Development of an ultrafast high throughput MALDI-triple quadrupole mass spectrometric method for the determination of 3,4-methylenedioxymethamphetamine (MDMA) in oral fluid

Michael Poetzsch[1], Andrea E. Steuer[1], Cedric M. Hysek[2], Matthias E. Liechti[2], Thomas Kraemer[1]*

[1]*Department of Forensic Pharmacology and Toxicology, ZIFM - Zurich Institute of Forensic Medicine, University of Zurich, Zurich (Switzerland)*

[2]*Departement of Biomedicine, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, University of Basel, Basel (Switzerland)*

*Corresponding author:

Prof. Dr. Thomas Kraemer

University of Zurich

ZIFM – Zurich Institute of Forensic Medicine

Department of Forensic Pharmacology & Toxicology

Winterthurerstrasse 190/52

CH-8057 Zurich

Switzerland

Tel.: 0041 446355641; Fax: 0041 446356852

E-mail address: thomas.kraemer@irm.uzh.ch (T. Kraemer)

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Abstract

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) still is a widely used illicit designer drug and its detection in different matrices is of major importance for forensic purposes (e.g. driving under the influence) as well as for work place drug testing or abstinence control. Established analytical methods for the determination of MDMA are mainly employing high performance liquid chromatography (HPLC) or gas chromatography (GC) coupled to mass spectrometric detection. MALDI-QqQ-MS/MS is so far rarely used and offers an ultrafast high throughput platform. The Quantisal™ Oral Fluid Collection Device was used for sample collection. After addition of the deuterated internal standard and a carbonate buffer (0.75 M Na_2CO_3), oral fluid samples were liquid-liquid extracted (ButOAc/EtOAc, 1:1). As little as 1 microliter of a mixture of this extract and the MALDI matrix (alpha-cyano-4-hydroxycinnamic acid) was spotted onto the MALDI plate and could directly be analyzed. With MALDI omitting chromatographic separation, very short analysis times of about 10 seconds per sample were possible. The method was developed and validated according to international guidelines including specificity, recovery, matrix effects, accuracy and precision, stabilities and limit of quantification. All validation criteria were fulfilled except for ion suppression/enhancement. Comparison with a routine LC-MS/MS method showed good agreement of the results. Applicability of the method was shown by analyzing about 250 oral fluid samples collected after controlled administration of 125 mg MDMA in a pharmacokinetic study. The whole lot of samples could be analyzed in less than one hour, proving the ultra-high speed of the method.

Introduction

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is an illicit drug of abuse that produces feelings of energy, friendliness, euphoria and empathychange refs! Include current Nr 20 and the ones newly noted in the reference section (Hysek *et al.*, 2011; Hysek *et al.*,

2014b)After decreasing numbers of MDMA seizures in recent years, most likely due to its non-availability on the illicit drug market, the Substance Abuse and Mental Health Services Administration and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) have reported on increasing MDMA consumption in the United States and Europe again since 2010 (Studies, 2010; (EMCDDA), 2013). For this reason its detection in biological matrices for the purpose of work place drug testing or forensic cases is still of main interest. Drug abstinence control can be performed with a variety of specimens. Blood and serum offer good quantitative correlation to the actual physical influence (Kolbrich *et al.*, 2008; Barnes *et al.*, 2011), whereas urine or sweat offer mainly qualitative information (Abraham *et al.*, 2009; Barnes *et al.*, 2009). In hair, drug consumption behavior from months to years can be monitored (Pragst *et al.*, 2006; Poetzsch *et al.*, 2014). Oral fluid (OF) sample collection offers a less invasive method, which is already widely distributed for abstinence control or driving under the influence of drugs (DUID) testing (Wille *et al.*, 2014). Furthermore, reference pharmacokinetic data for MDMA in OF are already available for method comparison (Barnes *et al.*, 2011). Analytical methods used for the determination of MDMA in different matrices are mainly employing high performance liquid chromatography (HPLC) or gas chromatography (GC) coupled to mass spectrometric detection (Scheidweiler *et al.*, 2006; Schwaninger *et al.*, 2011). Matrix assisted laser desorption/ionization triple quadrupole tandem mass spectrometry (MALDI-QqQ-MS/MS) is so far not very common and offers a novel high throughput platform (Meesters *et al.*, 2011b; Meesters *et al.*, 2011a). Biological samples are mixed with the MALDI matrix solution and spotted (0.25 – 1.5 µl) onto a MALDI stainless steel target plate. The matrix solution contains a small molecule that absorbs the energy of the laser and thus leads to soft ionization of the analyte. The analyte is afterwards analyzed in the multi reaction monitoring (MRM) mode, which is very selective and reduces the background noise caused by the matrix molecules. MALDI-QqQ-MS/MS offers very short sample analysis times, with about 10 seconds per sample in our procedure

and is therefore perfectly suitable for high throughput quantification as needed for forensic drug testing or in pharmacokinetic studies generating high numbers of samples.

The aim of the present study was therefore to develop a high throughput MALDI-QqQ-MS/MS method for analysis of MDMA and its main metabolite 3,4 methylenedioxyamphetamine (MDA) in oral fluid. The resulting method should be validated according to international guidelines. Furthermore, its applicability should be proven by analyzing OF samples from a double-blind, placebo-controlled, crossover study with ingestion of 125 mg of MDMA (Hysek *et al.*, 2014a).

Experimental

Chemicals and reagents

3,4-Methylenedioxymethamphetamine (MDMA); 3,4-methylenedioxyamphetamine (MDA); MDMA-D5; MDA-D5 and methylphenidate (MPh) were obtained from Lipomed (Arlesheim, Switzerland). Water was purified with a Purelab Ultra (Labtec, Villmergen, Switzerland) filtration unit. *Alpha*-cyano-4-hydroxycinnamic acid (CHCA), acetonitrile (ACN), methanol (MeOH), sodium carbonate (Na_2CO_3), ethyl acetate (EtOAc), butyl acetate (ButOAc) and trifluoroacetic acid (TFA) were purchased from Sigma Aldrich (Buchs, Switzerland). All other chemicals used were purchased from Merck (Zug, Switzerland) and of the highest grade available.

Sample preparation

MALDI-MS Oral Fluid Samples

To 80 µl *Quanti*sal™ (Alere toxicology, Abington, UK) buffer solution 10 µl deuterated internal standard (IS) solution was added and vortexed for 30 seconds. 100 µl 0.75 M Na_2CO_3 buffer pH 10 was added and vortexed for 30 sec. Afterwards 1 ml ButOAc/EtOAc (1:1) was

added and shaken for 10 min at 1400 rpm and then centrifuged at 10˙000 rpm for 10 min. Finally, the supernatant was evaporated under a gentle nitrogen stream after adding 50 µl 2% TFA at room temperature. The residue was reconstituted in 50 µl CHCA solution (10 mg/ml in 0.1 % TFA/ACN 1:1). One µl each was spotted into three wells of the MALDI target plate (OPTI TOF 384 well insert 123 x 81 mm, AB Sciex, Darmstadt, Germany) and was dried at room temperature before measurement.

Apparatus

MALDI-MS experiments were performed on a Flashquant® Workstation (AB Sciex, Darmstadt, Germany) fitted with a high repetition laser (Nd: YAG, λ = 355 nm, elliptic shape 100 x 200 µm). Measurements were acquired in MS/MS mode using positive ionization. Source operation conditions were: continuous mode (1 mm/s), laser power 40 %, laser frequency 1000 Hz. MS conditions were: unit resolution, vacuum gauge q2: 4.8 x 10-5 Torr (nitrogen as collision gas). Transitions and optimized MS parameters are shown in Table 1. Data acquisition and processing was performed with Analyst 1.4.2 software and Flashquant® software (AB Sciex, Darmstadt, Germany).

Comparison to micro flow liquid chromatography (MFLC) was performed using an AB Sciex Eksigent micro flow LC system (Redwood City, California, USA) coupled to an AB Sciex 4500 QTtrap linear ion trap (LIT) quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany). The MFLC settings were as follows: Halo Phenyl Hexyl column (eksigent; AB Sciex, Brugg, Switzerland) 50 x 0.5 mm, 2.7 µm, gradient elution with 10 mM ammonium formate buffer in water pH 3.5 (A) and acetonitrile containing 0.1 % (v/v) formic acid (B). The flow rate was 50 µL/min with the following gradient: 95% A for 0.1 min, 0.1-0.7 min decrease to 30 % A, 0.7-0.9 min hold at 30 % A, 0.9-1 min return to initial conditions. Reequilibrating is performed for 1 min before the next injection. Injection volume

was 5 µL. The Turbo V ion source, equipped with a hybrid electrode (50 µm internal diameter), was operated in positive ESI mode with the following MS conditions: gas 1: nitrogen (50 psi); gas 2: nitrogen (60 psi); ion spray voltage: 5.5 kV; ion-source temperature: 250 °C; curtain gas: nitrogen (30 psi); collision gas, medium. The MS settings for each analyte are given in Table 1. The MS was controlled by analyst 1.6.2 software.

Method Validation for MALDI-MS

Preparation of calibration and quality control (QC) samples

Separate stock solutions of MDMA, MDA (10 µg/ml) and MDMA-D5, MDA-D5 (1 µg/ml) were prepared in MeOH. Spiking solutions were prepared in MeOH by mixing appropriate amounts of the corresponding stock solution. All solutions were stored in aliquots at -20 °C. Calibration standards and QC samples (Low, High, above calibration range (ACR)) were prepared from 80 µl analyte-free *Quanti*sal™ buffer solution. The final MDMA and MDA calibrator concentrations were: 5; 50; 100; 150; 200; 300; 500; 1000; 2000 ng/ml and QC samples: 10 (Low); 1800 (High); 10000 (ACR) ng/ml, respectively. ACR QC samples were diluted 1:10 with analyte free *Quanti*sal™ buffer solution. IS concentrations were 50 ng/ml MDMA and MDA, respectively.

Selectivity and cross talk

80 µl of each analyte solution and each IS were analyzed for interferences in the other MRM transitions.

Specificity

Ten blank *Quanti*sal™ buffer solutions containing oral fluid from different donors were analyzed for peaks interfering with the detection of analytes or ISTD. Two zero samples (blank samples + IS) were analyzed to check for appropriate IS purity and presence of native analytes.

Matrix effects and recovery

Matrix effects and (ME) recovery (RE) were determined at QC Low and High concentration using 6 oral fluids from different donors according to the simplified approach described by Matuszewski et al (Matuszewski *et al.*, 2003). For investigating ion-suppression / enhancement, ten *Quanti*sal^{TM} buffer solutions were spiked with MPh and MDMA in combinations of different concentrations (table 1) and absolute areas under the curve were compared.

Calibration model

Replicates (n=6) at each concentration level were analyzed as described above. The regression lines were calculated using non-weighted, weighted 1/x and weighted $1/x^2$ regression models. The final choice of model was made after calculating validation data using these alternatives. Daily calibration curves were prepared with each batch of validation samples.

Accuracy and precision

QC samples (Low, High, ACR) were analyzed according to the procedures described above in duplicate on each of eight days. Accuracy was calculated in terms of bias as the percent deviation of the mean calculated concentration at each concentration level from the corresponding theoretical concentration. Intra-day and inter-day precision were calculated as relative standard deviation (RSD) according to ref. (Peters *et al.*, 2007)

Stability

Process sample stability were investigated at QC Low and High concentration (n=6) according to ref. (Peters *et al.*, 2007). For in source stability QC Low and High samples were stored in the MALDI source for 24h under vacuum condition and relative intensities compared.

Limits

The lowest point of the calibration curve was defined as the limit of quantitation (LOQ) of the method and fulfilled the requirement of LOQ with a signal to noise ratio of 10:1 determined by comparing background signal height after blank sample extraction and extraction of the

lowest calibrator. LOD was determined by dilution of the lowest calibrator until a signal to noise ratio of 3:1 was reached.

Comparison of MALDI-MS/MS and MFLC-MS/MS

For performance comparison of MALDI-MS/MS and MFLC-MS/MS, 15 authentic OF samples were quantified with both systems. For that purpose calibrators and authentic samples were prepared as described above. For LC-MS/MS quantitation, the residue was reconstituted in eluent A/B (95/5) instead of MALDI matrix solution. Obtained results were compared applying a Wilcoxon test with a 0.05 significance level.

Proof of applicability

The presented method was applied to about 250 samples of a pharmacokinetic study after controlled administration of 125 mg MDMA. 16 participants received MDMA or placebo in combination with methylphenidate and/or placebo (Hysek *et al.*, 2014a). Concentrations of MDMA in oral fluid were assessed.

Results and Discussion

MALDI-MS method development

MALDI-QqQ-MS/MS is a relatively new technique which achieves its high throughput ability mainly by direct ionization of the analyte without chromatographic separation. Due to the lack of chromatographic separation, retention time as one criterion for identification is missing as well as separation from other substances prior to the ionization process. Separation from other substances can only be achieved by changes in sample preparation. Therefore, the mass spectrometry specificities, inter-substance influences as well as ion suppression/enhancement effects are discussed in detail in the following.

Cross talk is a phenomenon which might occur in case of MS/MS acquisition. Precursor ions are fragmented in the collision cell most likely through collision induced dissociation (CID) followed by extraction of the ions out of the collision cell. In case of insufficient extraction,

fragments might still be present when the next ion is fragmented. Separation of isomers by tandem mass spectrometric detection without chromatographic separation is therefore difficult due to almost identical fragmentation. In our method, no isomers were included but MDMA and MDA both dissociate into the same ion fragment of *m/z* 105 (table 1) and therefore cross talk might occur in case of both substances being present in the same sample. However, cross talk could not be detected with the chosen 5 ms pause between MRM transitions.

MALDI process is still not completely understood so far. It is known, that the analyte of interest has to be co-crystallized with the appropriate MALDI matrix. Compounds like salts or proteins which may disturb the co-crystallization reduce the ionization yield dramatically. This phenomenon was seen when trying to mix the analyte containing *Quanti*sal^{TM} buffer solution directly with the MALDI matrix. The *Quanti*sal^{TM} oral fluid collection system contains an unknown buffer solution. A loss of intensity of up to 65 % compared to the finally performed LLE was measured. Salts from buffer or the sample itself lead to loss of signal intensity. This was also seen when LLE extraction was performed after pH adjustment using phosphate buffer or sodium hydroxide. Best results were finally achieved with the use of 0.75 M sodium carbonate buffer. Thus, LLE proved to be the best compromise between fast and cheap sample preparation and necessary sample clean-up for an optimized MALDI process.

The ionization process might also be influenced by other drugs of abuse or pharmaceuticals eventually present in the sample. This was investigated by analyzing spiked samples containing MPh and MDMA at different concentration ratios. MPh was chosen for that experiment because it was co-administered in the study from which the oral fluid samples for applicability testing were received (Hysek *et al.*, 2014a). MPh suppressed the MDMA signal intensity (Figure 1) but ion-suppression was still within guideline regulations. Surprisingly, decrease of signal intensity was independent from MPh concentration in a range expected

after MPh treatment. These aspects should be further evaluated for multi-analyte methods or when more drugs in oral fluid have to be expected.

Oral fluid MALDI-MS method validation

Specificity, Selectivity and Cross Talk

Blank oral fluid samples from ten different donors were analyzed for mass spectrometric interferences. No interfering transitions were detected caused by the internal standard or methylphenidate.

Matrix effects and Recovery

Recovery was efficient with 95.3 ± 12.3 % for MDMA and fulfilled guideline criteria. Matrix effects were 74.5 ± 6.4 % for QC low and 70.1 ± 3.0 % for QC high. Ion-suppression was greater than guideline limits (75-125 %) but reproducible with small standard deviations.

Calibration model

For quantitation the area ratio of analyte to internal deuterated standard was employed. Figure 2 illustrates the obtained MRM traces of three spots of the same sample. One transition was used as quantifier and second as qualifier. Six replicates of calibration curves were used to evaluate the calibration model. The calibration range for MDMA was 5 – 2000 ng/ml. Samples with higher concentrations were diluted with *Quanti*sal^{TM} buffer solution. An above calibration range (ACR) quality control was treated the same way and secured the procedure, already during validation. A 1/x weighted linear calibration model was used for unequal variances and showed best accuracy and precision data. Calibrator concentrations were within 30 % of target based on the full calibration curve.

Accuracy and precision

QC samples (Low; High; ACR) were analyzed in duplicates on each of eight days as proposed by Peters et al. (Peters *et al.*, 2007) and their concentrations determined from daily calibration curve. Accuracy, intra-day and inter-day precision were calculated as described above. All

validation parameters fulfilled the necessary criteria (Table 2). Nevertheless, some important experimental aspects must be considered. The laser can be employed in discrete shot or straight line mode. Applying discrete shot mode, the laser fires on the sample spot for a few milliseconds at one position and then moves to the next spot. This further increases sample throughput but decreases accuracy and precision. In straight line mode the laser moves continuously through the spot diameter at fixed laser speed. A laser speed of 1 mm/s was the best compromise between analysis time and optimal accuracy and precision in our experiment. Furthermore, so called hot spots might occur during sample drying, which means that ionization is increased or decreased by optimal or insufficient crystallization at certain spots in the spotted sample. These phenomena can be compensated for by use of an internal standard. It was clearly seen, that differences in crystallization, spot size and absence of chromatographic separation required compensation by internal standard use. Accuracy and precision were further improved by spotting three replicates of one sample on the MALDI plate and averaging intensities.

Stability

The used MALDI source is under the same vacuum as the Q0 region of the instrument. MDMA is known for fast evaporation in the deprotonated state. Evaporation of MDMA might therefore also occur when the spotted samples are kept in the vacuum source. No degradation was determined for samples spotted onto the MALDI target plate and stored in the MALDI source for 12 h. Normally, the acquisition of a 384 well plate takes approximately 28 min. (laser speed: 1 mm/sec). Also, no degradation of MDMA and MDA was observed after two freeze/thaw cycles in the Quantisal^{TM} collection system device. Long-term stability data on MDMA stability in the Quantisal^{TM} OF collection system have already been published (Walsh *et al.*, 2007)

Limits of quantification and detection

The LOQ for MDMA was consistent with the lowest calibrator with less than 30% bias compared to the target concentration. The necessary MALDI matrix always caused a little background noise, which had to be evaluated during method development. It is recommended to check all possible transitions for their signal to noise ratio. The choice of MALDI matrix can also influence background noise. For evaluation of LOD and LOQ the area ratio of a blank sample and a spiked sample was evaluated and area ratios were found to be greater than three for LOD and ten for LOQ, respectively.

MDA showed a significant in-source fragmentation which deteriorated the limit of detection dramatically. In-source fragmentation can occur due to multiple parameters. Plate voltage applied to the MALDI plate during acquisition accelerates ions towards the QO region and can cause source fragmentation in case of maladjustment. Ion source pressure is responsible for the cooling of the MALDI plume during ionization and can also influence fragmentation. Unfortunately, it was not possible to reduce the in-source fragmentation of MDA in a sufficient way to quantify this major metabolite of MDMA. Fragment monitoring of MDA was also not possible because MDMA in-source fragmented in the same manner but with less intensity. One possibility to avoid in-source fragmentation would be to derivatize MDA. However, this would be too time consuming, especially for a high throughput method.

Comparison of MALDI-MS/MS and LC-MS/MS

Quantitative results of 15 authentic OF samples measured with LC-MS/MS and MALDI-MS/MS were comparable. Statistical Wilcoxon matched pair test showed a P value of 0.89 and thus no statistically significant differences. This comparison substantiates the applicability of MALDI-QqQ-MS/MS as a high throughput platform comparable to LC-MS/MS methods.

Study results oral fluid

The validated method was successfully applied to the analysis of more than 250 authentic samples of a pharmacokinetic study. MDMA was detectable 3 and 24 hours after

administration. Concentrations of MDMA are shown in Figure 3. It could clearly be demonstrated that MALDI-QqQ-MS/MS use for routine quantification is a clear advantage in case of high sample numbers. The whole lot of 250 samples could be analyzed in approximately one hour including calibrators and QCs. Using the LC-MS/MS method which had been employed for the comparison above, the analysis time would have been around 6 hours. Furthermore, sample amount used for measurements could be further downscaled and costs for eluents required for LC-MS analysis could be omitted. MDA was detected in samples collected 3 h after administration at a time corresponding to the maximal plasma exposure of MDMA (Hysek *et al.*, 2014a) and when MDA levels close to maximum are already reached (Hysek *et al.*, 2011). Samples collected at the time of intake (t=0h) and 24 h after administration were negative for MDA. MDA could not be quantified.

Conclusion

A MALDI-QqQ-MS/MS high throughput method was developed with an analysis time of 10 seconds per sample for the validated quantification of MDMA in oral fluid samples. The method fulfilled the required validation criteria except for matrix effects. Ion suppression / enhancement phenomena were investigated in detail and showed small standard deviations despite the lack of chromatographic separation. The method was successfully applied to 250 oral fluid samples of a pharmacokinetic study.

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(EMCDDA) EMCfDaDA (2013). *European Drug Report 2013 - Trends and Developments*. Lisbon: European Monitoring Centre for Drugs and Drug Addiction (EMCDDA)

Abraham TT, Barnes AJ, Lowe RH, Kolbrich Spargo EA, Milman G, Pirnay SO*, et al.* (2009). Urinary MDMA, MDA, HMMA, and HMA excretion following controlled MDMA administration to humans. *Journal of analytical toxicology* 33**:** 439-446.

Barnes AJ, De Martinis BS, Gorelick DA, Goodwin RS, Kolbrich EA, Huestis MA (2009). Disposition of MDMA and metabolites in human sweat following controlled MDMA administration. *Clinical chemistry* 55**:** 454-462.

Barnes AJ, Scheidweiler KB, Kolbrich-Spargo EA, Gorelick DA, Goodwin RS, Huestis MA (2011). MDMA and metabolite disposition in expectorated oral fluid after controlled oral MDMA administration. *Therapeutic drug monitoring* 33**:** 602-608.

Hysek CM, Simmler LD, Ineichen M, Grouzmann E, Hoener MC, Brenneisen R*, et al.* (2011). The norepinephrine transporter inhibitor reboxetine reduces stimulant effects of MDMA ("ecstasy") in humans. *Clin Pharmacol Ther* 90**:** 246-255.

Hysek CM, Simmler LD, Schillinger N, Meyer N, Schmid Y, Donzelli M*, et al.* (2014a). Pharmacokinetic and pharmacodynamic effects of methylphenidate and MDMA administered alone or in combination. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum* 17**:** 371-381.

Hysek CM, Schmid Y, Simmler LD, Domes G, Heinrichs M, Eisenegger C*, et al.* (2014b). MDMA enhances emotional empathy and prosocial behavior. *Social cognitive and affective neuroscience* 9**:** 1645-1652.

Kolbrich EA, Goodwin RS, Gorelick DA, Hayes RJ, Stein EA, Huestis MA (2008). Plasma pharmacokinetics of 3,4-methylenedioxymethamphetamine after controlled oral administration to young adults. *Ther Drug Monit* 30**:** 320-332.

Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal Chem* 75**:** 3019-3030.

Meesters RJ, van Kampen JJ, Scheuer RD, van der Ende ME, Gruters RA, Luider TM (2011a). Determination of the antiretroviral drug tenofovir in plasma from HIV-infected adults by ultrafast isotope dilution MALDI-triple quadrupole tandem mass spectrometry. *Journal of mass spectrometry : JMS* 46**:** 282-289.

Meesters RJ, den Boer E, Mathot RA, de Jonge R, van Klaveren RJ, Lindemans J*, et al.* (2011b). Ultrafast selective quantification of methotrexate in human plasma by high-throughput MALDI-isotope dilution mass spectrometry. *Bioanalysis* 3**:** 1369-1378.

Peters FT, Drummer OH, Musshoff F (2007). Validation of new methods. *Forensic Sci Int* 165**:** 216-224.

Poetzsch M, Steuer AE, Roemmelt AT, Baumgartner MR, Kraemer T (2014). Single Hair Analysis of Small Molecules Using MALDI-Triple Quadrupole MS Imaging and LC-MS/MS: Investigations on Opportunities and Pitfalls. *Analytical chemistry* 86**:** 11758-11765.

Pragst F, Balikova MA (2006). State of the art in hair analysis for detection of drug and alcohol abuse. *Clinica chimica acta; international journal of clinical chemistry* 370**:** 17-49.

Scheidweiler KB, Huestis MA (2006). A validated gas chromatographic-electron impact ionization mass spectrometric method for methylenedioxymethamphetamine (MDMA), methamphetamine and metabolites in oral fluid. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* 835**:** 90-99.

Schwaninger AE, Meyer MR, Huestis MA, Maurer HH (2011). Development and validation of LC-HRMS and GC-NICI-MS methods for stereoselective determination of MDMA and its phase I and II metabolites in human urine. *J Mass Spectrom* 46**:** 603-614.

Studies USDoHaHS-SAaMHSAOoA (2010). Results from the 2009, National Survey on Drug Use and Health: Volume I. Summary of National Findings.

Walsh JM, Crouch DJ, Danaceau JP, Cangianelli L, Liddicoat L, Adkins R (2007). Evaluation of ten oral fluid point-of-collection drug-testing devices. *Journal of analytical toxicology* 31**:** 44-54.

Wille SM, Baumgartner MR, Fazio VD, Samyn N, Kraemer T (2014). Trends in drug testing in oral fluid and hair as alternative matrices. *Bioanalysis* 6**:** 2193-2209.

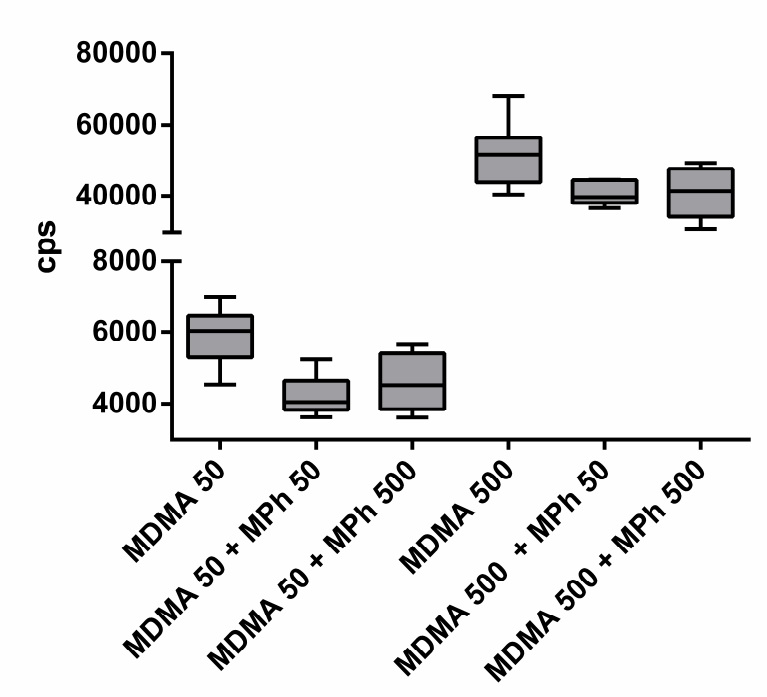


Figure 1: Influence of methylphenidate (MPh) on the ionization of MDMA; concentrations in ng/ml represented in the x-axis; cps: counts per second.

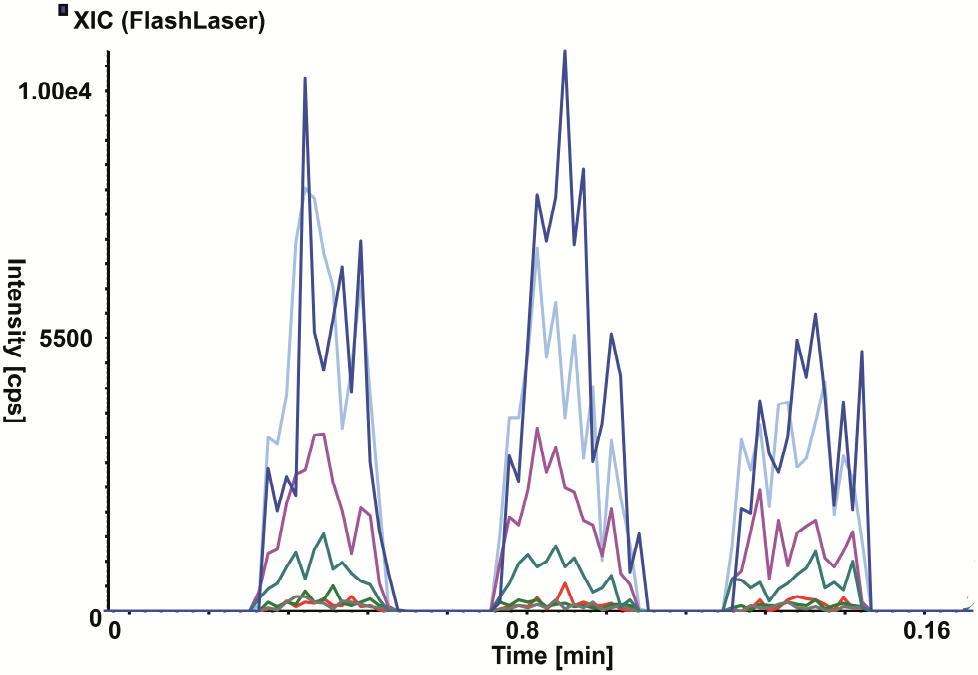
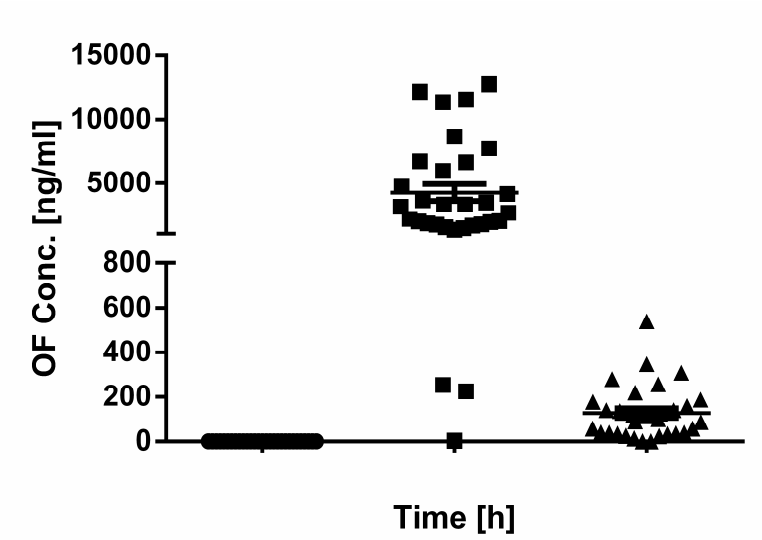


Figure 2: MALDI-QqQ-MS/MS transition trace of three spots of the same sample extract.



F**igure 3:** MDMA oral fluid concentrations after a controlled administration of 125 mg MDMA to healthy subjects. The data are represented by squares for individual concentrations and mean±SEM. Need to shown the time points in the graph!