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Grass as a biomonitor: impact of copper suplementation in the environment

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Abstract
In this work copper, molybdenum and sulfur determinations in grass, used as biomonitor of the soil, were performed during the period 2008-2009 and compared with the levels obtained in 2000-2001 in the same locations with the aim of observing environmental changes after the implementation of a program recommending oral and injected copper supplements in cows and after the use of fertilizers containing molybdenum and sulfur elements that can affect copper bioavailability. Several correlations with meteorological factors were also studied. The results showed that the copper level increased in grass but the concentrations remained below the recommended values. The mean copper concentrations in spring, summer, autumn and winter were 2.44, 3.12, 4.29 and 5.21 mg kg^{-1} DM, respectively, and for molybdenum were 0.20, 0.16, 0.22 and 0.17 mg kg^{-1} DM, respectively (DM = dry matter). In many samples, the molybdenum concentration was above the recommended range and consequently a secondary deficiency can be observed. In addition, a seasonal variation in grass, an inverse correlation between copper and molybdenum concentrations and a direct correlation between copper levels and the relative humidity were observed permitting visualization of the most critical periods of hypocuprosis.

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1. Introduction
The health of living organisms depends, among other factors, on their ability to obtain from the external chemical environment essential trace elements [1].

In Uruguay, where almost 90% of its land is devoted to raising livestock with extensive breeding, an important health problem related with copper deficiency was reported at the end of the twentieth century, in Salto Department [2] where a nosologic entity, named "growth syndrome", was detected in bovine and ovine herds showing anemia, low weight, bone deformities, depigmentation, persistent diarrhea, and low milk production, among others. Similar observations were performed in the nearby regions from Brazil and Argentina [3] [4].

Taking into account these observations, an interdisciplinary group was formed at the end of the nineties with the aim of studying, in Salto Department of Northern Uruguay (Fig. 1) [5], the copper lacking cattle, the causes of this deficiency and alleviation with copper complexes as supplements [6-8].

As a part of these studies, Cu, Mo and S levels in grass were determined in different locations of the milk, in production area Salto Department, particularly in Itapebí, San Antonio, EEFAS and Las Margaritas, in the period 2000-2001. The obtained results showed that copper content in grass was below adequate levels (10 mg kg^{-1}) most of the year [7]. Although there is no complete agreement with the copper requirements for cows, 10 mg kg^{-1} of dry matter (DM) is usually considered adequate or recommended [9]. Besides, as a conclusion of this previous study, a positive correlation between copper content in grass and serum copper level in cows was established [7], showing

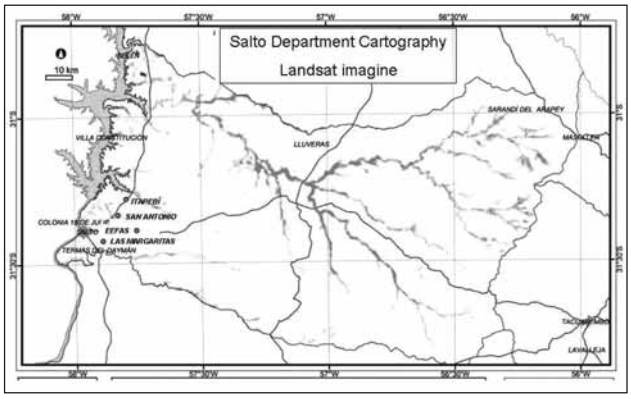

Figure 1. Salto Department cartography showing the sampling locations and their coordinates (Dirección General de Recursos Naturales Renovables Ministerio de Ganadería Agricultura y Pesca Renovables, 2010).

that the evaluation of copper levels in grass is a good tool to understand the copper status in cows.

On the other hand, it is well known that copper deficiency in cows can occur because of low copper content in grass (primary deficiency) but also due to other factors that impact copper bioavailability (secondary deficiency) [10]. Molybdenum and sulfur are the two most important factors that reduce copper absorption. Molybdenum can form copper compounds like $CuMoO_4$ and $CuMoS_4$, of low bioavailability, the latter with S^{2-} produced in the rumen from fresh grass [1][11]. For this reason it is important to evaluate Mo and S concentrations in grass.

Due to these observations, veterinarians of the region began to recommend oral copper supplements to alleviate the "growth syndrome" [12-13]. Oral supplementation with copper salts sometimes appears to be unsatisfactory for ruminants [14]. Especially, it is well known that only low amount of copper (near 5%) are absorbed in cows from feed and consequently most is excreted, principally through the feces, and remains in the environment [15].

Another important aspect about copper and environment is the use of fertilizers containing trace elements that could interfere with copper absorption. One of them is molybdenum, which can affect copper bioavailability.

In this work new Cu, Mo and S seasonal measurements in grass were performed during the period 2008-2009 with the aim of evaluating the bioavailable mineral levels that can be absorbed by grass after changes in the animal supplementation program. Then, a comparison with the results obtained in the period 2000-2001 was performed. Also, several correlations with meteorological factors were studied to understand the results observed in mineral content and consequently to advise the producers affected by the "growth syndrome".

2. Experimental

2.1. Materials

All reagents were of analytical grade. Purified water (ASTM Type I) was obtained from a Millipore (São Paulo, Brazil) Simplicity 185 purifier fed with glass-distilled water.

All bottles for storing samples and standard solutions were immersed in 10% (v/v) nitric acid for 24 h, rinsed with ultrapure water and dried before being used.

2.2. Grass samples

A total number of 35 samples of natural grass were obtained from four different locations named Itapebí, San Antonio, EEFAS and Las Margaritas (see Fig. 1). The minimum number of samples was 5 for each location with a minimum distance of 1 meter between each one. They were collected by the hand clipping method based on animal selectivity. The samples were collected in the period July 2008 – August 2009, washed with purified water and dried at 50 ºC until constant weight.

2.3. Analytical methods

Grass samples were prepared as follows: 10 g of dried grass was accurately weighed and ashed at 500 °C in an Atec furnace (Montevideo, Uruguay), model HFA10, until constant weight. Inorganic ashes were dissolved with 50 mL of 50 % v/v aqueous HCl solution according to AOAC International method 975.03 [16]. This sample treatment was run in duplicate. The solutions obtained were used for the analytical determination of Cu and Mo.

According to the preliminary information, Cu level in grass is usually in the order of mg kg^{-1}. For this reason the flame atomic absorption spectrometry (FAAS) technique is adequate, while for Mo determination (in the order of $\mu g\ kg^{-1}$) electrothermal atomic absorption spectrometry (ETAAS) was selected [17].

S levels in grass samples were also determined. For this purpose, 1 g of accurately weighed dried sample was mixed with a $Mg(NO_3)_2$ solution and heated in a furnace until samples were completely oxidized as described in AOAC International, method 923.01 [18].

Sulfur was determined as sulfate ion (SO_4^{2-}) in the final solution by a turbidimetric method using an Oakton TN-100 instrument according to the standard method 4500E-APHA [19].

2.3.1. Copper determination

The Cu determinations were carried out using the FAAS technique after sample preparation according to the AOAC International, method 975.03.

A Perkin Elmer (Norwalk, CT, USA) model 5000 flame atomic absorption spectrometer, with an air-acetylene flame and a copper hollow cathode lamp (Photron, Narre Warren, Australia) was used for Cu determination. The selected analytical wavelength was 324.8 nm.

The Cu stock solution (1000 mg L^{-1}) was prepared by dissolving electrolytic wire (purity > 99.7 %) in HNO_3 1 % (v/v).

Aqueous standards in the range of 1-5 mg L^{-1} were freshly prepared by appropriate dilution of the stock solution in purified water.

2.3.2. Molybdenum determination

The Mo determinations were carried out using the ETAAS technique after sample preparation according to the AOAC International, method 975.03.

These determinations were performed with a Varian AA-240 atomic absorption spectrometer with a deuterium background correction, a GTA-120 electrothermal atomizer and a PSD-120 autosampler (Victoria, Australia). Wall atomization with pyrolytic coated partitioned graphite tubes was used. The Mo hollow-cathode lamp (Photron, Narre Warren, Australia) was run under the conditions recommended by the manufacturer. The selected analytical wavelength was 313.3 nm. The graphite furnace temperature program for this analyte was 1000 ºC for pyrolysis and 2800 ºC for

atomization. All measurements were performed using integrated absorbance (peak area).

A 1000 mg L^{-1} stock standard solution of Mo was prepared from ammonium molybdate tetrahydrate (J.T. Baker, NJ, USA) in NH_4OH 1% (v/v). Working standards in the range of 4-80 μg L^{-1} were freshly prepared by appropriate dilution of the stock solution in 0.1 % (v/v) HNO_3.

2.3.3. Sulfur determination

Turbidimetric determinations where performed by an external laboratory. This laboratory was selected because it meets the quality standards to provide reliable results. The determinations were performed as was explained in section 2.3 with no modifications to the standard method referenced.

2.4. Meteorological factors

Temperature, relative humidity and rainfall data were obtained from INIA (Instituto Nacional de Investigación Agropecuaria, Salto, Uruguay), measured in the studied region [20].

3. Results and discussion

3.1. Copper and molybdenum determinations

Regarding the Cu determinations we investigated the possible existence of interferences. It was found that the slopes did not show significant differences. Consequently the measurements were carried out by direct calibration since no evidence of matrix interferences was found. Reagent blanks were also run.

The mean figures of merit were detection limit 10 μg L^{-1} (LD, 3s) in solutions, corresponding to 0.050 mg kg^{-1} in grass samples, linear range up to 5 mg L^{-1} and the day - to - day analytical precision, expressed as RSD (%), was better than 5%.

In order to evaluate the sample preparation procedure a spike/recovery approach was carried out. For the spiked samples analyzed, recoveries were in the range 95%-105%, showing no losses of the analyte during the procedure.

The same study was performed for Mo analytical determinations, after investigating the possible existence of multiplicative interferences by comparing the slope of the calibration curve with that of the standard additions curve by means of statistical hypothesis testing. It was found that the samples showed significant differences in the slopes. This suggests the existence of matrix interferences.

For this reason standard additions on samples were carried out. Reagent blanks were also run.

The mean figures of merit were detection limit 4.3 μg L^{-1} (LD, 3s) in solution, corresponding to 0.022 mg kg^{-1} in grass samples, linear range up to 80 μg L^{-1} and the day - to - day analytical precision, expressed as RSD (%), was better than 10 %.

The method was verified for grass samples by a spike/recovery approach. For the spiked samples analyzed, recoveries were in the range 90%-110%.

3.2. Cu, Mo and S levels in the period 2008-2009

Seasonal variation of mean Cu and Mo levels considering all grass samples from Itapebí, San Antonio, EEFAS and Las Margaritas locations are shown in Figure 2.

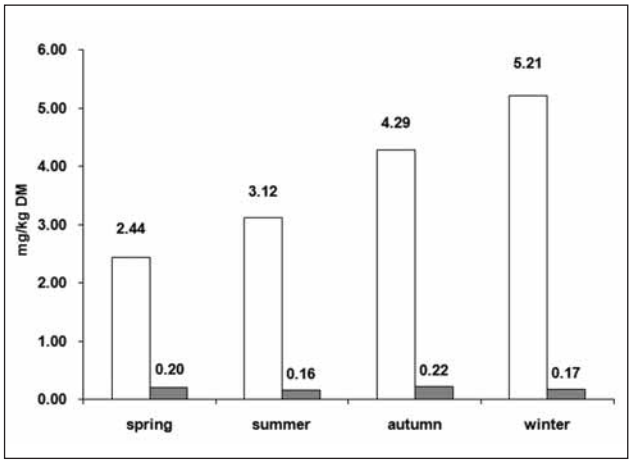

Figure 2. Seasonal variation of mean Cu (white bars) and Mo (gray bars) levels in grass samples from Itapebí, San Antonio, EEFAS and Las Margaritas locations in the period 2008-2009.

As Figure 2 shows, all the samples were well into the recommended level (10 mg kg^{-1} of dry matter (DM)). A seasonal fluctuation of Cu level in grass was observed, the lower values occurring in spring and summer, seasons with low humidity and high temperatures [20]. The correlation between humidity and copper levels in grass will be presented in section 3.4.

These results permitted better understanding of the "growth syndrome" in several cows, the nosological entity still reported in this region.

The recommended molybdenum levels in grass are under discussion and are not well established, oscillating between 0.03 and 0.15 mg kg^{-1} DM [21-22].

The obtained results in the region were in the range of 0.063-0.480 mg kg^{-1} DM. Fifteen samples were in the recommended range and twenty samples above this range. Nowadays, some producers use several fertilizers containing Mo, such as $Na_2MoO_4 \cdot 2H_2O$, MoO_3, or $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$, as a nutrient that can be applied to soil or foliage. Usually, Mo concentration in these products is in the range 0.0008-0.025% and they can also contain other trace elements, including Cu.

This Mo supplementation might be the cause of the number of samples out of the recommended range.

The S levels were, in this period, in the 0.1-0.3 % DM range, below the recommended values (0.3-0.5 % DM) [23-24]. This behavior discards the possibility of S interference in Cu absorption [25]. In spite of the fact that S deficiency is a growth limiting factor that should be treated, it is likely that correcting any sulfur deficit would decrease the Cu status.

3.3. Correlations

3.3.1. Cu and Mo monitoring during the periods 2000-2001 and 2008-2009

To evaluate changes in Cu, Mo and S levels in grass after veterinarian prescription of supplementing the animals, Las Margaritas and San Antonio locations were selected for monitoring Cu and Mo levels based on our previous research. In the other locations (Itapebí and EEFAS) copper and molybdenum seasonal variation was not studied in the period 2000-2001.

Figures 3 A and 3 B show the comparative seasonal copper levels in grass during the period 2000-2001 (previously reported) [7] and 2008-2009, in Margaritas and San Antonio locations, respectively.

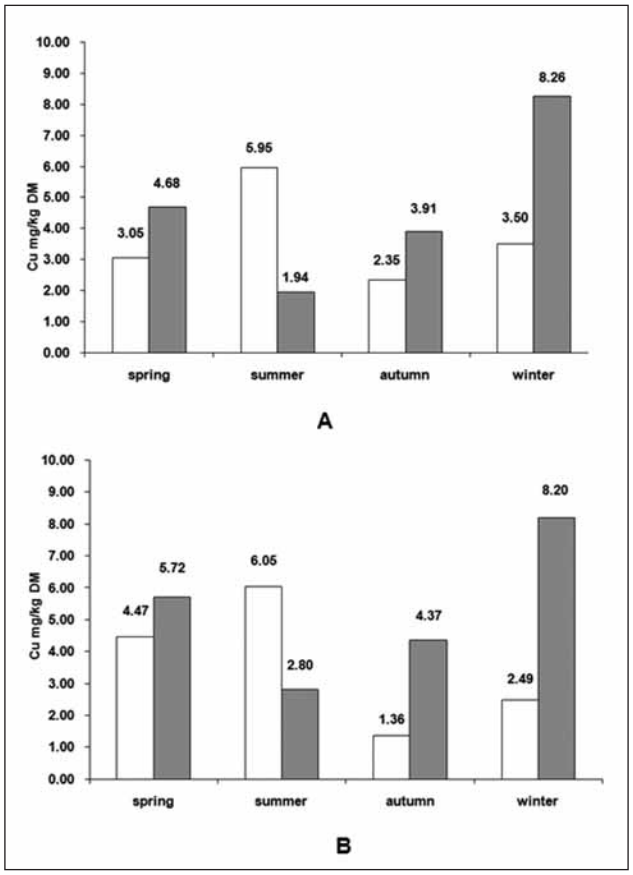

Figure 3. A) Cu levels in the periods 2000-2001 (white bars) and 2008-2009 (gray bars) in Las Margaritas; B) Cu levels in the periods 2000-2001 (white bars) and 2008-2009 (gray bars) in San Antonio.

As shown in Figures 3 A and 3 B copper increase was observed in the period 2008-2009 compared to that observed in the period 2000-2001, except in summer. This general behavior might be due to the copper supplementation of animals in the studied locations. This is in agreement with the fact that only a low copper percentage is absorbed in cows from feed as explained in section 1 [15].

The copper level decrease observed in summer could be related with the significant drought during this season in the period 2008-2009 that decreased the concentration of soluble Cu species and consequently the absorption [20]. Apart from this factor, the stage of development of plants could also affect the root uptake and hence the mineral content of grass in this season [1].

On the other hand, Figs. 4 A and 4 B show the comparative seasonal Mo levels in both periods, in Las Margaritas and San Antonio locations respectively.

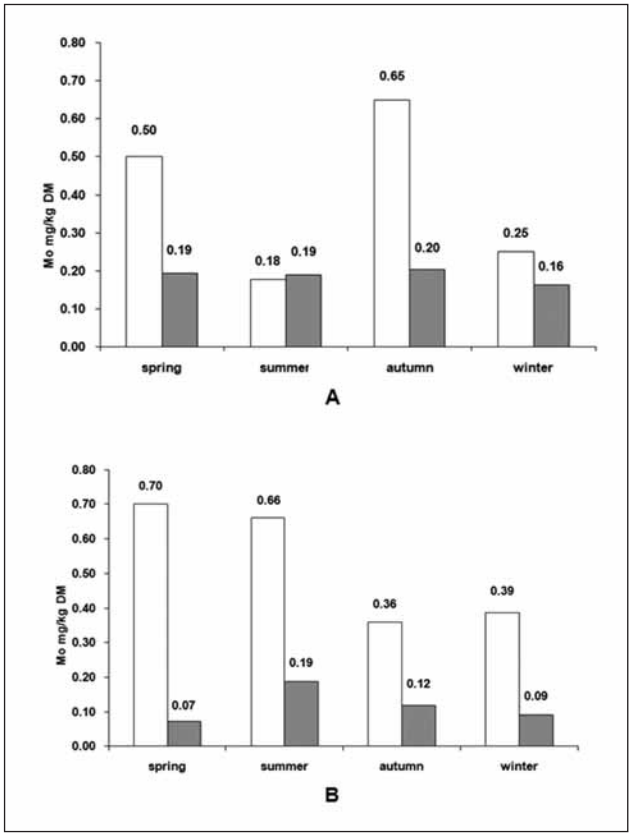

Figure 4. A) Mo levels in the periods 2000-2001 (white bars) and 2008-2009 (gray bars) in Las Margaritas; B) Mo levels in the periods 2000-2001(white bars) and 2008-2009 (gray bars) in San Antonio.

As figures 4 A and 4 B show, there was an important and unexpected Mo decrease in grass during the period 2008-2009 in almost all the seasons (except in summer in Las Margaritas location) even falling below the recommended limit for grass, in some cases.

This behavior could respond to the general soil impoverishment or to the formation in the soil of not bioavailable species that grass could not absorb. If we consider that some producers often used fertilizers with products containing molybdenum species, the second hypothesis would be the most appropriate.

3.3.2. Mo *vs* Cu levels

A Mo *vs* Cu correlation was analyzed using the statistic

table from Pearson et al. for a unilateral test with two degrees of freedom (N-2). The correlation coefficient (R) obtained for the analytical data was compared with the critical values of the Pearson table with a probability of 5% [26].

In Figs. 5A and 5B, the Mo concentrations *vs* Cu concentrations in Las Margaritas and San Antonio locations were plotted.

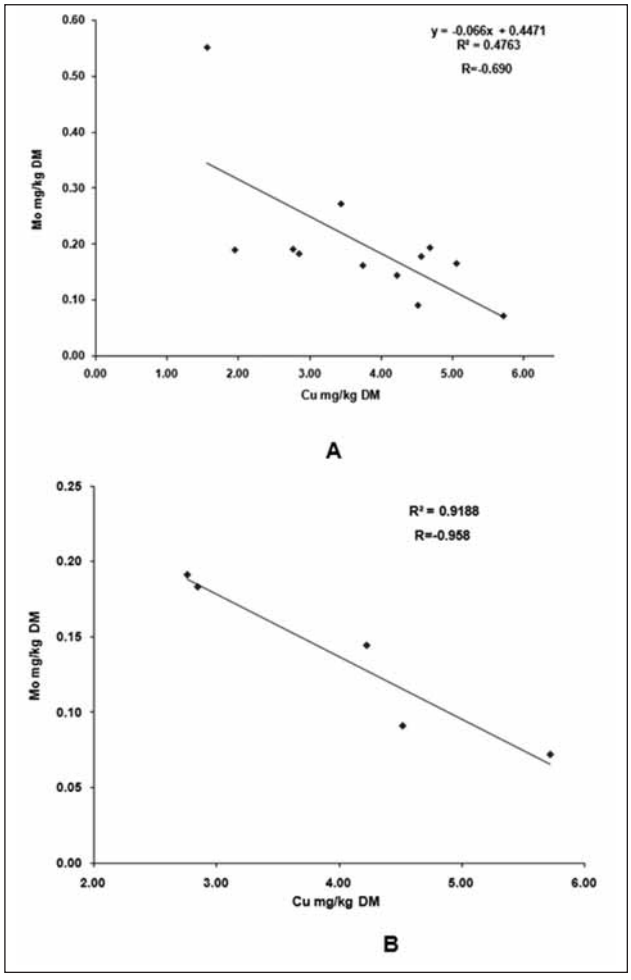

Figure 5. A) Correlation between Cu and Mo concentrations in grass obtained from Las Margaritas and San Antonio locations; B) Correlation between Cu and Mo concentrations only for San Antonio.

As expected taking into account previous reports [1] [23], Fig. 5 shows an inverse correlation between both metals (R = -0.690, critical absolute value: 0.576) when San Antonio and Las Margaritas data are plotted together. A better correlation was obtained for individual data from San Antonio (R = -0.958, critical absolute value: 0.878) (see Fig. 5 B) [26].

3.4. Cu and Mo levels vs meteorological factors

As explained in 3.3.2. the correlation was analyzed using the statistical table from Pearson *et al*. [26].

The correlations of Cu and Mo concentration with meteorological factors like temperature, relative humidity and rainfall data were performed [20].

Fig. 6 shows Cu levels from Las Margaritas and San Antonio locations *vs* mean daily relative humidity (%).

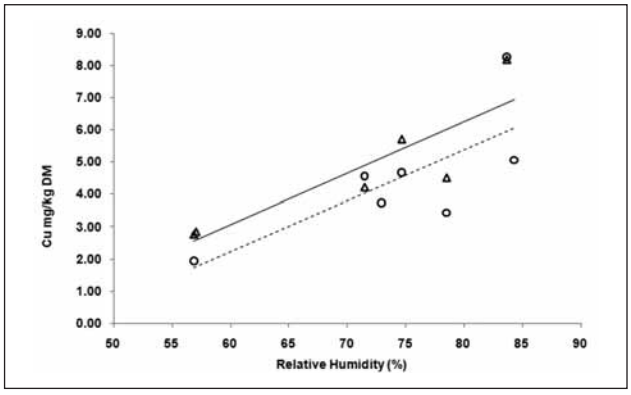

Figure 6. Cu levels from Las Margaritas (o) and San Antonio locations (Δ) vs mean daily relative humidity (%).

As Fig. 6 shows, a good positive correlation was observed in both locations, obtaining R = 0.752 and critical value 0.754 in Las Margaritas, and R = 0.878 and critical value 0.811 in San Antonio [26].

This result is in accordance with the fact that the increase of the environmental humidity could produce an increase of soluble Cu compounds and consequently higher Cu levels in grass.

No correlation was found with Mo levels and relative humidity or with Cu and Mo vs other meteorological factors.

4. Conclusions

The study of Cu levels in grass permitted better understanding of the "growth syndrome" in cows, nosological entity still reported in the region of Salto Department. This is a very important problem not only in Uruguay but also in other countries in the region devoted to livestock production, like Argentine and Brazil.

The monitoring of mineral levels in the periods 2000-2001 and 2008-2009 produced interesting data and show that supplementation with Cu compounds, promoted by veterinarians of the region in the first period, ten years ago, increased the Cu level in grass. However, the concentrations remained below the recommended values. Besides, the Mo levels decreased with time but several samples remained above the recommended range and consequently they can affect copper bioavailability, producing a secondary deficiency. For this reason it is important to control the addition of fertilizers containing Mo compounds. The low S levels obtained in grass samples discard the possibility of interference in copper absorption.

In addition, the seasonal mineral variation in grass, the inverse correlation between Cu and Mo concentrations and the direct correlation between Cu levels and the relative

humidity permitted visualization of the most critical periods for animal health.

Taking this work as a base, our future aim is to investigate other analytes such as Zn, Mg and Se and to perform similar studies in other regions and for a longer period.

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5. References

1. Selinus O.; Alloway B.J.; Centeno J.A.; Finkelman R.B.; Fuge R.; Lindh U.; Smedley P.; *Essentials of Medical Geology: Impacts of The Natural Environment On Public Health*; Amsterdam; Academic Press, 2004.
2. Pigurina G.; Soares de Lima J.M.; Berretta E. *Serie Técnica de INIA Tacuarembó*. 1998, Vol 102, 113.
3. Marques A.P.; Riet-Correa F.; Pereira Soares M.; Lippi Ortolani E.; Giuliodori M.J.; *Pesq. Vet. Bras*. 2003, 23, 21.
4. Mattioli, G.A.; Ramírez, C.E.; Giuliodori, M.J.; Tittarelli, C.M.; Yano, H.; Matsui, T.; *Livest. Prod Sci.* 1996, 47, 7.
5. Dirección General de Recursos Naturales Renovables, Ministerio de Ganadería Agricultura y Pesca: http://www.renare.gub.uy/suelos/ map_separate-legend.phtml accessed July 2010.
6. Kremer E.; Torre M.H.; Viera I.; Facchin G.; Cuevas A.; Baran E.J.; Bussi J.; Ohanian M.; Irigoyen J.; Porochin T.; di Donato V.; Irigoyen C.; Romero J.; in *Metal Ions in Biology and Medicine*. Centeno J.A., Collery P.; Vernet G.; Finkelman R.B.; Gibb H.; Etienne J. (Eds.), Paris, John Libbey, 2006, 6, 537.
7. Torre M.H.; Viera I.; Facchin G.; Kremer E.; Baran E.J.; Porochin T.; di Donato V.; Irigoyen C.; Irigoyen J.; Saldaña S.; Bussi J.; Ohanian M.; Fuentes J. *Livest. Prod. Sci.* 2005, 95, 49.
8. Torre M.H.; Viera I.; Kremer E.; Facchin G.; Bruné V.; Irigoyen J.; Porochin T.; di Donato V.; Saldaña S.; Dol I.; De Nigris A.; Fuentes J. in *Trace Elements in the Food Chain. Deficiency or excess of trace elements in the environment as a risk of health*. Working Committee on Trace Elements of the Hungarian Academy of Science (HAS) and Institute of Materials and Environmental Chemistry of the HAS. Szilágyi K.S.M.; Szentmihalyi K.; (Eds.), Budapest, 2009, 3, 118.
9. National Research Council (NRC) (Ed.) *Nutrient Requirements of Dairy Cattle*, 7th ed., Washington, National Academic Press, 2001.
10. Reid R.L.; Horvath D.J. *Anim. Feed Sci. Technol*. 1980, 5, 95.
11. Reid R.S.; Attaelmannan M.A. *J Inorg. Biochem*.1998, 69, 59.
12. Smart M.E.; Cymbaluk N.F.; Christensen D.A. *Can. Vet. J.* 1992, 33,163.
13. Ledoux D.R.; Henry P.R.; Ammerman C.B. *Nutr. Res.* 1996, 16, 69.
14. Frank A.; Danielsson R.; Selinus O. *Sci. Total Environ*, 2004, 330, 131.
15. http://ec.europa.eu/food/fs/sc/scan/out115_en.pdf accessed January 2011.
16. Isaac, R.A. *Official Methods of Analysis of AOAC International*, AOAC International, Gaithersburg MD, 1996, 3.
17. Welz B., Sperling M. *Atomic Absorption Spectrometry*, Weinheim, Wiley-VCH, 1999.
18. Isaac R.A. (Ed.), *Official Methods of Analysis of AOAC International*, AOAC International, Gaithersburg MD, 1996, 22.
19. American Public Health Association (APHA). *Standard Methods for the Examination of Water and Wastewater*, 20th ed., Washington, APHA, 1998.
20. http://www.inia.org.uy/online/site/gras.php?idEst=5 accessed January 2011.
21. Baker D.E.; Senft J.P. In Alloway B.J. Ed. *Heavy Metals in Soils*, 2nd ed. Glasgow, Chapman & Hall, 1992,179.
22. Dean J.R. *Methods for Environmental Trace Analysis*, Chichester, John Wiley & Sons, 2003.
23. Boila R.J.; Devlin T.J., Drysdale R.A.; Lillie L.E. *Can. J. Anim. Sci.* 1984, 64, 675.
24. Rayburn E.B. *Forage Quality – Minerals*, Virginia, West Virginia University Extension Service, 1997.
25. Suttle N.F. *Br. J. Nutr.*, 1975, 34, 411.
26. Snedecor G.W.; Cochran W.G. *Métodos Estadísticos*. México, C.E.C.S.A, 1980.