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Method Article

Rapid and simultaneous electrochemical method to measure copper and lead in canine liver biopsy



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A B S T R A C T

The conventional analytical techniques used for the quantitative analysis of heavy metals in animal tissues are the atomic absorption spectroscopy (AAS) and the inductively-coupled plasma mass spectrometry (ICP-MS). These methods involve high cost, skilled personnel and long analysis times. Several studies have shown the applicability of electrochemical transducer coupled to disposable screen-printed electrodes (SPE) for measuring metals. The aim of the present study was to applicate and validate a simple and fast protocol for the simultaneous measurement of Cu and Pb concentrations in canine liver biopsy, coupling a simple digestion procedure with electrochemical stripping analysis. Square wave anodic stripping voltammetry (SWASV) coupled to disposable SPE was employed as fast and sensitive method for metals detection. Samples were digested with hydrogen peroxide/hydrochloric acid mixture for 1 h coupled to solid phase purification with carbon columns. Instrumental precision, digestion procedure accuracy and limits of detections were evaluated. To validate the proposed method the metal concentrations have also been determined by AAS. Instrumental precision and digestion method accuracy were less than 15.3% and 16.8% for Cu and Pb, respectively. The limits of detection were 10 and 6 mg/kg dry weight in liver samples for Cu and Pb, respectively. The results obtained by the electrochemical method are in agreement with those of the reference method. The obtained results showed a reliable approach for Cu and Pb detection simultaneously with good sensitivity, accuracy and precision. The simple sample pre-treatment together with the low cost makes this approach particularly appealing for Cu and Pb quantification for diagnostic purposes.

- Simultaneous determination of Cu and Pb concentrations in canine liver biopsy.
- Square wave anodic stripping voltammetry (SWASV) with screen-printed electrodes (SPE) coupled to an acid digestion protocol.
- Cu and Pb were measured with a very good sensitivity and accuracy.
- Good agreement with AAS reference method.
- Low cost, fast and sensitive method for diagnostic purposes.

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ARTICLE INFO

Method name: Square wave anodic stripping voltammetry (SWASV) coupled to acid digestion determination of Cu and Pb in canine liver samples

Keywords: Copper, Lead, Liver, Dog, Square wave anodic stripping voltammetry (SWASV), Screen printed electrodes (SPE)

Article history: Received 7 September 2020; Accepted 16 November 2020; Available online 20 November 2020

Specifications table

Subject Area:	Veterinary Science and Veterinary Medicine
More specific subject area:	Analytical Methods
Method name:	Square wave anodic stripping voltammetry (SWASV) coupled to acid digestion determination of Cu and Pb in canine liver samples
Name and reference of original method:	The present method is the application and validation of a simple and fast protocol for the simultaneous measurement of Cu and Pb concentrations in canine liver biopsy, coupling a simple digestion procedure with electrochemical stripping analysis. The method applied is based on the reference: Meucci V, et al. An optimized digestion method coupled to electrochemical sensor for the determination of Cd, Cu, Pb and Hg in fish by square wave anodic stripping voltammetry. <i>Talanta</i> 2009; 77:1143–1148.
Resource availability:	N.A.

Method details

Background

Copper (Cu) is an essential metal involved in several biochemical processes but, in excess, can become extremely toxic. Cu concentrations above physiological concentrations overload the detoxifying capacity of the liver to bind this metal to metallothioneins. The free Cu in the cytosol may cause intense oxidative damage and cellular death [7,26,30].

Cu toxicity have been observed in several species, including dogs, in which hepatic Cu accumulation is associated with various inflammatory hepatopathies, in particular copper-associated chronic hepatitis [5]. Copper-associated chronic hepatitis (CACH) was first identified in Bedlington terriers as an inherited autosomal recessive defect of the *COMMD1* gene, which results in reduced biliary excretion of Cu because of hepatic metallothioneins sequestration of the metal in hepatic lysosomes [9,31]. Others various pure-breed dogs and most recently also mixed-breed dogs have been associated with CACH [5]. The pathological mechanisms involved for most of these breed, are not explained. Hepatic Cu accumulation may be the result of either a primary metabolic defect in Cu metabolism or a secondary event from abnormal hepatic function altering biliary Cu excretion. An early diagnosis of Cu accumulation might help to set the correct therapeutic management and to prevent progression of the hepatopathy [32]. The methods used in veterinary medicine to determine liver Cu concentrations are the semiquantitative evaluation on formalin fixed and paraffin embedded histological sections stained with rubeanic acid or rhodamine, and the quantitative analysis on biopsy specimens with the atomic absorption spectrometry (AAS) or inductively-coupled plasma mass spectrometry (ICP-MS) [5].

Lead (Pb) is a non-essential metal and an important environmental contaminant. Depending on environmental pollution variable Pb concentrations can be observed in foods derived from plants and animals [8,30]. Exposure to Pb in humans is associated with specific health consequences, resulting, among others, from the mutagenic, carcinogenic or immunosuppressive properties of this element. Gastrointestinal and neurological symptoms are the most common signs of Pb poisoning in dogs, with colic and agitation as clinical outcome. Other pathological manifestation in dogs are nephropathy and adverse effects of Pb on the haematological system are mainly the result of its perturbation of the heme biosynthesis pathway [10]. Exposure to Pb in dogs most often occurs as a result of oral exposure through contaminated water, Pb-containing paint, or other Pb-containing items [3]. Determination of Pb concentrations in organs could provide not only important reference data for clinical diagnostics but also an indication of Pb exposure of dogs through environment and food.

Conventional and most used analytical methods to measuring Pb concentrations in canine tissues, as for the Cu, are ICP-MS and AAS [5]. Although these methods have high sensitivity and precision, they are laboratory-based and require trained personnel and expensive equipment. They are time-consuming, involve high costs, often require large amounts of hazardous reagents and conditions [14,16].

Hence, there is a need of new methods that can overcome the disadvantages of conventional techniques. Also, rapid and low cost alternatives to the specialized lab-based analysis should be developed to facilitate better diagnosis for copper-associated hepatitis or monitoring heavy metals exposure.

Electrochemical methods have become one of the most used methods in the field of heavy metals detection due to their portability, rapidity, simultaneous measurement, and low cost.

Electrochemical stripping analysis is a useful technique for the determination of trace heavy metals in environmental, clinical and industrial origin samples. Stripping analysis has a first step that consists of the electrodeposition of the heavy metal ions at constant potential to preconcentrate them onto the surface of a working electrode. The second step is a linear potential sweep in the anodic direction to reoxidize or strip out the plated metals [27]. Among the electrochemical detection techniques, anodic stripping voltammetry (ASV) is the most commonly used method [14]. Stripping analysis has been often performed by pocket-size portable electrochemical analyzers coupled to disposable screen-printed electrodes (SPE). SPE are planar devices, based on different layers of inks printed on a plastic or ceramic substrate. Screen-printing technology is particularly attractive for the production of disposable sensors [14]. SPE are cheap disposable electrochemical devices that are easy to use and suitable for on-site and real-time sensing. The coupling of ASV to SPE allows low-power, low-cost, rapid and sensitive field analysis [13,15,23] as demonstrated by their use in environmental analysis for heavy metals and other pollutant detection [1,2,6,19–21,24,28].

The aim of the present study was to apply and validate a simple and fast protocol for the simultaneous measurement of Cu and Pb concentrations in canine liver biopsy, coupling a simple digestion procedure with electrochemical stripping analysis. The electrochemical technique used was the Square Wave Anodic Stripping Voltammetry (SWASV), based on the use of portable instrumentation and SPEs.

Material and standard solution

Reagents included suprapur grade hydrochloric acid and hydrogen peroxide (Merck). The water used for the preparation of solutions was from a Milli-Q System (Millipore). Heavy metal stock solutions were prepared by diluting AAS grade Pb(II) and Cu(II) standard solutions (Fluka). Superclean ENVI-CarbTM columns were used for samples purification (Supelco).

Methods and characterization tools

Electrodes used are screen-printed graphite-based mercury-modified electrodes (EcoBioServices & Researches). Electrodes are serigraphically screen-printed with a shape similar to as previously reported [23]. They consist of a round-shaped working electrode (diameter 3 mm), of a graphite counter electrode and of a silver pseudo-reference electrode. In addition, the silver electrical contacts were covered by a graphite layer in order to prevent oxidation phenomena during storage. Two working electrode materials were used in this work, depending on the two different measurement applications. Graphite-based Hg-modified screen-printed electrodes are based on the use of a special coating cellulose-derivative film deposited onto the graphite working electrodes containing a Hg(II) salt [23]; Hg(II) is reduced from the salt to the metallic form and the modified sensor can be then used for heavy metal accumulation and stripping. The use of this strategy allows to avoid the use of large amounts of Hg solutions, by keeping the high sensitivity which characterize mercury-coated electrodes.

For measurements, SPEs are interfaced with a portable electrochemical transducer (PalmSens 4, Palm Instrument) for the acquisition of the analytical signal, connected to a computer. The entire

methods, which includes the use of a magnetic stirrer for the mixing of the samples, was managed by the PalmSens PC software. The applied electrochemical technique was the SWASV.

Square wave (SW) voltammetry conditions for Pb(II) and Cu(II) analysis were conditioning potential -0.15 V for 60 s, deposition potential -1.1 V for 120 s, equilibration time 30 s, SW amplitude (E_{amp}) 28 mV, step potential (E_{step}) 3 mV and frequency (f) 15 Hz. Each mercury-coated electrode is pre-treated, before using, by applying -1.1 V for 300 s and then SW voltammetric scans were carried out until low and stable background were obtained, as suggested in literature [22]. HCl 0.1 M was used as supporting electrolyte for all measurements. All measurements were performed without removing oxygen from the solution. The measurements were performed immersing the sensor in 5.0 ml of solution; stirring conditions were used during the conditioning and the accumulation steps, whereas the square wave scan was performed in quiescent solution.

Samples treatment

Concentrations of Cu and Pb were evaluated by using an electroanalytical method based on square wave anodic stripping voltammetry (SWASV) coupled to an acid digestion protocol partially modified [20]. Fifty mg of liver were treated with 2.5 ml of H_2O_2 33%; samples were heated up at 50 °C for 1 h, cooled and 2.5 ml of HCl 30% were added. Samples were then heated up at 70 °C for 1 h until a limpid solution was obtained. The solution was made up to 5 ml with ultra-pure water and purified by elution with Superclean ENVI- Carb™ column to remove any organic residue. The carb columns were pre-conditioned with 2 ml of methanol followed by 2 ml of ultra-pure water, then the digested samples were loaded and column eluate collected in clean tubes.

Sample collection and analysis

Samples of liver were collected from 10 dogs, died from different causes at the Department of Veterinary Science of Pisa, Italy. All dogs were patients of the Veterinary teaching Hospital of the Department of Veterinary Science, that died or were euthanized and written consent was obtained from all owners. Three tissue samples of approximately 50 mg were collected from the left lateral liver lobe in order to simulate sample collection during percutaneous ultrasound-guided needle biopsy. All samples were taken from near the center of the lobe and stored at -20 °C until chemical analysis.

Analytical parameters

The quantitative analysis was done by standard addition method [11,15,18]. The instrumental precision parameters were indicated as mean relative standard deviations (r.s.d.%) of three measurements of each metal, performed on the same digested sample of canine liver. The digestion method accuracy was calculated by digesting each matrices three time and determining each metal three times in the same digested sample: the accuracy is represented by the mean r.s.d.% of all the measurements

The linearity, range and sensitivity were obtained from a calibration curve with 10 concentration levels (range 1 μ g/L to 1 mg/L), with triplicate analysis, employing HCl 0.1 N as supporting electrolyte. The limits of detection (LOD) of the studied metals were estimated as the concentration corresponding to the three times the standard deviation of the blank divided by the slope of the calibration curve. To validate the proposed SWASV method the metal concentrations have also been determined by atomic absorption spectroscopy. Tissues were digested in 70% nitric acid, 30% hydrogen peroxide and of ultrapure water for atomic absorption spectroscopy in a microwave digestion system under high pressure and temperature at 190 °C. Pb and Cu were determined by atomic absorption spectrophotometer equipped with graphite furnace atomizer. The precision of the AAS determination was approximately 10%.

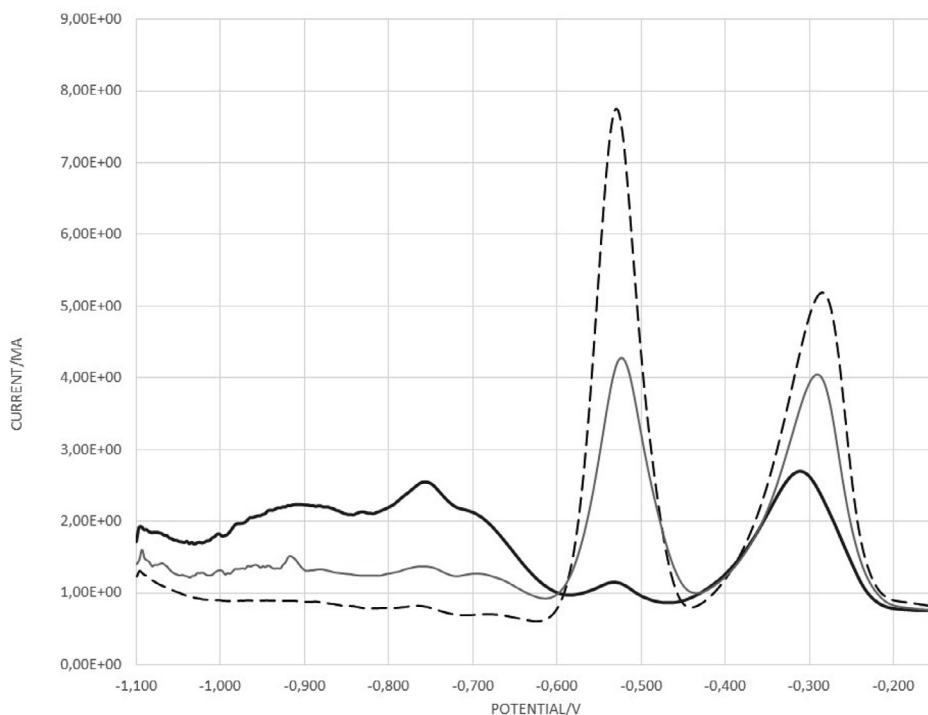


Fig. 1. SWASV scans for Cu (first peak from left) and Pb (second peak) detection in dog liver sample. Graphite-based Hg-modified screen-printed electrodes were used. Applied digestion procedure: H₂O₂/HCl treatment with carb-column purification. The experimental conditions were conditioning potential -0.15 V for 60 s, deposition potential -1.1 V for 120 s, equilibration time 30 s, SW amplitude 28 mV, step 3 mV and frequency 15 Hz. The electrode was conditioned before using by applying -1.1 V for 300 s; black line Sample; gray line first addition +20 ppb; dashed line second addition +40 ppb.

Statistical analysis

The statistical analysis of the data was performed using GraphPad Prism software (version 8.4.3). Reported statistics were mean \pm standard deviation in mg/kg on a dry weight basis. Pearson test was performed to evaluate correlation between SWASV and AAS methods.

Results

Several studies reported the analysis of heavy metals concentrations in the liver and kidneys of dogs. Gender- and age-associated differences were also identified [4,12,25,29]. These studies have useful significance since they can serve as an indication of the exposure of the dogs to heavy metals either through the environment or food. Copper-associated hepatitis has been increasingly recognized in dogs, and supposition exists that hereditary defects in Cu metabolism have been worsened by increased environmental Cu exposure. Currently the only way to diagnose copper-associated hepatitis in dogs and to monitor treatment efficacy, is by evaluation of hepatic biopsies [5]. There is a need to develop fast and low cost method to measure metals for diagnostic purposes in veterinary medicine.

The present electrochemical method allows the simultaneous determination of Cu and Pb in dog liver sample (Fig. 1) by using a fast sample treatment protocol also applicable in non-highly specialized laboratories.

The instrumental precision parameters were indicated as mean r.s.d.% of 3 measurements of each metal, performed on the same digested sample were within 15.3% and 16.8% for Cu and

Table 1
Cu concentrations (mg/kg wet weight) found in dogs liver samples ($n = 15$); a instrumental; b digestion method accuracy.

Sample	Cu (Mean \pm SD)	a	b
Dog 1	202.4 \pm 7.1	3.5	7.3
	221.9 \pm 5.2	2.4	
	235.3 \pm 11.1	4.7	
Dog 2	307.7 \pm 23.6	7.6	9.6
	335.0 \pm 16.2	4.8	
	321.5 \pm 49.4	15.3	
Dog 3	332.8 \pm 28.6	8.6	8.4
	364.9 \pm 28.2	7.7	
	383.5 \pm 9.1	2.4	
Dog 4	798.7 \pm 10.6	1.3	2.9
	802.3 \pm 17.5	2.2	
	793.0 \pm 39.9	5.0	
Dog 5	699.4 \pm 30.0	4.3	5.4
	742.7 \pm 44.0	5.9	
	747.9 \pm 35.9	4.8	

^aThe instrumental precision parameters were indicated as mean r.s.d.% of 3 measurements of each metal, performed on the same digested liver sample.

^bThe digestion method accuracy was calculated by digesting the 3 samples obtained from the same dog and determining each metal 3 time in the same digested sample: the accuracy is represented by the mean r.s.d.% of all the measurements.

Pb, respectively (Tables 1 and 2). The method accuracy was calculated by digesting each samples three time and determining each metal three times in the same digested sample: the accuracy is represented by the mean r.s.d.% of all the measurements and ranged from 2.9 to 15.1% for Cu and Pb, respectively (Tables 1 and 2). The linearity of the method, assessed by using Pb and Cu standard solutions, showed a correlation ≥ 0.99 for both metals. The LOD of the studied metals were 0.5 and 0.3 $\mu\text{g/L}$ for Cu and Pb, respectively. Considering a sample of 50 mg of liver sample, the detection limit was 10 mg/kg for Cu and 6 mg/kg for Pb.

The electrochemical detection, based on SWASV with disposable screen-printed electrodes has been successfully applied to Cu and Pb concentrations determination in canine liver samples. The method showed very good reproducibility and precision and a good correlation with reference AAS method. The main advantages of the electrochemical approach are: low analysis costs, fast response time, very low detection limits, simultaneous analysis of different metals. For all of these reasons can be proposed as new analytical tool heavy metals analysis in animal tissues. The results obtained by the electrochemical method are in agreement with those of the reference method (AAS) (Table 3). Pearson test showed a significant correlation between SWASV and AAS method ($p < 0.0001$, r of Pearson = 0.98). Moreover, the present method may be a good alternative to spectroscopy, which, in the case of determination of metals in complex matrices, needs expensive equipment.

Furthermore, previous study [19] found a positive linear correlation among Cu levels in liver biopsy and needle aspirates, suggesting the use of latter specimens as non-invasive diagnostic sample for measuring Cu hepatic levels. Future studies are needing to asses and validate the ultrasound-guided fine-needle biopsies as non-invasive sampling to quantify Cu for diagnostic purposes. Finally, as reported by other authors [17] pets could be suggested as bioindicators of human metal exposure and this method could be applied to measure metals with this aim.

Table 2

Pb concentrations (mg/kg wet weight) found in dogs liver samples ($n = 15$); a instrumental; b digestion method accuracy.

Sample	Pb (Mean \pm SD)	a	b
Dog 1	42.0 \pm 7.6	14.2	15.1
	47.2 \pm 7.2	15.1	
	52.4 \pm 5.9	11.2	
Dog 2	84.1 \pm 7.2	8.5	11.3
	92.7 \pm 7.9	8.4	
	85.8 \pm 14.6	16.8	
Dog 3	118.3 \pm 9.4	7.9	7.7
	124.4 \pm 6.7	5.4	
	119.2 \pm 13.3	11.1	
Dog 4	66.7 \pm 4.2	6.3	14.8
	73.2 \pm 13.3	16.2	
	67.2 \pm 13.5	16.1	
Dog 5	101.8 \pm 13.1	12.8	11.4
	107.9 \pm 14.7	6.5	
	118.3 \pm 5.7	4.8	

^aThe instrumental precision parameters were indicated as mean r.s.d.% of 3 measurements of each metal, performed on the same digested liver sample.

^bThe digestion method accuracy was calculated by digesting the 3 samples obtained from the same dog and determining each metal 3 time in the same digested sample: the accuracy is represented by the mean r.s.d.% of all the measurements.

Table 3

Comparison of SWASV and AAS results testing canine liver samples ($n = 5$).

Sample	SWASV		AAS	
	Cu (mg/kg wet weight)	Pb (mg/kg wet weight)	Cu (mg/kg wet weight)	Pb (mg/kg wet weight)
Dog 6	235.3 \pm 11.1	114.8 \pm 13.0	244.4	95.3
Dog 7	375.3 \pm 13.1	124.4 \pm 7.6	380.5	114.8
Dog 8	506.1 \pm 14.3	92.7 \pm 16.2	481.0	83.2
Dog 9	288.7 \pm 10.1	95.7 \pm 5.9	298.8	90.2
Dog 10	393.3 \pm 11.0	71.3 \pm 12.5	380.2	60.9

Conclusions

Coupling a simple digestion procedure with electrochemical stripping analysis has been applied to small pieces of canine liver showing that the simple sample pre-treatment together with the low cost makes this approach particularly appealing for Cu detection and quantification for diagnostic purposes in non-specialized laboratory. This method has been showed also to be very rapid; the time to digest a sample is 1 h and the time to measure it is less than 1 h.

Extension of this fast, simple, sensitive and cheap method for heavy metal analysis to other tissue matrices can be expected. The portability of the instrumentation together with simple sample pre-treatment makes this approach interesting for multi-analytes detection i.e. for inspection purposes and environmental analysis.

Declaration of Competing Interest

The Authors confirm that there are no conflicts of interest.

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