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Voltammetric sensing using an array of modified SPCE coupled with machine learning strategies for the improved identification of opioids in presence of cutting agents

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ABSTRACT

This work reports the use of modified screen-printed carbon electrodes (SPCEs) for the identification of three drugs of abuse and two habitual cutting agents, caffeine and paracetamol, combining voltammetric sensing and chemometrics. In order to achieve this goal, codeine, heroin and morphine were subjected to Square Wave Voltammetry (SWV) at pH 7, in order to elucidate their electrochemical fingerprints. The optimized SPCEs electrode array, which have a differentiated response for the three oxidizable compounds, was derived from Carbon, Prussian blue, Cobalt (II) phthalocyanine, Copper (II) oxide, Polypyrrole and Palladium nanoparticles ink-modified carbon electrodes. Finally, Principal Component Analysis (PCA) coupled with Silhouette parameter assessment was used to select the most suitable combination of sensors for identification of drugs of abuse in presence of cutting agents.

1. Introduction

Heroin (3,6-diacetylmorphine, diamorphine, Fig. 1) is a potent synthetic opiate drug synthesized by acetylation of morphine, typically obtained from poppy seeds. The appearance of this illicit drug changes from white (pure form) to dark brown due to impurities formed during the manufacturing process or adulterants and cutting agents. The common impurities come from opium alkaloids or by-products from the fabrication process (morphine, monoacetylmorphine, codeine, acetylcodeine, noscapine, papaverine or lead) [1–2]. Apart from impurities, it is frequent in illegal commerce to adulterate the narcotic with some cuttings agents; these can be pharmacologically inactive, just like sugars or starch or, otherwise, with pharmacological activity such as paracetamol, caffeine, phenobarbital, quinine, clenbuterol, procaine or levamisole, among others [3]. In this work, two mentioned examples were used as cutting agents, paracetamol and caffeine, as certain side-effects are considered for their choice. In the case of paracetamol, it simulates the analgesic effect of heroin; in the case of caffeine, it helps vaporizing heroin at lower temperature, facilitating its smoking.

Morphine (Fig. 1) is the opiate alkaloid which effectively causes disruption in the central nervous system, that is why it is pharmacologically used to relieve pain in patients [4]. The interest of analysis of this compound relies, first, in the monitoring of therapeutic levels in patients. Secondly, the analysis of morphine is relevant for epidemiological purposes of drug abuse control and also in forensic cases; in this field it can be an evidence of heroin usage and can help identifying causes of intoxication or death in situations of clinical and pathological interest. Codeine (3-methylmorphine, Fig. 1) is a second main alkaloid separated from opium. This drug is extensively used to treat mild to moderate pain and for cough suppression in clinical practice. Despite its medical applications, the abuse of this narcotic can also create health risks.

The widespread use of illicit drugs has led to an increase effort toward developing and improving methods for their detection per example in a seizure of a smuggled consignment, or in biological samples, which is still a very challenging task from an analytical point of view. Several analytical methods have been developed for individual determination of these compounds such as capillary electrophoresis [5–6] chemiluminescence [7–8], diffuse reflectance near-infrared spectroscopy [9], high-performance liquid chromatography, gas chromatography [10] and surface plasmon resonance based on immunosensors [11]. Drawbacks associated are the high costs and time-consuming nature of these methods, accompanied by a need of complex procedures such as sample pre-treatment step to obtain satis-

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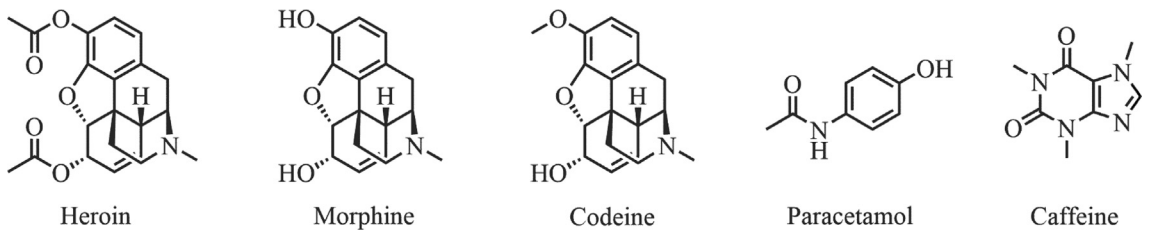

Fig. 1. Chemical structure of the three drugs of abuse considered in this study (heroin, morphine and codeine) and their corresponding cutting agents (paracetamol and caffeine).

factory results. For these reasons, the development of cheap, effective, rapid and simultaneous determination procedures in pharmaceutical and illicit samples is still a big challenge in analytical chemistry. One approach to overcome the above shortcomings can be the use of electrochemical sensors, as most of these drugs of abuse are electroactive substances. Electrochemical sensors may provide some advantages, such as a cheap and simple use, low detection limits, wide linear response ranges, good stability and reproducibility.

Electrochemical methods, in special voltammetry, have been already used for individual determination of opiate alkaloids, because of their advantages in applicability. However, certain difficulties arise when the simultaneous determination of these three compounds is attempted, due to the overlapping of the different voltammograms obtained at traditional electrodes.

In this work, the alternative proposed to tackle the problem is the use of modified electrodes [12], which have fascinated many researchers due to their simplicity, high sensitivity and low cost. This kind of devices provides improvement based on electrocatalysis, avoiding fouling effect and preventing undesirable reactions which can compete kinetically with the desired electrode process [13]. Modified electrodes can be prepared by different techniques based on adsorbing or attaching specific molecules (e.g. peptides [14] or complexing agents [15] to the surface; this may be achieved by self-assembled monolayers [16], chemical grafting, coating and entrapment, e.g. in the form of conductive ink [17]. The last strategy has become interesting in recent times, because the deliberate and controlled modification of the electrode surface can produce new surfaces with interesting properties employed for new devices and applications in electrochemistry. As a further step in electrochemical sensing, a newly, nature-inspired way to proceed, intended to enrich the usable departure information has become popularized: in this, different electrodes may be used in array form, work with them in parallel, and produce advanced applications at insignificant increase of effort or cost [18]. This approach will employ then a set of cross-sensitive, chemical sensors that will provide a complex information-rich response from a sample. This set of complex electrochemical signals generated are next processed using intelligent chemometric algorithms (e.g., machine learning strategies) to allow for the qualitative identification or the quantitative determination of the compounds [19]. As the main character of this work here is the qualitative identification of the compounds considered, firstly, the multivariate data generated was examined using Principal Component Analysis (PCA) [20]; this is normally the first strategy used to visualize similarity between samples. From this PCA analysis, a first grouping of samples could be established and a measure of clustering could be calculated using the Silhouette parameter [21].

The set of SPCE sensors to form the array considered the ensuing six modifiers: Graphite-Ink, Prussian blue-Ink, Cobalt (II) phthalocyanine-Ink, Copper (II) oxide-Ink, Palladium nanoparticles-Ink and Polypyrrole-Ink. In the procedure, these were incorporated one at a time in the array in a step way manner, for which after PCA analysis and calculation of the Silhouette parameter, the improvement of the latter determined the benefits of including the contemplated sensor. In this way, the best combination of electrodes to form the sensor array could be defined from their actual performance in the identification of the opioids and cutting agents. Eventually, some machine learning strategies were tested and their performance examined, choosing finally a K-nearest neighbor classifier (kNN) as the preferred algorithm.

2. Experimental

2.1. Chemical and reagents

Codeine and heroin were provided by the National Institute of Criminalistics and Criminology (NICC, Belgium). Morphine hydrochloride, potassium monophosphate, potassium chloride and potassium hydroxide were purchased from Sigma-Aldrich (Overijse, Belgium). Cobalt (II) phthalocyanine (CoPc), Copper (II) oxide (CuO) nanopowder (<50 nm), Polypyrrole doped (PP) and Palladium, powder submicron 99.9+% (Pd), which were used as modifiers, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Prussian blue (PB) was obtained from Acros Organics (Geel, Belgium). The preparation of the ink composite was done using mesitylene and polystyrene, obtained from Sigma-Aldrich (St. Louis, MO, USA). Graphite powder (particle size < 50 μm) was received from BDH (BDH Laboratory Supplies, Poole, UK). Potassium chloride was purchased from Merck (Darmstadt, Germany).

All aqueous solutions were prepared using MilliQ water (R > 18 $M\Omega \cdot cm^{-1}$). The reagents were of analytical reagent grade and used without further purification. Fresh stock solutions were prepared the same day of the measurements, to reduce day to day variability.

2.2. Instrumentation and apparatus

SWV measurements were performed using a Multi-channel Potentiostat/Galvanostat/Impedance Analyzer (MultiPalmSens4, The Netherlands) controlled by Multitrace software. ItalSens graphite screen-printed electrodes containing a graphite working electrode (3 mm diameter), a carbon counter electrode and a (pseudo)silver reference electrode (PalmSens, The Netherlands) were used for the measurements, as received or after modification.

2.3. Modification of the electrode surface

The material used for the modification of the SPCE is a graphite-based ink-like composite. The corresponding modifier, graphite and polystyrene were thoroughly dispersed with mesitylene for 2 hours [17]. After that, 2 minutes of sonication was performed in order to obtain a medium thick solution. The ink-like composite was dropped (1 μL) onto the surface (Fig. 2) of a graphite SPCE and dried at 40 °C for at least 1 hour in order to remove the solvent and let the SPCE operative.

Fig. 2. Scheme of the experimental procedure for the electrode surface modification. Firstly, an ink-like solution was prepared incorporating the corresponding modifier. Then, 1 μL of ink was dropped on the electrode surface and dried a 40 °C.

2.4. Characterization of the electrodes by scanning electron microscopy

The morphological characterization of the modified SPCE electrodes was performed by Field Emission Gun-Scanning Electron Microscope (FEG-SEM) of Zeiss, model MERLIN SM0087 and Energy Dispersive X-Ray Analysis (EDX). Imaging was performed based on secondary, back-scattered electrons.

2.5. Procedure

Samples were prepared in phosphate buffer 20 mM containing 100 mM KCl (PBS). This media was used as supporting electrolyte for electrochemical measurements; its pH was adjusted to desired value using a 100 mM KOH solution using a CyberScan 510 pH-meter from Eutech Instruments (Landsmeer, The Netherlands) equipped with a HI-1131 glass bodied pH electrode from Hanna Instruments (Bedfordshire, United Kingdom). SWV measurements were performed by placing a volume of 50 μL of sample solution onto the printed part of the SPCE, which was maintained in horizontal position. The technique employed for the determination of the considered substances was Square Wave Voltammetry (SWV). The single scan SWV parameters were as follows: potential range 0 V to 1.2 V, step potential 5 mV, amplitude 25 mV and frequency 10 Hz, as employed in former studies in the laboratory [22].

2.6. Calculation of Silhouette parameter

PCA is a suitable linear visualization method of multivariate data, that allows the reduction of the dimensionality of a multivariate problem and facilitates the visualization of the groupings of the multivariate profiles by remarking similarities and differences between them, forming sample clusters. PCA is very useful to identify these clusters, but it is normally hard to interpret and to validate the grouping. For this reason, the Silhouette calculation [21] was introduced as a measure of clustering, i.e. how easy is to distinguish between the clusters associated to the different compounds. This strategy refers to a method of interpretation and validation of consistency within clusters of data, providing a numerical figure of how well each object matches its cluster.

The Silhouette is based on the calculation of two parameters: a and b. For each sample i, $a(i)$ is the average distance between i and all other samples within the same cluster. In the case of $b(i)$ is the smallest average distance of i to all samples in any other cluster, of which i is not a member. Silhouette parameter is calculated then as:

$$s(i) = \frac{b(i) - a(i)}{max\{a(i), b(i)\}} \quad (1)$$

Which can be also written as:

$$s(i) = \begin{cases} 1 - \frac{a(i)}{b(i)}; if\ a(i) < b(i) \\ 0; if\ a(i) = b(i) \\ \frac{b(i)}{a(i)} - 1; if\ a(i) > b(i) \end{cases} \quad (2)$$

The Silhouette value is a measure of how similar an object to its own cluster (cohesion) compared to other clusters (separation). The Silhouette ranges from -1 to $+1$, where a high value (close to $+1$) indicates that the object is well matched to its own cluster. If most samples have a high Silhouette value, then the clustering configuration is appropriate. If many points have a low or negative value, then the clustering configuration may have too many or too few clusters. The average of the Silhouette parameter for the whole set of samples can then be employed as an index to evaluate the overall clustering ability of the selected sensor configuration, in a procedure to obtain the best identification ability.

2.7. Data treatment

The web page Clustvis [23] was the tool used for online PCA calculation; Sigmaplot (Systat Software Inc., San Jose, CA, USA) was used to graphically represent and analyze the results. Microsoft Excel 2016 and Orange open source programming language (University of Ljubljana, Slovenia) [24–25] were used to perform some Silhouette calculations and to generate the identification models for which K-nearest neighbor classifier (kNN), Random Forest, Naive Bayes and Support Vector Machine (SVM) algorithms were employed and compared [26].

3. Results and discussion

This research depicts an intelligent sensor strategy, in which a given opioid is identified, with the possibility of being confounded by a cutting agent, and where the involved technique used is voltammetry. In short, the strategy combines the use of multiple sensor electrochemistry to extract the fingerprint of each compound on each sensor, followed by advanced data processing for interpreting the multi-dimensional generated data.

The study started defining a procedure for the ink modification of the SPCE with their initial characterization and operation. Next, the sensor array was optimized by step by step systematic evaluation of the incorporation of an additional sensor electrode, till maximum identification ability was achieved. To evaluate this, it was used PCA to visualize clustering of target substances and how easily these could be distinguished, together with calculation of a clustering metrics, the Silhouette parameter, to provide a numeric criterion for the optimization of the best sensor array configuration. Finally, kNN and

additional classifiers were used as pattern recognition model to perform automatic identification of the substances considered in our study case.

3.1. Characterization of the electrode surface

Characterization of the ink-modified working electrodes (WE) employed in this work was done using microscopy studies and electrochemical techniques.

A SEM characterization was performed in order to investigate the spatial distribution of the ink-nanoparticles and to verify if the nanoparticles were all on the external surface or in the inner layers. As can be observed in Fig. 3, the different modifiers are distributed quasi-homogeneously between the graphite layers verifying the presence of the expected metals, on their respective inks through their EDX spectra (Fig. 4); electrodes modified with Polypyrrole or unmodified are not shown in the manuscript because of absence of distinctive metallic signals. However, their spectra can be checked in the supplementary material.

After microscopy studies, the effective surface area of bare and modified electrodes was evaluated according to the Randles–Sevcik equation (eq (3)) [27], where n is the number of transferred electrons for the redox reaction (in this case 1), F is the Faraday's constant (96485 C·mol^{-1}), c the concentration of electroactive substance (mol·cm^{-3}), A is the effective area in cm^2, ν the scan rate (V·s^{-1}), R the gas constant (8.314 J·mol^{-1}·K^{-1}), T the temperature in K and D is the diffusion coefficient for ferrocyanide (6.32·10^{-6} cm^2·s^{-1}). For that, CV experiments using 20 mM KH_2PO_4 and 100 mM KCl containing 5 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ solution in the potential range of −0.4 to 0.8 V were performed. Applying 7 different scan rates (0.01, 0.025, 0.05, 0.1, 0.2, 0.3 and 0.5 V·s^{-1}) it could be calculated the active area of each WE from the slope of the regression line of $\nu^{1/2}$ (V·s^{-1}) vs. $Ip \cdot c^{-1}$ (A·cm^3·mol^{-1}). The details of the performed voltammograms can be found in Supplementary Info. Concerning the calculated active areas, these were: 11.7 mm^2 for the bare electrode, 8.2 mm^2 for Graphite/SPCE-Ink, 8.5 mm^2 for Copper (II) oxide/SPCE-Ink and 8.1 mm^2 for Prussian blue/SPCE-Ink. 9.3 mm^2 for Cobalt (II) phthalocyanine/SPCE-Ink, 9.4 mm^2 for Pd nanoparticles/SPCE-Ink and 6.1 mm^2 for Polypyrrole/SPCE-Ink, whereas the geometric area was 7.1 mm^2 (Ø = 3 mm).

$$Ip = 0.4463 \cdot n \cdot F \cdot c \cdot A \cdot \sqrt{\nu} \cdot \left(\frac{nDF}{RT}\right)^{\frac{1}{2}} \quad (3)$$

Summarizing, this section verifies the proper modification of the electrodes via ink-deposit, showing a quasi-homogeneous distribution of the metal nanoparticles between the graphite particles, and yielding the different prepared electrodes comparable active surfaces.

3.2. Electrochemical response

The voltammetric responses for each of the modified screen-printed electrodes were first evaluated towards the five individual compounds (three drugs and two cutting agents), to assure that the generated signals were different enough for the desired application.

As a result, and as described under conditions in Section 2.5, individual stock solutions of 300 μmol·L^{-1} of heroin, morphine, codeine, paracetamol and caffeine in PBS at pH 7 were determined using SWV. It was decided to perform the electrochemical measurements at neutral pH because heroin and morphine undergo some hydrolysis reactions at alkaline pH [3,28]. Measurements were done in random sequence to avoid any structure in the signals.

Heroin gives rise to an irreversible oxidation split peak at +0.81 V on SPCE at pH 7 due to the oxidation of the amino group, which is in concordance with the literature [4,28–30]. An additional oxidation peak was observed at a lower potential +0.40 V due to the oxidation of the phenol group of 6-monoacetylmorphine (6-MAM) a trace constituent present in the sample (typical content, 3 wt%). In detail, 6-MAM is an impurity from heroin synthesis, resulting in the incomplete acetylation of morphine and also a product of hydrolysis of the alkaloid present in most heroin samples [1]. As can be seen in Fig. 5, the peak corresponding to the oxidation of the phenol group of 6-MAM and morphine is overlapped with the oxidation peak of paracetamol. The same situation occurs between the second oxidation peaks of heroin and morphine with codeine, foreseeing a case with difficult signal resolution.

As shown in Fig. 5, complex and highly overlapped signals are obtained along the whole voltammograms and with the different sensors considered. The fingerprint of each compound from a single sensor is not enough information, which can represent a limitation for the identification of the considered substances alone. For better assess-

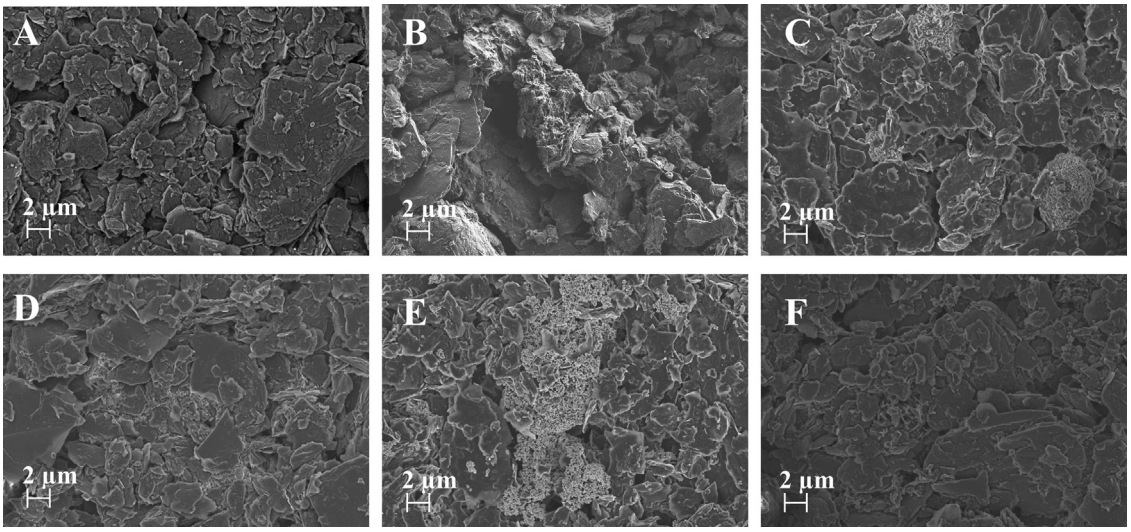

Fig. 3. SEM characterization of (A) Graphite/SPCE-Ink, (B) Cobalt (II) phthalocyanine/SPCE-Ink, (C) Copper (II) oxide/SPCE- Ink, (D) Prussian blue/SPCE-Ink (E) Pd nanoparticles/SPCE-Ink and (F) Polypyrrole/SPCE-Ink.

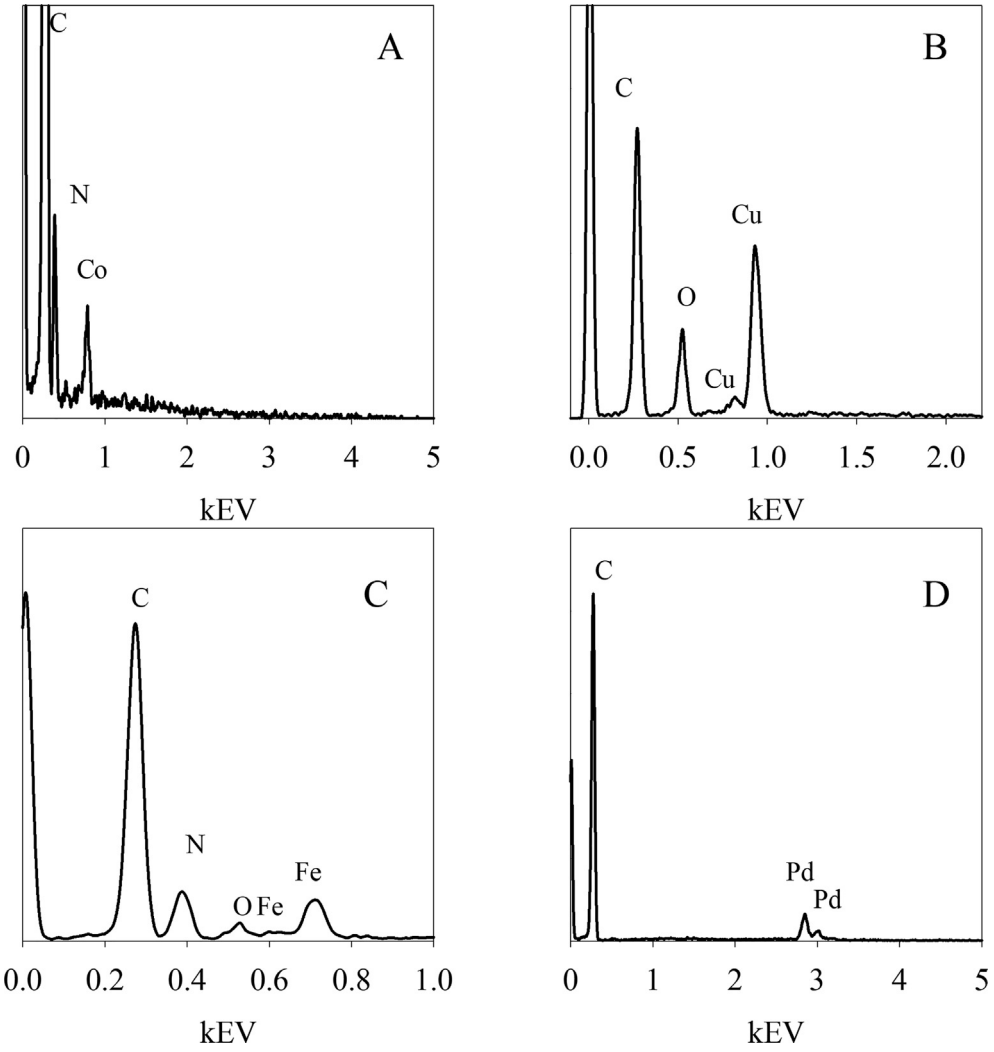

Fig. 4. EDX analysis of (A) Cobalt (II) phthalocyanine/SPCE-Ink, (B) Copper oxide (II)/SPCE-Ink, (C) Prussian blue/SPCE-Ink and (D) Pd nanoparticles/SPCE-Ink.

ment, a chemometric assay was further done: PCA was carried out to better evaluate mathematically the similarities and the complementarities between the voltammetric responses of the compounds of interest.

3.3. Selection of the working electrodes using Principal ComponentAnalysis strategies

Once the voltammograms of the five compounds of interest under study were collected with the six modified screen-printed electrodes, a PCA was performed. Each sample was measured in four replicates to check any drift or memory effect in electrodes. The complete set of samples were measured in random order, to roll out any structure in the data. The information gathered in this case to perform the mathematical calculation was one voltammogram per each sample, and per each electrode, and strategy was to check for similarities and differences among these. With PCA strategy it is expected that the redundant electrodes (electrodes that contribute with the same information) would appear superimposed in the scores space, while electrodes with different responses will manifest in distinction in it. Moreover, this strategy may allow to detect if electrodes can discriminate the studied compounds and how similar are the replicates of one sample.

The scores of samples corresponding to the two first principal components (PC) (the coordinates of each sample/electrode combination in the new space defined by the transformation defined for the PCA) for the five compounds of interest are represented in Fig. 6. In Fig. 6A, it can be seen that the major part of the variability among the samples, the most relevant information is explained for PC1 (81.1%). In this plot, it is clear to observe that Polypyrrole (PP) dominated the response in comparison with the other modifiers, also with a high dispersion for the replicas, distorting all the system. This argument was applied to discard it from the set of modified electrodes. The discrepancies in the voltammograms for this electrode can be also observed in Fig. 5D. In there, the voltammogram from the PP modified ink did not show very different shapes for the different compounds under study, on the contrary, a high non-specific variability, e.g. the baseline was observed. For all the mentioned reasons, a refinement of the calculation was performed removing the PP modified electrode to evaluate the rest of candidates. The results are shown in Fig. 6B. As it can be seen, the relevant information of the samples using the two first PCs made 60.7% of the total variability. Regarding to this plot, it is possible to notice that the purple sensor samples, which correspond to the Prussian blue (PB) modifier, presented large dispersion in comparison with the others, mainly a lack of stability in the voltammetric responses. Because of this inconsistence, the Prussian blue modifier was also discarded from the system. These undesired features can be observed in the different elongated purple clusters (symbol shape replicas) shown in Fig. 6B.

At this point, a new PCA was calculated with the remaining four modifier inks: Carbon, Cobalt phthalocyanine (II), Copper (II) oxide and Pd nanoparticles (Fig. 7A). Applying the previous criteria commented for the PP and PB modifiers, it was decided to remove

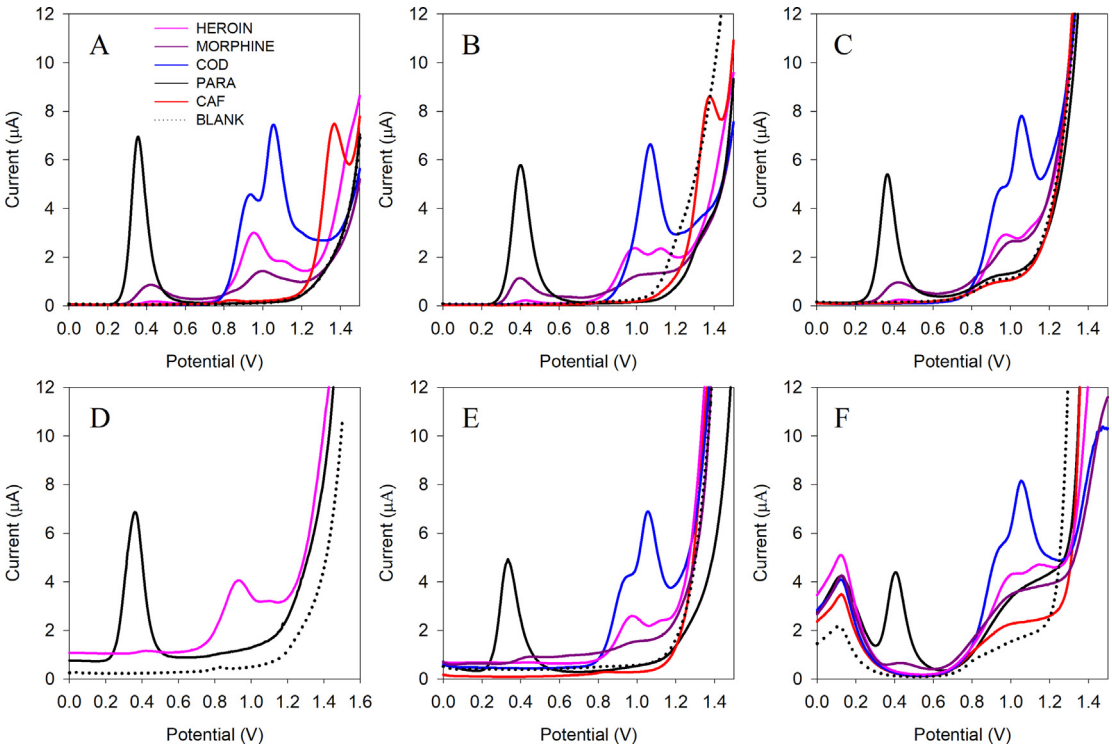

Fig. 5. Voltammetric response for Heroin (pink), Morphine (purple), Codeine (blue), Paracetamol (black) and Caffeine (red) using the six modified electrodes. (A) Graphite/SPCE-Ink; (B) Cobalt (II) phthalocyanine/SPCE-Ink; (C) Pd nanoparticles/SPCE-Ink; (D) Polypyrrole/SPCE-Ink; (E) Copper (II) oxide/SPCE-Ink; (F) Prussian blue/SPCE-Ink. SWV measurements were performed by placing 50 µL solution onto SPCE. The single scan SWV parameters were as follows: potential range 0 V to 1.2 V, step potential 5 mV, amplitude 25 mV and frequency 10 Hz. The scan rate was 50 $mV{\cdot}s^{-1}$. A 300 $\mu mol{\cdot}L^{-1}$ individual solution was employed for the six modified screen-printed electrodes.

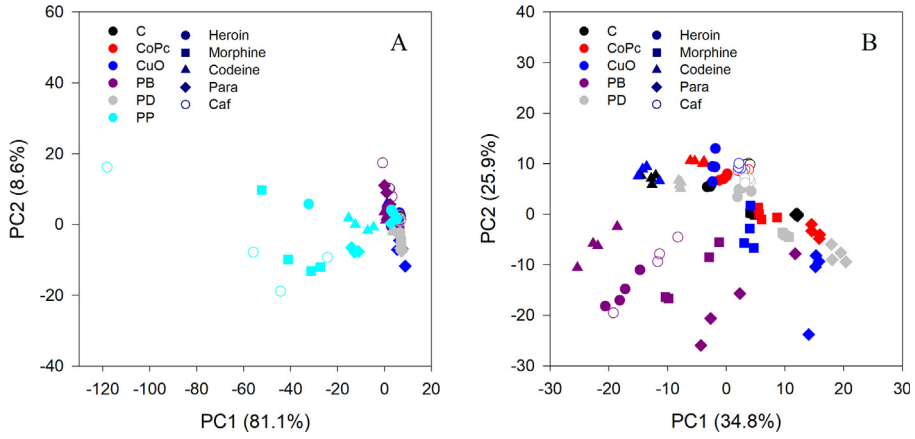

Fig. 6. Score plot of the two components obtained after PCA analysis. 4 replicates for each sensor were done determining the five compounds of interest: heroin, morphine, codeine, paracetamol and caffeine with a concentration of 300 $\mu mol{\cdot}L^{-1}$. (A) Use of an array with six SPCEs: Graphite/SPCE-Ink; Cobalt (II) phthalocyanine/SPCE-Ink; Copper oxide (II)/SPCE-Ink; Prussian blue/SPCE-Ink; Pd nanoparticles/SPCE-Ink and Polypyrrole/SPCE-Ink. (B) Use of the optimized sensor array: Graphite/SPCE-Ink; Cobalt (II) phthalocyanine/SPCE-Ink; Copper oxide (II)/SPCE-Ink; Prussian blue/SPCE-Ink and Pd nanoparticles/SPCE-Ink.

the Copper (II) oxide electrode from the sensor array. This sensor presented some drift among the four replicates of almost all the studied compounds (like PP and PB), causing a distortion in the clusters of the pure compounds. But at this point, the PCA strategy was able to differentiate clearly the cutting agents and the drugs of abuse (see how the symbols group together in Fig. 7A and 7B. Finally, the last PCA with the three modifiers selected is shown in Fig. 7B. As it can be observed, these candidates showed different response towards the studied molecules, and with limited dispersion, facilitating the assignment of substances to its class.

3.4. Optimization of the sensor array from the Silhouette parameter

Once a first assessment of sensors was done, the final optimization was developed, in this case with use of an objective numeric criteria. This second part consisted to determine which combination of the selected sensors is more suited to obtain the best performance in the identification of the studied compounds. To reply this question, a new strategy, which was the calculation of the Silhouette parameter as a measure of the clustering degree was applied for the first time in our group to deal with the problem. And from this point here, the

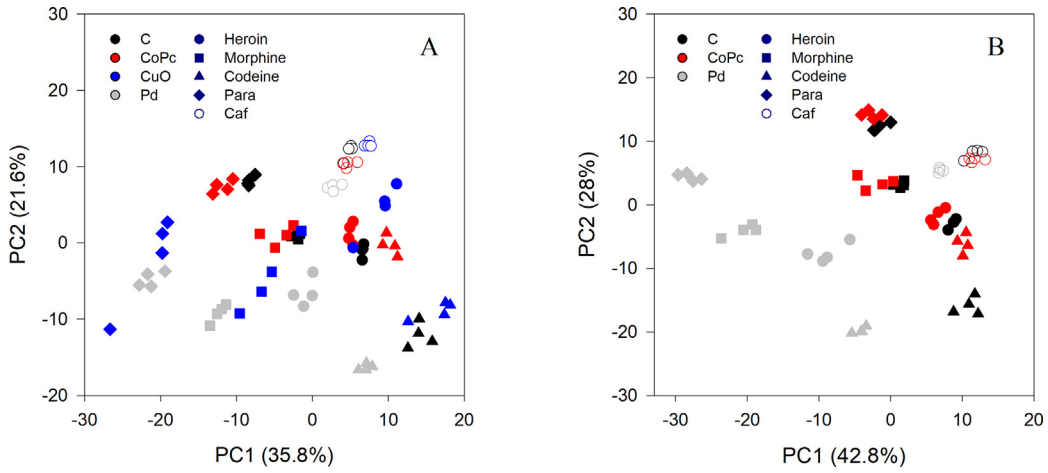

Fig. 7. Score plot of the two components obtained after PCA analysis. 4 replicates for each sensor were done determining the five compounds of interest: heroin, morphine, codeine, paracetamol and caffeine with a concentration of 300 μmol·L^{-1}. (A) With the 4 SPCE array: Graphite/SPCE-Ink; Cobalt (II) phthalocyanine/SPCE-Ink; Pd nanoparticles/SPCE-Ink; Copper (II) oxide/SPCE-Ink. (B) With the 3 SPCE array: Graphite/SPCE-Ink; Cobalt (II) phthalocyanine/SPCE-Ink and Pd nanoparticles/SPCE-Ink.

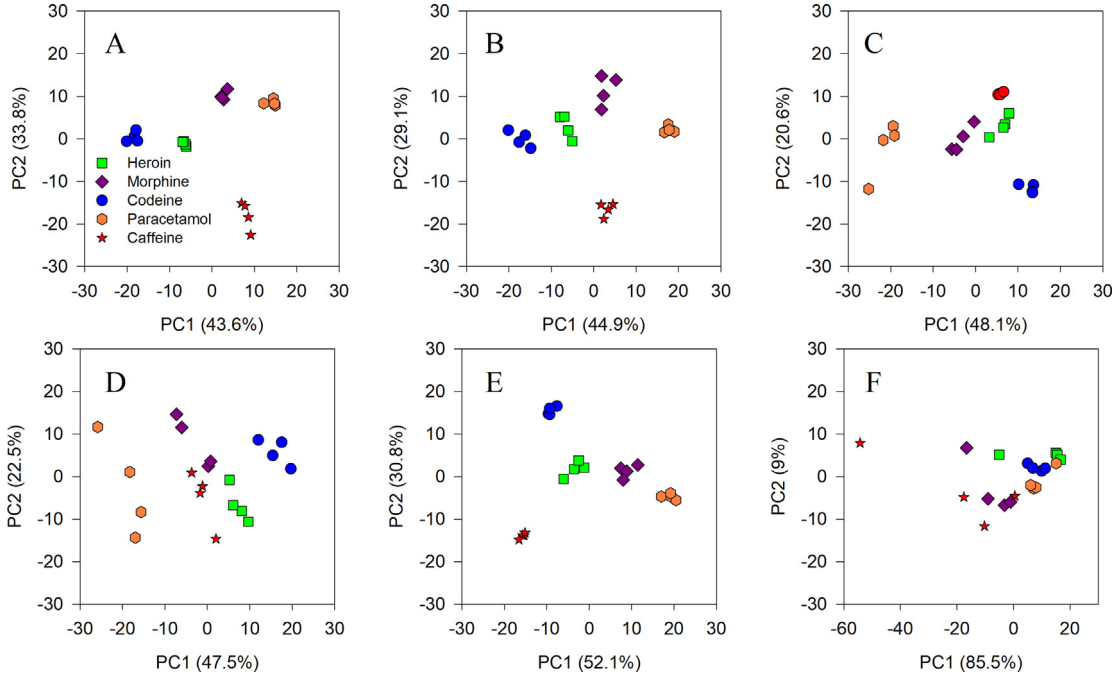

Fig. 8. Score plot of the two first components obtained after PCA analysis of information provided by each single SPCE electrode. A total of 20 samples were analyzed corresponding to quadruplicate determination of 300 μmol·L^{-1} of heroin, morphine, codeine, paracetamol and caffeine, using: (A)Graphite/SPCE-Ink, (B) Cobalt (II) phthalocyanine/SPCE-Ink, (C) Copper (II) oxide/SPCE-Ink, (D) Prussian blue/SPCE-Ink, (E) Pd nanoparticles/SPCE-Ink and (F) Polypyrrole/SPCE-Ink.

complete set of voltammograms from a given sample, as determined by the specific sensors forming the array, was used for the data processing. The first step for this was to do the unfolding of the data, i.e. the different voltammograms corresponding to a sample were concatenated in a unique column and used as available information for the processing. This is the usual way to cope with this limitation of PCA, which works vectorially, not matrix-like [31]. Alternatively, there are *N*-way alternatives to this treatment, but are much less spread in the field, and are of more difficult use [32].

With this multiple information approach, a first PCA analysis was done, which performed an initial, unsupervised clustering of the data according to their similarity in the multivariate space. Next, the Silhouette parameter was calculated to assess the goodness of the accomplished clustering, whereas a better clustering will embrace an easier identification of a given sample. In fact, the Silhouette calculation provides a parameter for each sample, based on *a(i)* and *b(i)*, which shows the intra-cluster compared with the inter-cluster variability. In this way, it is possible to quantify numerically which cluster is better

Table 1
Average of Silhouette parameter for the stepwise optimization of the sensor array.

Number of SPCE in the array	Modified SPCE in the array	Silhouette parameter
1	C	+0.849*
	CoPc	+0.735
	CuO	+0.640
	PB	+0.328
	Pd	+0.817
	PP	+0.041
2	C-CoPc	+0.841
	C-Pd	+0.863*
	CoPc-Pd	+0.848
3	C-CoPc-Pd	+0.877*

* Optimal configuration obtained after systematic evaluation on each step.

discriminated in comparison with all the clusters involved in the system. It is important to highlight that the data collected to perform this kind of analysis were the scores of the two first principal components (PC1 and PC2) obtained previously in the PCA score graphs from the unfolded voltammograms data. This treatment is useful to reduce the dimensionality in the case of voltammetric data; essentially it just transforms the voltammograms from a multidimensional matrix to a 2D matrix with condensed and simplified info.

Therefore, the procedure to afford this case was the calculation of the Silhouette parameter for different situations, in the stepwise strategy for the optimization of the sensor array. Firstly, the calculation was done for each sensor individually, that is, from the six sensors prepared initially.

As it can be observed in PCA score plots on Fig. 8, the sensors which produced worst clustering were CuO, PB and PP. This fact can be verified with the calculation of the Silhouette parameter, as summarized in Table 1. In this case, the three electrodes mentioned presented the worst Silhouette parameter with values of +0.640 for CuO, +0.328 for PB and +0.041 for PP. This first assessment, showing that best option with a single sensor is using the SPCE with graphite ink, yielded an average Silhouette parameter of +0.849. With this information, it is important to remark that with any of the three sensors alone (C, CoPc and Pd) the identification application could be carried out, since the Silhouette parameter can be considered acceptable. However, our primary objective is to complement the information from different sensors in an electronic tongue approach to improve final performance and reach a better degree of clustering. This may be of help in scenarios with unfavorable S/N ratio, as would be the case with lower concentration of the species sought, or with additional interference effects. Therefore, in the stepwise process, a second electrode is incorporated in the array, and the Silhouette parameter is calculated to provide the best combination of sensors from the set C, CoPc and Pd.

The results of these combinations are collected in Fig. 9 . Apparently, the visualization of the clusters for the different compounds in the three cases is quite good, so the crucial argument to decide which combination is the most suitable for the case of study is the Silhouette parameter. As it can be observed in Fig. 9, the global clustering for the three cases is largely similar, showing the best combination for the couple C + Pd with a calculated s of +0.863 (Fig. 9B). To finally take the decision, the last possibility combining the three modified sensors was evaluated. The PCA obtained for the combination using three SPCE sensors, and s = +0.877 is shown in Fig. 10B.

As a result, it can be concluded that the use of the combination of the modifying inks with these three modifiers allowed the optimal individual determination of the compounds under study. As it can be observed in Fig. 10B, the different sensors proportionated the particular response toward the individual compounds, showing five clusters, with close grouping, and clear differentiation. The PCA obtained is appropriate, representing the relevant information between PC1 and PC2 with a variance of 76.5%. The average Silhouette parameter finally obtained, +0.877 is a high value, and close to the highest attainable value, +1.00, forecasting an easy identification in the final 'intelligent' identification of the selected compounds. It is also clear that the information provided by the combination of the three modifiers previously commented is very similar to the combination of graphite and Pd nanoparticles with a Silhouette global parameter of +0.863, and a simpler setup of only two sensors in the array. For future applications, it was decided to maintain Pd in the sensor array in order to collect the information it provided. As it can be seen in Fig. 7B, the electrochemical response proportionated by this modifier sensor supplied differentiated response (grey points in a rather separate region) in comparison with the remaining working electrodes. The performed analysis would be also a base criterion to ascertain if the application can be performed with just a single sensor (the one with graphite ink, in this case), or the complexity involved in the use of a sensor array balances the obtained gains. Lastly, the use of CoPc alone was completely discarded exhibiting a value of Silhouette global parameter of +0.735.

3.5. K-nearest neighbor classifier

To conclude the final section of this study, a kNN classifier method was used to perform the final automated and intelligent operation. KNN [33] is one of the most fundamental and simple unsupervised classification methods and should be one of the first choices for a classification study when there is little or no prior knowledge about the distribution of the data. KNN just stores all the available cases and classifies the new data or case based on a similarity measure. The only parameter to tune is the number of closest neighbors to consider (the variable k) which can be obtained examining which is the best perfor-

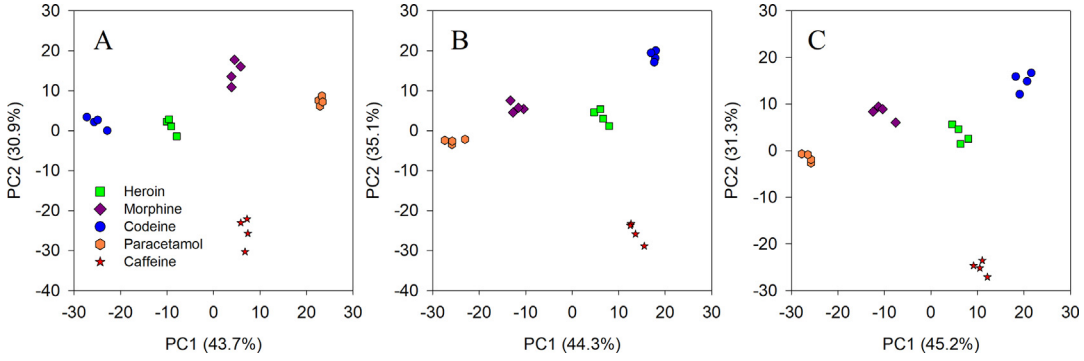

Fig. 9. Score plot of the two first components obtained after PCA analysis. A total of 20 samples were analyzed corresponding to quadruplicate determination of 300 μmol·L^{-1} of heroin, morphine, codeine, paracetamol and caffeine, with pair of SPCE electrodes: (A) C-CoPc, (B) C-Pd (C) CoPc-Pd.

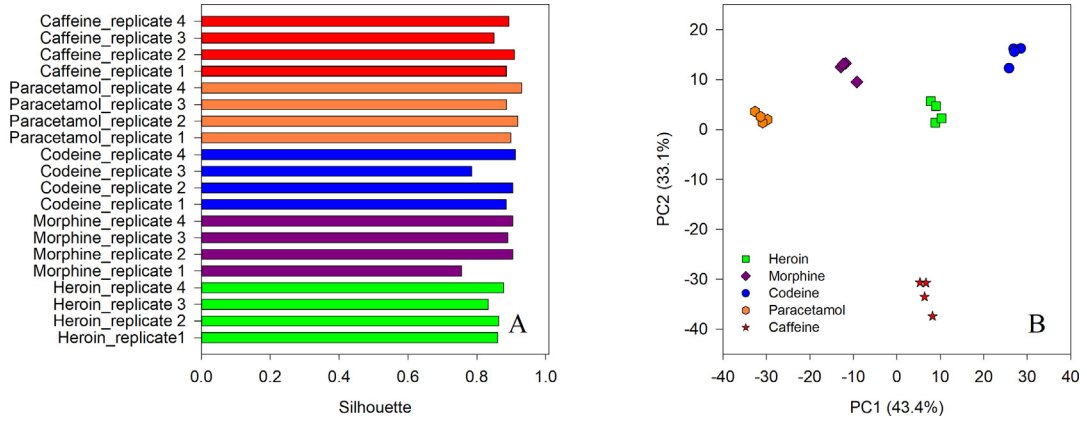

Fig. 10. (A) Silhouette plot for the different samples considered using the best combination of three SPCE sensors (C-CoPc-Pd). (B) Score plot of the two components obtained after PCA analysis. A total of 20 samples were analysed corresponding to quadruplicate determination of 300 μmol·L^{-1} of heroin, morphine, codeine, paracetamol and caffeine using the three SPCE sensors.

Table 2
Confusion matrix after applying the kNN algorithm, using leave-one-out cross-validation and k = 4.

	Predicted						
Actual		Heroin	Morphine	Codeine	Paracetamol	Caffeine	**Σ**
	Heroin	4	0	0	0	0	**4**
	Morphine	0	4	0	0	0	**4**
	Codeine	0	0	4	0	0	**4**
	Paracetamol	0	0	0	4	0	**4**
	Caffeine	0	0	0	0	4	**4**
	Σ	**4**	**4**	**4**	**4**	**4**	**20**

Table 3
Results of the statistical calculation using some machine learning strategies as kNN (k = 4), Forest, Naive Bayes and SVM employing leave-one-out cross-validation.

Model	Compound	Classification accuracy	Precision	Sensitivity	Specificity
kNN	Heroin	1.0	1.0	1.0	1.0
	Morphine	1.0	1.0	1.0	1.0
	Codeine	1.0	1.0	1.0	1.0
	Paracetamol	1.0	1.0	1.0	1.0
	Caffeine	1.0	1.0	1.0	1.0
Random Forest	Heroin	1.0	1.0	1.0	1.0
	Morphine	1.0	1.0	1.0	1.0
	Codeine	1.0	1.0	1.0	1.0
	Paracetamol	1.0	1.0	1.0	1.0
	Caffeine	1.0	1.0	1.0	1.0
Naive Bayes	Heroin	1.0	1.0	1.0	1.0
	Morphine	1.0	1.0	1.0	1.0
	Codeine	1.0	1.0	1.0	1.0
	Paracetamol	1.0	1.0	1.0	1.0
	Caffeine	1.0	1.0	1.0	1.0

mance when k is varied. In this particular case, the number of integrands in the cluster is known beforehand, as it corresponds to the number of replicas of each substance tested, k = 4.

Table 2 shows the confusion matrix of the identification accomplished. Cross validation of the identification model was done with the leave one out variant, as there were not many samples in the data set. With these excellent identification performance, statistic indicators of goodness of identification were also excellent in all instances, with indicators of classification accuracy, precision, sensitivity and specificity, all 100%. Additional machine learning strategies were tested in order to compare the obtained results. The identification algorithms tested were Random Forest, Naive Bayes and Support Vector Machines (SVM). As it can be observed in Table 3, Random Forest and Naive Bayes produce proper results in all the indicators previously mentioned. In the case of SVM, certain degree of misclassification of particular samples is observed, specifically heroin and horphine. In other words, the vast majority of algorithms employed demonstrated a correct identification of the samples, thanks to the excellent degree of clustering achieved by the optimized sensor system.

4. Conclusions

The presented work reports for a first time the qualitative analysis for the determination of the following drugs of abuse: heroin, morphine and codeine and their corresponding cutting agents (caffeine

and paracetamol) combining the use of modified screen-printed electrodes with chemometrics tools, in what has been named a multisensory analysis system or electronic tongue approach.

The samples were analyzed by SWV technique for extracting the fingerprint of the individual substances, coupled with advanced data treatment such as PCA and Silhouette parameter calculation. The use of PCA allowed firstly the pre-selection of the best sensors to define the candidates for the sensor array and secondly, after calculation of the Silhouette parameter, permitted its accurate optimization, showing the most suitable combination of working electrodes. Thanks to the application of both tools, the final combination selected was with electrodes modified with Graphite, Cobalt (II) phthalocyanine and Pd nanoparticle inks. With the optimized sensor array, different identification models were tested demonstrating that kNN could be easily developed, and showing performance among the best.

The reported work demonstrates the advantages of the modification through an ink-like solution composite of screen-printed electrochemical sensors for on-field analysis results in a promising methodology that could substitute the classical time-consuming methods. Future works are directed to equivalent case studies, but with quantification purposes of arbitrary mixtures of opioids and cutting agents.

CRediT authorship contribution statement

Dionisia Ortiz-Aguayo: Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing – original draft, Software. **Karolien De Wael:** Formal analysis, Funding acquisition. **Manel del Valle:** Conceptualization, Methodology, Software, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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