Inter-laboratory study to determine the copper concentration in three plants from the wild flora, with the view to evaluate their potential as natural source of minerals for piglets

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SUMMARY
An inter-laboratory study was conducted with 7 laboratories in the field of “food / feed / soil”. The main objective of the study was an accurate determination of the copper concentration of 3 plants from the wild flora (Origanum vulgare; Vaccinium myrtillus L.; Tribulus terrestris). The exact determination of the copper concentration is the first step necessary in order to evaluate the potential of these plants as natural sources of trace elements for weaned piglets. The three medicinal herbs used in our study, grow in Oltenia County, Romania. A homogeneity test was applied to the samples and they were distributed to each participant on sealed plastic bags. All laboratories accomplished ten measurements for each sample. The determination method was established by protocol, flame atomic absorption spectrometry. For the reference value we choose consensus values from three expert laboratories. The appearance frequency of the results was determined by Kernel distribution plot and the remove of the doubtful results was done by the t test. In the case of laboratories with Z score satisfactory and a Zeta score unsatisfactory the International Harmonised Protocol for the Proficiency Testing of Analytical Chemistry Laboratories offer as possible explanation of unsatisfactory Zeta score an underestimation of the uncertainty. The assigned values calculated were: Vaccinium myrtillus L. 4.54 ppm Cu; for Origanum vulgare 10.87 ppm Cu; for Tribulus Terrestris 9.12 ppm Cu

Keywords: inter-laboratory study, copper, wild flora, piglets
INTRODUCTION

The animal production is one of the agricultural operations that may generate, via the excreta, a surplus of trace elements that may pollute the environment. Supplementation of trace minerals with a large safety margin in animal nutrition has resulted in a high level of mineral excretion that ends up in the environment. Copper (Cu), one of the heavy metals, potentially toxic, is used excessively particularly in the diets of weaned pigs because of its growth promoting role and because of the safety margins employed by the nutritionists. The high loads of Cu and Zn from pig manure are directly proportional with their dietary level (Zielinska et al., 2009). The problem is that the sources of oxides, sulphates and other inorganic combinations are sources with low bioavailability of the minerals, which produces large amounts of ingested minerals which are excreted directly into animal wastes. The surplus of minerals from the dejections builds up directly in the soil and surface water. Solutions have to be found which to decrease the concentration of trace elements (Cu, Zn, Mn, Fe, etc.) in animal excreta. Nutritionally, the simplest way is to reduce the level of dietary trace elements by using mineral sources with higher bioavailability. Within this context, the evaluation of the phytogenic feed additives as natural sources of trace elements is yet little approached even though the plants and their extracts are studied as means to control the diarrhoea and other gut disfunctionalities (Han et at, 2006). The question is whether these plants can be, as alternatives to the inorganic premixes, a natural source of trace elements (Rozica, 2005) available biologically in the gut, so that the excreted mineral ballast diminishes. On the other hand, the plants from the wild flora seem to have a higher level of minerals than the cultured plants (Dogan, 2004).

Having in view the above context, we undertook to conduct an inter-laboratory study to determine the copper concentration of 3 plants from the wild flora (Origanum vulgare; Vaccinium myrtillus L.; Tribulus terrestris) is the first step necessary in order to evaluate the potential of these plants as natural sources of trace elements for weaned piglets.

Seven laboratories participated in our inter-laboratory study for determination of copper in medicinal herbs by flame atomic absorption spectrometry (FAAS). The inter-laboratory test was organized because it is very difficult to make a proper mineral characterization for the wild herbs. The herbs chosen as samples for the inter-laboratory study can be feed supplements used to enhance the Cu status in animal feeding trials and to avoid the environmental pollution through faeces.

The method was tested on three wild herbs: Origanum vulgare; Tribulus terrestris; Vaccinium myrtillus L.

- Origanum vulgare (sovarv in Romanian) is a species of Origanum, of the mint family, native to Europe, the Mediterranean region and southern and central Asia.
- Tribulus terrestris (coltul babei in Romanian) is a flowering herb in the family Zygophyllaceae, native to warm temperate and tropical regions of the
World (southern Europe, southern Asia, throughout Africa and in northern Australia).

- Bilberry is any of several species of low-growing shrubs in the genus *Vaccinium* (family *Ericaceae*), bearing edible fruits. The species most often referred to is *Vaccinium myrtillus* L. (*afina* in Romanian), but there are several other closely related species.

Among the analytical methods reported for determining the copper content in herbs are those using FAAS (Soylak, 2006, De Sergio, 2007), inductively coupled plasma atomic emission spectrometry (Pugh et al, 2002), inductively coupled plasma/mass spectrometry (Curdova, 1998). Possible interference with the absorption of essential nutrients is one of the areas of uncertainty (Harmuth–Hoene, 1980).

**Material and Methods**

The three medicinal herbs used in our study, were grown in Oltenia County, Romania:

- *Origanum vulgare* grows on edge of forests in mountains region; the total area for harvest was 64 km². The climate is continental temperate with annual rainfall – 600 l/m². We used a mix of stems, leaves and flowers.
- *Tribulus terrestris* grows on grass-land, fields, gardens, grapevines; the total area for harvest was 400 m² and the climate is continental temperate with annual rainfall – 400 l/m². We used the fruits of *Tribulus terrestris*.
- *Vaccinium myrtillus* grows in the mountain region; the total area for harvest was 1 km² and the climate is arctic-alpine with annual rainfall – 1000 l/m². We used the fruits of *Vaccinium myrtillus* L.

The institutions participated in the interlaboratory study for Cu determination were: National Research Development Institute for Biology and Animal Nutrition (IBNA Balotesti); Institute for Food Bioresources (IBA Bucharest); Institute for Food Chemistry (ICA Bucharest); Institute for Hygiene and Veterinary Public Health, Bucharest; Research Institute for Soil Science and Agrochemistry, Bucharest; Research Development Institute for Nonferrous and Rare Metals-Brăniştei (with two distinct laboratories participating in the testing).

The samples were conditioned by the organizer (National Research Development Institute for Biology and Animal Nutrition) and distributed to the participants.

The method used by all the participants was FAAS after wet digestion, using a microwave oven technique. The working protocol accepted by all laboratories is presented subsequently.

**Principle**

The samples were weight with 10-4 precision and quantitatively transferred in the reaction flasks. Concentrated nitric acid and hydrogen peroxide were
added to the samples, which were then digested in a microwave oven. After complete cooling the digested solutions were quantitatively transferred in volumetric flasks. The concentrations of iron and zinc were determined by flame AAS.

**Chemicals and reagents**
- Concentrated nitric acid 65%, (Merck, Germany).
- Hydrogen peroxide 30%, analytical quality.
- Ultrapure water (Milli-Q Millipore, 18.2 MΩ/cm)
- Zinc stock solution.-1000 mg/L Zn(NO₃)₂ in HNO₃ 0.5 mol / L
- Iron stock solution.-1000 mg/L Fe(NO₃)₂ in HNO₃ 0.5 mol / L

**Equipment and gases**
- Analytical balance
- Stove
- Laboratory microwave oven
- Flame atomic absorption spectrometer with deuterium lamp

The samples were conditioned (dried at 65°C and grinded according to the Official Journal of the European Union, Regulation (CE) nr. 152/2009, Annex III) by the organizer (National Research Development Institute for Biology and Animal Nutrition). In the particular case of the *Tribulus terrestris* samples, after grinding, they were passed two times through sieves with 850, and 500 μm mesh. The samples were send to the participants in the study as blind duplicates, and the participants were asked to perform 10 determinations for each sample.

**Statistical parameters**
The assigned value, its incertitude and the statistical methods for calculating the consensus mean and the standard deviation can be determined with one of the following possibilities:
1. Knowing the value from a formula of calculation
2. Certified reference values
3. Reference values
4. Consensus values between the reference laboratories
5. Consensus values between the participants in the test (Koch, 2009).

In this case, the first 3 possibilities can not be taken into consideration, the determination of the assumed value being the main purpose of the study.

The number of participants in the study being low, a limited number of reference laboratories, we have chosen to set the assigned value by consensus of all the participating laboratories (Koch, 2001).

The population mean and the standard deviation were established using robust means, using the Huber estimation (algorithm A).
Using algorithm A, we consider our set of data characterized by a robust mean \( X^* \) and a robust std dev \( S^* \). As initial values for \( X^* \) we took the median, and for \( S^* \) we calculated the median of the absolute deviation (\( S^* = 1.483 \times \text{med}(X_i - X^*) \)).

The limits of the range are:

\[
\begin{align*}
X_i^* &= X^* - \delta \text{ if } X_i < X^* - \delta; \\
X_i^* &= X^* + \delta \text{ if } X_i > X^* + \delta; \\
\delta &= 1.5 \times S^*
\end{align*}
\]

With these data we calculated a new \( X^* \) and a new \( S^* \).

\[
S^* = 1.134 \sqrt{\frac{(X_i^* - X^*)^2}{p - 1}}
\]

For the statistical performance we applied \( Z \), \( Z' \) and Zeta scores

\[
Z = \frac{x - \mu}{S}
\]

\( Z \) score:
- \( x \) = laboratory mean
- \( \mu \) = assigned value
- \( S \) = standard deviation

\[
Z' = \frac{(x - \mu)}{\sqrt{S^2 + u_x^2}}
\]

\( Z' \) score:
- \( u_x \) = laboratory uncertainty
- \( uX \) = uncertainty of assigned value

\[
\xi = \frac{(x - \mu)}{\sqrt{u_x^2 + uX^2}}
\]

\( \xi \) = Zeta score

\[
|z| \leq 2, \text{ satisfactory, probability - 95.46%;}
\]

\[
2 < |z| < 3, \text{ questionable;}
\]

\[
|z| \geq 3, \text{ unsatisfactory.}
\]

RESULTS AND DISCUSSION

The results reported by the participants form data strings which cover a quite wide range of values (Table 1). Given the specificity of the data strings the fact that they are contaminated with values which obviously do not belong to the same family, we need a calculation algorithm to determine the robust mean (Thompson, 2006). On the other hand, the large variation of the data in the string shows the usefulness of an inter-laboratory study, particularly under the conditions in which the analysed samples are from the wild flora.

Consensus from all participant laboratories is the most common method for establish the assigned value, but for our study, we choose consensus values from expert laboratories. We consider expert laboratories: Lab 1, Lab 3 and Lab 6.
Table 1 Analytical results reported by each laboratory participating in the study (mg/kg)

<table>
<thead>
<tr>
<th></th>
<th>Lab 1</th>
<th>Lab 2</th>
<th>Lab 3</th>
<th>Lab 4</th>
<th>Lab 5</th>
<th>Lab 6</th>
<th>Lab 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Origanum vulgare</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>9.07</td>
<td>7.11</td>
<td>11.38</td>
<td>8.94</td>
<td>9.34</td>
<td>12.15</td>
<td>11.30</td>
</tr>
<tr>
<td>Std dev</td>
<td>0.51</td>
<td>0.33</td>
<td>0.38</td>
<td>0.68</td>
<td>0.25</td>
<td>0.66</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>Tribulus terrestris</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>7.32</td>
<td>3.39</td>
<td>9.97</td>
<td>5.00</td>
<td>5.60</td>
<td>9.96</td>
<td>9.10</td>
</tr>
<tr>
<td>Std dev</td>
<td>0.66</td>
<td>0.24</td>
<td>0.42</td>
<td>0.45</td>
<td>0.31</td>
<td>0.19</td>
<td>0.71</td>
</tr>
<tr>
<td><strong>Vaccinium myrtillus L.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3.14</td>
<td>3.68</td>
<td>3.86</td>
<td>3.99</td>
<td>4.39</td>
<td>6.66</td>
<td>7.02</td>
</tr>
<tr>
<td>Std dev</td>
<td>0.24</td>
<td>0.20</td>
<td>0.81</td>
<td>0.44</td>
<td>0.36</td>
<td>0.34</td>
<td>0.46</td>
</tr>
</tbody>
</table>

The estimation of the density functions for the considered data strings (table 1) was done using the Kernel density function (fig 1). Density estimation is the starting point of any analysis. There are several methods that can be employed to obtain an estimator of the density functions. The simplest method is to plot a histogram. The factors that affect the shape of the histogram are the origin point of the interval and the size of the interval, which show the smoothness of the histogram curve. Histogram dependency on these two factors is a disadvantage of them. For our data strings we have chosen the Kernel density in order to avoid the functional shapes with different weights (Chu, 1991).

![Graphs of Origanum vulgare and Tribulus terrestris](image)

**Vaccinium myrtillus L**

Fig. 1 Kernel density
As expected, given the range of values reported by the laboratories, (table 1), the Kernel density for Cu determination shows a non homogenous set of results for *Vaccinium myrtillus L.*, while for *Origanum vulgare* and *Tribulus Terrestris*, the Kernel density doesn’t have a Gaussian shape. Therefore we used another test to determine the assigned value. The population means and standard deviations were calculated using Algorithm A.

Table 2- Comparison of the initial data string and of the data string taken to be real (after applying Algorithm A)

<table>
<thead>
<tr>
<th></th>
<th><em>Vaccinium myrtillus L.</em></th>
<th><em>Origanum vulgare</em></th>
<th><em>Tribulus terrestris</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (mg/kg)</td>
<td>4.68</td>
<td>9.90</td>
<td>7.19</td>
</tr>
<tr>
<td>Assigned value</td>
<td>4.54</td>
<td>10.87</td>
<td>9.12</td>
</tr>
<tr>
<td>Std. Dev.</td>
<td>1.49</td>
<td>1.75</td>
<td>2.48</td>
</tr>
<tr>
<td>Sample variance</td>
<td>2.23</td>
<td>3.05</td>
<td>6.13</td>
</tr>
<tr>
<td>Uncertainty</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

From the data of Table 2, one can notice an important difference between the arithmetic mean and the assigned value for *Origanum vulgare* and *Tribulus terrestris*, and a smaller difference for *Vaccinium myrtillus L.*, but for all three plants the interpretation of the Kernel graphs is supported by the variance of the initial data string. Compared to the variance of the data string taken to be real after applying Algorithm A, a higher initial variance is noticed, which characterises a less homogenous string.

Also the results displayed in Table 2 show that *Origanum vulgare* and *Tribulus terrestris* are important sources of Cu. The concentrations that we determined make these plants valuable sources of Cu that can be used in animal feeding. According to the National Research Council (1998), the Cu requirement of the weaned piglets (category which uses the richest supplements of trace elements) is 5 ppm. Currently, in order to have this concentration in the feeds, the diets for weaned piglets are supplemented with mineral salts (copper sulphate) which exceed NRC recommendations. Using these natural sources of minerals in pig diets (1 or 3% inclusion rate) will meet animal requirements for Cu while decreasing or even excluding the premix from the feed. For instance, excluding the inorganic source of Cu and adding 3% *Origanum vulgare* in the diets for weaned piglets (14 kg) we can obtain a concentration of 9.46 ppm Cu in the feed, against just 5 ppm as per NRC recommendations. The efficiency of these wild plants can only be assayed in studies of mineral bioavailability.

The analytical capacity of the participating laboratories was evaluated using Z, Z’ and Zeta scores. The results are shown in table 3.

Zeta score confirm the unsatisfactory results of Z score in four cases and for another two cases, 2 < zeta value 3 and no other test confirm the hypothesis of unsatisfactory results. The International Harmonised Protocol for the Proficiency Testing of Analytical Chemistry Laboratories offer as possible explanation of unsatisfactory zeta score an underestimation of the uncertainty.
This hypothesis is confirmed in case of laboratories with z score satisfactory and a zeta score unsatisfactory.

Table 3. Results of scoring Z, Z’ and Zeta

<table>
<thead>
<tr>
<th></th>
<th>Lab 1</th>
<th>Lab 2</th>
<th>Lab 3</th>
<th>Lab 4</th>
<th>Lab 5</th>
<th>Lab 6</th>
<th>Lab 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Origanum vulgare</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z score</td>
<td>-1.10</td>
<td>-2.30</td>
<td>0.31</td>
<td>-1.18</td>
<td>-0.94</td>
<td>0.79</td>
<td>0.26</td>
</tr>
<tr>
<td>Z’ score</td>
<td>-0.85</td>
<td>-2.26</td>
<td>0.18</td>
<td>-0.70</td>
<td>-0.84</td>
<td>0.43</td>
<td>0.23</td>
</tr>
<tr>
<td>Zeta score</td>
<td>-1.28</td>
<td>-7.76</td>
<td>0.22</td>
<td>-0.86</td>
<td>-1.73</td>
<td>0.51</td>
<td>0.71</td>
</tr>
<tr>
<td><strong>Tribulus terrestris</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z score</td>
<td>-1.24</td>
<td>-3.92</td>
<td>0.58</td>
<td>-2.82</td>
<td>-2.41</td>
<td>0.58</td>
<td>-0.01</td>
</tr>
<tr>
<td>Z’ score</td>
<td>-0.99</td>
<td>-3.83</td>
<td>0.34</td>
<td>-1.97</td>
<td>-2.00</td>
<td>0.33</td>
<td>-0.01</td>
</tr>
<tr>
<td>Zeta score</td>
<td>-1.57</td>
<td>-12.49</td>
<td>0.42</td>
<td>-2.68</td>
<td>-3.40</td>
<td>0.41</td>
<td>-0.03</td>
</tr>
<tr>
<td><strong>Vaccinium myrtillus L.</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z score</td>
<td>-0.76</td>
<td>-0.47</td>
<td>-0.37</td>
<td>-0.30</td>
<td>-0.08</td>
<td>1.15</td>
<td>1.35</td>
</tr>
<tr>
<td>Z’ score</td>
<td>-0.74</td>
<td>-0.46</td>
<td>-0.34</td>
<td>-0.23</td>
<td>-0.07</td>
<td>0.93</td>
<td>1.35</td>
</tr>
<tr>
<td>Zeta score</td>
<td>-2.22</td>
<td>-1.63</td>
<td>-0.77</td>
<td>-0.35</td>
<td>-0.12</td>
<td>1.51</td>
<td>3.89</td>
</tr>
</tbody>
</table>

Figure 2 shows comparatively the average values of the copper concentrations in all three studied plants against the reference values. By matching the values found by the participants with the reference domain ± standard deviation (**Origanum vulgare**: 10.87 ppm ± 1.63; **Tribulus terrestris**: 9.12 ppm ± 1.46; **Vaccinium myrtillus L.**: 4.54 ppm ± 1.84) we may consider the result as satisfactory.
Monitoring the individual results of the laboratories using algorithm A, we can notice the following situations of exceeding the range:

- Lab 2, 4, 5 for *Origanum vulgare* sample
- Lab 1, 2, 4 and Lab 5 for *Tribulus terrestris* sample
- Lab 2 and Lab 7 for *Vaccinium myrtillus L.* sample

The data of Table 3, Figure 1 and Figure 2 show that the sample of *Tribulus terrestris* caused the most serious problems to the laboratories. This plant required a more laborious preparation, particularly due to its thorny fruits which turned in a very hard matter after drying. This might have an adverse effect on its ingestion by the piglets.
CONCLUSIONS

Because there was no certified reference value, the samples being wild plants, we calculated reference values from the analytical results from expert laboratories.

We used the robust mean to estimate the mean population of values, using the Huber estimate (algorithm A). The assigned values calculated with algorithm A were: for *Vaccinium myrtillus* L. 4.54 ppm Cu; for *Origanum vulgare* 10.87 ppm Cu; for *Tribulus Terrestris* 9.12 ppm Cu.

The analytical data show that *Origanum vulgare* and *Tribulus Terrestris* have an outstanding concentration of Cu. The particularities of *Tribulus Terrestris* caused problems during sample analysis in four of the laboratories.

The data justify the intention to test *Origanum vulgare* as source Cu in weaned piglets feeding.

REFERENCES


