

## Original Article

Effect of lacquer on altered elemental proportions  
in the superficial layer of bone, using handheld X-ray fluorescenceKittisak Buddhachat<sup>1,2</sup>, Promporn Piboon<sup>1</sup>, and Korakot Nganvongpanit<sup>1,3\*</sup><sup>1</sup> Animal Bone and Joint Research Laboratory, Department of Veterinary Biosciences and Public Health,  
Faculty of Veterinary Medicine and Excellent Center in Veterinary Bioscience,  
Chiang Mai University, Mueang, Chiang Mai, 50100 Thailand<sup>2</sup> Department of Biology, Faculty of Science,  
Naresuan University, Mueang, Phitsanulok, 65000 Thailand<sup>3</sup> Excellence Center in Veterinary Bioscience, Chiang Mai University,  
Mueang, Chiang Mai, 50200 Thailand

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**Abstract**

We established a method to test if bones have been varnished with lacquer. Handheld X-ray fluorescence was employed to detect the elemental at the surface of porcine femoral bones (n=10) that served as lacquer-free bones (control group). Eight elements – silicon (Si), phosphorus (P), sulfur (S), calcium (Ca), titanium (Ti), zinc (Zn), cadmium (Cd) and tin (Sn) – plus light elements (LE) showed a significant discrepancy between lacquer-varnished and lacquer-free bones ( $p < 0.01$ ). The ratio of Ca to P (Ca/P) served as an excellent index for preliminary evaluation of lacquer contamination of bones. Ultimately we created an effective equation, using a stepwise discriminant analysis, to test for contamination of lacquer at the surface of bones. In conclusion, this method could serve as an effective tool for preliminary screening of lacquer contamination on the surface of bones.

**Keywords:** bone, discriminant analysis, element, forensic science, lacquer

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**1. Introduction**

Wild animals have been immensely threatened by human activities, leading to the illegal wildlife trade (Rosen & Smith, 2010; Yi-Ming, Zenxiang, Xinhai, Sung, & Niemelä, 2000) One of the most common purposes is for decorative items: e.g. the ivory or teeth of several species, such as tiger, walrus, dugong and elephant (Ellis, 2013; Nijman & Nekaris, 2014; Stiles, 2003; Wilson-Wilde, 2010; Wu & Phipps, 2002) For these reasons, the decline in the population of these wild animals is still ongoing (Wilson-Wilde, 2010; Wittemyer *et*

*al.*, 2014) necessary to urgently combat the international illegal wildlife trade in order to prevent the extinction of wild animals (Oldfield, 2014; Zimmerman, 2003).

Thus far, thousands of elephants have been victims of ivory hunting, and the elephant population has consequently been decimated (Burn, Underwood, & Blanc, 2011; Wittemyer *et al.*, 2014). In 1989 the ivory trade was banned by the Convention on International Trade in Endangered Species (CITES), except for captive elephants or those that died naturally, whose ivory is approved for sale with authorization (Sands & Bedecarre, 1989). To defend against illegal poaching, our previous studies have established an effective tool, handheld X-ray fluorescence (HH-XRF), for multi-element analyses to achieve two definitive goals: (i) identification of whether it is real or fake ivory (Buddhachat, Brown, Thita-ram, Klinhom, & Nganvongpanit, 2017), and

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\*Corresponding author

Email address: korakot.n@cmu.ac.th

(ii) differentiation of whether it is Asian or African ivory (Buddhachat *et al.*, 2016b). Furthermore, HH-XRF has been useful for distinguishing dugong tusks, Asian elephant tusks and tiger teeth with a high accuracy (Nganvongpanit *et al.*, 2017a). The designed algorithms gave predictive results with high accuracy and precision, accompanied by a quick report requiring less than 30 min per sample (Buddhachat *et al.*, 2016; Buddhachat *et al.*, 2017; Nganvongpanit *et al.*, 2017). In addition to being portable, a notable advantage of this technique is that it is a non-invasive sampling method.

Often, suspected samples of elephant ivory and others (tiger teeth, walrus and dugong tusks) are coated with lacquer. When measuring the elemental content by HH-XRF, the chemical contamination on the surface of the samples alters the elemental proportions to some degree, leading to incorrect estimations for both authentication of real ivory and differentiation of tusk origin (e.g. Asian or African). The possibility of misidentification presents a problem for law enforcement.

Because ivory, tiger teeth, walrus or dugong tusk are such an expensive materials and their possession must be proved and registered by the governmental authorities, it is a hard task to take these materials for doing the experiment. Another way is borrowing these materials from the authorized owner; however, it cannot be done because, in this study, we needed to coat the materials with acrylic lacquers. Bone and teeth are a similar type of dense connective tissue with mineralization (calcium hydroxyapatite). Our previous study exhibited that several elements in bones and teeth are highly similar despite of the difference in amounts (Buddhachat *et al.*, 2016a). Eventually, the porcine bones were used as the model to test our hypothesis due to its easy availability in our local area. This study compared the elemental content of porcine bones with and without lacquer coating, in order to look for elements which could serve as markers for preliminary screening of whether a bone has been coated with lacquer.

## 2. Materials and Methods

### 2.1 Bone samples and experimental groups

Fresh porcine femoral bones (n=10) from a slaughter-house were used in this study. After removing all muscle and connective tissue, bones were dried under natural conditions (in sunlight). Two types of acrylic lacquers, spray and liquid (TOA paint (Thailand) Co. Ltd.), were used for varnishing, five bones for each type. All bones were scanned before applying the lacquers, to serve as a control group.

### 2.2 X-ray fluorescence (XRF) measurement

Sample elemental analyses were conducted using a handheld XRF analyzer (DELTA Premium, Olympus, USA) with a silicon drift detector that can detect elements from magnesium (12 Mg) through bismuth (83 Bi) on the periodic table. The elements below Mg were designated as light elements (LE). The collimator size was set at 0.3 mm for analysis-area diameter, and operating voltages of 10 and 40 kV with 2 min scans were used as the source of incident radiation.

### 2.3 Statistical analyses

The data was calculated as mean with standard deviation, and depicted the kernel density as estimated by the lattice package in the R program. Kruskal–Wallis and Mann–Whitney test was used for comparing the proportions of each element among spray-type or liquid-type lacquer-coated porcine bones and lacquer-free porcine bones. The data set of elements exhibiting remarkably significant differences ( $p < 0.001$ ) was used to establish a potential algorithm by a stepwise discriminant analysis with leave-one-out classification for determining whether the suspected mineralized tissues (bones, teeth, etc.) had been varnished with lacquer.

## 3. Results

Porcine bones with and without spray-type and liquid-type acrylic lacquer finishes were scanned by handheld XRF to detect the presence of multiple elements. A total of 20 elements plus light elements (LE) were observed. The elements were classified into three groups relating to their quantity, including large-quantity elements (Mg, Ca, P, S, Cl, K and LE), trace elements (Fe, Si, Mn, Zn, V, Cr and Co) and non-essential elements (Ti, Sb, Sn, Zr, Ag and Cd). Nine elements with a considerably significant difference ( $p < 0.01$ ) comprised four quantity elements, phosphorus (P), sulfur (S), calcium (Ca) and LE (Figure 1), two trace elements, silicon (Si) and zinc (Zn) (Figure 2), and three non-essential elements, titanium (Ti), cadmium (Cd) and tin (Sn) (Figure 3). The proportions of Si and Ti appeared to be higher in spray-type rather than liquid-type lacquer, whereas the proportion of LE seemed to be incrementally higher for both lacquer types compared with lacquer-free bones (Table 1). Furthermore, we found that the proportion of Si for spray-type lacquer (mean = 0.757%, SD = 0.134%) did not overlap that for lacquer-free bones (mean = 0.277%, SD = 0.206%) (Table 1). A decrease in the proportion of P, S, Ca, Zn, Cd and Sn was observed in both spray and liquid-type lacquer when compared with the control (Figures 1–3). Otherwise, the proportion of many elements was not affected by the lacquer finish, including magnesium (Mg), chlorine (Cl), potassium (K), chromium (Cr), manganese (Mn) and iron (Fe), as shown in Table 1. Cobalt (Co) and zirconium (Zr) were detectable in all of the spray-type varnished bones, whereas these elements were absent in the other bones (Table 1 and Figures 2, 3). More interestingly, the ratio of Ca to P (Ca/P) showed a remarkable difference between groups. The highest ratio of Ca/P (14.23) was observed in liquid-type varnished bones, followed by spray-type varnished bones (6.54) and lacquer-free bones (3.87) (Table 1).

The data set of eight elements plus LE showing a considerable significance was expanded to create an algorithm using a stepwise discriminant analysis for testing whether a bone is lacquer-finished. The analysis revealed that all elements except Zn could be included to create a function, as follows:  $4.41Si - 1.37P - 5.75S + 0.77Ca - 10.52Ti - 393.34Cd + 117.71Sn + 0.37LE - 29.18$ . This function was tested for accuracy by the leave-one-out classification method, and showed predictability with absolute correctness (100%) (Figure 4).

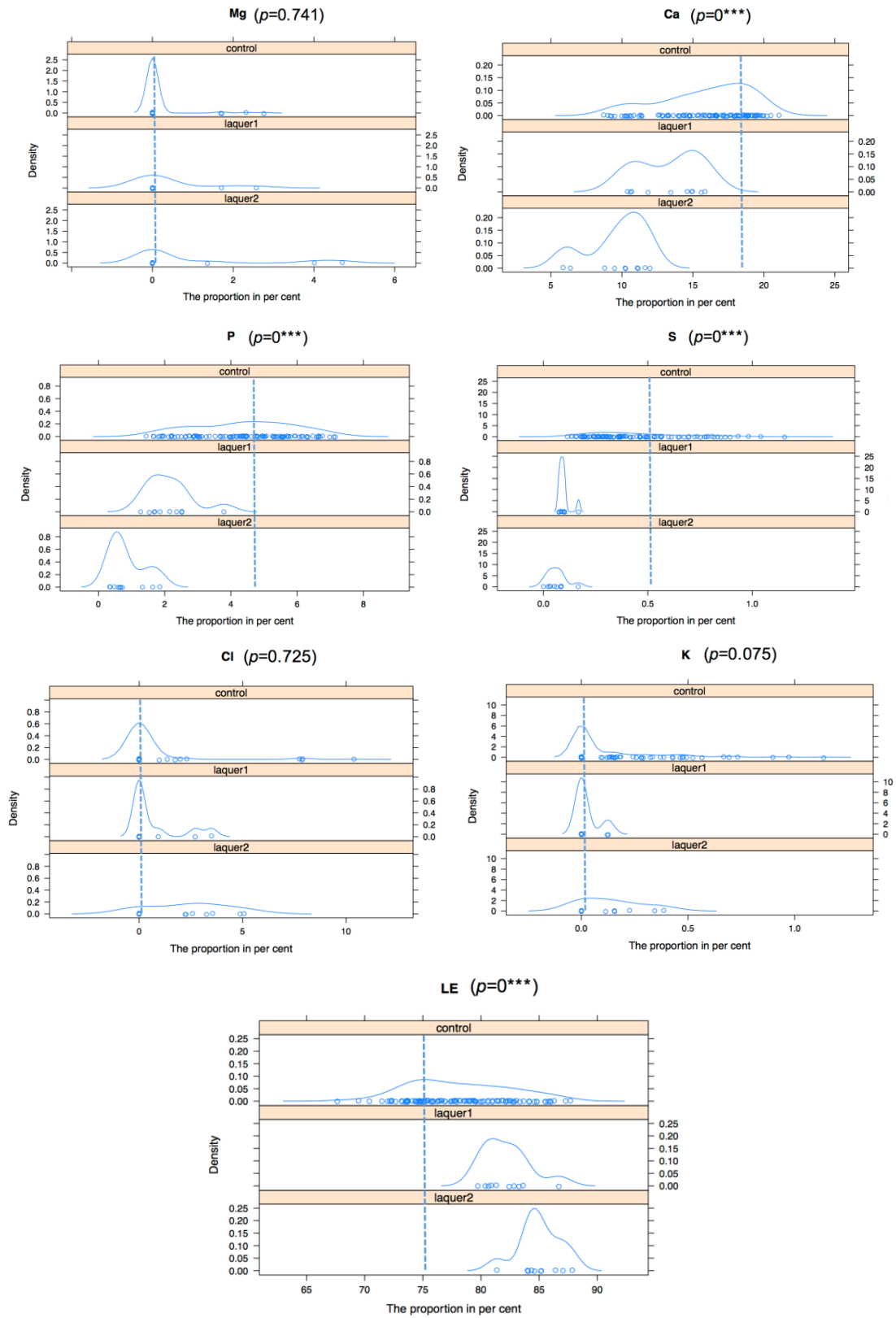


Figure 1. Distribution of the proportions of quantity elements from varnish-free bones (control), spray-lacquer varnished bones (lacquer1) and liquid-lacquer varnished bones (lacquer2). \*, \*\* and \*\*\* indicate a significant difference at  $p < 0.05$ ,  $0.01$  and  $0.001$ , respectively. Not available (N.A.) means it could not be analyzed.

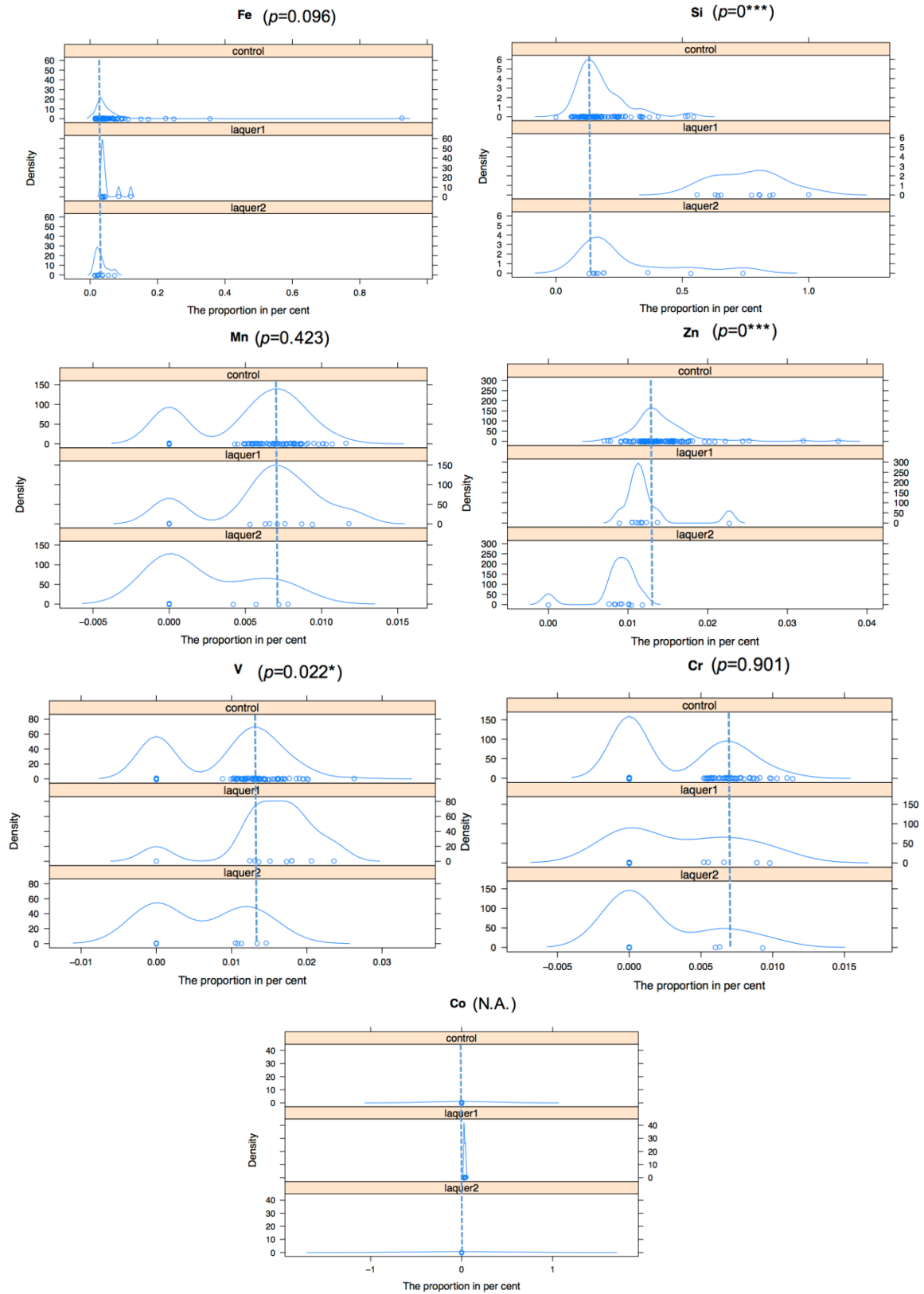


Figure 2. Distribution of the proportions of trace elements from varnish-free bones (control), spray-lacquer varnished bones (lacquer1) and liquid-lacquer varnished bones (lacquer2). \*, \*\* and \*\*\* indicate a significant difference at  $p < 0.05$ ,  $0.01$  and  $0.001$ , respectively. Not available (N.A.) means it could not be analyzed.

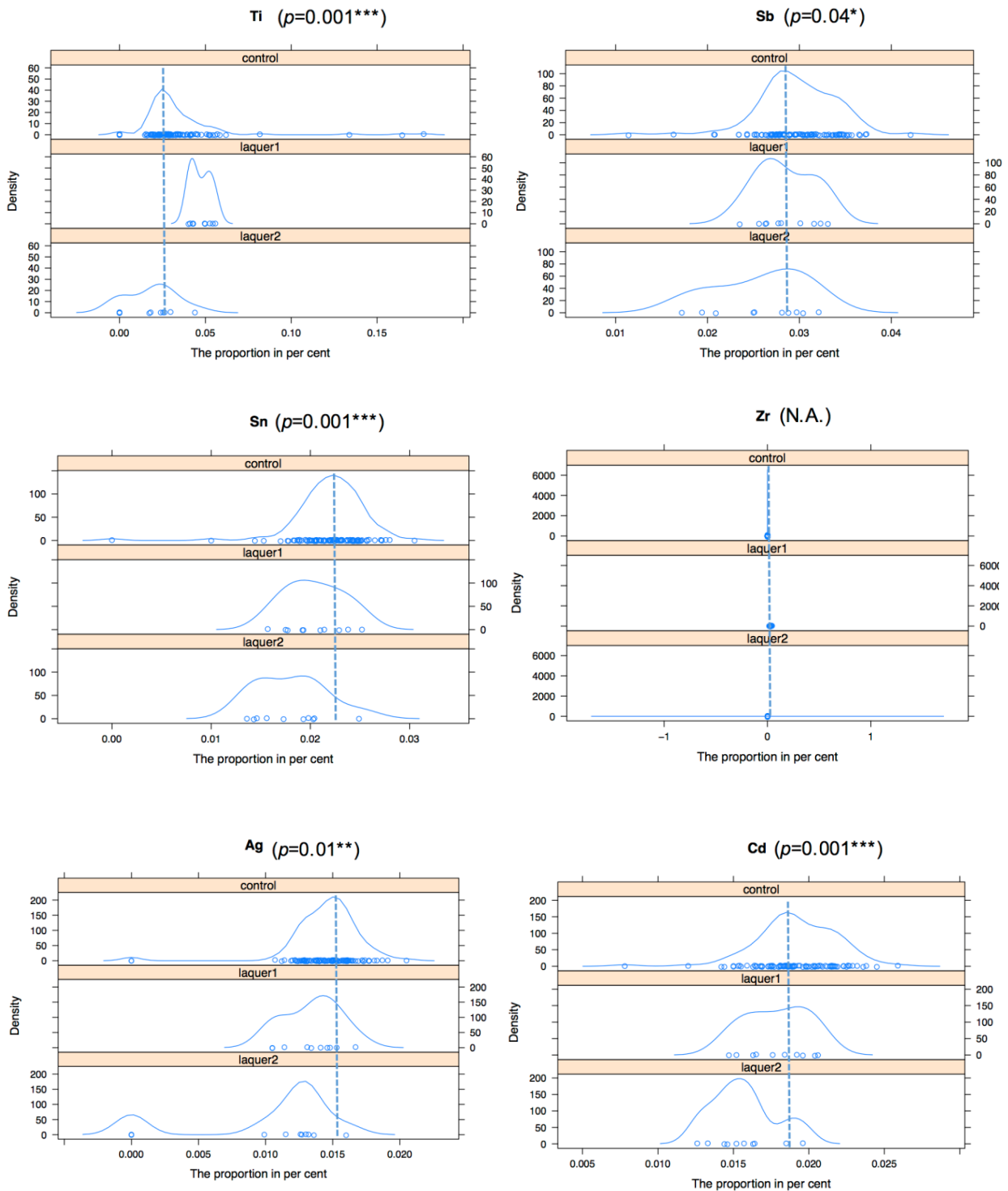


Figure 3. Distribution of the proportions of non-essential elements from varnish-free bones (control), spray-lacquer varnished bones (lacquer1) and liquid-lacquer varnished bones (lacquer2). \*, \*\* and \*\*\* indicate a significant difference at  $p < 0.05$ , 0.01 and 0.001, respectively. Not available (N.A.) means it could not be analyzed.

Table 1. Elemental comparison among bones varnished with liquid- and spray-type lacquers and lacquer-free bones (control)

Element	Type of lacquer	Mean	S.D.	P-value
Quantity elements				
Mg	liquid	1.007	1.822	0.741
	spray	0.428	0.923	
	control	0.081	0.417	
Ca	liquid	9.679 <sup>a</sup>	2.114	0
	spray	13.288 <sup>b</sup>	2.201	
	control	15.828 <sup>c</sup>	3.189	
P	liquid	0.862 <sup>a</sup>	0.542	0
	spray	2.139 <sup>b</sup>	0.718	
	control	4.377 <sup>c</sup>	1.493	
S	liquid	0.069 <sup>a</sup>	0.044	0
	spray	0.096 <sup>b</sup>	0.027	
	control	0.439 <sup>c</sup>	0.227	
Cl	liquid	2.383	1.906	0.725
	spray	0.713	1.306	
	control	0.402	1.67	
K	liquid	0.138	0.111	0.074
	spray	0.025	0.003	
	control	0.129	0.262	
LE	liquid	84.998 <sup>a</sup>	1.826	0
	spray	82.176 <sup>b</sup>	2.061	
	control	78.157 <sup>c</sup>	4.369	
Ca/P	liquid	14.226 <sup>c</sup>	5.704	0
	spray	6.544 <sup>b</sup>	1.329	
	control	3.866 <sup>a</sup>	0.847	
Trace elements				
Fe	liquid	0.031	0.019	0.096
	spray	0.049	0.029	
	control	0.059	0.059	
Si	liquid	0.277 <sup>a</sup>	0.206	0
	spray	0.757 <sup>b</sup>	0.134	
	control	0.178 <sup>a</sup>	0.095	
Mn	liquid	0.003	0.005	0.423
	spray	0.008	0.004	
	control	0.007	0.005	
Zn	liquid	0.009 <sup>a</sup>	0.003	0
	spray	0.011 <sup>b</sup>	0.003	
	control	0.015 <sup>c</sup>	0.014	
V	liquid	0.005	0.005	0.022
	spray	0.015	0.007	
	control	0.008	0.007	
Cr	liquid	0.003	0.005	0.901
	spray	0.005	0.005	
	control	0.005	0.005	
Co	liquid	0	0	N.A.
	spray	0.033	0.009	
	control	0	0	

Table 1. Continued.

Element	Type of lacquer	Mean	S.D.	P-value
Non-essential elements				
Ti	liquid	0.026 <sup>a</sup>	0.009	0.001
	spray	0.047 <sup>b</sup>	0.006	
	control	0.035 <sup>a</sup>	0.025	
Sb	liquid	0.027	0.005	0.04
	spray	0.029	0.003	
	control	0.03	0.004	
Sn	liquid	0.017 <sup>a</sup>	0.005	0.001
	spray	0.021 <sup>ab</sup>	0.003	
	control	0.021 <sup>b</sup>	0.004	
Zr	liquid	0	0	N.A.
	spray	0.029	0.009	
	control	0	0	
Ag	liquid	0.009	0.006	0.01
	spray	0.012	0.004	
	control	0.014	0.005	
Cd	liquid	0.016 <sup>a</sup>	0.005	0.001
	spray	0.019 <sup>ab</sup>	0.003	
	control	0.02 <sup>b</sup>	0.002	

Superscript letters (<sup>a, b, c</sup>) indicate a significant difference at  $p < 0.05$  obtained from Mann-Whitney test, based on the elements exhibiting a significant difference at  $p \leq 0.001$  by Kruskal-Wallis test

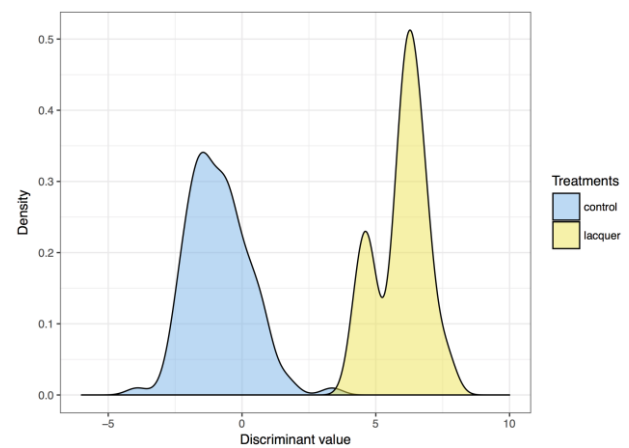


Figure 4. Distribution of discriminant values between lacquer-free bones (control) and lacquer-varnished bones (including spray- and liquid-type lacquer)

#### 4. Discussion

Several wild animals, such as elephant, tiger, dugong, etc., are still being poached (Ellis, 2013; Nijman & Nekaris, 2014; Stiles, 2003; Wilson-Wilde, 2010; Wu & Phipps, 2002). Often the illegal trade in these animals is processed as decorative items that are varnished with lacquer

to achieve a shiny finish. Although our recent studies have been successful in establishing an accurate, reliable and rapid tool for determining a fake or real tusk (Buddhachat *et al.*, 2017) and identifying the origin of a tusk (Africa or Asia) by handheld XRF (Buddhachat *et al.*, 2016), as well as differentiating dugong tusks, elephant tusks and tiger teeth (Nganvongpanit *et al.*, 2017a), misidentification of these items also has occurred due to the tusk being varnished with lacquer. To address this issue, it is necessary to perform a preliminary test by handheld XRF to detect whether the suspected materials are coated with lacquer, prior to testing for the authenticity or origin of a tusk.

When considering the effect of lacquer on the change in elemental proportions, our results revealed that porcine bones varnished with lacquer exhibited a change in the proportion of some elements, especially Si and Ti. Both elements were significantly incremented in spray-lacquer varnished bones when compared with the control and even liquid-lacquer varnished bones. Yet the liquid and spray-type lacquers led to a decrease of P, S, Ca, Zn, Cd and Sn. Furthermore, Co and Zr appeared in the elemental composition only when the bones were coated with spray-type lacquer. Unfortunately, the entire components of both spray and liquid-type lacquers could not be defined because of trademark rights. However, we postulated that the composition of spray-type lacquer may include Si, Ti, Co and Zr.

Based on the elemental composition of porcine bones, we noted that the Ca/P ratio, an average of 3.87, was relatively high, similar to that in dolphins (3.58), whereas in other species (dog, horse, human and monkey) it was 2.3–2.7 (Buddhachat *et al.*, 2016). However, it may be difficult to arrive at a conclusion on this point due to the difference in preservation period; fresh porcine bones were utilized in this study, whereas dolphin bones were preserved for a longer period. The influence of preservation period and condition, therefore, is one of the important issues for further study on the change of elemental composition in bones or other mineralized tissues. More importantly, a considerable difference in ratio of Ca/P among spray and liquid-type varnished and lacquer-free bones was observed, possibly indicating that Ca/P might serve as a specific ratio for a preliminary test of the surface contamination of lacquer. Typically, the Ca/P ratio, representative of calcium hydroxyapatite ( $\text{Ca}_5(\text{PO}_4)_3$ ) in bones and teeth, ranges from 1.65–3.58, as measured by XRF (Buddhachat *et al.*, 2016; Christensen, Smith, & Thomas, 2012; Dickerson, 1962; Legros, Balmain, & Bonel, 1987; Nganvongpanit, Buddhachat, Piboon, Euppayo, & Mahakkanukrauh, 2017b; Nganvongpanit, Buddhachat, Piboon, & Klinhom, 2016). We noted that bones that were varnished with lacquer showed more than twice as high a Ca/P ratio as lacquer-free bones. This was due to the influence of lacquer, which greatly decreased the proportion of P, contributing to a significant increase in the Ca/P ratio, especially in the case of liquid-type lacquer. The observed changes in elemental composition led to the creation of an effective equation by a stepwise discriminant analysis. Seven elements (Si, P, S, Ca, Ti, Cd and Sn) plus LE exhibited high accuracy (100%) for assessment of lacquer contamination on bone surfaces.

In conclusion, handheld XRF may serve as an effective tool for detection of lacquer on bones. Prior to authentication of tusks or other biological materials and even

identification of their origin, suspected specimens should be examined by handheld XRF to detect lacquer contamination in the superficial layer of bone, possibly leading to increased reliability of the obtained results.

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