
	3	13.03	12.89	14.2	12.26	14.28	15.56
	4	17.61	13.96	12.15	14.91	14.53	16.18
	5	15.47	12.75	16.02	13.17	15.85	14.87
	6	14.53	14.85	16.07	12.24	17.31	13.7
72	1	15.51	14.21	11.24	12.13	12.52	14.57
	2	17.78	12.89	10.46	11.41	11.59	14.22
	3	15.38	13	14.3	10.62	13.3	12.73
	4	18.35	9.84	14.23	13.79	12.91	12.07
	5	17.79	13.59	11.75	13.5	13.16	11.52
	6	18.86	8.64	11.2	12.31	12.71	13.56

75 ng/mL in pH 4.5 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	61.98	69.28	73.91	71.09	77	63.93
	2	63.45	82.4	65.89	100.03	71.98	72.57
	3	64.65	64.88	78.3	65.6	75.51	64.54
	4	59.95	64.61	88.83	76.88	83.28	76.3
	5	63.04	69.07	88.93	75.62	62.83	73.24
	6	69.07	67.82	61.19	82.67	62.95	63.4
4	1	41.79	39.65	31.28	43.33	36.86	25.68
	2	47.42	43.04	32.61	43.41	37.24	33.06
	3	42.74	37.16	35.19	32.84	31.4	33.34
	4	39.07	37.48	34.01	31.3	29.93	29.58
	5	42.67	30.64	40.48	32.39	34.85	34.11
	6	47.68	36.9	42.21	30.63	32.78	33.22
6	1	33.17	26.15	29.58	31.26	25.99	26.38
	2	29.98	29.31	24.86	28.94	27.59	25.93
	3	32.9	28.91	26.09	26.96	28.03	24.86
	4	32.57	30.15	34.03	26.59	26.98	27.52
	5	31.02	24.7	33.51	27.51	28.09	28.54
	6	32.13	29.13	28.43	27.56	28.86	23.04
24	1	38.34	24.71	30.33	30.03	24.74	24.35
	2	43.9	28.89	27.64	34.93	25.72	23.03
	3	37.63	27.75	29.26	28.8	28.71	28.88
	4	47.27	27.39	29.99	25.27	27.67	22.97
	5	37.22	27.36	38.26	25.47	26.06	26.17
	6	42.2	29.15	28.41	28.03	22.74	25.89
72	1	27.95	29.47	24.58	31.48	32.21	29.74
	2	37.59	25.46	19.44	32.39	28.83	29.25
	3	31.7	23.85	24.58	28.66	29.85	27.05
	4	35.05	23.57	32.96	26.32	38.23	23.91
	5	32.7	27.95	28.94	37.66	31.22	32.45
	6	33.04	23.92	25.83	33.37	32.79	18.62

100 ng/mL in pH 4.5 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	88.5	109.8	102.0	93.3	85.8	108.3
	2	98.1	93.5	102.6	100.0	117.9	108.4
	3	97.2	123.5	98.4	97.5	115.0	109.3
	4	97.7	104.5	103.7	94.6	119.7	97.0
	5	95.0	106.0	100.1	81.7	125.1	102.3
	6	94.9	86.9	113.1	94.4	121.6	104.6
4	1	40.7	43.1	35.4	40.3	40.8	39.9
	2	39.4	43.1	41.1	46.9	38.6	39.8
	3	41.8	46.4	44.1	43.1	38.0	42.1
	4	45.6	44.4	40.8	38.5	38.1	39.4
	5	43.1	43.4	41.1	41.2	45.7	46.7
	6	46.3	40.9	40.2	39.7	37.7	46.1
6	1	43.0	38.3	34.1	32.1	32.4	34.9
	2	42.6	39.7	37.1	35.0	32.2	30.7
	3	42.9	43.3	34.5	38.1	29.9	25.7
	4	44.7	41.7	37.5	32.3	30.6	33.6
	5	45.1	40.8	31.8	33.9	34.4	33.0
	6	38.3	37.3	35.2	31.9	34.3	27.7
24	1	55.2	44.0	38.1	40.2	28.4	35.4
	2	47.8	42.5	41.2	36.3	30.4	30.7
	3	48.4	43.0	39.3	38.4	32.8	26.2
	4	48.7	43.0	37.5	32.2	35.8	26.3
	5	50.5	45.3	37.5	32.5	35.0	30.7
	6	54.6	39.9	32.0	30.1	35.1	28.6
72	1	45.1	39.8	34.2	37.1	33.8	29.6
	2	46.7	36.8	37.4	33.8	29.2	29.9
	3	51.0	39.7	35.0	40.2	32.9	27.9
	4	46.0	39.3	38.9	31.5	41.1	38.9
	5	50.3	39.7	34.1	32.0	35.5	32.3
	6	49.6	40.1	32.2	37.7	28.2	31.8

250 ng/mL in pH 4.5 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	125.1	104.2	101.3	103.3	114.2	148.1
	2	131.0	112.4	104.2	95.4	123.1	138.6
	3	120.0	117.6	102.1	90.0	121.7	109.2
	4	132.9	111.4	105.0	118.2	90.5	106.0
	5	132.9	114.4	108.8	97.9	101.7	110.4
	6	121.6	84.4	91.3	100.3	104.3	101.3
4	1	168.8	112.6	112.5	90.8	95.0	92.8
	2	176.1	114.9	87.6	101.6	68.4	59.6
	3	139.7	96.5	96.4	66.2	61.9	65.3
	4	188.0	112.4	108.7	73.1	67.6	72.4
	5	195.4	112.0	109.4	65.4	62.9	61.6
	6	166.7	150.2	90.8	56.7	50.2	60.3
6	1	120.6	74.7	54.9	71.0	48.4	53.0
	2	102.2	73.3	58.0	49.7	40.1	47.4
	3	100.3	93.0	54.6	51.3	35.6	44.3
	4	105.1	78.6	51.7	54.4	40.0	45.4
	5	106.8	66.8	69.4	44.9	37.8	50.5
	6	108.1	73.3	59.6	38.7	38.9	37.8
24	1	101.1	65.8	67.5	59.5	65.8	56.8
	2	102.2	53.7	56.1	48.0	48.2	51.9
	3	96.6	55.0	49.5	51.2	53.5	55.2
	4	122.3	60.2	53.3	52.8	54.9	49.0
	5	101.6	57.2	53.5	36.7	50.7	62.7
	6	81.8	45.0	53.1	43.8	44.9	60.4
72	1	91.5	81.9	86.5	60.5	80.7	74.3
	2	101.7	65.2	60.9	55.1	53.3	73.0
	3	77.0	64.7	72.1	34.2	48.0	48.1
	4	97.2	66.6	65.4	55.8	63.4	51.3
	5	85.6	56.1	65.4	51.6	90.1	33.7
	6	75.6	50.7	44.1	37.7	44.1	59.1

500 ng/mL in pH 4.5 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	297.3	252.8	231.4	201.9	257.5	215.0
	2	243.5	263.3	258.9	243.7	263.1	276.7
	3	242.0	217.1	250.6	256.6	258.1	380.9
	4	284.0	244.0	263.5	255.7	272.3	313.7
	5	272.9	254.7	254.8	247.2	528.6	230.9
	6	226.6	305.0	253.7	229.7	226.6	261.1
4	1	219.7	202.0	167.9	133.0	118.4	203.5
	2	170.9	197.6	171.4	178.0	144.8	117.6
	3	234.2	217.2	208.8	154.2	128.7	136.8
	4	319.2	189.1	169.7	157.3	139.2	113.8
	5	250.5	254.1	194.4	182.6	152.4	123.6
	6	245.5	202.8	219.6	168.3	152.5	135.1
6	1	376.1	318.2	289.9	195.6	218.9	173.4
	2	411.0	351.4	300.1	211.6	160.9	136.1
	3	406.0	345.3	302.0	229.7	172.0	143.3
	4	452.0	383.1	274.1	297.7	141.2	127.8
	5	425.2	354.8	312.9	272.8	165.6	141.3
	6	433.4	412.3	450.4	345.1	164.3	144.2
24	1	350.1	326.3	306.2	259.6	259.2	214.2
	2	368.9	288.2	292.1	226.2	217.4	213.0
	3	335.3	274.3	351.4	223.1	250.1	198.9
	4	364.3	300.6	254.6	236.0	197.8	184.4
	5	364.8	255.4	248.8	282.0	262.4	243.8
	6	403.3	301.4	275.3	275.3	214.4	214.8
72	1	274.5	344.3	174.6	155.9	339.8	108.5
	2	328.9	178.7	177.3	127.8	91.3	80.0
	3	302.3	205.7	176.8	111.0	85.4	80.0
	4	215.6	209.8	154.9	127.3	85.8	69.0
	5	301.1	289.7	132.1	118.6	93.2	92.2
	6	381.3	201.2	206.6	111.2	87.2	67.7

25 ng/mL in pH 6.2 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	24.61	22.35	25.42	25.25	24.35	22.28
	2	25.11	22.54	23.37	24.31	25.78	23.16
	3	23.17	19.75	23.84	25.11	24.53	28.57
	4	22.37	22.94	21.88	24.4	26.65	26.56
	5	23.44	24.53	21.04	25.49	22.75	23.48
	6	26.2	24.92	24.63	24.28	23.58	19.93
4	1	21.34	16.94	17.74	18.67	17.05	13.79
	2	22.58	18.39	18.17	16.63	17.2	14.22
	3	20.09	16.46	19.06	18.53	18.36	16.39
	4	21.54	18.71	18.2	18.31	17.3	13.54
	5	24.27	21.18	20.18	18.1	16.84	13.28
	6	26.43	18.37	20.38	19.11	17.46	17.16
6	1	16.82	17.76	18.09	18.27	14.66	13.62
	2	20.58	17.09	18.25	15.06	13.06	9.93
	3	23.3	16.89	16.84	17.05	14.89	10.95
	4	22.38	19.17	18.31	17.53	13.28	12.08
	5	22.9	18.14	19.29	17.19	13.38	10.99
	6	20.61	16.46	18.17	17.99	14.23	11.24
24	1	24.07	11.59	17.87	14.82	13.02	11.44
	2	18.31	13.24	17.12	15.4	15.15	13.1
	3	22.75	14.96	19.2	14.95	16.1	13.46
	4	22.55	18.17	18.14	19.72	15.77	12.3
	5	23.75	17.6	20.07	17.96	14.86	13.04
	6	20.93	17.16	12.96	19.17	14.88	12.16
72	1	22.15	32.59	33.38	30.71	23.63	11.46
	2	23.22	29.5	27.86	28.71	18.63	11.32
	3	23.76	31.26	31.42	27.63	17.16	11.8
	4	29.51	31.94	30.92	27.72	18.9	11.21
	5	22.53	31.34	35.03	29.28	18.77	15.29
	6	26.98	30.35	32.2	29.15	18.38	13.04

75 ng/mL in pH 6.2 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	72.32	72.6	77.4	77.24	79.16	86.75
	2	80.23	69.24	74.1	79.73	71.86	76.12
	3	80.29	68.99	74.38	81.91	82.55	82.19
	4	83.67	74.7	77.97	75.48	77.75	76.68
	5	86.59	69.77	74.72	91.22	80.04	84.04
	6	82.81	71.28	88	79.64	84.52	79.45
4	1	89.19	58.53	49.63	49.44	41.21	30.9
	2	92.19	53.83	48.87	62.57	45.93	27.19
	3	89.34	49.97	46.04	59.24	38.31	25.4
	4	98.2	68.33	52.54	59.96	39.78	27.08
	5	101.55	52.09	49.93	53.98	44.29	36.1
	6	97.02	50.93	51.39	54.9	39.38	23.44
24	1	60.52	43.64	45.67	48.05	33.94	29.47
	2	74.06	43.77	44.83	42.82	30.54	28.77
	3	72.04	50.49	46.05	39.09	32.43	28.06
	4	73.25	47.99	57.88	43.88	31.64	27.42
	5	74.91	47.98	49.81	43.17	35.36	31.36
	6	64.02	44.57	42.99	48.66	35.94	27.07
72	1	55.87	39.2	34	29.8	22.64	19
	2	61.76	38.02	30.73	28.55	23.69	19.46
	3	60.7	43.53	32.96	29.18	19.38	24.93
	4	62.95	38.88	38.2	28	19.85	19.03
	5	66.24	41.91	34.98	27.92	20.56	19.55
	6	63.98	42.1	35.02	29.87	24.07	16.36

100 ng/mL in pH 6.2 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	81.0	99.0	94.9	98.7	101.2	93.3
	2	100.3	82.3	87.8	108.4	105.5	96.6
	3	93.8	76.4	87.4	108.7	99.9	88.6
	4	90.9	78.7	113.5	105.3	130.1	86.0
	5	105.5	88.1	105.0	103.9	106.0	87.4
	6	95.0	87.3	114.0	108.4	103.6	82.8
4	1	89.2	71.4	62.2	49.4	42.8	29.0
	2	92.2	67.3	60.4	62.6	49.5	30.7
	3	89.3	59.8	55.1	59.2	38.8	36.7
	4	98.2	62.6	74.5	60.0	51.7	33.3
	5	101.6	64.7	60.7	54.0	41.0	34.4
	6	97.0	64.4	62.6	54.9	48.3	32.3
24	1	72.9	56.2	58.6	43.6	41.9	26.9
	2	66.6	56.2	51.9	43.4	39.7	31.0
	3	72.2	53.8	52.7	45.7	43.9	28.3
	4	75.8	58.3	51.1	49.9	37.6	31.3
	5	80.5	62.6	50.5	48.4	40.1	31.4
	6	70.8	53.9	49.6	49.2	38.2	23.9
72	1	80.7	38.7	44.5	36.5	31.5	23.4
	2	68.8	42.9	45.5	35.6	27.9	25.7
	3	76.4	39.5	40.1	34.4	33.7	27.6
	4	69.0	39.7	41.3	35.2	37.4	26.7
	5	78.4	49.2	39.8	35.8	26.8	24.7
	6	80.4	51.6	40.8	38.3	27.4	24.8

250 ng/mL in pH 6.2 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	180.3	162.8	201.5	183.2	214.7	168.7
	2	201.4	156.0	181.5	205.5	212.4	199.2
	3	221.0	159.9	246.9	236.7	230.9	209.2
	4	210.7	173.1	199.9	226.5	215.8	205.4
	5	201.2	206.2	200.5	246.7	226.1	216.3
	6	200.9	211.3	188.3	238.1	191.2	189.4
4	1	208.8	180.8	157.8	125.3	95.1	68.6
	2	220.7	184.9	166.9	117.7	75.6	67.6
	3	226.1	182.0	158.5	139.3	92.0	80.5
	4	239.6	186.7	172.8	123.5	117.9	77.9
	5	243.0	199.8	179.8	129.8	105.3	75.2
	6	229.8	181.9	167.2	114.9	102.8	80.8
6	1	199.5	164.2	161.0	124.4	84.6	105.6
	2	245.6	164.0	173.2	137.1	97.7	117.5
	3	251.7	164.9	156.4	147.0	105.0	103.4
	4	254.6	175.6	147.3	111.8	107.0	99.8
	5	254.9	175.6	157.2	153.9	102.7	112.2
	6	248.2	181.8	132.9	132.5	82.9	103.0
24	1	143.9	238.2	123.0	87.8	67.3	49.9
	2	175.1	132.6	115.1	97.5	66.9	53.7
	3	168.7	140.8	111.1	108.7	69.2	55.8
	4	188.8	139.4	105.6	86.5	73.2	56.6
	5	200.3	139.6	147.0	117.6	77.1	54.6
	6	202.9	148.5	131.8	114.3	73.7	55.6
72	1	144.1	104.7	83.8	82.4	58.5	45.8
	2	112.7	84.5	95.4	82.3	61.1	51.3
	3	186.0	85.2	83.6	71.5	64.3	48.6
	4	184.7	104.2	113.2	78.7	69.7	52.4
	5	190.7	109.7	110.1	69.4	69.7	48.0
	6	189.1	116.3	101.4	70.5	82.1	49.9

500 ng/mL in pH 6.2 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	302.6	328.4	304.0	262.6	262.4	185.7
	2	347.5	323.7	344.5	269.7	244.6	268.3
	3	331.0	389.7	327.6	338.3	303.2	262.5
	4	365.0	353.3	320.2	289.4	277.7	281.1
	5	422.4	376.1	293.3	325.7	244.3	326.8
	6	436.3	402.1	279.0	295.3	283.4	293.0
4	1	336.1			249.6	183.8	141.8
	2	416.7	309.8	246.7	291.3	223.9	164.8
	3	369.4	331.8	312.8	243.1	231.6	176.7
	4	423.2	309.6	304.0	268.2	183.8	179.9
	5	444.6	290.9	315.8	275.6	236.8	163.0
	6	394.2	303.8	361.3	285.6	236.4	170.8
6	1	399.3			219.3	182.8	127.1
	2	475.9	293.4	232.8	297.1	208.4	137.7
	3	486.5	356.9	250.8	271.1	205.4	136.1
	4	449.0	326.4	330.3	269.6	221.3	108.7
	5	498.6	298.0	258.1	277.6	179.6	133.5
	6	434.3	319.7	360.7	284.9	204.4	139.4
24	1	379.1			169.1	117.8	87.0
	2	376.1	216.6	167.6	154.8	127.7	79.0
	3	467.5	248.2	192.6	214.9	113.4	101.9
	4	397.2	225.0	196.8	229.0	144.8	93.5
	5	438.9	230.2	210.8	194.0	164.9	90.0
	6	403.4	238.1	232.6	178.5	160.5	103.7
72	1	426.1			157.8	98.0	84.9
	2	489.7	219.1	168.2	163.3	102.3	112.5
	3	526.1	265.2	199.5	167.0	112.9	78.8
	4	487.0	228.3	218.6	162.8	108.7	88.3
	5	450.3	207.0	209.1	144.3	110.5	78.8
	6	427.9	216.0	228.9	145.8	100.7	81.3

25 ng/mL in pH 8.0 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	25.3	26.7	29.3	29.8	27.2	21.3
	2	24.8	28.0	29.0	29.4	28.9	21.8
	3	23.0	23.9	31.5	30.9	27.6	20.7
	4	29.5	27.8	29.7	30.7	29.9	27.0
	5	28.5	25.1	32.0	32.2	30.3	22.8
	6	26.8	28.0	30.6	33.6	29.2	22.6
4	1	22.1	26.6	26.6	28.1	23.2	16.9
	2	22.7	24.0	29.3	25.1	21.1	14.2
	3	25.1	27.3	28.6	28.6	20.3	15.7
	4	26.4	23.8	27.7	28.8	23.4	17.5
	5	28.0	27.6	29.9	28.4	24.9	18.0
	6	29.4	27.1	28.8	26.8	18.8	18.0
6	1	25.2	25.4	28.3	26.4	20.6	15.8
	2	22.7	26.5	29.5	24.5	19.8	12.8
	3	23.6	26.3	28.0	28.2	18.5	14.8
	4	25.4	26.5	28.0	26.6	19.2	15.5
	5	26.1	26.7	30.5	29.1	21.1	16.1
	6	25.1	26.9	24.3	26.4	18.4	15.8
24	1	23.1	27.6	29.1	25.0	23.4	16.2
	2	20.9	26.6	28.2	28.3	20.0	18.7
	3	22.2	28.6	28.7	29.9	19.1	15.9
	4	24.3	30.6	28.8	30.9	19.0	15.1
	5	26.7	28.2	30.6	29.0	23.0	16.3
	6	24.7	28.9	27.3	26.6	21.8	16.0
72	1	20.7	30.5	32.9	25.9	22.7	15.7
	2	23.3	29.0	29.4	24.2	17.8	17.0
	3	21.0	29.2	35.2	29.6	18.2	15.7
	4	26.8	28.5	32.2	29.7	19.2	12.4
	5	27.3	32.6	33.6	30.2	19.5	12.6
	6	28.1	29.6	35.0	28.1	18.7	14.5

75 ng/mL in pH 8.0 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	62.1	60.0	71.5	61.4	83.6	73.4
	2	62.8	63.6	71.9	50.9	85.5	70.5
	3	63.7	63.7	57.3	85.6	80.4	72.0
	4	69.6	72.6	61.8	86.6	81.2	67.9
	5	72.0	74.5	61.1	87.2	74.1	68.9
	6	63.6	82.5	66.1	85.6	85.8	71.3
4	1	75.4	65.7	60.8	45.3	59.1	38.9
	2	70.9	67.1	62.7	40.8	60.0	35.9
	3	80.9	66.1	48.6	60.5	61.4	38.8
	4	81.0	65.3	55.3	71.0	62.6	39.8
	5	84.4	63.3	52.8	70.5	57.7	35.4
	6	82.5	64.0	56.5	69.6	65.0	43.8
6	1	78.4	62.2	58.6	42.8	52.3	33.6
	2	75.0	67.8	55.0	41.6	67.7	30.9
	3	76.9	67.7	47.7	52.6	59.4	36.9
	4	77.9	62.8	51.1	66.3	58.5	38.6
	5	82.1	61.5	48.5	59.4	53.1	34.0
	6	84.5	63.1	52.4	65.1	54.9	43.3
24	1	63.3	57.9	65.0	38.8	38.4	21.1
	2	66.7	65.5	60.0	33.7	37.8	23.2
	3	72.9	63.7	51.5	57.4	39.3	26.4
	4	68.9	68.2	55.6	60.7	40.0	24.6
	5	72.9	59.3	54.2	56.7	39.9	22.7
	6	79.9	62.2	56.0	61.4	36.7	25.9
72	1	69.5	65.5	68.3	44.5	37.5	19.9
	2	64.3	57.6	63.9	40.1	35.0	18.5
	3	69.0	54.7	66.7	61.5	40.3	16.2
	4	74.0	60.9	55.7	64.1	46.0	22.6
	5	75.6	75.1	59.3	57.5	36.8	20.4
	6	70.5	77.5	60.7	63.7	36.9	22.9

100 ng/mL in pH 8.0 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	82.1	93.1	98.2	109.5	84.2	70.4
	2	86.8	90.3	104.6	105.4	75.1	78.0
	3	86.7	97.4	106.6	99.4	95.0	65.5
	4	91.7	116.2	105.4	109.5	88.0	71.9
	5	93.7	113.5	111.2	112.9	85.9	82.2
	6	90.4	105.8	102.2	108.6	87.9	86.7
4	1	90.4	96.4	103.0	100.2	80.4	51.5
	2	94.5	88.8	106.7	107.3	63.6	55.7
	3	95.3	104.1	107.7	99.2	71.9	41.7
	4	100.9	110.7	107.5	110.9	71.7	54.3
	5	102.5	99.7	111.6	110.8	87.0	55.8
	6	102.7	116.5	117.4	107.7	76.2	66.1
6	1	87.0	109.0	116.1	106.1	64.4	35.5
	2	86.8	118.6	114.6	105.9	57.0	41.6
	3	93.7	115.8	121.5	92.9	63.9	42.5
	4	98.7	117.7	124.5	89.3	65.6	35.4
	5	91.6	115.3	109.9	100.6	64.1	44.3
	6	96.8	112.9	121.0	106.9	58.3	47.7
24	1	83.5	114.8	121.2	91.7	54.5	35.4
	2	81.5	121.8	121.4	110.7	49.7	30.7
	3	88.1	133.4	116.2	100.8	52.8	37.1
	4	92.7	119.0	120.0	90.3	62.3	33.5
	5	93.9	119.5	115.7	100.4	58.8	36.2
	6	96.4	120.3	117.8	111.6	55.4	36.9
72	1	67.7	119.1		108.5	54.6	30.8
	2	67.7	120.3	130.9	100.2	44.1	28.2
	3	66.6	125.6	124.8	105.6	49.3	32.5
	4	83.9	126.8	121.4	96.0	52.7	29.1
	5	78.7	118.2	123.7	99.6	49.2	35.4
	6	78.8	124.5	128.2	116.0	46.6	39.7

250 ng/mL in pH 8.0 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	287.1	251.7	260.4	251.9	237.1	193.1
	2	279.1	261.0	254.0	242.2	243.6	233.4
	3	277.1	239.9	226.9	250.6	234.6	200.9
	4	276.5	247.9	253.1	249.6	241.2	214.4
	5	281.5	261.0	233.7	239.5	230.0	216.4
	6	284.8	235.8	244.8	237.7	220.7	229.3
4	1	242.0	235.1	219.8	204.9	181.0	135.6
	2	249.6	231.3	223.9	238.0	179.6	124.0
	3	252.7	230.3	236.4	208.4	195.5	134.0
	4	257.0	243.4	212.2	250.7	173.8	136.4
	5	370.0	235.8	232.8	218.5	189.4	151.2
	6	289.9	239.6	246.5	219.5	187.5	140.9
6	1	195.2	201.8	191.6	173.3	161.6	103.6
	2	231.2	222.4	197.0	173.8	138.7	88.7
	3	221.6	225.4	216.9	195.6	158.1	108.2
	4	237.7	221.0	214.3	194.0	145.9	93.5
	5	241.1	216.6	215.4	173.4	151.4	106.2
	6	223.1	234.8	205.6	183.7	152.7	99.1
24	1	191.0	198.0	211.2	181.7	143.0	75.6
	2	217.1	205.3	159.9	167.3	122.5	74.8
	3	202.7	204.3	201.7	174.7	222.9	85.3
	4	193.6	208.2	198.4	178.9	125.9	79.8
	5	216.6	207.7	190.1	173.3	130.1	82.5
	6	227.6	212.0	205.1	173.8	135.9	73.1
72	1	176.4	232.4	230.9	208.0	129.8	77.8
	2	202.5	211.5	193.7	170.9	125.3	80.4
	3	216.1	192.6	185.4	198.4	124.1	80.3
	4	206.1	220.4	200.4	182.7	115.4	72.9
	5	190.5	217.4	208.1	183.9	118.3	75.6
	6	214.3	230.9	223.9	186.2	123.0	71.3

500 ng/mL in pH 8.0 synthetic urine

time (h)	replicate #	papain concentration (mg/mL)					
		0	0.5	1	2	5	10
0	1	396.0	453.8	390.8	435.8	412.2	382.3
	2	380.9	407.5	431.0	463.5	439.1	377.6
	3	496.3	427.8	398.0	441.1	409.6	372.7
	4	401.2	436.0	413.4	426.6	431.9	385.2
	5	463.7	381.7	447.5	436.2	392.6	394.0
	6	430.4	393.4	426.1	470.0	431.2	378.5
4	1	383.8	358.4	376.1	356.1	303.4	251.4
	2	435.4	398.0	359.8	373.1	312.6	211.5
	3	402.8	398.0	369.2	367.9	308.9	208.0
	4	422.6	394.4	414.2	352.1	282.2	205.1
	5	464.9	380.2	417.6	396.2	306.2	201.3
	6	439.7	399.4	440.8	412.8	290.5	214.9
6	1	501.2	504.4	360.1	386.0	439.2	261.5
	2	460.2	378.8	401.0	411.4	302.8	284.6
	3	471.9	414.0	402.5	421.3	372.5	170.4
	4	484.4	443.2	406.9	372.8	335.9	183.5
	5	492.8	410.3	388.3	366.7	264.2	183.3
	6	494.0	423.2	429.5	400.6	268.2	209.3
24	1	380.6	427.6	362.7	381.5	257.9	202.6
	2	374.5	485.9	396.9	366.5	290.2	154.3
	3	343.6	398.2	387.3	346.3	265.0	191.5
	4	397.4	357.2	402.3	359.8	230.4	188.0
	5	424.1	408.1	420.2	364.0	281.4	173.6
	6	369.5	388.0	432.0	370.7	273.4	159.1
72	1	367.7	382.5	369.1	350.6	242.2	145.6
	2	351.8	436.9	434.4	309.7	258.7	121.0
	3	385.6	422.5	399.1	315.2	246.3	141.8
	4	387.0	223.9	380.8	336.5	220.3	156.7
	5	462.7	400.5	362.0	352.6	241.4	135.1
	6	398.1	370.6	362.6	428.2	285.1	114.2

FPIA Assays for Other Drugs of Abuse

Negative (-50% cutoff) Solutions

Time (h)	Rep #	Assay (Papain,mg/mL)											
		cocn (0)	cocn (10)	opt (0)	opt (10)	pcp (0)	pcp (10)	amp (0)	amp (10)	barb (0)	barb (10)	benz (0)	benz (10)
0	1	90.8	103.4	137.8	158.6	7.5	9.7	600.0	673.0	47.5	95.1	106.9	95.1
	2	105.9	98.8	154.0	167.4	8.6	10.9	590.9	696.0	88.8	129.6	103.2	99.1
	3	101.9	111.0	152.3	178.6	9.1	8.7	537.3	612.7	73.3	90.4	112.1	99.3
	4	104.0	101.9	158.7	165.8	7.3	11.8	592.4	618.0	84.5	93.0	109.2	99.1
	5	104.3	112.6	161.2	176.1	10.9	11.7	630.6	699.1	110.1	71.6	114.1	102.4
	6	106.9	118.9	155.2	174.4	12.0	12.4	555.3	604.0	99.9	104.5	106.1	101.4
6	1	90.7	118.1	152.0	161.1	10.0	11.3	553.3	656.1	84.7	103.8	110.6	85.8
	2	99.5	99.9	167.2	153.0	9.7	11.2	573.5	734.9	72.8	82.1	111.7	92.8
	3	101.2	110.6	156.5	162.4	11.7	11.8	578.5	683.0	74.1	90.6	114.8	85.5
	4	111.1	108.8	166.1	162.4	10.9	12.2	663.0	701.1	70.3	108.0	112.1	97.0
	5	105.5	131.1	176.9	163.2	13.8	12.1	639.9	695.3	99.3	113.5	109.5	92.7
	6	98.8	97.2	164.2	157.6	11.6	11.9	700.8	669.0	90.4	104.8	119.5	97.4
72	1	83.9	105.4	147.0	160.9	8.2	12.1	581.2	695.6	49.3	82.4	97.8	85.7
	2	98.5	113.3	164.0	158.0	9.2	15.4	574.1	658.7	64.7	63.0	103.6	84.7
	3	97.0	115.7	157.6	143.8	8.5	10.0	596.3	635.7	71.1	79.9	100.1	83.8
	4	114.6	103.0	171.6	166.6	10.6	13.1	615.2	681.0	91.7	72.5	99.9	96.6
	5	108.1	111.4	149.7	161.5	10.5	14.6	612.0	576.8	75.1	70.6	105.5	86.6
	6	109.3	102.4	165.3	156.4	7.9	13.0	647.9	626.1	93.6	76.0	107.9	88.5

Positive (+50% cutoff) Solutions

Time (h)	Rep #	Assay (Papain,mg/mL)											
		cocn (0)	cocn (10)	opt (0)	opt (10)	pcp (0)	pcp (10)	amp (0)	amp (10)	barb (0)	barb (10)	benz (0)	benz (10)
0	1	413.2	429.7	458.2	479.8	32.8	33.1	1539.1	1585.6	286.1	240.4	309.8	250.6
	2	451.0	469.3	512.1	519.6	33.9	35.4	1375.7	1619.4	255.6	245.2	290.5	257.3
	3	418.0	483.2	452.4	452.2	35.4	35.3	1557.1	1584.1	253.9	264.5	302.3	252.9
	4	431.9	458.1	476.8	377.6	33.4	35.3	1626.4	1643.0	276.4	263.2	311.1	259.2
	5	436.6	437.9	530.8	426.5	36.1	31.0	1589.4	1621.8	275.6	249.8	293.2	254.4
	6	468.7	427.6	475.3	523.4	31.7	35.1	1773.6	1484.6	280.2	248.3	289.8	271.6
6	1	404.3	404.5	472.6	511.5	32.0	35.4	1329.2	1484.6	290.4	247.1	297.5	237.5
	2	423.4	459.3	376.8	563.4	34.9	36.9	1778.8	1673.3	290.0	265.6	300.7	233.2
	3	419.4	457.8	441.9	510.3	34.9	35.8	1692.8	1458.1	291.7	284.6	304.2	246.9
	4	490.1	462.1	470.9	475.1	32.6	37.6	1612.4	1356.4	259.3	305.4	299.2	241.6
	5	457.6	432.6	447.2	556.4	37.3	34.6	1618.6	1573.5	260.2	285.5	299.3	252.5
	6	449.3	439.0	461.6	500.4	35.6	36.7	1597.0	1574.3	274.9	288.6	304.3	246.0
72	1	459.1	457.0	422.5	464.9	38.7	38.3	1741.1	1454.6	232.4	265.6	268.0	206.3
	2	439.7	437.8	440.8	443.8	42.1	36.9	1652.5	1687.9	243.8	242.0	301.4	211.1
	3	447.8	444.8	529.5	481.6	33.0	40.3	1765.8	1584.1	243.6	295.5	276.1	222.3
	4	435.0	476.5	419.8	441.3	39.6	37.0	1532.0	1629.6	254.1	289.0	291.2	224.1
	5	427.2	441.3	440.8	423.9	37.5	42.5	1672.5	1768.4	262.4	296.3	280.3	222.3
	6	451.3	408.7	446.5	487.9	37.1	38.1	1635.9	1549.6	266.7	323.2	287.9	222.2

RP and DRP

BANI Assay of RP Inhibition

Preparation	Replicate #	delta-mabs
Blank	1	1
1 mg/mL papain	1	38
	2	37
	3	35
1 mg/mL papain+1 mg/mL E-64	1	0
	2	0
	3	1

FPIA of 60 mg/mL THC-COOH with RP and DRP

Specimen	Replicate #	Result
Control (0 mg/mL RP)	1	63.44
	2	62.38
	3	66.91
	4	70.08
	5	65.12
	6	71.19
1 mg/mL RP	1	39.28
	2	43.73
	3	44.24
	4	42.06
	5	42.98
	6	46.19
1 mg/mL DRP	1	43.65
	2	40.78
	3	40.25
	4	42.72
	5	41.49
	6	38.85

FPIA of 250 mg/mL nordiazepam with RP and DRP

Specimen	Replicate #	Result
Control (0 mg/mL RP)	1	196.08
	2	210.12
	3	212.35
	4	217.9
	5	207.46
	6	215.93
1 mg/mL RP	1	209.76
	2	201.19
	3	223.64
	4	205.93
	5	214.98
	6	218.36
1 mg/mL DRP	1	227.79
	2	212.99
	3	214.91
	4	211.28
	5	220.31
	6	221.27

APPENDIX E

GC/MS of THC-COOH

THC-COOH (ng/mL)	THC-COOH area	d_3-THC-COOH area
Standard Curve		
0	578	11558
100	44327	20567
200	120520	22500
300	266254	33843
400	626387	57495
500	882399	64039
Specimens w/ 10 mg/mL papain		
500	108912	19663
500	61606	16315
500	46820	12798
500	61923	16864
500	91118	20028
500	48939	9268

APPENDIX F

HPLC/UV of Nordiazepam

Nordiazepam (ng/mL)	Nordiazepam area	Prazepam area
Standard Curve		
0	0	4911435
200	278920	3293260
250	266769	2265366
300	624154	4439322
400	925367	5144384
Specimens w/ 10 mg/mL papain		
500	58123	511652
500	381144	3694503
500	462029	4367027
500	490568	4887712
500	493657	4839268
500	506353	5189691

APPENDIX G

Specimen Validity Testing

Creatinine Standard Curve

Specimen	Absorb. (mabs/min)
0	2
3	9.1
5	13.5
7	20.5
12	36.3
23	75.2

Creatinine assay of 30 random urine specimens

Specimen	Dilution Factor	Absorb. (mabs/min) in the unadulterated specimens	Absorb. (mabs/min) in the specimens adulterated with 10 mg/mL papain
1	5	26.9	31.9
2	5	35.8	39.1
3	2	19.4	13
4	20	26.4	27.2
5	5	20.5	19.5
6	10	36.1	33.5
7	5	37.3	40.2
8	10	59.5	74.9
9	20	44.8	36.6
10	10	49.8	49
11	5	38.7	43.9
12	5	39.9	43.1
13	5	33.2	33.8
14	10	30.5	28.3
15	5	24.7	37
16	10	36.1	34.1
17	5	30.9	35.9
18	10	57.7	61.5
19	20	34.1	41.4
20	10	41.1	40.6
21	5	56.4	58.6
22	5	57.3	62.5
23	10	45.7	44.4
24	5	64.1	74.9
25	5	33.3	31.9
26	10	41.7	40.7
27	20	46.5	57
28	5	7.9	7.4
29	5	15.9	20.3
30	2	16.9	14.9

Creatinine assay of 6 random urine specimens

Time (h)	Specimen	DF	Absorb. (mabs/min) in the unadulterated specimens	Absorb. (mabs/min) in the specimens adulterated with 10 mg/mL papain
0	30	2	15.1	12.6
	17	5	25.7	25.1
	12	5	38.1	34.2
	6	10	24.9	21.8
	18	10	52.5	47
	27	20	36.9	38.3
6	30	2	13.4	5.3
	17	5	18	22.5
	12	5	39.1	30.7
	6	10	24.7	24.7
	18	10	48.6	48.2
	27	20	44.8	41.8
72	30	2	12	12.1
	17	5	26.4	26.6
	12	5	39.7	37.9
	6	10	30.7	29
	18	10	54.8	54.7
	27	20	43.3	44.2

Specific Gravity Standard Curve

Specimen	Absorb. (mabs/min)
1.000	1.000
1.002	1.002
1.005	1.005
1.030	1.034

Specific gravity of 30 random urine specimens

Specimen	Specific Gravity of the unadulterated specimens	Specific Gravity of the specimens adulterated with 10 mg/mL papain
1	1.008	1.011
2	1.030	1.034
3	1.026	1.030
4	1.024	1.028
5	1.019	1.023
6	1.019	1.023
7	1.014	1.018
8	1.013	1.017
9	1.009	1.013
10	1.009	1.013
11	1.007	1.011
12	1.028	1.033
13	1.023	1.028
14	1.021	1.026
15	1.019	1.024
16	1.018	1.023
17	1.014	1.019
18	1.012	1.017
19	1.009	1.014
20	1.009	1.014
21	1.020	1.026
22	1.016	1.022
23	1.010	1.016
24	1.010	1.016
25	1.009	1.015
26	1.003	1.009
27	1.002	1.008
28	1.001	1.007
29	1.034	1.041
30	1.015	1.022

Specific gravity of 6 random urine specimens

Time (h)	Specimen	Specific Gravity of the unadulterated specimens	Specific Gravity of the specimens adulterated with 10 mg/mL papain
0	30	1.002	1.004
	17	1.009	1.010
	12	1.009	1.011
	6	1.021	1.023
	18	1.021	1.023
	27	1.035	1.038
6	30	1.002	1.005
	17	1.009	1.012
	12	1.009	1.013
	6	1.021	1.024
	18	1.021	1.024
	27	1.035	1.038
72	30	1.002	1.005
	17	1.009	1.012
	12	1.010	1.013
	6	1.020	1.024
	18	1.020	1.026
	27	1.036	1.038

pH of 30 random urine specimens

Specimen	pH of the unadulterated specimens	pH of the specimens adulterated with 10 mg/mL papain
1	7.0	7.0
2	7.0	6.6
3	7.3	6.3
4	6.7	6.0
5	7.1	6.7
6	5.5	5.4
7	6.5	6.3
8	5.9	5.8
9	5.7	5.6
10	6.5	6.5
11	6.4	6.2
12	5.7	5.1
13	6.5	6.5
14	6.8	6.8
15	6.0	5.9
16	7.9	8.2
17	6.2	5.9
18	5.1	5.5
19	5.3	5.4
20	5.3	5.2
21	5.5	5.4
22	5.8	5.6
23	5.3	5.2
24	6.0	5.9
25	5.6	5.4
26	5.2	5.2
27	5.6	5.5
28	6.9	6.7
29	6.2	5.9
30	7.6	6.7

Specific gravity of 6 random urine specimens

Time (h)	Specimen	pH of the unadulterated specimens	pH of the specimens adulterated with 10 mg/mL papain
0	18	5.5	5.5
	6	5.5	5.5
	22	5.7	5.7
	10	6.6	6.6
	1	7.0	7.0
	16	7.9	7.9
6	18	5.5	5.5
	6	5.5	5.4
	22	5.8	5.8
	10	6.7	6.6
	1	7.1	7.0
	16	8.0	8.0
72	18	5.4	5.4
	6	5.4	5.4
	22	5.8	5.8
	10	6.6	6.5
	1	7.1	6.8
	16	8.0	7.7

POC test strips

Specimen		Nitrite	Glutaraldehyde	Oxidants
Control				
	L1	neg	pos	neg
	L2	neg	neg	neg
	L3	pos	neg	pos
Unadult				
	30	neg	neg	neg
	17	100 mg/dL	neg	neg
	12	neg	neg	neg
	6	neg	neg	neg
	18	neg	neg	neg
	27	neg	neg	neg
Adult (10mg/mL papain)				
	30	neg	neg	neg
	17	100 mg/dL	neg	neg
	12	neg	neg	neg
	6	neg	neg	neg
	18	neg	neg	neg
	27	neg	neg	neg

VITA

DAVID L. BURROWS

Personal Data:

Date of Birth: May 14, 1975
Place of Birth: Bethesda, Maryland
Marital Status: Married

Education:

Public Schools, Palm Bay, Florida
Florida State University, Tallahassee, Florida
Chemistry, B.S., 1997
East Tennessee State University, Johnson City, Tennessee
Inorganic Chemistry, M.S., 2000
East Tennessee State University, Johnson City, Tennessee
Biomedical Science/Pharmacology/Toxicology, Ph.D., 2004

Professional Experience:

Rehabilitative Nursing Assistant, HealthSouth Rehabilitation Hospital; Tallahassee, Florida, 1995-1996
Inorganic Chemist, Flowers Chemical Laboratory; Altamonte Springs, Florida, 1997-1998
Graduate Assistant, East Tennessee State University; College of Arts and Science, 1998-2000
Pharmacology Laboratory Facilitator, East Tennessee State University; College of Medicine, 2003-2004

Publications:

Burrows, David L.; Nicolaides, Andrea; Stephens, Gretel C.; Ferslew Ken E. (2004). "Sevoflurane Analysis in Various Biological Matrices by Headspace Gas Chromatography." J. Analytical Toxicology. Niles, IL: Preston Publications. Accepted for Publication.

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Honors and Awards:

Received an Educational Research Award at the Society of Forensic Toxicologist Annual Meeting, Washington, DC, August 2004, for an ERA presentation of "Papain, A Novel Urine Adulterant."

Received a First Place Award at the Southeastern Regional Chapter of the Society of Toxicology Annual Symposium, Chattanooga, Tennessee, October 2003, for a platform presentation of "Sevoflurane Analysis by Headspace Gas Chromatography."

Received a Third Place Award at the Southeastern Regional Chapter of the Society of Toxicology Annual Symposium, Athens, Georgia, October 2002, for a poster presentation of "A Fatal Drug Interaction Between Oxycodone and Clonazepam."