USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHYTODETECT THE PRESENCE OF CATHINONE AND CATHINE IN FALSE-POSITIVE SAMPLES FOR AMPHETAMINE BY IMMUNOASSAY TECHNIQUES

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ABSTRACT

Introduction: The use of Khat trend is increasing among higher education students in Jazan region. Immunoassay techniques are used in the poison control and forensic medical chemistry center in Assir region to screen the urine samples for the presence of amphetamine. Immunoassays are sensitive, fairly inexpensive and are relatively easy to perform. Poor specificity is a concern with the first step of the Urine Drug Screen (UDS) process, so a confirmatory test is recommended for immunoassay positive result. **Aim of the work:** This study was conducted to assess the cross-reacting effect of Cathinone and Cathine (the active principles of Khat) with amphetamine when analysed by immunoassay analysers and the efficacy of High Performance Thin Layer Chromatography (HPTLC) in differentiating between these chemicals and at what concentration. **Materials and Methods:** Twelve different concentrations from the standards of both Cathinone and Cathine were prepared. The samples were tested for Amphetamine Assay using two immunoassay analysers; Abbott AxSYM and Abbott Architect. The samples were then examined by HPTLC. **Results:** No cross-reactivity was detected on AxSYM while the samples examined by the Architect showed cross-reactivity at 5ug/ml for cathine and 70ug/ml for cathinone. **Conclusion:** HPTLC was unable to detect the difference between cathinone and cathine from Amphetamine at all prepared concentrations. **Recommendations:** We recommend the use of other confirmatory techniques such as; HPLC, GC-MS, or LC-MS, to examine all amphetamine positive samples by immunoassay analysers to detect the false positive samples and to detect the presence of Cathinone and Cathine especially in areas where Khat is frequently used.

Key wards: Cathine, Cathinone, Immunoassay techniques, Amphetamine, Cross-reactivity, Khat.

INTRODUCTION

Khat chewing is a common habit among all segments of southwest Arabic peninsula. Khat chewing produces psycho stimulation effect in the form of euphoria and excitement because of cathinone contents **(Feyissaand Kelly, 2008)**. It is well documented that Khat has many severe public health and social problems **(Al-Habori et al., 2002; Daifalah and Santavy, 2004; and Al-Motarreb et al., 2010)**. The use of Khat trend is increasing among higher education students in Jazan region, and it reflects the social and legal status of Khat in the community. Although Khat is illegal in Saudi Arabia, it is both socially acceptable and easily available and accessible by the majority of the population **(Al-Motarreb et al., 2002).**

Immunoassay techniques are used in the poison control and forensic medical chemistry center in Assir region to screen the urine samples for the presence of amphetamine. The most frequently used techniques work by homogenous immunoassay, which, rather than detecting for the presence of the unbound labeled substance, compares the concentration of the labeled free versus the concentration of the labeled attached substance **(Darwish, 2006)**.

Types of homogeneous immunoassays used in Assir poison control center include enzyme-multiplied immunoassay technique (EMIT) used by ViVa Analyzer ofSiemens Corporation, fluorescence polarization immunoassay (FPIA) used by AxSYM of Abbott Corporation (USA), and enzyme immunoassay technique (EIA) used by Architect Corporation by Abbott (USA).

Immunoassays are sensitive, fairly inexpensive tests and are relatively easy to perform because they are easily collected and automated. Poor specificity is a concern with the first step of the Urine Drug Screen (UDS) process, so a confirmatory test is recommended for immunoassay positive results **(Darwish, 2006)**.

Confirmatory tests are frequently done via the second step, gas chromatography–mass spectrometry or high-performance liquid chromatography. Chromatographic techniques work by separation of substances between a mobile and a stationary phase. These testing methods are more cumbersome than the other methods; however, they are the most sensitive and specific tests to help exclude false positive results **(Hand and Baldwin, 2004).**

THE AIM OF THIS WORK

The aim of this work was to detect the possible cross-reactivity between Cathine and Cathinone containing samples and amphetamine when examined by the available immunoassay analyzers and the efficacy of HPTLC in differentiating between cathine, cathinone and amphetaminequalitatively and quantitatively.

MATERIALS AND METHODS

The Standard Samples of Amphetamine, Cathinone and Cathinewere provided by UCT, Bristol, PA, USA. All the solutions, reagents, controls and Calibrators needed for AxSYM and Architect were provided by Abbott Corporation.The solutions and reagents used for HPTLC (Methanol, Ninhydrine, Butanol, and Acetic Acid) were provided by Thermo Scientific, USA.

Laboratory analyzers used in the study:

- **AxSYManalyzer** (by Abbott Corporation, USA), depending on FBIA (Fluorescence Polarization Immunoassay) technique.
- **Architectanalyzer** (by Abbott Corporation, USA), depending on EIA (Enzyme Immunoassay) technique.
- **HPTLC** (by CAMAG Corporation, Switzerland)

Methods:

This research was conducted from April 1st, 2013 to May 30th 2013 in Assir Poison Control and Forensic Chemistry Centre, Saudi Arabia. Twelve different concentrations from the standards of both Cathinone and Cathine were prepared by diluting the Standard from 1mg/ml in methanol to concentrations (1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ug/ml). The standard samples (12 concentrations of Cathinone and 12 concentrations of Cathine) were tested for Amphetamine Assay using two immunoassay analyzers; Abbott AxSYM and Abbott Architect. Any concentration measured above 300 ng/ml of amphetamine was considered positive as recommended by the directorate of poison control and forensic chemistry in ministry of health, Saudi Arabia.

To confirm the presence of amphetamine the standard samples (12 concentrations of Cathinone and 12 concentrations of Cathine) were tested by High PerformanceThin Layer Chromatography (HPTLC) manufactured by CAMAG.

RESULTS

Table (1): The results of examining different concentrations of Cathinone and Cathine by AxSYM and Architect.

Cathine and Cathinone Concentrations*	Cathinone (ng/ml) **		Cathine (ng/ml) ***	
	AxSYM	Architect	AxSYM	Architect
1µg/ml	6.0	<100	18.8	<100
5µg/ml	24.22	<100	22.8	436.0
10µg/ml	33.6	<100	33.5	998.5
20µg/ml	54.7	<100	40.5	1450.0
30µg/ml	77.8	<100	66.0	1760.0
40µg/ml	99.5	<100	91.2	1990.0
50µg/ml	130.6	217.8	110.4	>2000.0
60µg/ml	157.7	278.0	133.5	>2000.0
70µg/ml	177.9	337.9	150.7	>2000.0
80µg/ml	189.1	400.1	169.2	>2000.0
90µg/ml	221.8	471.6	199.3	>2000.0
100µg/ml	278.3	521.0	262.1	>2000.0

N.B.: any concentration > 300 ng/ml was considered amphetamine positive

** The readings of the immunoassay analyzer when examiningcathinone standards using amphetamine reagents.

*** The readings of the immunoassay analyzer when examiningcathine standards using amphetamine reagents.

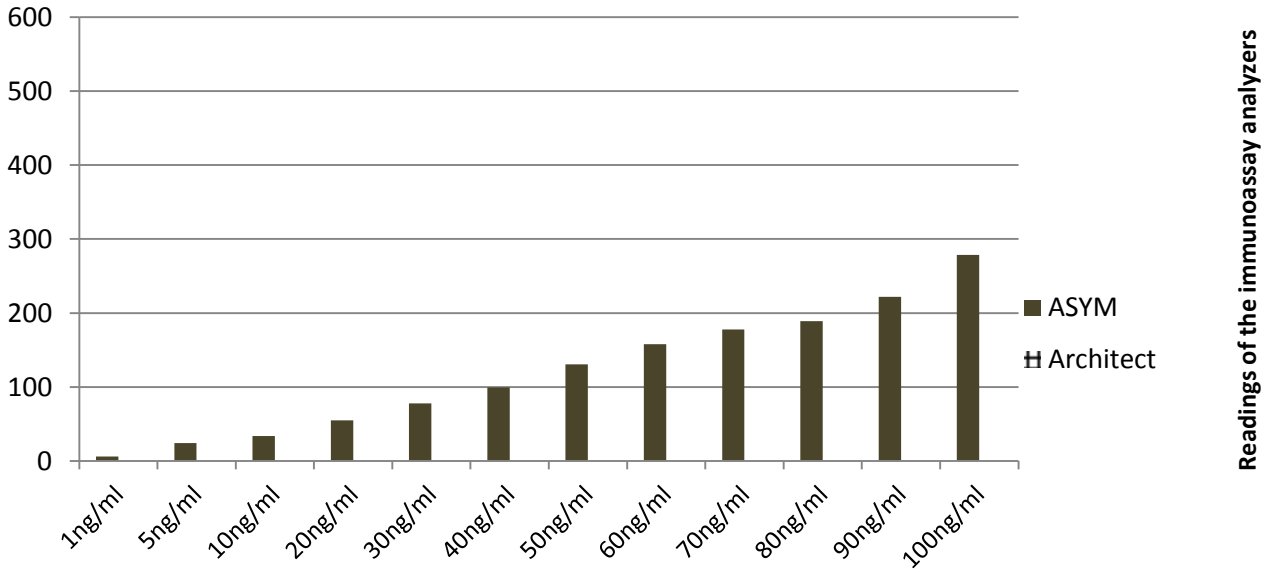


Figure (1): The results of examining different concentrations of cathinoneby AxSYM and Architect Analyzers.

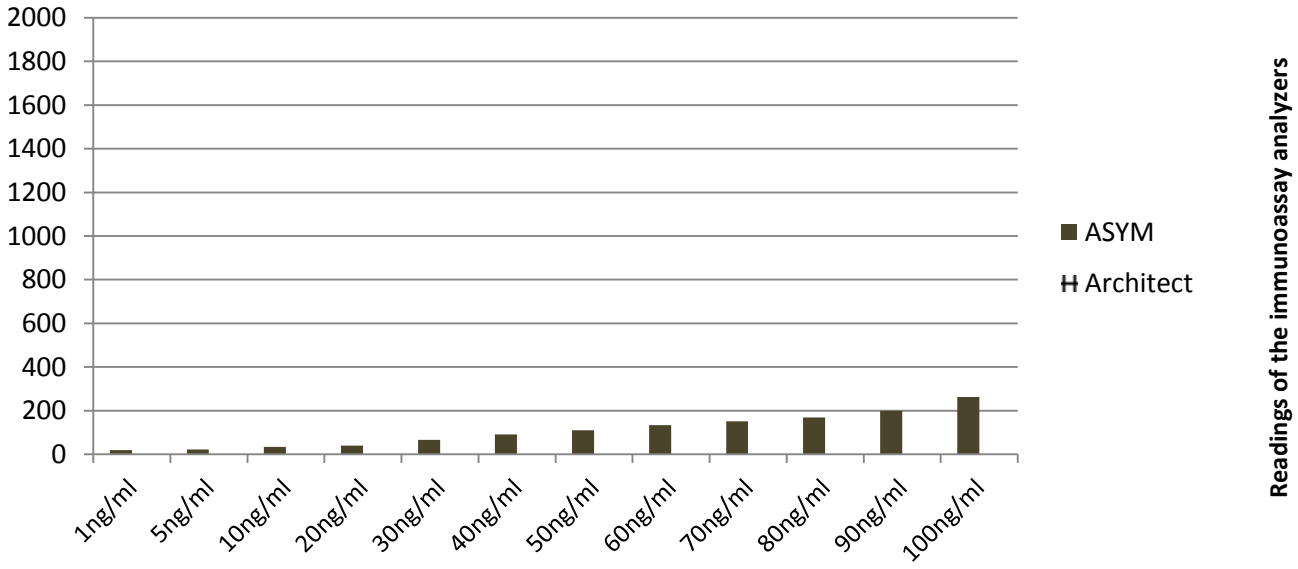


Figure (2): The results of examining different concentrations of cathine by AxSYM and Architect Analyzers.

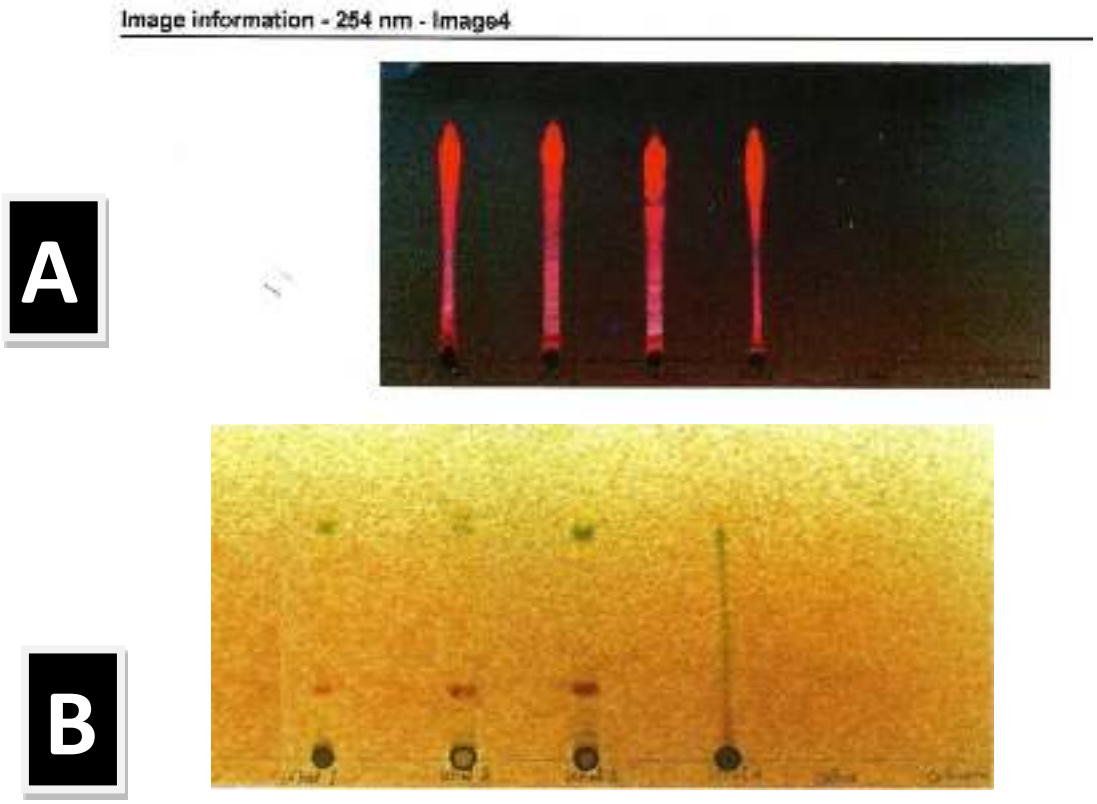
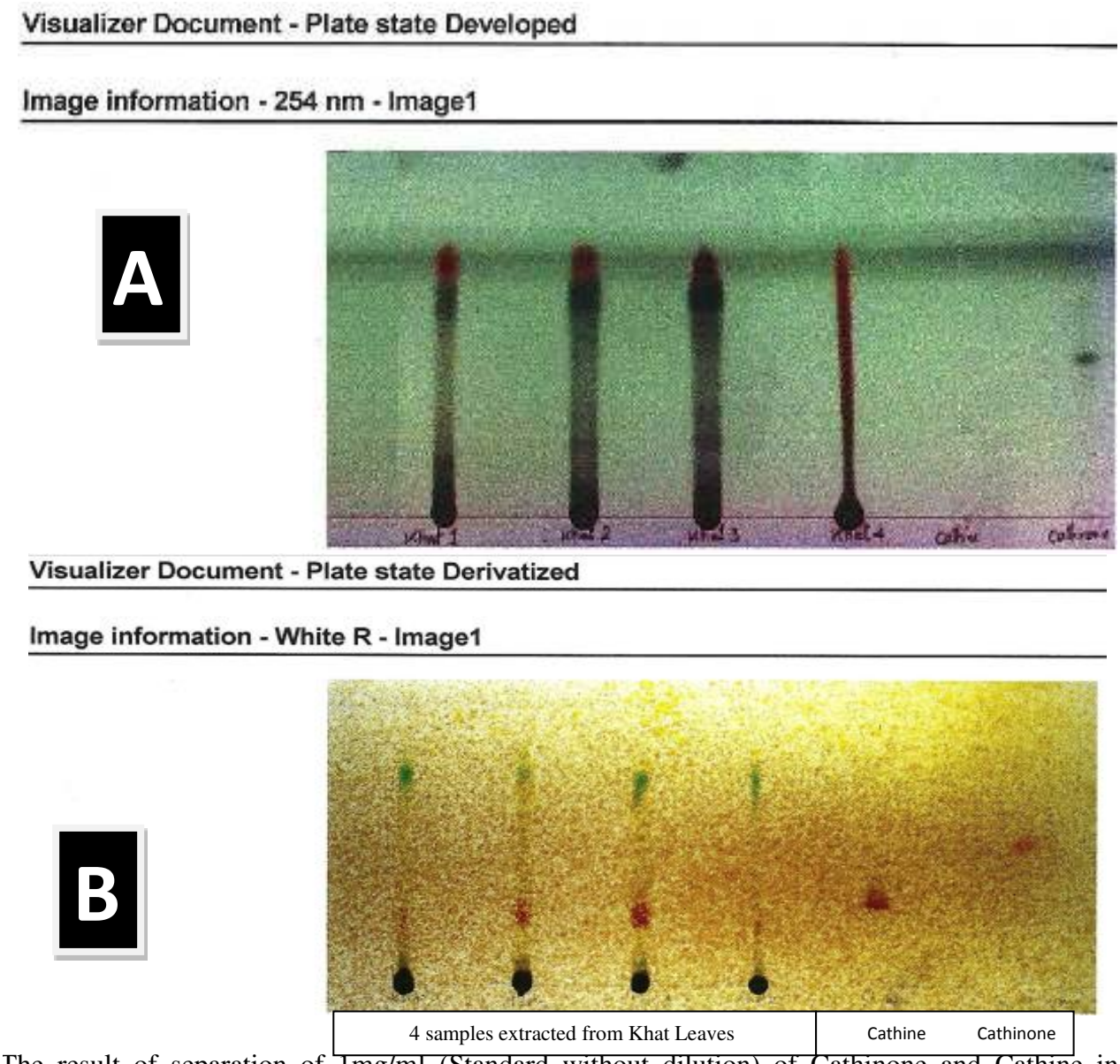


Figure (3): The result of separation of 100ug/ml of Cathinone and Cathine in comparison with 4 samples extracted from Khat leaves. (A) using UV 254, (B) Ninhydrine 0.3%



The result of separation of 1mg/ml (Standard without dilution) of Cathinone and Cathine in comparison with 4 samples extracted from Khat leaves. (A) using UV 254, (B) Ninhydrine 0.3%

DISCUSSION

By examining table (1) we conclude that:

- The standard samples of both cathine and cathinone with different concentrations (1-100ug/ml) did not reveal any cross-reactivity with amphetamine on AxSym (FPIA).
- Concentrations of cathine with above 5ug/ml and concentrationof cathinoneabove 70ug/ml reported false positive results for amphetamine when examined by Architect analyzer (EIA).

The results of our study is consistent with the results of several other studies that stated that a large number of drugs cross-react with amphetamine when these drugs are analyzed using immunoassay techniques especially those depending on (EMIT) Enzyme Multiplier Immunoassay Technique which is similar to the method used in Architect (EIA) of Abbott.

Roberge et al., (2001) stated that samples containing Trazodone cross-react with

amphetamine when the test is done by Triage Drugs of Abuse Panel.

Vorce et al., (2011) stated that using two methods of immunoassay examination with two different principals to screen for amphetamine will reduce the number of samples need to be confirmed.

The result of this study is contrary to the work done by **Paul and Cole, (2001)** who recommended using EMIT technique to examine urine samples containing amphetamine even if they contain Cathinone ormethcathinone. They also reported that false positive results for amphetamine occur with 1ug/ml phenylpropanolamine or dl-Ephedrine, or 10ug/ml of dl-pseudoephedrine.

The results of the current study was in agreement with, **Toennes and Kauert(2002)** who stated that immunoassay analyzers using FPIA technique proved to have negative results for amphetamine when examining all the urine samples containingCathine or Cathinone. But other immunoassay techniques resulted in false positive results for amphetamine when examining urine samples containing 50mg/L cathine or cathinone.

In our study; HPTLC could not separate the highest concentration prepared of both Cathine and Cathinone (100ug/ml) (figure 3). We used Khat extract (extracted according to the method of **Lee et al., 1995)** to be separated on a TLC plate to assess the efficacy of mobile phase used. In figure (4) we notice that standard concentrated samples (1mg/ml) of cathine and cathinone were separated. The extracted khat leaves showed cathine positive spot while cathinone did not appear because the khat leaves are not fresh enough to show the cathinone.

Thin Layer Chromatography is considered one of the fast screening methods to determine the presence of Khat constituents. When examining the separated samples by UV light, the spot of Cathine (R_f 0.48) and cathinone (R_f 0.26) were brown and when ninhydrine was sprayed cathine spot became purple and cathinone spot became orange **(Poole, 2004).**

Lee, 1995, stated that the results of examining samples of cathinone and cathine by Infrared was not consistent and was not accurate and could not be compared with those of mass spectrometry. In this study Lee, used mobile phase of Ethyl acetate: Methanol: Ammonia (85:10:5), and the examination was done by UV_{254}nm, and Ninhydrine 0.5%. After heating cathinone appeared as a burnt orange spot with R_f 0.46 and cathine appeared as purple spot with R_f 0.25.

Lehmann et al., 1990, determined the lowest concentration that could be detected by TLC is 250ng after using UV_{254}, and this amount could reach 300ng by usingninhydrine as a spraying agent, but these results must be confirmed by other chromatographic techniques such as: HPLC, GC-MS or LC-MS.

CONCLUSION

From the above mentioned results we concluded that HPTLC should not be used to confirm samples positive for amphetamine by immunoassay techniques and Fluorescence Polarization immunoassay techniques is more sensitive than EIA technique in detecting khat containing samples.

RECOMMENDATIONS

We recommend the use of chromatographic techniques other than TLC to confirm the positive Amphetamine results by immunoassay. Fluorescence Polarization immunoassay techniques have to be one of the recommended techniques to examine urine samples proposed to contain amphetamine especially in areas using Khat.

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استخدام الفصل اللوني بالطبقة الرقيقة عالي الكفاءة للكشف عن الكاثينون والكاثين في العينات الايجابية الخاطئة للأمفيتامين بواسطة طرق الفحص المناعي

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تهدف هذه الدراسة لتقييم التداخل بين الكاثينون والكاثين (المواد الفعالة في نبات القات) مع الامفيتامين عند تحليل العينات بواسطة اجهزة الفحص المعتمدة على تقنية الفحص المناعي وكذلك قدرة جهاز الفصل اللوني بالطبقة الرقيقة عالي الكفاءة على التفرقة بين هذه المركبات وعند أي تركيز.

تم استخدام ١٢ تركيز مختلف من العينات القياسية للكاثينون والكاثين، وتم فحصها علي جهازين من الاجهزة المعتمدة على تقنية الفحص المناعي باستخدام كواشف الامفيتامين.

لم نسجل اي ايجابية خاطئة للعينات القياسية بكافة تركيزاتها على جهاز الـ AxSYM، اما جهاز الـ Architectفقد تم تسجيل ايجابية خاطئة للامفيتامين بدءا من تركيز ٥ ميكروجرام لكل مل في عينات الكاثين و ٧٠ ميكروجرام لكل مل في عينات الكاثينون. لم يستطع جهاز الطبقة الرقيقة عالي الكفاءة من فصل عينات الكاثينون والكاثين عن الامفيتامين بالتركيزات المستخدمة. ولذا ينصح باستخدام طرق تأكيدية اخرى لفحص العينات الايجابية للامفيتامين بواسطة طرق الفحص المناعي خاصة في المناطق التي يكثر فيها استخدام نبات القات.