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High Performance Thin Layer Chromatography method for analysis of 3,4-methylenedioxymethamphetamine in seized tablets

[Método de análisis de 3,4-metilendioximetanfetamina por Cromatografía Planar de Alta Eficiencia en comprimidos incautados]

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Abstract

Context: Consumption of synthetic drugs had increased in recent years, used as a recreational drug by young people who presume that consumption of this drug is harmless for health; however clinical studies have shown that this stimulant and its metabolites are toxic. Due to these reasons, chemical analysis of this illicit drug is crucial from the points of view of occupational medicine, toxicology, and law enforcement with the aim of pursuit the traffic of illegal drug.

Aims: Implement and fully validate a rapid and simple method for detection and quantitation of MDMA by High-Performance Thin Layer Chromatography in seized samples.

Methods: With the implemented method was analyzed 12 positive samples seized by Chilean police, to found the concentration of MDMA in ecstasy tablets.

Results: The method was fully validated, the linearity of the method was evaluated by the calibration curve between 51.0 - 510.0 µg/band (R^2 0.9977); limit of detection was 12.1 µg per band, and limit of quantitation was 36.8 µg per band. The precision of the method (RSD) was lower than 5.0%. Accuracy was evaluated by determination of the percentage of MDMA recovered by the assay (99.13%), and relative Uncertainty was 6.66%. With this method, it was analyzed real seized samples of MDMA, results showed that all samples contained MDMA and concentration was between 18.15 - 59.84 % w/w.

Conclusions: The method is selective, sensitive, and specific, with possible application in forensic analysis. To the best of our knowledge, this is the first report about concentration of MDMA in ecstasy pills in Chile.

Keywords: 3,4-methylenedioxymethamphetamine; ecstasy; HPTLC; MDMA.

Resumen

Contexto: El consumo de drogas sintéticas se ha incrementado en los últimos años. Estas son utilizadas principalmente por jóvenes quienes asumen que su consumo es inofensivo para la salud; sin embargo, estudios clínicos han demostrado que, particularmente el MDMA y sus metabolitos, son tóxicos. Debido a estas razones, el análisis químico de esta droga ilícita es muy importante desde el punto de vista de la toxicología, medicina forense y en la aplicación de la ley con el propósito de perseguir el tráfico ilícito de drogas.

Objetivos: Implementar y validar un método rápido y simple para la detección y cuantificación de MDMA por Cromatografía Planar de Alta Eficiencia en muestras incautadas.

Métodos: Con el método implementado se analizaron 12 muestras reales incautadas por la policía chilena, con el fin de encontrar la concentración de MDMA en comprimidos de éxtasis.

Resultados: El método fue completamente validado, la linealidad del método fue evaluada mediante la curva de calibración entre 51.0 - 510.0 µg/banda (R^2 0.9977); el límite de detección fue 12.1 µg por banda y el límite de cuantificación fue 36.8 µg por banda. La precisión del método (RSD) fue menor que el 5.0%. La exactitud fue evaluada mediante la determinación del porcentaje de MDMA recuperado en el ensayo (99.13%), y la incertidumbre relativa fue 6.66%. Con este método se analizaron muestras reales de incautaciones de MDMA, los resultados revelaron que todas las muestras contenían MDMA en el rango de concentración entre 18.15 - 59.84% p/p.

Conclusiones: El método es selectivo, sensible y específico, con aplicaciones posibles en el análisis forense. Según nuestro conocimiento, este es el primer reporte acerca de la concentración de MDMA en comprimidos de éxtasis en Chile.

Palabras Clave: 3,4-metilendioximetanfetamina; éxtasis; HPTLC; MDMA.

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INTRODUCTION

The current wave of popularity of synthetic drugs between young teenagers has spread around the world (da Costa and da Matta Chasin, 2004). These drugs are produced in clandestine laboratories these compounds are commonly known as “designer drugs” and they are sold illegally in the streets. The typical constituents of these designer drugs are chemical substances derived from amphetamine, but with significant differences in effects caused and duration of these effects; in this group we found the 3,4-methylenedioxymethamphetamine known commonly as MDMA or “ecstasy” (Fig. 1) (Mitrevski and Zdravkovski, 2005).

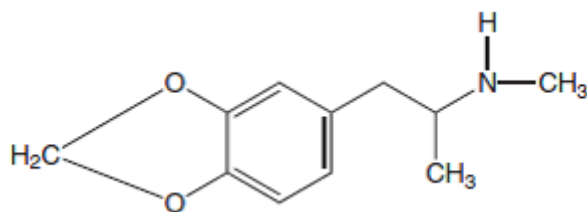


Figure 1. Chemical structure of MDMA.

The MDMA is a synthetic drug, with psychedelic and stimulant effects. This compound was synthesized in 1912 as an appetite suppressant drug, however at present time; is principally known a “club drug” is frequently used at rave parties (Castro et al., 2012) and is available illicitly in tablets, capsules, and powder forms, the most typical way of consumption is oral ingestion, but insufflation and intravenous injection also have been reported. The popularity of the methylenedioxyamphetamine derivatives can be attributed to their psychotropic effects called entactogenic effects (Bedi et al., 2010), that evoke mainly pleasant emotional effects of relaxation, feelings of happiness, increased empathy, and closeness to others (Clauwaert et al., 2000). The consumption of this substance, considered to be harmless by some young people (Falck et al., 2004), is extremely dangerous, especially by the hazardous effects on the liver, kidneys and the brain. This drug also constitutes a major class of central nervous system stimulants, also producing cardiovascular and behavioral effects. The symptoms noted in acute toxicity include anxiety, mydriasis, hyperten-

sion, tachycardia, tachypnea, hallucinations, bruxism, and diaphoresis, hyperthermia, arrhythmias, hyperreflexia, seizures, metabolic acidosis, ischemia, rhabdomyolysis, and in some cases renal failure may be seen in severe toxicity. The hyponatremia, (one of the most serious medical complications of ecstasy abuse) (Schwaninger et al., 2011) hyperkalemia, coagulopathies, pulmonary edema, adult respiratory distress syndrome and intracranial hemorrhage have been associated with ingestion of 'ecstasy' (Hughes et al., 1993); while the immense majority of these cases have spontaneous recovery, an increasing number of acute hepatic failure reports are now appearing in the literature (Henry et al., 1992; Campbell and Rosner, 2008). However, the number of deaths related to the use of Ecstasy pills is small when compared to the frequency of its use in many countries (Klys et al., 2007).

Illegal use of synthetic drugs is an increasing global problem and an important public health issue. Amphetamine-like stimulants are used in many countries (Gimeno et al., 2003); including Chile as illegal drugs. The analyzes of these substances have interest from the points of view of toxicology, occupational medicine, and law enforcement; in this case, a chemical profile can be used as a fingerprint, for the purpose of establishing a link between samples and their origin (Choe et al., 2012). Numerous chromatographic methods for the detection and determination of different amphetamines in tablets and biological samples have been published (Cheng et al., 2003; Bartos and Gorog, 2008; Giebink and Smith, 2011).

In this scenario, an increasing technique is High-Performance Thin Layer Chromatography (HPTLC), that has become in a powerful tool for the analysis of food, pharmaceutical formulations and forensic samples (Santhana Lakshmi and Lakshmi, 2012; Shewiyo et al., 2012) offers many advantages (Aranda et al., 2007). This technique is fast, reliable and reproducible with high resolution and sensitivity (Kato et al., 2008). In consequence, in this work was developed and validated a rapid and simple method for detection and quantitation of methylenedioxyamphetamine (MDMA) in tablets. Besides validation parameters, the uncertainty of the method was also evaluated (González

and Herrador, 2007). With the selected and validated method, 12 positive samples were analyzed, to found the concentration of MDMA in ecstasy tablets, this solid, reliable and simple technique appears to be suitable for comparison of ecstasy tablets, impurity profiling, and routine use.

MATERIAL AND METHODS

Chemicals, reagents, and standards

Methanol, chloroform, concentrated ammonia, sodium hydroxide were analytical grade and purchased from Merck, Darmstadt, Germany. Methylenedioxymethamphetamine (MDMA HCl) was purchased from Lipomed, Wienerbergstrasse, Vienna.

Chromatography

HPTLC was performed on 20 x 10 cm precoated silica gel F254 plates, (Merck, Darmstadt, Germany) previously activated at 80° C for 30 minutes. Six µL of standards and 2 µL of samples were applied in bands of 3 mm with a CAMAG ATS 4 automatic TLC sampler, using a spray band technique, the first application x-axis is 15 mm, the y-axis is 8.0 mm, and the distance between tracks was 5.6 mm. Plates were developed with an automatic developing chamber ADC-2 to a distance of 70 mm. with methanol/ammonia 100:1.5 as mobile phase (10 mL), without saturation, drying time of 5.0 min; the spots were scanned with a CAMAG TLC Scanner 4 densitometer by absorbance at 210 nm. Spectra of each peak were recorded in the range of 190 – 400 nm on all detected peaks mode. Slit dimension 4.00 x 0.30 mm, scanning speed 20 nm/s, data resolution 100 µm/step, reference spectrum x = 10.0 mm, y = 5.0 mm, were controlled by the software Wincats Planar Chromatography Manager version 1.4.7 (CAMAG, Switzerland).

Extraction method

Approximately 5-10 mg of homogenized sample (with a mortar and pestle) was dissolved in 10 mL of methanol with 4N sodium hydroxide until pH of the solution almost 10. Then, using an ultrasonic bath the sample was extracted for 15 min, the extract was filtered using a syringe filter (Millex® pore

size 0.22 µm) to a glass vial for chromatographic analysis.

Statistical analysis

As recommended by ICH guidelines the validation was performed during the development of the procedure (Thompson et al., 2002). The proposed method was validated for specificity, linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness, uncertainty was also estimated. The statistical parameters were evaluated using the software STATGRAPHICS Centurion XVI.I.®.

RESULTS

Specificity

The specificity of the method was determined by analyzing standard drug and samples. The band for MDMA was confirmed by comparing the R_f (0.34) (see Fig. 2) and spectrum (190-400 nm) of each band (Fig. 3). The peak purity of MDMA was determined by comparing the spectrum in three different regions of the spot [i.e. peak start (S), peak apex (M) and peak end (E)], results of purity and spectrum comparison of real samples were at least 0.999 of correlation. It was found an acceptable correlation between UV spectra acquired from the standard, and the real samples and no other peaks were present at the R_f of MDMA. Contaminants and/or excipients did not alter the separation and identification process, and false negatives or positives were not found in real samples.

Linearity and range

The calibration plot for the HPTLC method was constructed by examination of ten independent solutions prepared by dissolving appropriate amounts of the standard of MDMA HCl to give concentrations of 51.0 - 510.0 µg per band of MDMA each point applied in triplicate. Data were fitted by a linear equation $y = bx + a$ (Fig. 4), the coefficient of determination (R²) and ANOVA with Lack-of-Fit of regression were also performed. The linearity of the method was evaluated by the calibration curve (Renger et al., 2011). Data were best fitted by a linear equation $Y = 121.287 + 3.629 * X$. The

lack of fit test was designed to define if the selected model was adequate to describe the observed data, or other more complicated model should be used. This test revealed that *p*-value for lack-of-fit in the ANOVA test was greater than 0.05 (0.4308) then the model was satisfactory for the observed data at the 95% confidence level. The *F*-value obtained was (6107.68); consequently, there was a strong relationship between concentration ($\mu\text{g}/\text{band}$) and response (area). The correlation coefficient was 0.9977.

Detection and quantification limits

LOD and LOQ were calculated by the formulas:

$$\text{LOD} = 3.3 \times \text{Sa}/b$$

$$\text{LOQ} = 10 \times \text{Sa}/b$$

Where,

Sa was the standard deviation of the intercept,
b was the slope of the calibration curve.

In this case, the LOD was 12.1 μg per band, and LOQ was 36.8 μg per band.

Precision

Precision was measured in terms of relative standard deviation (RSD) in conditions of repeatability and reproducibility (intermediate precision). RSD of repeatability (intraday) was obtained by evaluating ten independent replicates of one previously homogenized sample during the same day, on the same plate and by the same analyst. Intermediate precision (RSD) was investigated by analyzing the same sample in three different days by two different analysts each in triplicate. RSD of repeatability (intraday) was 2.14%, and intermediate precision (RSD interday) is presented in Table 1.

Table 1. Results of intermediate precision (RSD interday).

Analyst	RSD	RSD	RSD
	Day 1	Day 2	Day 3
1	0.82%	2.80%	3.73%
2	0.66%	4.30%	2.98%

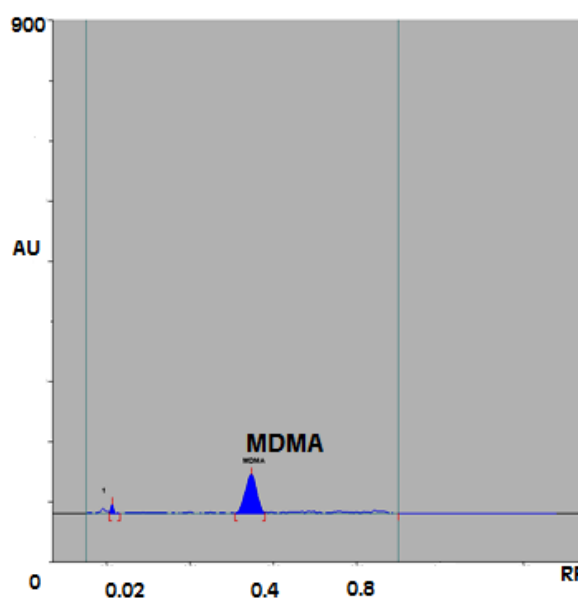
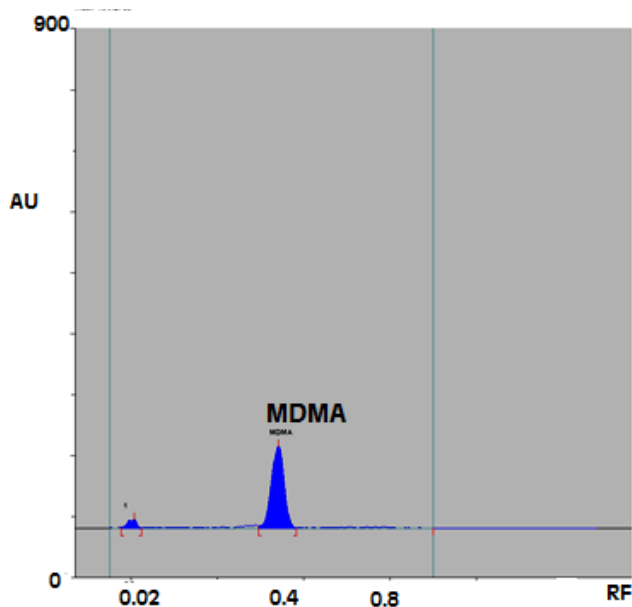


Figure 2. Densitogram of MDMA (left) and real sample (right).

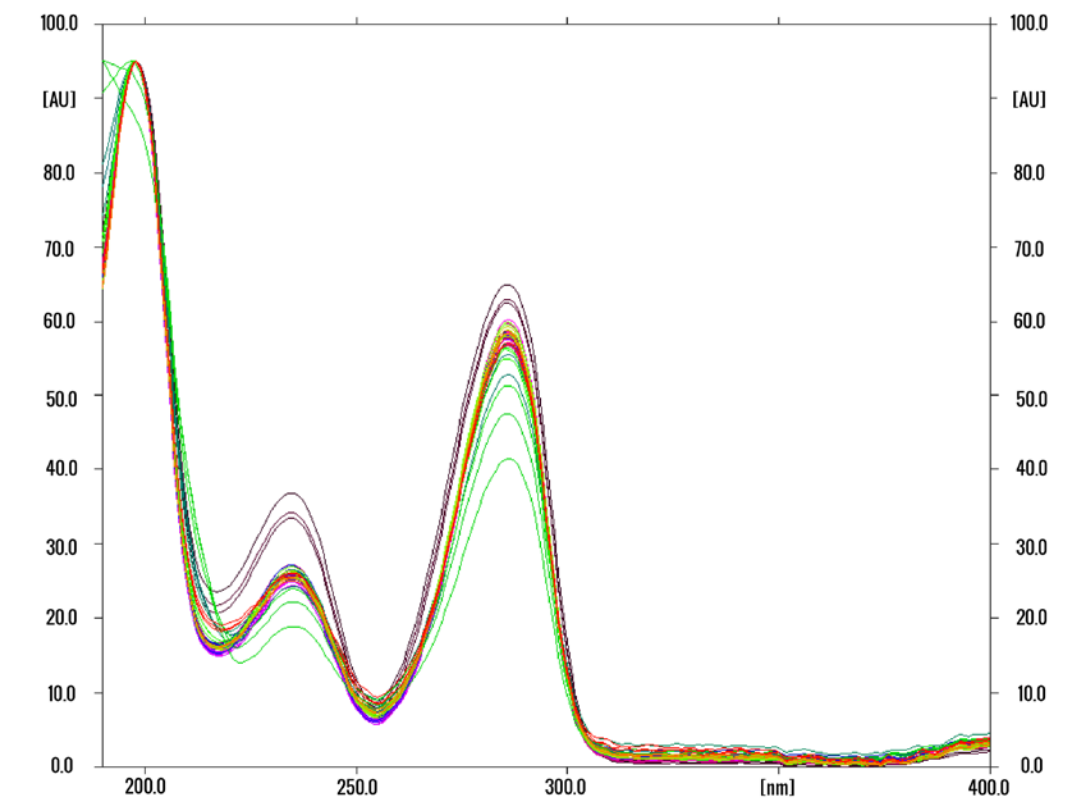


Figure 3. UV spectra of standard (in black color) and real samples (190-400 nm).

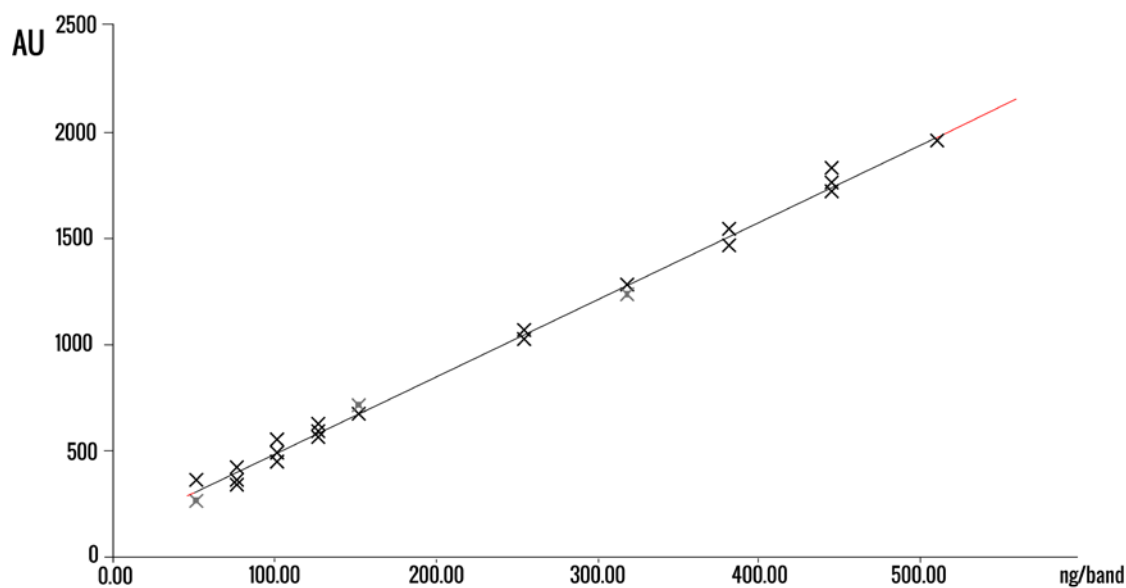


Figure 4. Calibration plot of MDMA.

Substance MDMA @ 210 nm. Regression mode: Linear. $Y = 121.287 + 3.629 * X$, $r = 0.9977$, $sdv = 3.98\%$

Accuracy

Accuracy was evaluated by determination of the percentage of MDMA recovered by the assay, for this purpose was used an external quality control sample of 36.6% w/w of MDMA supplied by proficiency test supplier (United Nations UNODC Round 2, 2012). The average of percentage recovery was 99.13% (9 independent replicates); Student *t* test revealed no statistical difference between the observed result and the reference value at 95% of confidence level and 8 degrees of freedom (*t* calculated was 0.31 and *t* tabulated was 2.31).

Robustness

Robustness is a measure of the capacity of a method to persist unaffected by small but deliberate variation of the method settings, and is an indication of the reliability of the methodology; in the case of different specificity plates from the other manufacturer (Macherey-Nagel, Germany) did not alter the separation process. Chromatographic specificity, precision, linearity, and accuracy were not influenced by the time of preparation of mobile phase while it was stored in the refrigerator at 4°C.

Uncertainty

Uncertainty, in general, comprises multiple components and is calculated from the estimation of the errors associated with various steps of the analytical process. For example, homogenization, volumetric material, injection, extraction, recoveries, calibration curves, and data obtained from method validation such as accuracy and precision under repeatability and reproducibility conditions. In this case, it was applied the results from validation of the method and uncertainty was calculated with the equation (1) (Müller and Windberg, 2005):

$$U = 2 \times \sqrt{CV \text{ Repet}^2 + CV \text{ Reprod}^2 + CV \text{ Cal}^2} \quad (1)$$

Where,

U was the estimated uncertainty as RSD;

CV Repet corresponded to uncertainty of repeatability and was equals to 2.14%,

CV Reprod was uncertainty of intermediate precision (2.55%), and

CV Cal was the uncertainty of calibration and was estimated from uncertainty of MDMA standard expressed in the certificate of analysis (0.002%),

The value of relative expanded uncertainty (*U*, *k*=2) was 6.66%

Application of the method

Twelve positive samples of ecstasy tablets were tested with the validated method; all samples were from different seizures. The tablets generally have a distinctive imprint; these designs are not limited only to MDMA tablets (may be found on amphetamine and other illicit products). Results of the samples are summarized in Table 2 and revealed that all samples had MDMA, with a broad range of concentration (18.15 to 59.84% w/w as MDMA base); the distribution of the samples (scatterplot of %MDMA) is shown in Fig. 5. A brief summary of statistical analysis is presented in Table 3; and includes measures of central tendency, measures of variability, and measures of shape. Of particular interest here were the standardized skewness and standardized kurtosis, which can be used to determine whether the sample comes from a normal distribution. Values of these statistics outside the range of -2 to +2 indicate significant departures from normality, which would tend to invalidate any statistical test regarding the standard deviation. In this case, the standardized skewness value was within the range expected for data from a normal distribution. The standardized kurtosis value was within the range expected for data from a normal distribution. To evaluate links between real samples we applied cluster analysis; this procedure created one cluster from the 12 observations supplied. The clusters are groups of observations with similar characteristics, in this case, the amount of MDMA. To form the clusters, the procedure begins with each observation in a separate group, then are combined the two observations, which were closest together to form a new group. After re-computing the distance between the groups, the two groups then closest together were combined. This process was repeated until only one group remained; the Fig. 6 illustrate a dendrogram of correlation between related samples (Euclidean Nearest Neighbor Method) per-

formed using the software STATGRAPHICS Centurion XVI.I.® from the analysis visualized that samples with “Smile” imprint contained a comparable amount of MDMA.

Table 2. Results of analysis of 12 positive samples.

Sample	%MDMA Base	Design	Color
1	45.89	No	Light blue
2	23.07	No	White
3	35.19	Omega	Beige
4	40.16	Smile	White
5	59.84	Apple	Green
6	50.43	Michelin	White
7	30.82	Hyundai	Pink
8	43.33	Kangaroo	White
9	40.51	Smile	White
10	28.47	Omega	Beige
11	19.26	No	White
12	18.15	Omega	Beige
Mean: 36.26% SD: 12.30 RSD: 33.92%			

Table 3. Summary statistics for content of MDMA in real samples.

Count	12
Average	36.26%
Standard deviation	12.85%
Coeff. of variation	35.43%
Minimum	18.15
Maximum	59.84
Range	41.69
Standard skewness	0.26
Standard kurtosis	-0.45

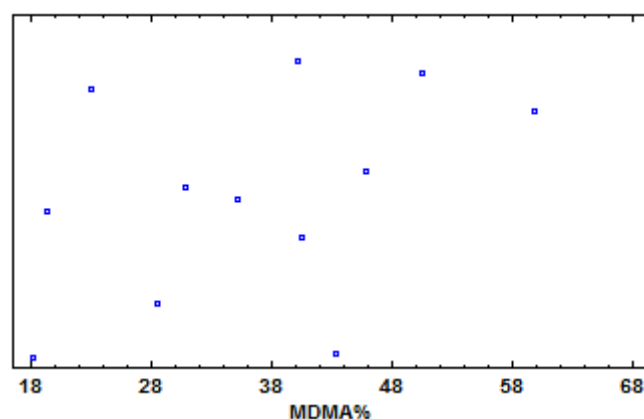


Figure 5. Scatterplot of content of MDMA in real samples.

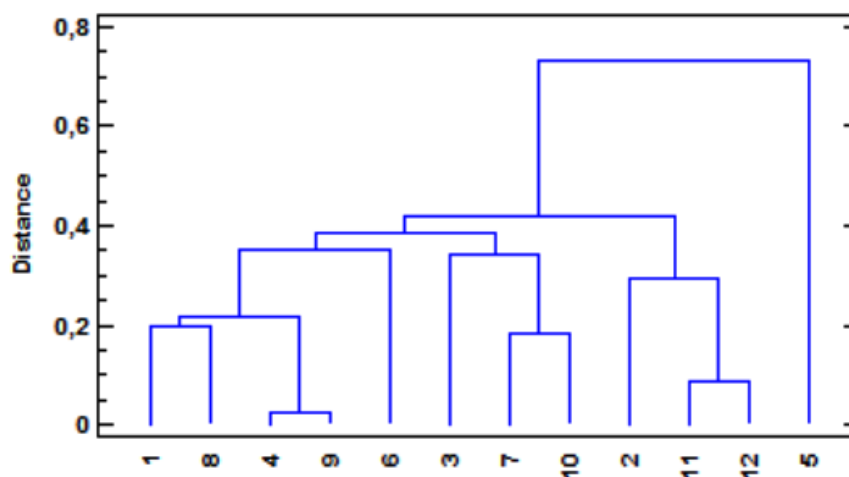


Figure 6. Dendrogram of correlation between related samples (Euclidean Nearest Neighbor Method).

DISCUSSION

The proposed method was fully validated according ICH guidelines, and is suitable for a rapid qualitative and quantitative analysis of the content of MDMA in seized tablets, offering fast and reliable results, with good specificity, linearity, accuracy, precision, detection and quantitation limits and low relative uncertainty, and allowed us to determine the content of MDMA in ecstasy tablets with very low manipulation of samples, without use of internal standard, spraying or staining reagents, due to in this method the detection mode was performed by absorbance at 210 nm showing a good performance and sensitivity. With this technique, there is no need of the "scraping off" method of the plate, in the same manner there is no need for any sample pre-treatment, so these aspects involve a small cost of development.

The method presented in this study can be easily implemented for routine analyzes of seized MDMA in forensic laboratories. The examination of real samples revealed that all seized samples analyzed in this study contained MDMA; the tablets with an imprint on its surface contain more concentration of MDMA than samples without any symbol, and two samples of the same design had a very similar concentration of MDMA. This information could be helpful to authorities to establish the source, suppliers, and route of illicit traffic of MDMA in Chile. With cluster analysis of the concentration of MDMA of each sample, we visualized a correlation between samples with the same inscription or figure. Probably due to that, these seized tablets were manufactured by the same process or by the same manufacturer, samples without any imprinting on its surface shown a wide range of concentration without any apparent correlation between the values. Because one of the most important reasons for death from abusing ecstasy pills is their MDMA content, constant monitoring of MDMA contents of illicit ecstasy pills will help authorities to pay more attention to eradicating high-dose ecstasy tablets from the illegal drug market.

This is the first study about the concentration of MDMA in tablets in Chile and offers valuable information about the content of this illegal drug

consumed in Chile; this evidence can also be useful for toxicologist, prosecutors and police enforcement.

CONCLUSIONS

The proposed method was fit for purpose, with a good accuracy and short time of development, with this method a forensic laboratory can carry out the analysis of MDMA in seized pills in short time and with low cost of development. These aspects are very important in order to determine the presence of illicit drugs in seized pills. In terms of concentration, the MDMA seized in Chile contains a wide range of concentration; this fact could be alarming from the point of view of toxicology and health of consumers of these synthetic drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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