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Novel multiplex capacitive sensor based on molecularly imprinted polymers: a promising tool for tracing specific amphetamine synthesis markers in sewage water.

Esther De Rycke^{1,2*}, Olivier Leman³, Peter Dubruel², Martin Hedström⁴, Matthias Völker³,
Natalia Beloglazova^{1,5**}, Sarah De Saeger^{1,6**}

¹ *Centre of Excellence in Mycotoxicology and Public Health, Department of Bioanalysis, Ghent University, Ottergemsesteenweg 460, B-9000 Ghent, Belgium.*

² *Polymer Chemistry & Biomaterials Research Group, Centre of Macromolecular Chemistry (CMaC), Department of Organic and Macromolecular Chemistry, Ghent University, Krijgslaan 281, Building S4-Bis, B-9000 Ghent, Belgium.*

³ *Fraunhofer Institute for Integrated Circuits IIS, Smart Sensing and Electronics Division, Department for Integrated Sensor Systems, D-91058 Erlangen, Germany.*

⁴ *Department of Biotechnology, Lund University, Box 124, 221 00 Lund, Sweden.*

⁵ *Nanotechnology Education and Research Center, South Ural State University, 454080 Chelyabinsk, Russia.*

⁶ *Department of Biotechnology and Food Technology, Faculty of Science, University of Johannesburg, Doornfontein Campus, Gauteng, South Africa.*

* Corresponding author: esther.derycke@ugent.be

** [Authors contributed equally to this work](#)

Abstract

The development of a sensing system for amphetamine (AMP), N-formyl amphetamine (NFA), and benzyl methyl ketone (BMK) in sewage is a strict requirement for enabling the on-site detection and tracing of the consumption of AMP, and the production and/or transportation of these target analytes. The present research is therefore devoted to the development of an on-site capacitive sensing system, based on molecularly imprinted polymers (MIPs) as recognition elements.

To this end, the commercially available CapSenze capacitive sensor system was miniaturized by implementing an application-specific integrated circuit (ASIC), dedicated to the bias and readout of the chemical sensor. MIPs towards AMP were purchased, whereas the ones towards NFA and BMK were synthesized in house. Gold transducers, consisting of six working electrodes with their corresponding reference electrodes and one common auxiliary electrode, were designed together with a flow cell to enable analyses. The applied water samples were filtered through a 20 micron filter before application in the sensors' flow cell. The limits of detection in filtered sewage water were determined to be 25 µM for NFA and BMK and 50 µM

for AMP. The overall performance of the sensing system was tested by analysis of blind-coded sewage samples, provided by legal authorities.

To the best of our knowledge, this is the first research presenting multiplex MIP-based detection of amphetamine synthesis markers using a capacitive sensor, miniaturized via ASIC technology. The presented technique is undoubtedly a potential solution for any analysis requiring constant reliable on-site monitoring of a substance of interest.

Key words

Capacitive sensor, molecularly imprinted polymers, amphetamine type stimulants, ASIC, sewage water analysis.

1. Introduction

Amphetamine-type stimulants (ATS) are a group of synthetic illicit drugs, which include amphetamine (AMP), methamphetamine (MA) and ecstasy, including 3,4-methylenedioxymethamphetamine (MDMA), but also less popular compounds like methcathinone, fenetylline, methylphedrine, and methylphenidate. For ATS detection, also their precursors (alpha-phenylacetoacetonitrile (APAAN), benzyl methyl ketone (BMK)), intermediates (N-formyl amphetamine (NFA)), and impurities (4-methyl-5-phenyl pyrimidine (4M5PP), di(β -phenylisopropyl)amine (DPIA)) are of interest (UNODC, 2015). Although cannabis remains the most popular drug worldwide, ATS are second in place (UNODC, 2011). According to the World Drug report of 2007, the illicit drug trade is still a booming global business, worth approximately \$322 billion (United Nations Office on Drugs and Crime, 2007). While the legal market for these drugs has shrunk over the last decades, the black market is thriving. Approximately half of the ATS seizures at 2000-2005 were MA, AMP, and MDMA. Over the recent years, this trend has been clearly shifting towards AMP since the improved legal control of two main MA precursors, ephedrine and pseudo-ephedrine (United Nations Office on Drugs and Crime, 2007). In 2016, more than 70 tons of AMP were seized (UNODC, 2018). AMP production is primarily located in Europe, notably in Poland and the Netherlands, followed by Belgium and the Baltic region. Smaller production sites were also found in North America and South-East Asia (United Nations Office on Drugs and Crime, 2007). The most common way to produce AMP is the Leuckart synthesis. In this two-step process, the well-known precursor BMK is reductively aminated to NFA by adding ammonium formate or a mixture of ammonia and formic acid (Leuckart step 1). NFA is then hydrolysed in the presence of a strong acid (e.g. hydrochloride, sulphuric acid) to the corresponding AMP salt. After

addition of an alkaline compound (e.g. sodium hydroxide), the salt is converted into the AMP freebase (Leuckart step 2). The obtained product can contain several impurities, originating from impure start product(s), products formed from those impurities, by-products from the reactions, or from incomplete conversion of intermediates or reagents (Aalberg et al., 2005; Hauser et al., 2018). DPIA, for example, is marked as a specific impurity of the Leuckart reaction and is often found as the main ingredient in poor quality AMP tablets sold on the illicit market (Ottaviano et al., 2002). Although the purchase and consumption of BMK is internationally controlled (Di Giovanni et al., 2012), it is still available relatively cheap on the black market or can be easily synthesized from BMK bisulphite (EMCDDA-Europol joint publications, 2011), APAAN (Hauser et al., 2018), alpha-phenylacetoacetamide (APAA) (Emke et al., 2018a) or BMK glycidate (Vijlbrief, 2012). Therefore, in the present manuscript, these compounds (BMK, NFA, and AMP) were selected as possible indicators for suspicious ATS activity (production and transportation).

The most widely used techniques to trace ATS include gas chromatography (GC-) and liquid chromatography tandem mass spectrometry (LC-MS/MS) (Fernández et al., 2004; Hernández et al., 2014; Kala et al., 2008). Although chromatography is known for its high accuracy, it is disadvantaged by its high price, long and complicated measuring procedure and sophisticated sample pre-treatment (Lurie, 1997). Electrochemical sensors, such as amperometric, voltammetric, impedimetric, and capacitive, could offer an easy and cheaper alternative. Despite the fact that since the last decade sensors are gaining popularity in pharmaceutical (Beitollahi and Mostafavi, 2014; Goyal et al., 2012), biomedical (Chen and Chatterjee, 2013; Llp, 2005), environmental (Hart et al., 2004), and industrial applications (Malhotra and Chaubey, 2003), development of electrochemical sensors towards illicit drugs, and more specifically towards ATS, stays rather limited (Florea et al., 2018). MA and cocaine are the most popular target analytes, and their analysis is often performed in biological fluids like urine and serum to trace consumption (De Rycke et al., 2020). To the best of our knowledge, only two papers devoted to an electrochemical sensor monitoring illicit drugs in sewage water were published, more specifically a DNA-directed aptamer sensor using impedance spectroscopy (EIS) (Yang et al., 2016) and a molecularly imprinted polymer (MIP)-based screen printed carbon electrode (SPCE) sensor using square wave voltammetry (SWV), both for the detection of cocaine (Florea et al., 2019). Furthermore, multiplex detection systems for illicit drugs were rarely developed (De Rycke et al., 2020).

In this research, a multiplex capacitive system was developed for the detection of ATS markers in sewage water. Sewage water is a quite harsh matrix since algae, bacteria, organic compounds,

including solvents, solid particles or any type of human waste can be present. Moreover, the temperature can fluctuate in a wide range (10-25°C), not only dependent on the season but also on individual household activities (laundry, shower, etc). Finally, the pH can vary as well. Indeed, a pH of approximately 7 to 8 is generally found whereas the pH of the waste produced at different steps of the Leuckart reaction changes from extreme low (<3) to high (>12) (Hauser et al., 2019). Since natural recognition elements like antibodies would denature under these conditions and lose their functionality, artificially created MIPs are the optimal recognition element for this application. MIPs are cross-linked polymers with specific recognition sites which are complementary in shape, size, and functionality to a template molecule (Vasapollo et al., 2011). They are characterized by a high mechanical and thermal stability and show an excellent chemical resistance in a broad pH-range (Yu and Mosbach, 1997). Our previous work focussed on the computational design and following syntheses of MIPs towards NFA and BMK, and their use as recognition elements in the capacitive sensor for the single target detection in water for environmental control (De Rycke et al., 2021; Graniczowska et al., 2016). Both articles described the choice of necessary reagents (monomers and crosslinkers) and optimal polymerisation techniques. The applied commercially available gold electrodes consisted of one working electrode each, therefore, they were used for single target analysis. Capacitive sensing was performed by a commercially available benchtop sensor system, which can operate only in a laboratory environment. In this study, the MIPs towards BMK and NFA were used, together with the commercially available MIP towards AMP, for the multiplex on-site detection of these compounds in sewage water. For this, the commercially available CapSenze capacitive sensor system was miniaturized by implementing an application-specific integrated circuit (ASIC), enabling on-site simultaneous detection of the three target analytes. The MIPs were immobilized on the new gold transducers by electropolymerisation using tyramine as a linker. The amount of cycles was optimized, while the success of the immobilization was checked by cyclic voltammetry (CV) and optical microscopy. After selecting an appropriate inlet filter, the limits of detection (LODs) were determined and the overall analytical method was validated.

2. Experimental

2.1 Materials

Methanol (MeOH, LC-MS grade) was obtained from Biosolve BV (Valkenswaard, The Netherlands), acetone (C₃H₆O) from Fiers (Kuurne, Belgium), and ethanol (EtOH absolute, Analar Normapure) from VWR International (Leuven, Belgium). Sulfuric acid (H₂SO₄, 95–97%), potassium dihydrogen phosphate (KH₂PO₄, p.a.), and potassium chloride (KCl, p.a.)

were bought from Merck (Darmstadt, Germany). Dipotassium hydrogen phosphate (K_2HPO_4 , $\geq 98\%$), 1-dodecanethiol ($\text{C}_{12}\text{H}_{26}\text{S}$, 98%), hydrogen peroxide (H_2O_2 , 30 wt%), potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$, $\geq 99.0\%$), trimethylamine ($\text{C}_6\text{H}_{15}\text{N}$, $\geq 99\%$), tyramine ($\text{C}_8\text{H}_{11}\text{NO}$, 99%) were purchased from Sigma Aldrich (Bornem, Belgium). Disolol was purchased from Chem Lab (Zedelgem, Belgium). Standards of amphetamine hydrochloride (AMP.HCl) were provided by Lipomed AG (Arlesheim, Switzerland). Commercially available MIPs towards AMP were bought from MIP technologies (Lund, Sweden). Standards of NFA, BMK, and blind-coded samples were kindly provided by Bundeskriminalamt (Wiesbaden, Germany). Ultrapure water was obtained with the arium® pro system from Sartorius (Schaarbeek, Belgium). Sputtered gold electrodes were provided by CapSense AB (Lund, Sweden).

Piranha solution consisted of H_2SO_4 and H_2O_2 , in a ratio of 3/1 (v/v). Regeneration buffer was made from MeOH/phosphate-diphosphate buffer/triethyl amine in a ratio 47.5/47.5/5 (v/v/v). Running buffer consisted of a 10 mM phosphate-biphosphate buffer, $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ in ultrapure water with a pH of 7.2. Tyramine solution (10 mM) was prepared by dissolving 0.0137 g (0.100 mmol) of tyramine in 1.25 mL ethanol and further diluted with 3.75 mL of the running buffer. The 1-dodecanethiol solution (10 mM) was prepared by dissolving 60 μL of 1-dodecanethiol in 25 mL EtOH.

Optical measurements were performed using the Axiotech 100, Axiocam 105 optical microscopy with a colour camera from (Carl) Zeiss (Göttingen, Germany). Cyclic voltammetry (CV) was performed with an Autolab PGSTAT2014 compact potentiostat/galvanostat from Metrohm (Antwerp, Belgium), the used glassy carbon auxiliary electrode (AE) and Ag/AgCl reference electrode (RE) were also purchased from Metrohm (Antwerp, Belgium). The original capacitive sensor was developed by CapSense, the miniaturization was performed by Fraunhofer Institute for Integrated Circuits (IIS) in close collaboration with CapSense. Software for measuring and post-processing obtained data was designed under GNU Octave by IIS as well. Following commercial parts were incorporated in the sensor device: a mp-6 piezoelectric membrane micro-pump (PMP1), and its driver module mp6-original equipment manufacturer (OEM) from Bartels Mikrotechnik GmbH (Dortmund, Germany), the valves, miniature thermally actuated shape-memory alloy devices, from the SMV-series, Takasago Fluidic Systems (Massachusetts, USA). Sewage water was filtered by the frit in-line assemble system with filters with various pore sizes (20, 10, 5, 1, and 0.1 μm) of Analytical Scientific Instruments (ASI, California, USA).

2.2 Miniaturized capacitive sensor system

The applied device (Figure 1) implements an overall functionality close to the system described by Erlandsson et al. (2014), but in a highly miniaturized format and with low-cost parts in order to achieve an economically viable sensor. In comparison to earlier published research on capacitive-based sensing platforms, this device was especially developed for miniaturized multichannel detection (Beloglazova et al., 2018; Foubert et al., 2019; Labib et al., 2009; Lenain et al., 2015; Teeparuksapun et al., 2010).

Figure 1. Picture of the developed capacitive sensor.

Figure 2a shows the architecture of the sensing system. The system includes three tanks; each one could be selected via a respective valve (VLV1, 2 and 3). Fluid (sample, running or regenerating buffer) is pumped from a selected tank by the pump (PMP1) and pushed through the flow cell (FC1, Figure 2c) down to the waste tank. The sensor electrodes are biased by the ASIC-based readout system calculating a capacitive value for each working electrode in the sensor, whenever requested by the host computer. The host computer controls all these processes/elements via a microcontroller (Figure 2b, black package on the left side of the printed circuit board (PCB)) of the instrumentation mainboard. More information on the construction of the PCB can be found in Appendix Figure A1.

Figure 2. Architecture of the miniaturized chemical sensor system (a). Custom manufactured miniaturized devices of the proposed system: instrumentation mainboard (b), and 6-working-electrodes flow cell (c) where the electrodes are visible through the window.

The printed circuit board with the ASIC (Figure 2b, QFN48 package on the right side of the PCB) implements the functionalities for the sensor bias and readout that would otherwise require a large number of general-purpose integrated circuits. In addition, a limited amount of external passive devices (i.e. capacitors and resistors) are used, thus reducing the area footprint on the printed-circuit-board, production costs and increasing reliability of the measurements. The electronic system also includes drivers for the piezoelectric micro-pump and valves. The sensor bias and readout principle appeal to the current-cycle method which consists of a sequence of positive/negative pulses with a precisely regulated current (e.g. 10 μA), as presented in Appendix Figure A.2. Each current pulse results in a voltage slope between a RE and a working electrode (WE), translating the capacitive behaviour of the WE interface with the fluid. Therefore, fluctuations of this voltage slope contain information about the sensor response to the analyte(s) i.e. binding of the molecules.

2.3 Production and pre-treatment of the multiplex gold electrodes

CapSense previously developed several gold working electrodes, based on silica, glass or ceramic substrates, in order to perform capacitive measurements (Figure 3a) (Limbut et al., 2007; Teeparuksapun et al., 2012, 2010). These electrodes hold one WE, meaning that only one type of recognition element can be immobilized on this surface. In order to switch to multiplex detection, new gold electrodes, consisting of six gold WE's, each with their own RE, were designed and developed. In addition, one common AE was utilized per electrode chip according to Figure 3b. The selected electrode substrates were 2" Si wafers, n-doped to 5-10 Ohm-cm. These wafers were initially wet oxidized to a silicon oxide thickness of 200 nm. The WE's were

made out of 185 nm gold (99,999% purity), deposited by thermal evaporation. The RE and AE, contact pads and connecting lanes, consisted of a platinum/palladium alloy (80/20), with a thickness of 150 nm, deposited by sputter coating in vacuum. Furthermore, the adhesive layer between the silica surface and the noble metals was made of evaporated chromium with a thickness of 30 Å. The transducer chip was used directly in the intermediate flow cell chamber using pogo pins as electrical connectors to the control system. For cleaning, gold transducers were submerged and sonicated for 15 minutes in subsequently acetone, absolute ethanol, and piranha solution (composition, see higher). Between the washing steps, the electrodes were rinsed with water and disolol and dried under a stream of nitrogen.

Figure 3. Single target gold electrodes (silica, glass and ceramic) (a), and the multiplex gold electrode (b).

2.4 Immobilisation of MIP beads on the gold transducers

MIPs towards NFA were synthesized and fully characterized in water by Graniczowska et al. (2016), the MIPs towards BMK were described by De Rycke et al. (2021). The overall performance of the polymers was investigated during a batch rebinding study (an equilibrium experiment), based on a LC-MS/MS analysis, as described in the Appendix B. The aim of this research was to apply these MIPs on the newly developed gold transducers for the on-site detection of three ATS markers in sewage water, using the developed miniaturized capacitive sensing system.

To achieve immobilisation of the MIPs on the transducer's surface, 2.5 mg of the MIPs were suspended by sonication in 5 mL of the 10 mM conductive tyramine solution as described before by Lenain et al. (2015). Clean electrodes were placed in a reaction cell filled with 300 µL of the MIP suspension. The particles were allowed to sediment for 15 minutes. A wafer gold electrode was employed as the WE, the common internal AE of the transducer as the AE, and an external glassy carbon electrode was used as the RE. Electrooxidation of tyramine was

performed by 10 potential sweeps in a potential range from 0 V to 1.5 V, with a scan rate of 50 mV/s. In this study, the same convention was always used: WE1 and WE2 were modified with AMP-MIPs, WE3 and WE4 with NFA-MIPs, and WE5 and WE6 with BMK-MIPs. The transducer was rinsed with disolol and deionized water, and dried under nitrogen in between the modification of the different WE's. Afterwards, the transducer was again rinsed thoroughly with ultrapure water and ethanol to remove any non-polymerized tyramine monomers and dried. To insulate all remaining pinholes, the transducer was kept in 10 mM 1-dodecanethiol in ethanol for 15 min.

The successful insulation of the transducers was verified with CV. Measurements were performed in 10 mM $K_3[Fe(CN)_6]$ with 0.1 M KCl in H_2O , with a scan rate of 50 mV/s, and a step potential of 0.001 V in a potential range from -0.250 to 0.700 V. An Ag/AgCl electrode was employed as RE, an external glassy carbon electrode as AE.

2.5 Running scheme of the capacitive sensor

All analyses were performed using the same measuring scheme. Different fluids were pumped at a similar flow rate. This assumption relies on the fact that the membrane pump is operated at a frequency much lower than its resonant frequency i.e. the flow rate is nearly proportional to the pulse frequency. It was verified that the differences in dynamic viscosity between each fluid did not significantly interfere with the flow rate. A constant flow rate of 500 $\mu L/min$ was set up, and the fluidic volume, to be pumped for each phase of the measuring scheme, was controlled by timing of the pump's activity and (des)activation of the corresponding valves. The scheme started with the regeneration for 8 seconds where regeneration buffer was pumped through the flow cell. This step cleaned the surface of the transducer, making sure that the MIPs were free for (re)binding. This was followed by pumping of the running buffer for 120 seconds, which removed the regeneration buffer from the flow cell, and prepared the electrode for sample analysis. The sample analysis was performed for 8 seconds, ensuring that the binding event could successfully proceed within the flow cell. The sample inlet held an in-line filter with a 20 micron pore size. Afterwards, the running buffer was again pumped through the flow cell, flushing away the unbound components from the flow cell. A capacitive drop, as a result of the binding event between the MIPs and the targets, was investigated.

3. Results and discussion

The present study aims to develop a novel capacitive sensor for the multiplex detection of AMP markers in sewage based on MIPs for specific binding with the target analytes. The development and validation of the overall sensing system is divided in several consecutive steps. Prior to electrochemical analysis, the immobilisation process of the MIPs on the multichannel transducers was optimized and an appropriate sensor inlet was selected to prevent clogging. Afterwards the affinity was investigated and blind-coded ATS artificially-spiked sewage water samples and waste stream samples from clandestine laboratories were analysed.

3.1 Multichannel functionalized transducers

The chemical sensor was equipped with a transducer chip with six parallel channels produced inhouse by CapSense. The chip was manufactured from a silica wafer using regular metal sputter coating/evaporation techniques. The connector lanes between the different electrode surfaces and connector pads were sputtered with a platinum/palladium alloy with a chromium layer between the silica surface and the noble metals. The working electrodes were functionalized with three different MIPs, synthesized towards compounds specific for ATS markers (AMP, NFA, and BMK), each in duplicate. The immobilisation process was optimized by changing the amount of potential sweeps (between 5 and 15) during the electropolymerization, while the potential range and the scan rate remained the same, and its influence on the layer thickness and the baseline stability was investigated. The voltammograms showing the influence of the amount of potential sweeps on the thickness of the insulation layer, are shown in Appendix C (Figure C.1 and C.2). Figure 4a represents the electropolymerisation using 15 potential sweeps of tyramine as an example. As the process continued from cycle 1 to 15, the current dropped due to the formation of an additional layer and an increase of the total thickness of the insulation layer on the electrode's surface. Figure 4b shows the cyclic voltammograms of the WE's immobilized with a different amount of potential sweeps. As anticipated, a higher amount of potential sweeps resulted in a thicker layer on the micro-electrode surface, and therefore to a higher insulation rate. After blocking of the electrode with 10 mM 1-dodecanethiol, the baseline stability was tested. Using 15 potential sweeps, a zero capacitive baseline was found.

The assumption is that this layer is too thick to be used for this application and was therefore excluded from further study. Three and 5 potential sweeps were not sufficient to insulate the surface, and they were excluded as well. From these results, we opted to apply 10 potential

sweeps. The selectivity of the immobilisation process was investigated by both CV and optical microscopy. As it can be seen from the voltammograms of Figure 4, selective MIP immobilisation was possible.

Figure 4. Cyclic voltammogram before (dark) and after (lighter) functionalization of one of the working electrodes. All measurements were recorded in 10 mM $K_3[Fe(CN)_6]$ with 0.1 M KCl in H_2O , a scan rate of 50 mV/s, and a step potential of 0.001 V in a potential range from -0.250 to 0.700 V. Ag/AgCl was used as a RE, and the internal AE. Optical measurement, performed by optical microscopy, of a bare WE (b), surrounded by the corresponding RE (c), and the MIP-functionalized WE (d).

As derived from Figure 4, only the voltammogram of the selected WE, in this case WE III, changed upon the functionalization while the voltammetric profiles of the other WE's remained unaltered. This could be confirmed by the naked eye given an orange/yellowish coloration of the functionalized WE upon the deposition of the polytyramine layer. There can thus be concluded that immobilization of different MIPs on the same transducer chip is feasible. These results were confirmed by optical microscopy. After modification of the selected WE, an orange/yellowish color was observed on the surface where aggregates of MIPs could be observed (Figure 4d). Due to the small size of the MIPs (200-350 nm), it was not possible to distinguish single particles. In comparison, the surfaces of the unfunctionalized WE's remain clear (Figure 4b-c).

3.2 Selection of the sample pretreatment

The sensor was intended for sewage water analysis. In order to perform real life measurements, the sewage water filtered in-line. Therefore, it was of crucial importance to find a compromise for the pore size of the filter. On one hand, the pore size must be large enough, preventing filter

clogging. On the other hand, the pore size must be small enough to prevent failure of the pump and protect the MIPs (200-350 nm sized) from blocking particles.

The manufacturer of the mp-6 piezoelectric micropump stated that these pumps work optimally transporting any fluid containing particles smaller than 20 μm . Therefore, the choice was made to test filters of 20 micron, some with smaller pore sizes (<1, 1, 5, and 10) and one with a larger pore size (50 micron). Fifty micron filters were excluded since after 30 minutes of pumping sewage water, deposition and aspecific binding of matrix components occurred on the electrode's surface. The functionality of the electrode was affected. As an example, there was no longer a change in capacitive drop between 500 and 1000 μM . Filters with a pore size smaller than 1 micron could not be used in the final system being too small for pumping sewage water, as the filters were clogged very fast. Measurements of spiked samples (50 μM) resulted in an analytical signal using 5-, 10-, and 20 micron-filters. The smaller the filter size, the more stable the baseline became. As a compromise, 20 micron filters were used for all further experiments.

3.3 Affinity of the MIPs

In our previous work, the affinity of the MIPs towards the target compounds was tested analysing artificially spiked (tap) water samples using the commercially available capacitive sensor (CapSenze, Lund, Sweden (single target detection)). The limits of detection (LODs) were determined to be 10 μM for AMP and NFA, and 1 μM for BMK. (De Rycke et al., 2021; Graniczowska et al., 2016) These tests were repeated using the new gold electrodes and miniaturized capacitive sensing system in filtered (20 micron) sewage water. The LODs were determined to be 50 μM for AMP, and 25 μM for NFA and BMK.

Several studies have been performed where water samples, collected from sewage treatment plants, were quantitatively analysed to estimate the amount of AMP-users within a population. Table 1 summarizes several articles devoted to the detection of AMP in sewage water, published within the last two years. To the best of our knowledge, no information was found towards the detection of NFA and BMK in sewage water. Furthermore, also research on AMP was limited. Most sensing systems were based chromatography, while no portable devices were published.

Table 1. Comparison of analytical techniques and parameters for the detection of amphetamine in sewage water (published within the last two years). (Abbreviations: AMP, amphetamine; BMK, benzyl methyl ketone; (HR)MS, (high resolution) mass spectrometry; NFA, N-formyl amphetamine; (UHP)LC, (ultra-high performance) liquid chromatography.)

Reference	Method of detection	Range	LOD
(Centazzo et al., 2019)	LC-MS/MS	5 – 1000 ng/L	n.m.
(Deng et al., 2020)	LC-MS/MS	0.1-10 µg/L	0.01 µg/L
(Ruan et al., 2020)	LC-MS	0.1-150 ng/mL	0.025 ng/mL
(Emke et al., 2018b)	HRMS	Qualitative detection (presence and characterisation) of AMP, BMK and NFA	
(Löve et al., 2018)	UHPLC-MS	Obtained results: 100-350 mg/day/1000 people	
This work	Capacitive sensor	-	25 µM for NFA and BMK, 50 µM for AMP

AMP concentrations were found in a range from several to tens ng/L of sewage water (Li et al., 2014). These concentrations are too low to be detected by the described sensing system. The goal of this research is to investigate suspicious ATS synthesis activity, without focusing on the individual user. The synthesis of 1 kg AMP results in 20-30 kg waste and production sites are often scaled-up in order to synthesize more than 1 kg AMP per batch. Depending on the weather condition, this waste will be diluted 1/100 to 1/10.000 in the sewage pipelines. Therefore, even at the highest dilution, the present sensor will obtain a positive result.

3.4 Validation of the miniaturized capacitive sensor

In a final and very important part of our work, the developed sensor was validated. To this end, blind-coded ATS artificially-spiked sewage water samples and waste stream samples from clandestine laboratories were obtained from Bundeskriminalamt (Wiesbaden, Germany) and subsequently analysed in our facilities. Table 2 summarizes the exact sample details as well as the obtained results from the chemical sensor and the real content (confirmed by GC- or LC-MS/MS).

Table 2. Summary of the analysed samples, matrix: sewage water if not mentioned differently, filter: 20 micron. Results were confirmed by GC-MS analysis by Bundeskriminalamt or LC-MS/MS analysis in-house. The chemical sensor gave a correct or false results**. (Abbreviations: APAAN, alpha-phenylacetoacetanitrile; AMP, amphetamine; BMK, benzyl methyl ketone; DI, deionized water; DPIA, di(β-phenylisopropyl)amine; HCl, hydrochloride; NaCl, sodium chloride; NaOH, sodium hydroxide; NH₃, ammonia; NFA, N-formyl amphetamine; SW, sewage water; TW, tap water.)*

Sample	Sample content	Result of the chemical sensor	Sample	Sample content	Result of the chemical sensor
1*	Pure SW	Blanc	14**	DPIA in SW	AMP
2*	AMP in DI	AMP	15*	HCl in TW	Blanc
3*	BMK in DI	BMK	16**	HCl in DI (pH=3)	NFA
4*	BMK in DI	BMK	17*	NaCl in DI	Blanc
5**	NFA in DI	NFA and AMP	18**	Hydrochloric acid, (pH=2) in SW	NFA in low concentration
6*	AMP, NFA and BMK in DI	AMP, NFA and BMK	19**	Leuckart step 1 waste in TW	NFA and AMP
7*	NH ₃ in DI	Blanc	20*	Leuckart step 1 waste (diluted 1:50) in SW	NFA
8*	NH ₃ (pH=10) in SW	Blanc	21*	SW	Blanc
9*	NaOH (pH=12) in SW	Blanc	22*	AMP and NFA in SW	AMP and NFA
10*	NH ₃ (pH=14) in SW	Blanc	23*	Pipercleaner (pH=12) in ATS waste	AMP, NFA and BMK
11*	AMP in alkaline solution (NH ₃ in SW, pH=10)	AMP	24*	Waste from conversion APAAN to BMK	BMK
12**	Acetophenone in SW	AMP	25*	Leuckart step 1 waste (diluted 1:100)	NFA and BMK
13*	Pseudoephedrine in SW	Blanc	26**	Leuckart step 2 waste (diluted 1:100)	AMP, NFA and BMK

From the results in Table 2, it can be derived that the overall performance of the developed sensor was good: from the 26 samples, 19 were identified correctly* and 7 falsely**. It should be noted that all 7 samples were false positives and this sensor will be applied for a first-line on-site screening of aqueous samples. In case of a positive result, the sample will be taken to the laboratory and the result will be confirmed by GC-MS e.g. as proof in court. In this regard, having some false positives is better than false negatives.

Medium to strong alkaline solutions of NH₃ and NaOH were not negatively affecting the sensor performance (samples 7-11, and 23). However, for strongly acidic solutions (pH<3) several false positive results were obtained (samples 16 and 18 versus sample 15). The developed MIPs for NFA especially suffered under the harsh acidic conditions. In the applied experimental conditions, no interaction was found between the MIPs and salty solutions (sample 17). Furthermore, in a highly contaminated NFA-sample (sample 5 and 19), a capacitive drop was

observed for both NFA and AMP. A plausible explanation is that by aging of the sample, NFA is converted to AMP. However, since sample 19 represents waste of Leuckart step 1, which is strongly acidic, conversion from NFA to AMP is unlikely to happen in these conditions. A second reason might be found in the group-specificity of the commercially available AMP-MIPs revealing binding potential for both AMP, other ATS or compounds specific to ATS synthesis with similar structures. In samples of Leuckart 1, which contain NFA but also impurities and side-products, false positives of AMP, caused by one of a combination of those compounds, were also detected. This was also confirmed by a false positive of DPIA and acetophenone (sample 12 and 14, respectively). As shown in Appendix Figure D.1, DPIA and acetophenone have similar chemical structures and were, not surprisingly, also captured by the AMP-MIPs. In real-life conditions, all these components co-exist. Therefore, it can be considered as an advantage of the applied group-specific MIPs.

Leuckart step 1 represents waste of the first step of the Leuckart process, namely the conversion of BMK to NFA. Depending of each synthesis and from barrel to barrel, the percentage of BMK in the waste will change according to the yield of the reaction. Therefore, NFA should be found for sure, with additionally small amounts of BMK (sample 20 and 25). Leuckart step 2 represents waste from the reaction of NFA to AMP and both compounds should be present in this waste. The precursor BMK should already reacted and no longer be present in this waste. However the results show an additionally capacitive drop for BMK (sample 26). Since there is only a small possibility that BMK was still present in this barrel, the positive result is probably caused by the cross-contamination from NFA to the BMK-MIPs. This was not seen as a problem since the presence of NFA was confirmed so the additional BMK result actually confirmed that the Leuckart reaction occurred.

4. Conclusion

AMP, NFA and BMK were selected as possible indicators of the Leuckart synthesis of AMP in sewage water. In our previous work, MIPs towards NFA and BMK were successfully synthesized and validated in tap water. The current study highlights the use of these MIPs in a miniaturized capacitive sensor for multiplex, on-site detection of ATS markers in sewage waste water. The particles were selectively immobilized on gold electrodes by electropolymerization using tyramine as a linker. Due to the selective immobilisation process, it was possible to immobilize different MIPs on one single six-electrode transducer.

A low-cost miniaturized automated flow-injection immunosensor was successfully developed which uses ASIC technology to implement a simple, robust and low-power system able to readout the six electrodes of the miniaturized capacitive sensor. The operating system was tested in real sewage water samples, the filter size for the sample inlet was optimized to 20 micron. In filtered sewage water, the LOD was determined to be 50 μM for AMP and 25 μM for NFA and BMK. Several blind-coded samples were successfully analysed using the developed sensing system. The overall performance was good, the errors were found to be in the extreme low pH range ($\text{pH} < 3$), especially for the NFA-MIPs. The commercially available AMP-MIPs were group specific, and therefore gave some false-positive results for the high concentrations of NFA, acetophenone, and DPIA.

In our opinion, the presented operating system shows a great potential for environmental monitoring of ATS markers using our pioneering work in multiplex electrochemical MIP-based detection.

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Supplementary information

Appendix A. Miniaturized capacitive sensor

Figure A.1. Embedded printed circuit board (PCB).

Figure A.2. Current-cycle principle for the readout of a capacitive biosensor as proposed by Erlandsson et al., 2014.

Appendix B. Batch rebinding study

LC-MS/MS Method

LC-MS/MS analyses were performed with an Acquity UPLC system, coupled to a Waters Xevo[®] TQ-S triple quadrupole mass spectrometer (Waters Technologies, Zellik, Belgium), equipped with an electrospray interface. Chromatographic separation was obtained using an Acquity UPLC[®] HSS T3 (1.8 μm x 2.1 x 100 mm) column (Waters, Milford, MA, USA). The temperature of the column and autosampler was set at 40 °C and 10 °C, respectively. A modified procedure for the gradient elution was established based on Vazquez-Roig et al. (2010), with a flow rate of 0.4 mL/min, an injection volume of 10 μL , resulting in a total run time of 7 minutes. Masslynx and Targetlynx software 4.1 (Waters, Milford, MA, USA) were used for the data acquisition and processing. AMP was found at a retention time of 1.46 min, NFA at 4.80 min, and BMK at 5.20 min. The MS analyses were carried out using multiple reaction monitoring (MRM) with a positive electrospray ionization (ESI+).

Equilibrium experiment and Scatchard analysis for MIP characterization

Five mg MIPs were transferred to Eppendorf tubes, and 1 mL of the template solution (in water) with different target concentrations in a range of 0 to 1000 μM was added in each tube. After mixing and equilibrating, the supernatants were collected and directly analysed by LC-MS/MS. The amount of bound analyte was calculated as the difference between the initial template

concentration and the concentration of the template present in the supernatant after incubation (free analyte concentration). By plotting the concentration of the bound ATS ([B]) in function of the free concentration ([F]), the corresponding binding isotherms were found. In the Scatchard analysis, the concentration of bound/free ATS was plotted in function of the bound concentration, according to equation B.1:

$$\frac{[B]}{[F]} = \frac{B_{MAX} - [B]}{K_D}$$

Where B_{MAX} is the amount of apparent maximum binding sites and K_D the dissociation constant. Figure B.1 shows the Scatchard analysis for the commercially available AMP-MIPs. The K_D can be calculated from the coefficient of the curve and is determined to be 18.11 μM , while the B_{MAX} is 11.6 nmol/mg.

Figure B.1. Scatchard analysis of MIPs towards AMP.

The same procedure was applied on the BMK-MIPs towards BMK. The K_D and B_{MAX} were determined to be 114.4 nmol/mg and 173.1 μM respectively.

Appendix C. Cyclic voltammogram

Figure C.1. Cyclic voltammogram after 5 (pink), 7 (blue), 10 (orange), and 12 (yellow) potential sweeps. The analyses were performed in 10 mM $K_3[Fe(CN)_6]$ with 0.1 M KCl in H_2O , a scan rate of 50 mV/s, and a step potential of 0.001 V in the potential range from 0.250 to 0.700 V. Ag/AgCl was used as a reference electrode, a glassy carbon as auxiliary electrode, and the gold electrode as a working electrode (a). Voltammogram during MIP immobilization for 15 cycles using tyramine as a linker (b).

Appendix D. Identification of false positives

Figure D.1. Chemical structure of amphetamine (AMP, left), acetophenone (middle), and di(6-phenylisopropyl)amine (DPIA, right).

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