



A pencil-lead bismuth film electrode and chemometric tools for simultaneous determination of heavy metals in propolis samples



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ABSTRACT

A simultaneous determination of Zn, Cd, Pb and Cu in raw propolis samples, employing square wave anodic stripping voltammetry and chemometric tools, was developed. For this purpose, a pencil-lead bismuth film electrode was used as a working electrode. In order to optimize the parameters of square wave voltammetry a Box–Behnken design combined with desirability function was used. Different algorithms such Asymmetric Least Squares (AsLS) and Correlation Optimized Warping (COW) were employed in order to preprocess the raw data. Partial Least Squares (PLS) and Artificial Neuron Networks (ANN) were used to predict the concentration of the four metals in the samples. The method was tested successfully on raw propolis samples obtained from different localities of the Province of Buenos Aires, Argentina and the results were validated with recovery assays.

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1. Introduction

Argentina has extensive geographic areas sharply differentiated in terms of agricultural development, which makes possible the location of important apiaries. Among hive products, propolis deserves special attention. Propolis is a chemically complex product collected by bees from different plants and trees in their flight area. It is mainly composed for resins, waxes, balsams, essential oils, pollen, minerals, and mechanical impurities in different proportions depending on harvest time, as well as the kind of plant resin and the race of bees. A broad spectrum of biological activities for propolis has been discovered and investigated, such as anticancer, antioxidant, anti-inflammatory, antibiotic, antimicrobial, immunomodulatory, and hepatoprotective properties [1]. Mineral propolis composition is affected by the different types of soil, bee flora and climate and at the same time by the environmental pollution. Therefore, this leads to a diversity of mineral composition of propolis [2]. Within essential minerals this product includes zinc and copper [3]. These elements are required for a variety of biomolecules to maintain the normal structure, function, and proliferation of cells. On the other hand, these metals can be toxic in excessive amounts, causing certain genetic disorders [4]. The presence of toxic metals in this type of products comes from environmental pollution by heavy traffic, factories and industrial plants and produce toxic effects in humans [2]. Lead content in propolis may be due to atmospheric pollution or caused by the different harvest, extraction and processing methods [5]. Air pollution

from industry contaminates propolis with cadmium [6]. For this reason, bee products including propolis are considered as bioindicators of environmental pollution [7–9].

Propolis intake can provide significant levels of metals, thus in the literature there are many authors that determined their content in this product. Sales et al. employed the electrothermal atomic absorption spectrometry technique (ET-AAS) to determine lead in propolis samples [5]. Korn et al. evaluates two digestion procedures to determine inorganic elements such as Ba, Ca, Cd, Cr, Co, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, and Zn in Brazilian propolis by using inductively coupled plasma optical emission spectrometry [3]. On the other hand, Neutron Activation Analysis was used to classify Argentinean propolis from eight trace element content [10]. Gong et al. used an inductively coupled plasma atomic emission spectrometer to determine fifteen elements in thirty-two Chinese propolis samples [2]. The major disadvantage of these methods is that these require expensive instrumentation. On the other hand, electrochemical techniques have not been commonly used for determining metals in propolis samples. In our previous work, the square wave anodic stripping voltammetry method with bismuth electrodes to determine lead in propolis samples was employed [11]. The bismuth electrodes are a good alternative to replace the often used mercury electrodes since bismuth is considered a “green” element, with very low toxicity. However, this method involves a glassy carbon electrode modified with Nafion and multi-walled carbon nanotubes. Thus, the development of electrochemical methods with low cost and simple electrodes to determine several metals in these complex samples and that takes into account intermetallic interaction must be of interest. Low cost electrode as a pencil lead has been used as a working electrode to determine dopamine and uric acid [12], paclitaxel drug [13], and

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hydrazine [14]. Voulgaropoulos et al. used a Nafion-covered pencil-lead bismuth film electrode for cadmium, lead and zinc determination with anodic stripping voltammetry. However the method was applied to determine lead and zinc in a simple matrix as tap water [15].

In this paper the simultaneous determination of zinc, cadmium, lead and cooper in a chemically complex matrix as raw propolis from Argentina, was carried out. This sample is widely employed in natural therapeutic products for human use. For this proposed a square wave anodic stripping voltammetry method assisted by chemometric tools which consider the intermetallic interaction was developed.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical grade and ultra pure water (18.3 M Ω , Barnstead, Dubuque, USA) was used. Bismuth (III) standard stock solution was prepared by dissolving an appropriate amount of Bi (NO₃)₃·5H₂O (99.999% Sigma-Aldrich) in 5 mL of 20% (v/v) nitric acid and taken up to 25.0 mL with water. Pb(II), Cd(II), Cu(II) and Zn(II) working solutions were obtained by diluting the corresponding atomic absorption standard solution (MERCK). The supporting electrolyte was 0.1 M acetate buffer (pH 4.50) prepared by mixing the appropriate amounts of acetic acid and sodium acetate (Sigma). All the experiments were carried out at room temperature and without removing oxygen.

2.2. Propolis samples

The samples were collected from beehives of the Province of Buenos Aires in the period from September to March (spring–summer in Argentina), by employing the method of plastic mesh. These meshes were placed in different hives during the period mentioned above. Then, the propolis is extracted from the plastic mesh and impurities were removed. Then, 8.0 g of propolis was placed in a furnace at 400 ± 25 °C (scan heat 10 °C min⁻¹) until constant weight is achieved. The ashes were treated in HNO₃ 10% (v/v) and then filtered through a Whatman N° 42 filter paper to eliminate the carbonaceous residue. Finally, dilute to 25.0 mL with water.

2.3. Instrumentation and software

All measurements were performed using an Epsilon potentiostat (BASi-Bioanalytical System, USA) and run with electrochemical analysis software. A Ag/AgCl (3 M NaCl) and platinum foil as reference and counter electrodes respectively, were used. A pencil-lead, Faber-Castell HB 0.7 mm purchased from a local bookstore was used as a working electrode (PLWE). A RDE-2 Rotating Disk Electrode (BASi-Bioanalytical System, USA) device, coupled to the potentiostat, was employed with a laboratory-made holder of the PLWE. Partial Least Square (PLS) and Artificial Neural Networks (ANN) calculations were performed using MATLAB®, Version 5.3 high-level programming language. The UNSCRAMBLER® chemometrics software (CAMO A/S), Version 9.5, was used for PLS calculations.

2.4. Electrode rotating PLWE design

A laboratory-made holder for the pencil-lead to be assembled in the RDE-2 device was designed. This holder was built with a polytetrafluoroethylene (PTFE) tube which offers the following features: 3 cm length, a central hole with 0.7 mm diameter and a screw thread in the top of the tube to be assembled in the device. A section of 2.5 cm of pencil-lead was introduced in the hole leaving an exposed area of 1 cm of length (geometric area 0.228 cm²). This PLWE was polished with waterproof sandpaper and then was introduced in HNO₃ 20% (v/v) solution for 5 min to remove the plastic coating of the pencil-lead [15]. Successive voltammograms between -1.40 V and

0.50 V with a scan rate of 0.100 V s⁻¹ at pH 4.50 acetate buffer solution were registered in order to stabilize the surface of the electrode (Fig. 1).

2.5. Procedure for square wave anodic stripping voltammetry measurements (SWASV)

Square wave anodic stripping voltammetry measurements (SWASV) were performed by in situ deposition of bismuth. Voltammetric measurements were carried out using an electrochemical cell made up of three electrodes immersed in a solution containing 25 mL of 0.1 M acetate buffer (pH 4.50), 1000 $\mu\text{g L}^{-1}$ of Bi (III) and the studied analytes. The experiments were performed with a deposition potential of -1.4 V for 120 s and the working electrode rotation rate was kept at 8000 rpm. Following the deposition step, rotation of the working electrode was stopped and after 10 s of equilibration time, the voltammograms were recorder by applying a positive-going square wave voltammetric potential scan from -1.4 V to 0.5 V. Prior to each measurement, a 30 s conditioning step at 0.5 V with 8000 rpm of rotation rate was used in order to clean the electrode surface. Optimal preconcentration parameters as bismuth concentration, supporting electrolyte, deposition time and deposition potential have been established in previous studies [11,15–17]. Consistent with our previous work [11], in order to select the correct range of each parameter for the square wave voltammetry, an experimental design was development. A three-level three-factor Box-Behnken experimental design was applied to determine the best voltammetric response of 100 $\mu\text{g L}^{-1}$ Zn, 10 $\mu\text{g L}^{-1}$ Cd, 40 $\mu\text{g L}^{-1}$ Pb and 30 $\mu\text{g L}^{-1}$ Cu. These concentrations are within the range of each metal study. The studied factors were as follows: step potential (ΔE_s), frequency (f) and amplitude of the square wave (ΔE_{sw}). The levels to each factor are shown in Table 1.

Considering that the best voltammetry response for the four analytes should be obtained, the desirability function was used. The general approach of desirability function is to transform each response into an individual desirability function (d_i) that varies from 0 to 1 (lowest desirability to highest desirability) [18]. Then the design variables are chosen to maximize the overall desirability as:

$$D = (d_1 \times d_2 \times d_3 \dots \times d_n)^{1/n}$$

where n is the number of responses in the experiment to optimization. For simultaneous optimization each response must have a low and high value assigned to each goal. The meanings of the goal parameters are:

- $d_i = 0$ if response < low value.
- $0 \leq d_i \leq 1$ if response is between the low to high values.
- $d_i = 1$ if response > high value.

d_i is estimated for each analyte in function of the value of net current peak and then a desirability total function is calculated.

2.6. Calibration and validation sets

A calibration set was designed according to a fractional factorial design at several levels. This calibration set involves a series of four component mixtures at four concentration levels. A training set of sixteen standard solutions was prepared in the concentration range of 80 to 380 $\mu\text{g L}^{-1}$ for Zn, 5 to 11 $\mu\text{g L}^{-1}$ for Cd, 20 to 65 $\mu\text{g L}^{-1}$ for Pb and 30 to 60 $\mu\text{g L}^{-1}$ for Cu (Table 2). These values were established taking into account the concentration of the analytes in the samples.

The model is validated by using leave-one-out cross-validation when Partial Least Squares (PLS) was employed. The performance of the multivariate calibration models was evaluated in terms of the root mean square error of prediction (RMSEP) for a set of samples which were not employed in the model-building process (external prediction set). A validation set of 12 synthetic mixtures containing the four analytes (Zn, Cd, Pb, Cu), with concentrations that are included in the calibration set was studied.

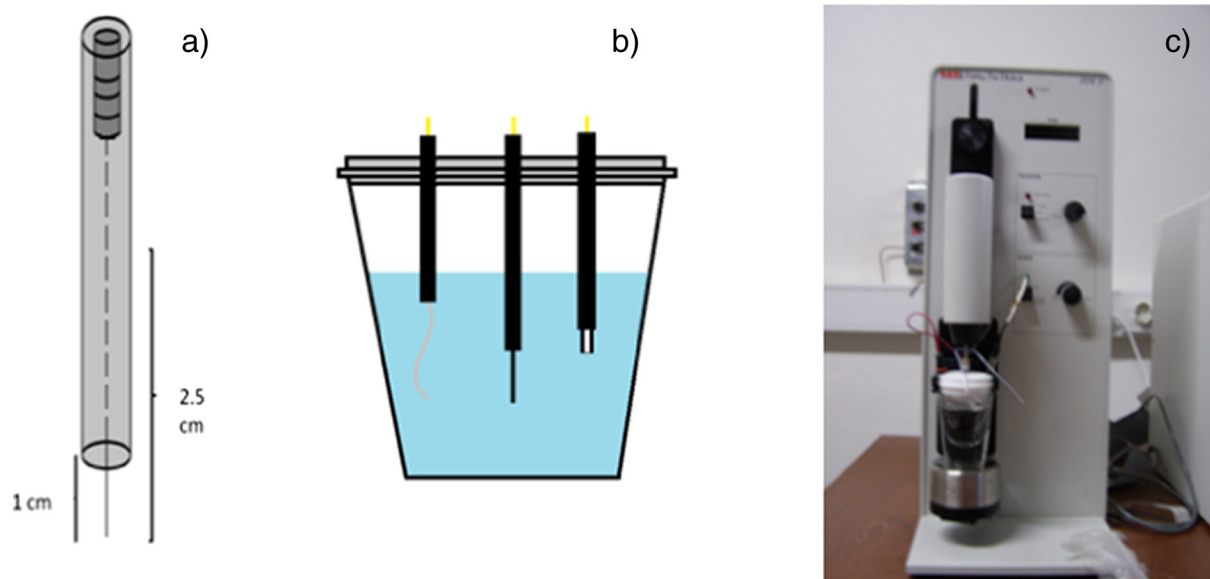


Fig. 1. Electrochemical cell. a) Pencil-lead work electrode, b) cell electrochemical with PLWE, counter electrode and reference electrode, c) RDE-2 Rotating Disk Electrode.

3. Results and discussion

3.1. Optimization of the experimental parameters

A graphical representation of the response surfaces applying desirability function was shown in Fig. 2.

These surfaces correspond to a polynomial equation of second order:

$$D = 0.17 \pm 0.09 + 0.26\Delta E_s \pm 0.11 + 0.16\Delta E_{sw} \pm 0.11 - 0.35f \pm 0.11 + 0.20\Delta E_s^2 \pm 0.17 - 0.35\Delta E_s f \pm 0.16 + 0.18f^2 \pm 0.17. \quad (1)$$

D is the value of overall desirability, which was calculated from the values of corresponding partial desirability for each metal. The individual desirability was estimated from the current values of net peak for each analyte. In the present model, we can see that the parameters most influential in the response were f and ΔE_s compared with ΔE_{sw} . This result can be explained in the fact that the combination of f and ΔE_s results in the scan rate. At a high scan rate (more than 100 mV s^{-1}), copper signal is overlapped by the discharge of bismuth. Then, the desirability function values (individual and total) are zero. For this reason, scan rates greater than 100 mV s^{-1} were not studied. The net current peak is directly proportional to the frequency ($I_{p,n} = nFAc_M^{*n+} (Df)^{1/2} \psi(\Delta E_s, \Delta E_{sw})$), where $I_{p,n}$ is the current, n is the number of exchanged electrons, F is the Faraday constant, A is the electrode surface area, c_M^{*n+} is the bulk concentration of the metal analyte ion M^{n+} (aq), D is the diffusion coefficient, f is the frequency of the potential modulation, and ψ is the dimensionless current, in square wave anodic stripping voltammetry using bismuth film substrates [22]. According to the results of Eq. (1), it can be seen that the most influential parameter is the frequency. On the other hand, high values of ΔE_{sw} were necessary to obtain best resolution for nearly all signals.

Table 1
Levels corresponding to the studied variables.

Factors	Levels		
Coded level	−1	0	1
ΔE_s (mV)	1	2	3
f (Hz)	10	20	30
ΔE_{sw} (mV)	50	60	70

The R-Squared statistic indicates that the model as fitted explains 82.92% of the variability in D , that is, how tight are the values obtained by the model compared to experimentally obtained data. The optimal values obtained are: ΔE_s : 3 mV, ΔE_{sw} : 70 mV, and f : 10 Hz.

3.2. Stability of pencil-lead bismuth film electrode

The pencil lead used in this paper as a work electrode, required a simple pretreatment, as can be seen in Section 2.4. Therefore a stable and reproducible bismuth film electrode was obtained. This was confirmed by evaluating the relative standard deviations (RSD %) for five replicate measurements of $100 \mu\text{g L}^{-1}$ Zn, $10 \mu\text{g L}^{-1}$ Cd, $40 \mu\text{g L}^{-1}$ Pb and $30 \mu\text{g L}^{-1}$ Cu. The obtained values were 1.86%, 4.43%, 1.48% and 8.31% respectively.

3.3. Preprocessing data and analysis of the models

In order to carry out baseline correction, the Asymmetric Least Squares (AsLS) method was employed [19,20]. This pretreatment allows avoiding the semi-manual and individual correction of baseline that is subjective and time consuming. The parameters of this algorithm,

Table 2
Calibration set.

N° experiment	Zn [$\mu\text{g L}^{-1}$]	Cd [$\mu\text{g L}^{-1}$]	Pb [$\mu\text{g L}^{-1}$]	Cu [$\mu\text{g L}^{-1}$]
1	180	7	35	40
2	280	7	50	30
3	80	9	50	50
4	280	9	20	40
5	180	5	50	60
6	380	9	35	60
7	380	7	65	50
8	280	11	65	60
9	380	11	50	40
10	180	9	65	30
11	80	11	35	30
12	80	7	20	60
13	380	5	20	30
14	80	5	65	40
15	180	11	20	50
16	280	5	35	50

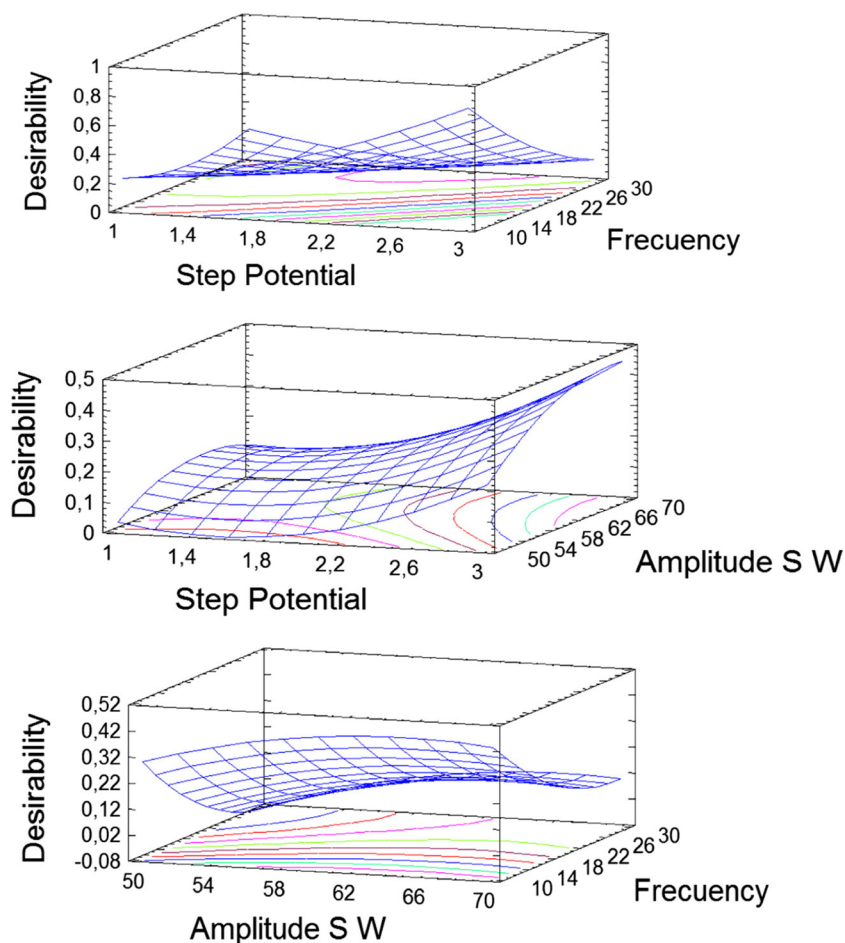


Fig. 2. Desirability function response surfaces.

asymmetry (p) and smoothness (λ) were tested visually for the best baseline and the obtained values were 0.001 and 10^5 respectively. On the other hand, the obtained voltammograms with bismuth film electrodes can show peak potential shifts towards less or more negative potentials. This is due to the presence of attractive or repulsive forces in the film formed, between the analyte and bismuth [21,22]. To resolve this problem, the Correlation Optimized Warping (COW) algorithm to

align the voltammograms was used [23]. Fig. 3 shows the corresponding voltammograms obtained from the calibration set, without and with preprocessing with AsLS-COW.

Partial Least Squares (PLS) is one of the algorithms commonly used in many fields of chemistry, mainly analytical chemistry [24,25]. Table 3 shows the prediction results employing PLS to the voltammograms pretreated without and with AsLS and AsLS-COW for each

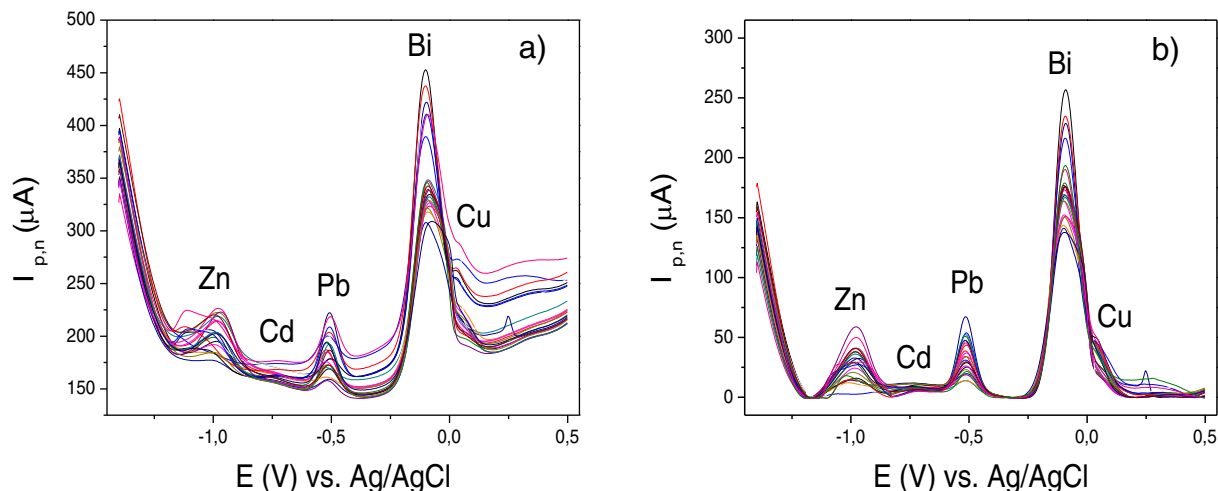


Fig. 3. Calibration set voltammograms, without (a) and with (b) preprocessing with AsLS-COW. (ΔE_s : 3 mV, ΔE_{sw} : 70 mV, f : 10 Hz and $1000 \mu\text{g L}^{-1}$ Bi).

Table 3
Statistical parameters for PLS models for each analyte.

Analyte	Statistical parameter	PLS	AsLS-PLS	AsLS-COW-PLS
Zn (80–380) [$\mu\text{g L}^{-1}$]	LV	6	7	8
	RMSECV	20.90	17.94	22.18
	RMSEP	64.62	52.18	34.53
	BIAS	14.16	5.893	−7.223
	SEP	67.40	55.42	36.09
	r	0.7228	0.8686	0.9339
Cd (5–11) [$\mu\text{g L}^{-1}$]	LV	20	16	12
	RMSECV	0.8474	1.020	1.097
	RMSEP	3.216	2.094	1.697
	BIAS	0.4309	−1.583	−0.8595
	SEP	3.407	1.465	1.565
	r	−0.1481	0.5938	0.4294
Pb (20–65) [$\mu\text{g L}^{-1}$]	LV	8	7	8
	RMSECV	2.940	3.015	2.264
	RMSEP	6.560	4.432	3.627
	BIAS	−2.617	−2.496	0.665
	SEP	6.431	3.916	3.812
	r	0.9000	0.9881	0.9730
Cu (30–60) [$\mu\text{g L}^{-1}$]	LV	8	9	9
	RMSECV	3.080	2.811	9.001
	RMSEP	13.94	7.249	8.317
	BIAS	2.680	0.9589	2.630
	SEP	13.94	7.681	8.434
	r	−0.1034	0.604	0.580

analyte. RMSEP values show that with the AsLS-COW model the results improved for lead and zinc regarding the PLS model, since COW treatment tends to increase the linearity of the data [26].

On the other hand, cadmium and copper showed a more pronounced non-linear behavior than zinc and lead, probably due to the formation of intermetallic compounds [27–29] which leads to non-linearity of the data. In this case the use of the AsLS-COW-PLS

model is not suitable (r values are lower than 0.8) and the use of PLS is not appropriate. Lead and zinc also form intermetallic compounds [27] and despite the existence of copper in solution, the use of PLS models is suitable due to AsLS-COW-PLS improving the results. Furthermore, copper concentration is lower than zinc and similar to lead in the propolis samples. Considering that the four analytes form intermetallic compounds, a non-linear model as Artificial Neural Networks (ANN) to predict their concentration was employed [30].

In order to optimize the number of neurons in the hidden layer, RMSEP for each analyte was calculated. The results were shown in Fig. 4. The optimal hidden layer neurons were: 5 for zinc, 20 for cadmium, 10 for lead and 10 for copper. To avoid overfitting in the prediction of cadmium, 10 neurons in the hidden layer were employed. The neurons were trained using the Levenberg–Marquardt backpropagation and the network was trained with the calibration data set, considering 70% of learning, 15% for monitoring, and another 15% to test the network [31].

Table 4 shows the obtained results for the different models generated by ANN, AsLS-ANN, and AsLS-COW-ANN. The obtained results showed that ANN is capable of modeling all analytes, due to the non-linear nature of the data. A correction of the baseline (AsLS-ANN) improves the prediction models. Furthermore the COW algorithm increased the linearity of the data; therefore application of ANN is not appropriate. Then, AsLS-ANN was employed.

3.4. Application to propolis samples

The proposed method was applied successfully in raw propolis samples to determine simultaneously zinc, cadmium, lead and copper using the optimum experimental conditions for each chemometric model (Table 5). To validate the proposed method a recovery study was

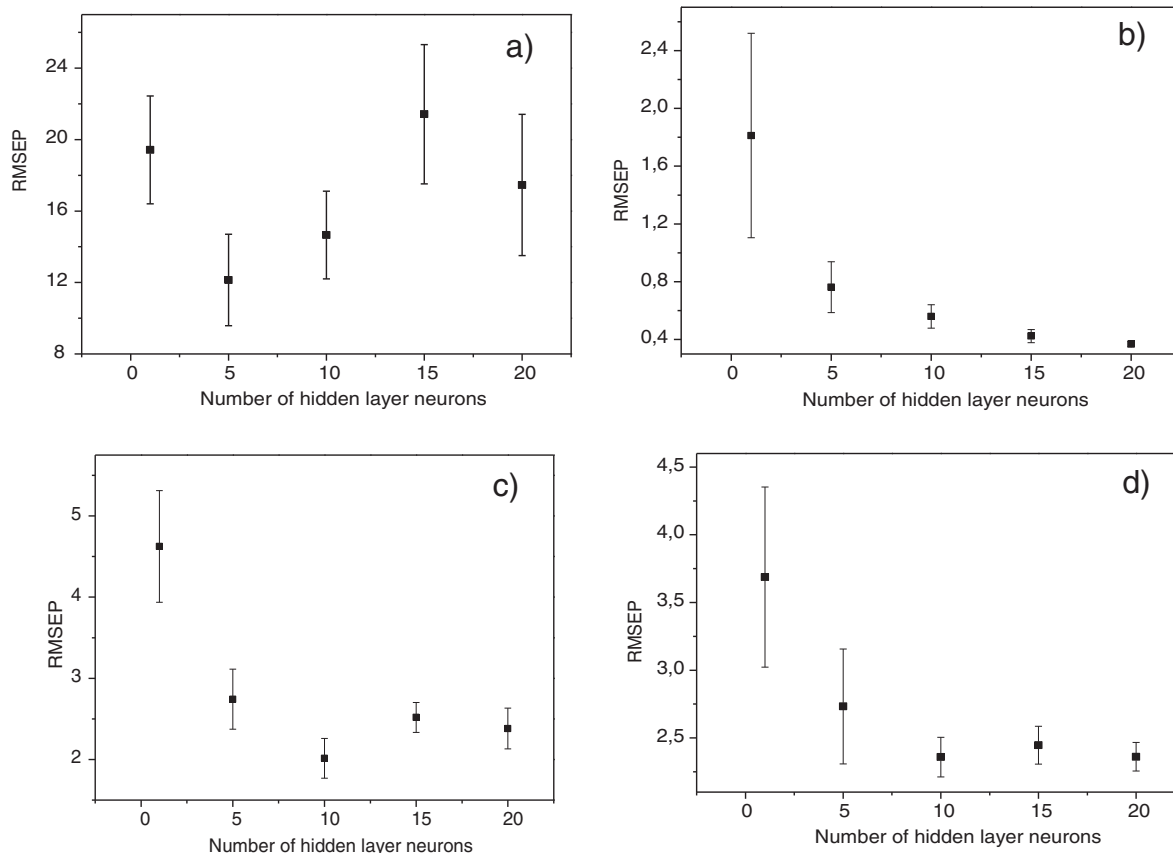


Fig. 4. Graphical of number of hidden layer neurons versus RMSEP for a) Zn, b) Cd, c) Pb and d) Cu.

Table 4

Statistical parameters for ANN models for each analyte. Optimal number of neurons of hidden layer was employed.

Analyte	Statistical parameter	ANN	AsLS-ANN	AsLS-COW-ANN
Zn (80–380) [$\mu\text{g L}^{-1}$]	RMSEV	12.80	4.921	17.65
	RMSEP	16.59	7.623	13.63
	Slope	0.9889	1.0094	0.9761
	Intercept	4.500	2.7591	7.193
	r_p	0.995	0.997	0.996
Cd (5–11) [$\mu\text{g L}^{-1}$]	RMSEV	0.7144	0.2674	0.6237
	RMSEP	0.7926	0.4852	0.3718
	Slope	1.001	0.9627	1.036
	Intercept	−0.08567	0.5797	−0.1562
	r_p	0.946	0.982	0.993
Pb (20–65) [$\mu\text{g L}^{-1}$]	RMSEV	5.172	3.087	4.157
	RMSEP	1.635	1.007	2.539
	Slope	0.9884	1.0158	0.929
	Intercept	0.5744	0.2472	1.457
	r_p	0.997	0.998	0.987
Cu (30–60) [$\mu\text{g L}^{-1}$]	RMSEV	1.169	1.625	1.382
	RMSEP	1.931	1.455	2.338
	Slope	0.9232	1.0042	0.9814
	Intercept	2.276	−0.2092	1.014
	r_p	0.982	0.991	0.975

Table 5

Analysis of real samples.

Sample	Zn [mg kg^{-1}]	Cd [mg kg^{-1}]	Pb [mg kg^{-1}]	Cu [mg kg^{-1}]
M1	14.8	nd	7.73	2.45
M2	15.2	nd	3.05	2.09

nd: not detected.

performed at two concentration levels. The accuracy of the AsLS-ANN performance was found to be in the range of 97.5–103.6% (Table 6).

4. Conclusions

A new analytical method for the multi-elemental determination of zinc, cadmium, lead and copper, in raw propolis samples, was developed. A bismuth-film electrode was applied to the square wave anodic stripping voltammetry in order to determine the four analytes. The present study uses the Artificial Neural Networks (ANN) that allow one to consider the metallic interactions. Asymmetric Least Squares (AsLS) for the baseline correction data and Correlation Optimized Warping (COW) to correct potential discharge were used. A multi-criteria decision strategy based on desirability function and Box-

Behnken design as response surface methodology have been used to optimize the square wave voltammetry parameters (step potential (ΔE_s), frequency (f) and amplitude of the square wave (ΔE_{sw})) for the multiple determinations of the metals.

The proposed method is simple and fast and presents the advantages of low consumption of reagents and sample and minimal waste generation. The use of this methodology is an eco-friendly proposal since it employs a bismuth film instead of mercury.

The method was applied in a chemical complex as raw propolis samples from the Province of Buenos Aires, Argentina, and the obtained results were validated successfully with recovery assays.

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Table 6

Recovery study. Comparative prediction of Zn, Cd, Pb and Cu by applying AsLS-ANN and AsLS-COW-PLS.

Sample	Zn [$\mu\text{g L}^{-1}$]			Zn [%]		Cd [$\mu\text{g L}^{-1}$]			Cd [%]	
	Added	Found AsLS-ANN	Found AsLS-COW-PLS	Recovery AsLS-ANN	Recovery AsLS-COW-PLS	Added	Found AsLS-ANN	Found AsLS-COW-PLS	Recovery AsLS-ANN	Recovery AsLS-COW-PLS
M1a	69.84	69.83	87.08	99.98	124.68	5.00	5.03	13.30	100.60	266.00
M1b	166.65	165.5	159.21	99.31	95.53	6.91	6.91	13.14	100.00	190.16
M2a	59.93	61.56	82.31	102.72	155.51	5.00	5.01	13.98	100.20	276.60
M2b	156.85	158.15	182.86	100.83	116.58	6.91	6.94	13.33	100.43	192.91
Sample	Pb [$\mu\text{g L}^{-1}$]			Pb [%]		Cu [$\mu\text{g L}^{-1}$]			Cu [%]	
	Added	Found AsLS-ANN	Found AsLS-COW-PLS	Recovery AsLS-ANN	Recovery AsLS-COW-PLS	Added	Found AsLS-ANN	Found AsLS-COW-PLS	Recovery AsLS-ANN	Recovery AsLS-COW-PLS
M1a	9.98	9.47	5.9	94.88	59.12	9.64	9.78	23.30	101.42	241.75
M1b	19.47	19.6	21.65	100.66	111.97	18.54	18.12	35.64	97.76	192.26
M2a	19.95	19.86	25.41	99.55	127.37	9.88	10.21	32.82	103.38	332.23
M2b	29.37	29.56	36.33	100.65	123.70	19.02	19.78	51.71	103.98	271.87

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