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Analytical Profiling of Airplane Wastewater - a New Matrix for Mapping Worldwide Patterns of Drug Use and Abuse

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Abstract:
There is limited knowledge on the global prescription and consumption patterns of therapeutic (TD) and illicit drugs (ID). Pooled urine analysis and wastewater-based epidemiology (WBE) has been used for local-based drug screening. It is, however, difficult to study the global epidemiology due to difficulties in obtaining samples. The aims of the study were to test the detectability of TD and ID in airplane wastewater samples categorized according to their geographical origin.
Wastewater samples (n= 17) were collected from long-distance flights and prepared with enzymatic conjugate cleaving followed by either precipitation or solid phase extraction. Aliquots were analysed on various liquid chromatography – mass spectrometers. TDs were grouped according to their Anatomical Therapeutic Chemical (ATC) codes.
Identification confidence was assigned to three levels based on variables including detection on multiple instruments and number of targets per compound. A total of 424 compounds were identified across all samples, distributed on 87 unique TD and 2 ID. Two principal components in a principal component analysis separated three clusters of wastewater samples corresponding to geographical origin of the airplanes with therapeutic subgroup ATC codes as variables. Airplane wastewater analysis is useful for identifying targets for WBE and toxicological analysis and explore drug use and abuse patterns.

Keywords:
wastewater profiling, drug screening, LC-MS, wastewater-based epidemiology, principal component analysis

INTRODUCTION

An increasing number of methods and matrices are being employed to monitor drug use and abuse [1]. Conventional methods include analysis of seized materials and biological samples such as urine, saliva, hair, and blood. Newer methods include wastewater-based epidemiology (WBE) [2], screening of pooled urine [3-5], urinated soil [4], and exhaled breath samples [6]. Low concentrations of analytical targets in pooled and diluted samples call for sophisticated sample work-up and/or highly sensitive and selective analytical equipment such as tandem mass spectrometry (MS/MS) or high resolution MS in target screening mode [2;5;7]. Identification confidence is then assigned based on the acquired analytical data, which was previously discussed for high resolution MS data in environmental or clinical samples [7;8]. Before being able to identify which drugs have been consumed in pooled biological samples, the analytical targets need to be identified preferably through comprehensive pharmacokinetic studies from controlled clinical trials, but as ethic committees rarely allow such studies on emerging illicit drugs (ID), alternatively by metabolism studies and/or *in silico* prediction [9]. Elimination through feces is a quantitatively relevant route of elimination for certain IDs and drugs with abuse potential, including buprenorphine [10], methadone [11], and Δ9-tetrahydrocannabinol (THC) [12]; this might also apply to e.g. synthetic cannabinoids. Pooled urine and urinated soil analysis can provide a snap-shot of which therapeutic drugs (TD) and ID are consumed in a smaller population and can itself be used in analytical target identification, whereas WBE can provide some spatial and temporal resolution to monitoring drug consumption with sensitive, targeted MS methods [2;13]. An approach for obtaining concentrated wastewater samples to screen for drug consumption with spatial resolution is by sampling airplane wastewater samples. Such samples could be used for identifying analytical targets for TD and ID use excreted in urine and feces, covering a more representative range of analytes in respect to WBE. Also, the findings can be used for investigating correlation between drug consumption amongst flight passengers from various regions of the globe.

The aims of the study were to analyse wastewater samples from flights arriving at Copenhagen airport, assign identification confidence of TDs and IDs, group identified compounds according the anatomic therapeutic chemical (ATC) classification system and investigate correlation between identified compounds and origin of the flights by principal component analysis (PCA).

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

Reference and internal standards were purchased from Lipomed (Bad Säckingen, Germany), Toronto research chemicals (Toronto, Canada), Cerilliant (Round Rock, Texas, USA), and pharmaceutical companies. Acetonitrile, methanol, and purified water (LC-MS grade) were from Fisher Scientific UK (Leicestershire, UK). Deconjugation enzyme (100 000 U/mL) of glucuronidase (EC No. 3.2.1.31) and arylsulphatase (EC No. 3.1.6.1), analytical grade formic acid (98 %), aqueous ammonia (25 %), ammonium acetate, and ammonium formate were from Merck (Darmstadt, Germany). Purified

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water was generated from a Millipore Synergy UV water purification system (Millipore A/S, Copenhagen, Denmark). SPE columns were Strata X-C Bond Elut 96 Square-well, Certify 100 mg (Agilent Technologies, Santa Clara, CA, USA).

Wastewater samples (n=17) were collected as previously described [14] from long-distance flights arriving at Copenhagen airport from nine cities, representing three regions (North America, North and South Asia). A deodorising agent based on glutaraldehyde and benzalkoniumchloride was used as disinfection agent in the flight wastewater containers. Samples were stored at – 20 °C until analysis.

Sample Preparation

Wastewater samples containing about 5 mL were thawed and centrifuged at 10,000 *g* for 10 min at 20 °C. 500 µL wastewater supernatant was mixed with 250 µL ammonium acetate buffer (pH 5.5; 1 M). Enzymatic conjugate cleaving was performed with the addition of 25 µL freshly prepared glucuronidase/arylsulphatase enzyme - millipore water (1:3, *v*/*v*) and thorough shaking. The mixture was incubated overnight at 40 °C. Further sample preparation was performed according to a fully-automated setup previously validated for whole blood samples on a Freedom Evo 200 platform (Tecan, Männedorf, Switzerland), with minor modifications, including precipitation and solid phase extraction (SPE), as previously described [15-17].

Instrumentation

All mass spectrometers were coupled to Acquity ultra high performance liquid chromatography (UHPLC) systems (Waters corporation, Milford, USA) for chromatographic separation. The screening was based on *in-house* developed methods and previously published methods for analysis of a wide range of TDs and IDs. UHPLC- time-of-flight (TOF) was used in data-independent acquisition mode (MS^E) at low collision energy at 4 eV (MS^L) and high collision energy function from 15 to 40 eV (MS^H) [16]. Samples were also analysed on various UHPLC-MS/MS methods in multiple reaction monitoring mode with negative and/or positive ionisation [15;17;18]. Processing of MS^E data was achieved using UNIFI v. 1.8.1 (Waters corporation, Milford, USA) with a 1,471 compound library for target screening, and MassLynx v.4.1 (Waters corporation, Milford, USA) for MS/MS data [16].

Data Analysis

Assignment of identification confidence based on analysed MS data was achieved using the classification system presented in table 1. Confirmed compounds were identified above or equal to the limit of detection (LOD) on the respective MS/MS method and by targeted screening in MS^E with two fragment ions (FI) in MS^H, a retention time error within 0.3 min from the library value, and a mass error within 3 mDa in MS^L. Tentatively identified compounds were equal to or below the LOD on the respective MS/MS method, and only one target was identified and one or no FI were observed in MS^H. Probable identifications of compounds were assigned when the analytical parameters ranged between the tentative and confirmed identification criteria (table 1). Identified compounds from the wastewater samples were grouped according to their ATC codes [19]. When more than one code was available per compound, the formulation with highest number of sold defined daily doses in Denmark in the latest quarter was chosen using the Danish register of Medicinal Product Statistics [20]. ATC codes for oral administration were chosen over topical administration when relevant drug metabolites, were identified, using excretion data on the drug from Baselt et al. [21].

Principal component analysis

A two-dimensional data matrix with wastewater samples against number of compounds with all identification confidences in each therapeutic main group as discrete variables was imported to Unscrambler X (Camo, Norway) for PCA analysis. Using the therapeutic main groups as variable for the PCA should reduce clustering based on different prescription patterns of compounds for the same indication. Variables were centered and scaled by dividing each sample result with the standard deviation of the variable prior to analysis.

RESULTS AND DISCUSSION

The prepared airplane wastewater samples were injected onto the UHPLC-MS/MS and UHPLC-TOF systems. Data from the UHPLC-MS/MS was extracted and categorised as ≥LOD or ≥ limit of quantification (LOQ). The following UHPLC-TOF variables were extracted: mass error in MS^L for protonated molecular mass, number of FIs per target, and number of targets per

Table 1: Identification confidence levels based on UHPLC-TOF and UHPLC-MS/MS data. LOD: limit of detection, LOQ: limit of quantification, MS: mass error of protonated molecular ion in low collision energy ≤ 3 mDa, t_R: retention time error ≤ 0.3 min from library value, FI: mass error of characteristic fragment ions in high collision energy function ≤ 3 mDa, Target: number of analytical targets per compound

	TOF screening			
	MS, t_R, ≥2 FI	MS, t_R OR ≥1 FI		<LOD or not analysed
MS/MS		≥ 2 targets	1 target	
≥ LOQ	Confirmed	Probable	Probable	Probable
≥ LOD	Confirmed	Probable	Tentative	-
<LOD or not analysed	**Probable**	**Probable**	**Tentative**	**-**

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compound. Retention time (t_R) error from the library value were additionally extracted for compounds in the target screening database.

Identification confidence levels

A system for assigning identification confidence was developed based on previous systems [8], for this given application, to weigh information provided by the complementary analytical approaches, and to ensure pharmacokinetic information of the TDs and IDs were taken into consideration (Table 1). When a metabolite of a drug is also a drug with unique ATC code, the compound which is not metabolically transformed to the other will be categorised as having two targets.

Confirmed compounds are identified with the highest identification confidence according to the instrumentation and method set-up used in present study. 89 unique compounds were identified. Across the wastewater samples, the number of identified compounds were 84 (confirmed), 146 (probable), and 194 (tentative); combined 424 compounds in 17 samples. The total number of identified unique compounds per sample was 25.4 ± 8.6 (standard deviation).

Compound identification

The ATC classification system was used to group drugs and metabolites. Compounds most likely originating from food, soft drinks and tobacco were excluded from the study.

An overview of confirmed to tentatively identified compounds is presented in relative numbers in the doughnut diagram in Fig. 1a and the same data in absolute numbers are presented in the histogram. The main detected groups of compounds belonged to the anatomical main groups for drugs acting on the neurological (N), respiratory (R) and cardiovascular (C) systems. The therapeutic main group level of the anatomical main groups N, R, and C are presented in Fig. 1b as doughnut diagrams and corresponding histograms in absolute numbers. The most common therapeutic main group of N were N02: the analgesics, (paracetamol/acetaminophen etc.) and N06: psychoanaleptics (citalopram, venlafaxine etc.). The most common therapeutic main group of R was R06: antihistamines for systemic use. The majority of the detected antihistamines were 1st generation, possibly consumed for their off-label sedating effects. The most common therapeutic main group of C were C07: the beta blocking agents (metoprolol etc.) and C09: agents acting on the renin-angiotensin system (valsartan etc.). The five most frequently detected compounds across the 17 wastewater samples are presented in table 2. Frequent detection needs not correlate with frequent consumption, as the LOD, degree of metabolism, and route of excretion varies across the detected compounds.

Table 2: Top-five detected compounds in the airplane wastewater samples.

Compound	ATC therapeutic main group	Number of detections
Paracetamol	N02	17
Pseudoephedrine	R01	16
Diphenhydramine	R06	16
Metformin	A10	15
Cetirizine	**R06**	**15**

N02: Analgesics, R01: Nasal preparations, R06: Antihistamines for systemic use, A10: Drugs used in diabetes

Tentatively identified compounds covered a wider range of therapeutic drugs compared to confirmed compounds. The in-house UHPLC-MS/MS methods are biased towards covering compounds of relevance in forensic toxicology. Cocaine and methamphetamine were both tentatively identified, each in two samples. Amphetamine was identified in two samples, and was grouped as being the metabolite of therapeutic ADHD medication; however, it could also originate from abused amphetamine. Several TDs mainly excreted in feces were identified, including dipyramidol, fexofenadine and irbesartan [21].

In a globalised world with increasing work-force mobility and affordable long-distance vacations, the consumption patterns of TD and IL could be affected on a local basis. Knowledge of which TD and ID have been consumed by passengers from other parts of the world could reveal new drug consumption trends. Routine analysis of airplane wastewater can alert local drug enforcement agencies and forensic laboratories on which TD and ID are consumed, even when the TD are not prescription drugs in the Scandinavian countries. Furthermore, analysis of wastewater from long-distance flights could reveal which potentially toxic xenobiotics the tourists are exposed to when travelling outside of the European Union.

The findings are in line with previous reports from target screening of influent wastewater samples in Norway, Italy and Spain, where several TDs and some IDs were detected by data-dependent and/or –independent acquisition [5;22]. However, a larger amount of unique TDs were detected in the airplane wastewater, most likely due to the size of the target screening database being used. The frequency of detected IDs in the airplane wastewater samples are low compared to previous screening studies of pooled urine and urinated soil from festivals or city centers [3;4]. This could be explained by the collected sample not being a true pooled sample, as the solid and semi-solid components would require mechanic mixing as opposed to the liquid pooled urine. Previously, chemical profiling of wastewater from wastewater treatment plant (WTP) serving the Schiphol airport and the WTP serving Amsterdam in the Netherlands showed similar loads of measured ID when taking population size into consideration [23], however, no distinction between travelers and airplane workforce can made from ground-level wastewater. Finally, absence of detected NPS can be explained by unknown urinary and/or fecal analytical targets or because they are not in the target screening databases. Glutaraldehyde from the disinfection agent can form covalent bonds with nucleophiles, particularly with primary amines [24]. Stability of analytical targets at concentrations of glutaraldehyde present in the wastewater are unknown, but glutaraldehyde could react with either the enzymes employed for conjugate cleaving during sample preparation, or form adducts with analytical targets for TDs and IDs containing primary amines. No conjugated metabolites were detected, so the enzymatic conjugate cleaving did not appear altered.

Principal component analysis

A PCA was performed to examine correlation in spatial separation of flight origin and qualitative, analytical screening results of number of TDs and IDs from therapeutic main group ATC codes from each flight as discrete variables. Fig. 2a shows origins of flights with color codes corresponding to bars in the histogram with number of identified compounds per sample in Fig 2b, and the PCA scores plot in Fig. 2c for 15 of the 17 samples.

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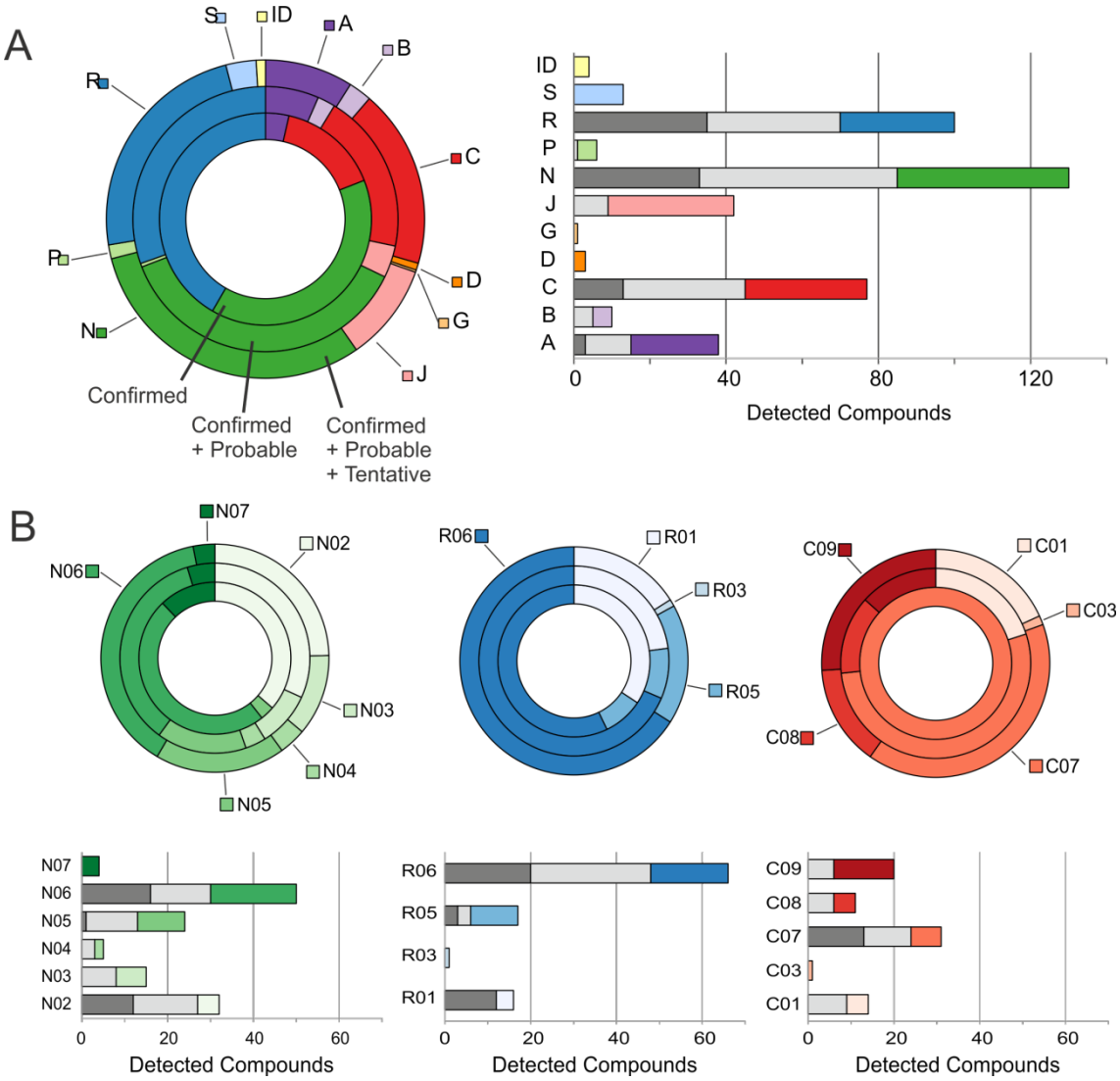

Fig. 1: Combined results from screening of wastewater samples in 3 confidence levels according to anatomical main group (Fig. 1A) and therapeutic main groups (Fig. 1B) for drugs acting on the nervous (N), respiratory (R), and cardiovascular (C) systems. Doughnut diagrams presenting compounds with confirmed identifications (inner circle), confirmed and probable identifications (middle circle), and confirmed, probable, and tentative identifications (outer circle). The horizontal histograms presenting the same data in absolute numbers with bars in dark grey, light grey, and color corresponding to number of confirmed, probable, and tentatively identified compounds, respectively.
ID: illicit drugs, ATC anatomical main groups: A: Alimentary tract and metabolism, B: Blood and blood forming organs, D: Dermatologicals, G: Genito-urinary system and sex hormones, J: Antiinfectives for systemic use, P: Antiparasitic products, insecticides and repellents, S: Sensory organs, ATC therapeutic main groups: N02: Analgesics, N03: Antiepileptics, N04: Anti-parkinson drugs, N05: Psycholeptics, N06: Psychoanaleptics, N07: Other nervous system drugs, R01: Nasal preparations, R03: Drugs for obstructive airway diseases, R05: Cough and cold preparations, R06: Antihistamines for systemic use, C01: Cardiac therapy, C03: Diuretics, C07: Beta-blocking agents, C08: Calcium channel blockers, C09: Agents acting on the renin-angiotensin system

The PCA does not explain variance in the Pakistan samples. Only one sample is available from Pakistan, and it is geographically separated from the remaining samples. Two samples were collected from flights originating from Singapore, one of these samples Singapore I clusters together with the North American samples (data not shown) and Singapore II clusters together with the South-east Asian samples. The explained variance in the model does not give a distinct grouping of the Singapore flights based on identified TCs and IDs on therapeutic main group level. The Pakistan and Singapore I samples were defined as outliers, and were not included in the PCA (Fig. 2c). It should be noted, relatively few flights were sampled from each geographical cluster. Analysis of additional flight wastewater samples could strengthen the model and possibly reveal if the Singapore I sample is truly unrepresentative for South-east Asian samples.

Plotting of wastewater samples (Fig. 2c) shows that the first two principal components separate three clusters: North American, Southeast Asian and Northeast Asian and explain a total of 58 % of the data set variance. The 1st principal component separates Northeast Asian (red) samples from Southeast Asian samples (blue), and the 2nd principal component separates

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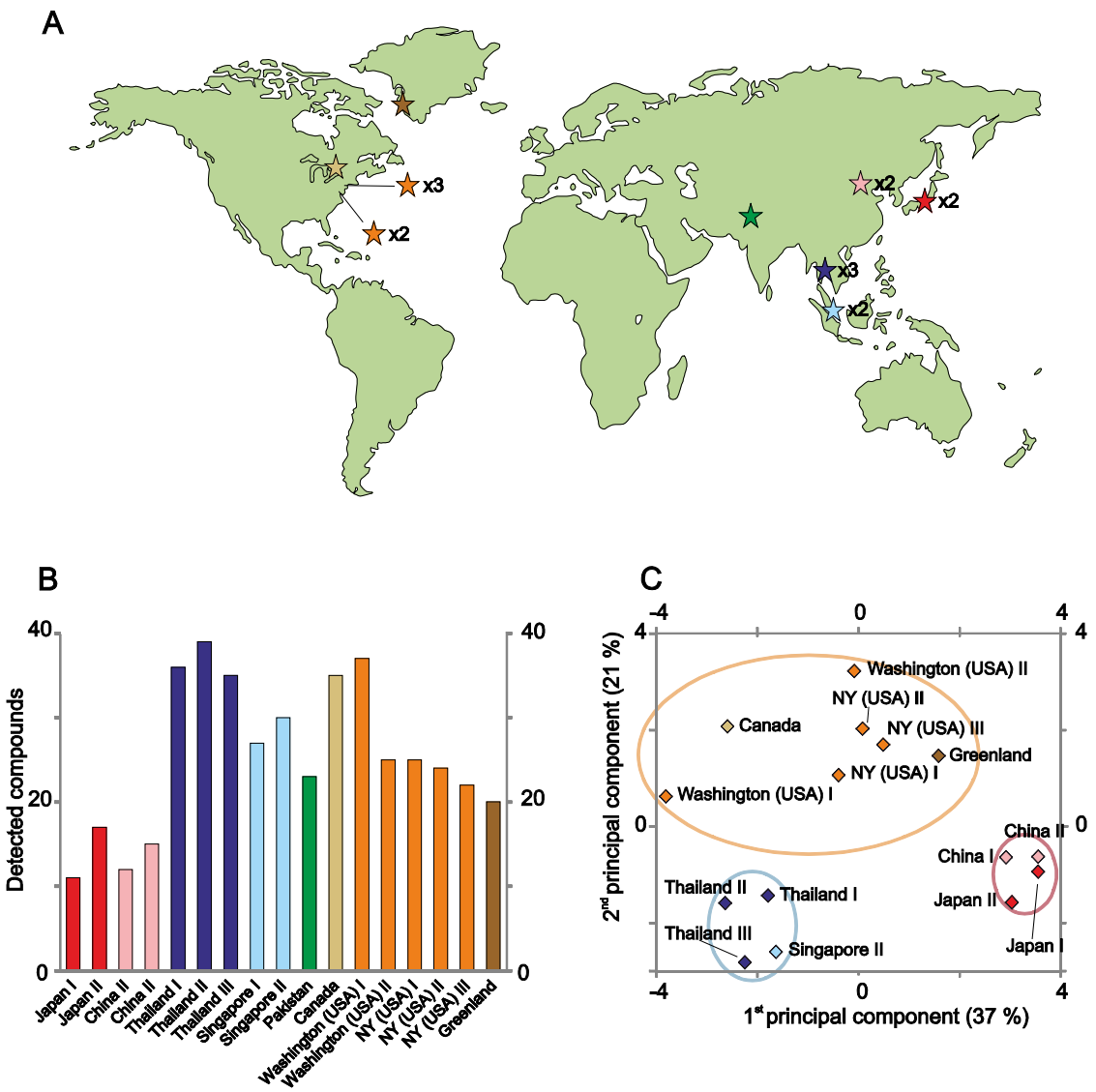

Fig. 2: A: World map showing origin of sampled flights represented by stars with unique colors for each country and numbers of independent flights from each origin. B: histogram showing the total number of compounds identified in each sample. C: Score plots from the first two principal components, with percent variance explained for each principal component at each axis, for 15 wastewater samples (B). Colored circles in Fig. 2C delimit geographical clusters based on geographical origin of the flights.

the American (yellow) samples from the Asian Samples. The variables C07 (beta blocking agents) and N06 (psychoanaleptics) are important loadings for the 2nd quadrant, whereas R06 (antihistamines for systemic use) and J01 (antibacterials for systemic use) have the highest loadings for the 3rd quadrant. Grouping of the samples from Northeast Asia was based on low number of compounds identified (Fig. 2b) rather than high scores in any variables. Accordingly, the samples from Canada and Washington (USA) I are observed with a low score on the 1st principal component (Fig. 2c) with a high total number of detected compounds (Fig. 2b), but remain separated from the Asian samples on the 2nd principal component.

CONCLUSION

Airplane wastewater samples were for the first time screened for analytical targets of TDs and IDs consumption. A system for assigning identification confidence was developed and implemented. A total of 89 unique TDs or IDs were identified across 17 wastewater samples, mainly belonging to the anatomical main groups for drugs acting on the neurological, respiratory and cardiovascular systems. A PCA revealed three clusters based on geographical origin of the flights from identified TDs and IDs in the wastewater samples as variables. The study demonstrates how analysis of airplane wastewater can reveal international drug use and abuse patterns using systematic toxicological analysis.

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REFERENCES

[1] Meyer MR. Trends in analyzing emerging drugs of abuse - from seized samples to body samples. Analytical and Bioanalytical Chemistry 2014; 406(25):6105-6110.

[2] Kinyua J, Covaci A, Maho W, McCall AK, Neels H, van Nuijs AL. Sewage-based epidemiology in monitoring the use of new psychoactive substances: Validation and application of an analytical method using LC-MS/MS. Drug Test Anal 2015; 7(9):812-818.

[3] Archer JRH, Dargan PI, Lee HMD, Hudson S, Wood DM. Trend analysis of anonymised pooled urine from portable street urinals in central London identifies variation in the use of novel psychoactive substances. Clinical Toxicology 2014; 52(3):160-165.

[4] Mardal M, Kinyua J, Ramin P, Miserez B, van Nuijs AL, Covaci A et al. Screening for illicit drugs in pooled human urine and urinated soil samples and studies on the stability of urinary excretion products of cocaine, MDMA, and MDEA in wastewater by hyphenated mass spectrometry techniques. Drug Test Anal 2016.

[5] Baz-Lomba JA, Reid MJ, Thomas KV. Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF. Anal Chim Acta 2016; 914:81-90.

[6] Stephanson N, Sandqvist S, Lambert MS, Beck O. Method validation and application of a liquid chromatography-tandem mass spectrometry method for drugs of abuse testing in exhaled breath. J Chromatogr B Analyt Technol Biomed Life Sci 2015; 985:189-196.

[7] Kinyua J, Negreira N, Ibanez M, Bijlsma L, Hernandez F, Covaci A et al. A data-independent acquisition workflow for qualitative screening of new psychoactive substances in biological samples. Anal Bioanal Chem 2015; 407(29):8773-8785.

[8] Schymanski EL, Jeon J, Gulde R, Fenner K, Ruff M, Singer HP et al. Identifying small molecules via high resolution mass spectrometry: communicating confidence. Environ Sci Technol 2014; 48(4):2097-2098.

[9] Nielsen LM, Linnet K, Olsen L, Rydberg P. Prediction of cytochrome p450 mediated metabolism of designer drugs. Curr Top Med Chem 2014; 14(11):1365-1373.

[10] Cone EJ, Gorodetzky CW, Yousefnejad D, Buchwald WF, Johnson RE. The metabolism and excretion of buprenorphine in humans. Drug Metab Dispos 1984; 12(5):577-581.

[11] Anggard E, Gunne LM, Homstrand J, McMahon RE, Sandberg CG, Sullivan HR. Disposition of methadone in methadone maintenance. Clin Pharmacol Ther 1975; 17(3):258-266.

[12] Wall ME, Sadler BM, Brine D, Taylor H, Perez-Reyes M. Metabolism, disposition, and kinetics of delta-9-tetrahydrocannabinol in men and women. Clin Pharmacol Ther 1983; 34(3):352-363.

[13] Castiglioni S, Thomas KV, Kasprzyk-Hordern B, Vandam L, Griffiths P. Testing wastewater to detect illicit drugs: state of the art, potential and research needs. Sci Total Environ 2014; 487:613-620.

[14] Petersen TN, Rasmussen S, Hasman H, Caroe C, Baelum J, Schultz AC et al. Meta-genomic analysis of toilet waste from long distance flights; a step towards global surveillance of infectious diseases and antimicrobial resistance. Scientific Reports 2015; 5.

[15] Andersen D, Rasmussen B, Linnet K. Validation of a fully automated robotic setup for preparation of whole blood samples for LC-MS toxicology analysis. J Anal Toxicol 2012; 36(4):280-287.

[16] Pedersen AJ, Dalsgaard PW, Rode AJ, Rasmussen BS, Muller IB, Johansen SS et al. Screening for illicit and medicinal drugs in whole blood using fully automated SPE and ultra-high-performance liquid chromatography with TOF-MS with data-independent acquisition. J Sep Sci 2013; 36(13):2081-2089.

[17] Bjork MK, Simonsen KW, Andersen DW, Dalsgaard PW, Siguroardottir SR, Linnet K et al. Quantification of 31 illicit and medicinal drugs and metabolites in whole blood by fully automated solid-phase extraction and ultra-performance liquid chromatography-tandem mass spectrometry. Analytical and Bioanalytical Chemistry 2013; 405(8):2607-2617.

[18] Simonsen KW, Steentoft A, Buck M, Hansen L, Linnet K. Screening and Quantitative Determination of Twelve Acidic and Neutral Pharmaceuticals in Whole Blood by Liquid-Liquid Extraction and Liquid Chromatography-Tandem Mass Spectrometry. Journal of Analytical Toxicology 2010; 34(7):367-373.

[19] World health organization. International Language for Drug Utilization Research. 25-3-2011. 4-5-2016.

[20] Sundhedsdatastyrelsen. Register of Medicinal Product Statistics. 1-1-2016. Copenhagen. 4-5-2016.

[21] Baselt RC. Disposition of Toxic Drugs and Chemicals in Man. ninth ed. Seal Beach (CA): Biomedical Publications; 2011.

[22] Bade R, Rousis NI, Bijlsma L, Gracia-Lor E, Castiglioni S, Sancho JV et al. Screening of pharmaceuticals and illicit drugs in wastewater and surface waters of Spain and Italy by high resolution mass spectrometry using UHPLC-QTOF MS and LC-LTQ-Orbitrap MS. Anal Bioanal Chem 2015; 407(30):8979-8988.

[23] Bijlsma L, Emke E, Hernandez F, de VP. Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry. Chemosphere 2012; 89(11):1399-1406.

[24] Hermanson GT. Bioconjugate techniques. 3rd ed. Oxford: Elsevier Inc.; 2013.