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DOCTORAL THESIS

(abstract)

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CHEMICAL-ANALYTICAL STUDY OF CORRELATIONS BETWEEN TRACE ELEMENTS CONTENT OF ANIMAL ORGANISM AND THEIR CONCENTRATIONS FROM DIETS ENRICHED WITH PLANTS AND PLANT EXTRACTS

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CONTENTS

INTRODUCTION

The aim of the thesis was to realize a chemical – analytical characterization of several types of biological samples of plant and animal origin in order to assess correlations between trace elements diets content and their retrieval in animal organism. Specifically, these correlations were evaluated for diets of weaned piglets, supplemented with some phytoadditives (plants and mixtures of plants harvested from wild flora and crops) in order to improve mineral status and protect the environment by reducing the amount of used mineral premixes.

The phytoadditives used as supplements in animal nutrition are plants or derived products from plants which are included in feeds with the aim of improving animal productivity or/and to enhance the quality of food obtained from these animals. Mineral premixes (mineral salts mixtures) are used currently as supplements in animal nutrition.

These trace elements in feed are absolutely indispensable for the growth performance and health. Elements such as Cu, Fe, Mn and Zn need to be added into feed diets for those animals which are raised conventionally to farm.

The problem is that the form in which these sources are added, especially oxides, sulfates and other inorganic combinations are less bioavailable sources of trace elements which makes a large amount of minerals ingested to reach directly into excreta. Moreover, due to their low bioavailability, large amounts of such salts are used, in order to assure the demand. The problem is acute in the case of copper and zinc, because, in accordance with European legislation, these are heavy metals and their accumulation in soils severely affects the quality of soil and groundwater.

There is a great variety of plant compounds used in animal nutrition and which are studied for their antioxidant properties, as well as for their antibacterial properties and for their vitamin or mineral potential. The phytoadditives field is developed due to the banning regarding the use of antibiotics in animal feed as a result of identifying the risk of bacterial resistance to antibiotics.

To assess the effects of phytoadditivs supplementation on animal organism, there was studied: a plant extract (inulin obtained from chicory), two crop plants (Jerusalem artichoke and buckthorn harvested from greenhouses of a producer of dietary supplements) and three plants harvested from wild flora (oregano, blueberries and caltrop). There were conducted a series of nutrition experiments on weaned piglets (2 experiments with 2 groups and 4 pigs / group, 2 experiments each with 3 groups and 4 pigs / group, 1 farm experiment with 2 groups and 8 piglets / group), in which these plants were included in animal diets. In these experiments, vegetal (plants, combined fodder) and animal (faeces, urine, serum, organs) samples were harvested.

Chapter 1

ESSENTIAL TRACE ELEMENTS

1.1. BIOLOGICAL VALUE OF ESSENTIAL TRACE ELEMENTS FOR ANIMAL ORGANISM

Minerals are found both in feed, as well in animal organism, in different quantities. They are present in biological tissues and also are in the ash obtained as a result of their burning.

Both, macro and micronutrients are absorbed better when are found as salts, relatively easy soluble form, in the acidic environment of the stomach: carbonates, sulfates, oxides. Mineral substances of vegetable feed have their assimilation coefficient (absorption) lower compared with inorganic salts given to animals as mineral supplements (blends, premixes) included in the compound feeds.

In case of an abnormal situation concerning mineral nutrition, for an accurate diagnosis of cause and effect, often it is resorting to mineral balance studies, associated with observations concerning the production and analysis concerning the mineral content values in serum and various tissues (organs, skin).

Indicative parameters of mineral metabolism are: absorption coefficients, nutrient concentrations in excreta and storage tissues, etc.interactions with other nutrients. The values of these parameters shows that the level of mineral substances present in feed is adequate (according to the requirements and standards). It follows that the analysis of mineral content can often be a clue to satisfy requirements relevant body minerals.

Biological functions of iron, copper, manganese and zinc

The main functions of iron are related to participation in blood oxygen transport and tissue respiration. Iron is a component of hemoglobin and red blood cells. It is found in muscles as myoglobin, as transferrin in serum, as uteroferină in placenta, as lactoferrin in milk and in liver as ferritin and hemosiderin (Zimmerman, 1980). It also plays an important role in the body as a component of several metabolic enzymes.

Physiological role of copper, component of ceruloplasmin, consists in participation in hematopoesis, therefore iron absorption and his utilization in the synthesis of porphyrin III and heme iron enzymes is conditioned. In the absence of copper in the body was found the occurrence of anemia that does not succumb to iron administration, but only in association with copper.

Metabolic role of manganese consist in his participation in various synthesis and, also in enzymatic systems: peptidases, phosphatase, arginase etc.. Manganese participates to the non-toxic function of the iron, vitamin C and the potentiation of the hypoglycaemic effect of adrenaline (Pârvu, 2003).

Zinc plays an important role in the structure of enzymes as: dehidraza carbon (0.33 to 0.34% zinc), urate oxidase (UO) or uricase (0.1% zinc), pancreatic carboxypeptidase, alcohol dehydrogenase (from yeast), tiroxinază, intestinal phosphatase, whose activity is favorised. It has a functional role as metaloenzyme hydrolase, lyase, isomerase, transferase etc. (Han, 2009). Zinc inactivates some enzymes, such as: pepsin, trypsin, etc.. Zinc has a potentiating role of some hormones such as insulin and vitamin B1 (Wash., 1994).

Trace elements requirements of weaned piglets

In mineral nutrition, to define the contribution of an essential element in a diet, first, it has to be answered, to the questions: how much and in what form? Great difficulty in finding answers to these questions is due to the fact that the recommendations must fit tightly in the complex of factors that influence the nutrition process: the complexity of diet and mineral source, the level of feed energy, the ambient temperature and the factors of stress (Criste, 2000).

Nutritionists consider that we need a safety margin between necessary and practical advices, recommending an "economic" waste of mineral elements (Diaz, 1986).

Lack of experimental data to sustain various recommendation systems led to the emergence of differences between concepts of: requirements, recommendation and supplementations (Paullauf, 1989). Usually, the recommended intakes indicated in tables, for each specie and type of animal represents a multiple of 2-5 times of the net requirements of the determined value.

For essential mineral elements, maintenance and production requirements are clearly defined for almost all species and therefore there is also the concern of the nutritionists to ensure that the requirements are met including mixtures of these minereal salts in combined fodder.

NRC requirements of micronutrients published in 1998 for different categories of pigs are presented in Table 1.1

Table 1.1. Trace elements requirements of weaned piglets according with NRC 1998

Trace element	**Body weight**					
	3-5 kg	**5-10 kg**	**10-20 kg**	**20-50 kg**	**50-80 kg**	**80-120 kg**
Fe (mg / kg)	100	100	80	60	50	40
Cu (mg / kg)	6	6	5	4	3.5	3
Mn (mg / kg)	4	4	3	2	2	2
Zn (mg / kg)	100	100	80	60	50	50

1.2. METHODS OF ESSENTIAL TRACE ELEMENTS ASSURANCE ON WEANED PIGLETS

Even though we are in the XXI century, there are not yet determined the optimal levels of micronutrients to be supplemented / added to basic ration. There are known physiological requirements, but it is unclear how much has to be supplemented / added, beside the existing content of components from the basic feed ration in order to achieve performant quantitatively, qualitatively, economically and environmentally productions.

Supplementation of the weaned piglets diet with essential trace elements

On weaned piglets (15-30 kg) main objective when establishing the requirements is optimizing growing performances during the first weeks after weaning. It is a time when serious problems can occur by: decreasing growth rate, lower feed consumption, increased morbidity and mortality.

In addition to other stressors that are installed during post-weaned period, the transition from milk to solid food is a major change in the life of a piglet. In practice, the supplementation of animal feed with micronutrients is made as recommended by manufacturers of premixes (concentrated mixtures) minerals. These recommendations often not based on scientific data that take into account various factors described above. Basically are 2-3 times higher than the requirements established scientifically.

Minerals bioavailability from supplementary sources of feed

After evaluating the amount of trace element present in the feed, bioavailability it is the most important aspect in providing the organism with minerals requirements. The key to reduce the quantities of minerals ingested by animals in their food is to increase their bioavailability from feed. Any source of micronutrients can be evaluated through bioavailability. For this it is necessary to make at least its absorbability study, concentrations in blood and storage organs, and livestock performance.

In vivo, several methods are used to determine the bioavailability of trace elements. Sometimes using balance studies, but because the elements are poorly absorbed, small errors in estimating intake or excretion may lead to large errors in the final balance. Another method for estimating the bioavailability is the concentration of trace elements in deposit organs.

Chapter 2

UTILIZATION OF SPECTROMETRIC METHODS FOR TRACE ELEMENTS DETERMINATION IN VEGETABLE AND ANIMAL ORIGIN SAMPLES

Some minerals are essential for health and animal productivity and nutritional and there is a well defined biochemical role. There is another group of minerals that occur in food and animal tissue in very small quantities and at the present level of knowledge, not nutritional role. These elements are known, conventional contaminants.

2.1. DETERMINATION OF TRACE ELEMENTS IN VEGETABLE SAMPLES

Determination of mineral elements in forage samples is weighted by the fact that they have a complex organic matrix containing chemical species whose concentration may vary from trace level to that of major level of components. This fact has required the development of a wide range of analytical methods (Emons, 2006): atomic absorption spectrometry (with flame - FAAS or graphite furnace - GFAAS); optical emission spectrometry with inductively coupled plasma (ICP-OES); inductively coupled plasma with mass spectrometry (ICP-MS) and voltammetry. However, atomic absorption spectrometry is considered the reference method (Regulation (EC) no. 152/2009) for analysis of trace elements in the feed. Therefore, it is used as a basis for comparison to other methods of analysis.

Microwave, dry and wet digestion are the most common methods of bringing the analytes from organic matrix into solution in order to determine the biological samples (Tuzen, 2007).

In Table 2.3. some bibliographic references are included to compare the methods of bringing into solution of the trace elements from different types of samples.

Tabel 2.3. Studies involving comparisons of decomposition methods for determining trace elements by spectrometric methods

Analized samples	**Trace elements determination**	**Determination methods**	**Bibliography**
Concentrate soupes	Cu, Zn, Mn, Fe, Al	FAAS	Soylac, 2006
Spices	Cu, Cd, Pb, Zn, Mn, Fe, Cr, Ni	FAAS	Soylac, 2010
Tea	Zn, Cu, Ni	FAAS	Soylac, 2007
Aliments	Cd, Fe, Pb, Zn	AAS	Da Col, 2009
Biscuits	Fe, Zn	FAAS	Doner, 2004

2.2. DETERMINATION OF TRACE ELEMENTS IN ANIMAL ORIGIN SAMPLES

Methods of bringing into solution of the analytes

Several studies concerning the determination of some trace elements from biological samples by spectometric methods are mentioned in table 2.11

Table 2.11. Studies concerning the determination of some trace elements from biological samples

Biological samples	Studied trace elements	Determination method	Bibliography
Chicken meat	Cu, Cd, Pb, Se, Mn, As	AAS	Uluozlu, 2009
Fish	Cu, Fe, Mn, Zn, Se, Cr	AAS	Tuzen, 2009
Liver	Cu, Zn	FAAS	Mesko, 2006
Pig kidney, cattle liver	Cd, Cu, Pb	FAAS	Pereira-Filho, 2002
Milk	Cu, Fe, Pb	FAAS	Florian, 2001
Crab	Cd, Cr, Cu, Fe, Mn, Ni, Zn	ICP-OES	Mutlu, 2011

2.4 SOURCES OF TRACE ELEMENTS USED TO SUPPLEMENT ANIMAL DIETS

Conventional sources – anorganic salts

On the market exists a extremly wide number of salt mixtures designated to animal nutrition. In some EU countries, there is a strict legislation concerning the usage at high levels of some mineral mixtures, formed of anorganic sources, which are suspected being potentially-polluting agents (Nicholson, 2008).

Unconventional sources – phytoadditives in animal feeding

In the last 50 years trace elements like Cu and Zn have started to be used, on large scale and at very high levels (Carlson, 1999; Hill, 2001; Poulsen, 1995) in piglets' feeding, replacing the antibiotics with growing stimulating effect. Using antibiotics in animal feeding is banned in EU, due to concernings referring to the instauration of resistant bacteries on human and animals (Lalles, 2007). But also, the practice of using high levels of trace elements has a negative and on long term impact on the environment (soil and phreatic water) situated next to large farms (Nicholson, 2008). In this context are enrolled these researches concerning the utilization of plants/phytoadditives. A question is raised: Can these plants be also a natural source of trace elements (Rozica, 2005), bioavailable at intestinal level, so that the excreted mineral waste to be diminished?

Chapter 3

PRACTICAL ASPECTS OF THE STUDY ON CORRELATION BETWEEN THE TRACE ELEMENTS CONTENT IN ANIMAL ORGANISM AND THEIR CONCENTRATIONS IN FEED DIETS ENRICHED WITH PLANTS AND PLANTS EXTRACTS

3.1. SAMPLING AND PREPARATION OF SAMPLES FOR ANALYSIS

In order to accomplish experiments in the study on correlations between the content of trace elements in animal organism and its concentrations in diets supplemented with plants or plant extracts, it was necessary to organize proper steps, in the Laboratory of Analytical Chemistry and Instrumental Analysis to determine concentrations of Fe, Cu, Mn, Zn from a large number of different types of biological samples and for organizing experiments.

Sampling and sample preparation of plant material

Plant material (plants used as supplements in nutrition experiments) was collected and dried naturally, crushed by cutting, grinding in the mill and sieved through a sieve with square openings of 1 mm as defined in Regulation (EC) no. 152/2009. Sampling of feeds and combined fodder were done using pooled samples (10 kg) reduced by quartering method to samples of 1 kg.

Samples of plants, plant extracts, feed materials and compound feed, dried and ground, were brought into solution by microwave digestion.

Sampling and sample preparation of biological material of animal origin

The samples of animal origin (faeces, urine, serum, organs) collected during the nutrition experiments were taken as follows:

- During experiments, faeces were collected quantitatively once a day. At the end of the balance period (one week), samples were weighed, mixed well and formed the average weekly samples. Samples of faeces were dried, ground and prepared for analysis by microwave digestion.
- The urine was collected daily and the eliminated amount was recorded. A sample of 10% of the amount was stored in plastic bottles with screw cap, which was added a solution of 0.1 N sulfuric acid to ensure a pH of 2-4.
- Serum - At the beginning and end of the experiment, 7 mL of blood from vena cava were collected from each animal into heparin tubes.
- Organs - At the end of experiment, pigs were slaughtered under the provisions of the Law on Animal Welfare (Dir. 93/119/CCE, Order 180/2006). From each pig slaughtered were collected samples of organs (liver, kidney, spleen, heart, lung, brain).

To establish the optimum digestion method for organ samples, it was performed a comparative study of results obtained using three methods: dry, wet and microwave digestion. This study was done on liver samples using a certified reference material: bovine liver CRM - BCR 185R. Following the comparison of experimental results the most effective and economical analytically method was chosen microwave digestion.

3.2. ASSESSMENT OF OPTIMUM WORKING CONDITIONS FOR THE DETERMINATION OF TRACE ELEMENTS Fe, Cu, Mn, Zn IN BIOLOGICAL SAMPLES

To establish working conditions for quantitative determination of trace elements Fe, Cu, Mn, Zn in biological samples by flame atomic absorption spectrometry it was used analytical grade reagents and appropriate laboratory equipment. For adapters, dilutions and storage it was used Class A glassware.

3.3. ORGANIZING OF ANIMAL NUTRITION EXPERIMENTS

Experimental technique used in the study on absorption and utilization of trace elements Fe, Cu, Mn, Zn from various plant sources used as feed additives, was based on metabolic cages for microtest and collective pens in macrotest type experiments.

Microtest type experiments were conducted on crossbred male piglets in appropriate physiological, healthy state with normal capacity of ingestion. Number of animals was 4 pigs / group. Experiments lasted approximately 30 days, depending on the period they reached 30 kg. The basic diet was the same for all pigs from all experiments.

Macrotest type experiments were used as biological material 16 crossbred piglets after weaning, housed in collective pens with manure drain grate. Excreta disposal was made on the cushion of water. The animals were housed by 8 in each box.

Pigs were individually weighed at the beginning of experiment, weekly and at the end of experiment. During the experiment to follow the zootechnical parameters: body weight, average daily gain, average daily feed consumption and feed to gain ratio.

Chapter 4

VALIDATION METHODS FOR DETERMINING TRACE ELEMENTS (Fe, Cu, Mn, Zn) IN BIOLOGICAL SAMPLES

In this chapter are presented the experimental results and the conclusions drawn from them, regarding the choosing and the validation of digestion and determination methods for Fe, Cu, Mn, Zn, from biological samples of animal and vegetable nature (**Untea, 2010a; Untea 2012a**).

4.1. CHOOSING OF Fe, Cu, Mn, Zn DIGESTION METHOD FROM LIVER SAMPLES

To choose the optimum method of digestion of liver samples in order to determine quantitatively trace elements Fe, Cu, Mn, Zn by FAAS, it were obtained results by using three methods were compared: dry, wet and microwave digestion. Measurements were done using as reference material: Certified bovine liver (CRM - BCR 185R) (**Untea, 2012a**). From the results obtained by applying three methods, the nearest percent mean recovery of 100% and the lowest values of standard deviation were obtained for the microwave digestion.

4.2. CHECKING THE LABORATORY METHOD FOR DETERMINING THE MICROELEMENTS Fe, Cu, Mn, Zn BY FLAME ATOMIC ABSORBTION SPECTROMETRY

The method for determining trace elements by flame atomic absorption spectrometry was verified in the laboratory, following a number of parameters: linearity, working range, sensitivity, detection limit and quantification limit (**Untea, 2010a, Untea 2012a**) in accordance with international regulations (Validation of Analitical Procedures, 1995).

Verifying of the performance parameters for the determination method of Fe, Cu, Mn and Zn by FAAS

Linearity, working range and sensitivity for determination method were evaluated using a calibration curve with 10 points, standard solutions with concentrations between 0.5 and 5 ppm for Fe, 0.4 and 4 ppm for Cu, 0.2 and 2 for Mn and 0.1 ppm and 1 ppm for Zn.

Detection limit and quantification limit for determination method of Fe, Cu, Mn, Zn by FAAS

In order to determine the detection limit, LOD and quantification limit, LOQ were prepared series of five solutions with the same concentration: Fe standard solution with the lowest concentration used for plotting the calibration curve (0.5 ppm) was diluted 50 times (LOD = 0.03

ppm and LOQ = 0.22 ppm); Cu standard solution with a concentration of 0.4 ppm was diluted 40 times (LOD = 0.014 ppm and LOQ = 0, 06 ppm); Mn standard solution with a concentration of 0.2 ppm was diluted 20 times (LOD = 0.04 ppm and LOQ = 0.16 ppm) and Zn standard solution with a concentration of 0.1 ppm was diluted 10 times (LOD = 0.008 ppm and LOQ = 0.03 ppm).

4.3. MICROWAVE DIGESTION METHOD VALIDATION

Validation of microwave digestion method for liver samples

The method of bringing into solution of trace elements Fe, Cu, Mn, Zn by microwave digestion was validated following the protocol specific parameters: accuracy, precision, repeatability, reproducibility and recovery. To calculate the performance parameters of the method, it was prepared a series of 8 samples of organic certified reference material (bovine liver CRM - BCR 185R) with: 184 ppm Fe, 277 ppm Cu, Mn and 138.6 ppm 11.07 ppm Zn (**Untea, 2012a**).

Evaluation of **accuracy** was done using 8 certified reference material samples and then it was determined the content of microelements Fe, Cu, Mn, Zn by atomic absorption spectrometry.

The values obtained for **accuracy** were 101.80% for Fe, 99.09% for Cu, 98.13% for Mn and 98.18% for Zn. The calculated values are in the range required by performance criteria (bias ± 2%), so the method can be considered validated for accuracy parameter.

Data obtained for **precision** of determination method for Fe, Cu, Mn, Zn in liver samples by FAAS after microwave digestion were:

- Fe - same day precision: RSD = 0.46%, 0.60%, 0.66% and on different days: RSD = 1.48%;
- Cu - same day precision: RSD = 1.50%, 1.23%, 1.03% and on different days: RSD = 1.18%;
- Mn - same day precision: RSD = 1.87%, 1.96%, 1.74% and on different days: RSD = 1.92%;
- Zn - same day precision: RSD = 1.68%, 1.95%, 1.62% and on different days: RSD = 1.81%.

Performance criteria: RSD correspond to Horwitz equation: RSD = 2 (1-0.5lgC). For a concentration of 1 ppm, RSD = 10.72%. For presented method, RSD values <2%, so the performance criteria was met.

Data for **reproducibility** of determination method for Fe, Cu, Mn, Zn in liver samples by FAAS after microwave digestion were :

- Fe - RSD = 1.93% for analyst 1 and RSD = 1.14% for analyst 2;
- Cu - RSD = 1.50% for analyst 1 and RSD = 1.47% for analyst 2;
- Mn - RSD = 1.98% for analyst 1 and RSD = 1.61% for analyst 2;
- Zn - RSD = 1.72% for analyst 1 and RSD = 1.82% for analyst 2.

For presented method, RSD values <2%, so the performance criteria was met.

The data obtained for **recovery** of determination method for Fe, Cu, Mn, Zn in liver samples by FAAS after microwave digestion were : 101.41% for Fe, 101.72% for Cu, 100,45% for Mn and 100,51% for Zn. The recovery was in the range of 100% ± 2%, so the method can be considered validated for this parameter.

Validation of microwave digestion method for plants samples

To calculate the performance parameters of the method, it was prepared a series of 8 samples of plant certified reference material (amestec botanic MIXED POLISH HERBS INCT-MPH-2) with: 460 ppm Fe, 7,77 ppm Cu, 191 ppm Mn şi 33,5 ppm Zn.

The values obtained for **accuracy** were 99.85% for Fe, 98.38% for Cu, 98.13% for Mn and 98.81% for Zn. The calculated values are in the range required by performance criteria (bias ± 2%), so the method can be considered validated for accuracy parameter.

Data obtained for **precision** of determination method for Fe, Cu, Mn, Zn in plant mix samples by FAAS after microwave digestion were:

- Fe - same day precision: RSD = 0.38%, 0.47%, 0.99% and on different days: RSD = 1.53%;
- Cu - same day precision: RSD = 1.47%, 1.66%, 1.64% and on different days: RSD = 1.85%;
- Mn - same day precision: RSD = 0.74%, 1.18%, 0.75% and on different days: RSD = 1.08%;
- Zn - same day precision: RSD = 1.68%, 0.94%, 1.40% and on different days: RSD = 1.84%.

For presented method, RSD values <2%, so the performance criteria was met.

Data for **reproducibility** of determination method for Fe, Cu, Mn, Zn in plant mix samples by FAAS after microwave digestion were :

- Fe - RSD = 1.93% for analyst 1 and RSD = 1.14% for analyst 2;
- Cu - RSD = 1.87% for analyst 1 and RSD = 1.10% for analyst 2;
- Mn - RSD = 1.83% for analyst 1 and RSD = 0.99% for analyst 2;
- Zn - RSD = 1.84% for analyst 1 and RSD = 1.83% for analyst 2.

For presented method, RSD values <2%, so the performance criteria was met.

The data obtained for **recovery** of determination method for Fe, Cu, Mn, Zn in plant mix samples by FAAS after microwave digestion were : 100.33% for Fe, 100.63% for Cu, 98.21% for Mn and 101.27% for Zn. The recovery was in the range of 100% ± 2%, so the method can be considered validated for this parameter.

4.4. CALCULATION OF MEASUREMENT UNCERTAINTY OF DETERMINATION OF TRACE ELEMENTS Fe, Cu, Mn, Zn FROM BIOLOGICAL SAMPLES BY FLAME ATOMIC ABSORPTION SPECTROMETRY AFTER MICROWAVE DIGESTION

Quantification of uncertainties

The purpose of this study was to quantify the uncertainties from every step of analyse. This can be done using own experimental data or data obtained from well-founded assumptions.

- **The volume (V)** - composed uncertainty using a 50 ml flask, consists in three individual uncertainties: calibration, repeatability, temperature

- **Mass (m)** - composed uncertainty associated to mass, consists of: linearity uncertainty, metrology uncertainty, repeatability uncertainty and resolution uncertainty.

- **Trace concentration (C0)** - Uncertainty associated to trace element concentration in animal and vegetable samples is calculated using the appropriate calibration curve.

In order to calculate the combined uncertainty of a product, u (r) / r , the standard uncertainty involved are composed in the final result. The data used for calculating measurement uncertainty for bovine liver samples (P1) and plant material (P2) are shown in Table 4.42.

Table 4.42. Data used for the uncertainty of Fe, Cu, Mn, Zn concentrations in bovine liver samples and plant material (reference materials)

	u(V)	**u(m)**	**S_{xx}**	**S**	**$u(c_0)$**	**r**	**U(r)**
IRON							
P1	0,029	0,00012	7,58	0,0076	0,089	**187,31**	0,179
P2	0,029	0,00012	7,58	0,0076	0,080	**459,33**	0,160
COPPER							
P1	0,029	0,00012	5,75	0,153	1,669	**274,48**	3,338
P2	0,029	0,00012	0,63	0,029	0,357	**7,65**	0,713
MANGANESE							
P1	0,029	0,00012	0,40	0,049	0,632	**10,86**	1,263
P2	0,029	0,00012	1,44	0,156	1,582	**188,73**	3,164
ZINC							
P1	0,029	0,00012	0,32	0,178	2,294	**136,07**	4,588
P2	0,029	0,00012	0,32	0,178	2,315	**33,10**	4,630

From Table 4.42 it can be observed high values of measurement uncertainty for results corresponding to low levels of trace elements in the sample. One of the factors that influence the final amount of uncertainty is the working range.

4.5. INTERLABORATORY STUDY TO VALIDATE THE DETERMINATION METHOD FOR MICROELEMENTS Fe, Cu, Mn, Zn IN THE PLANT SAMPLE AND DETERMINING THE TRACE ELEMENT CONTENT

For precise knowledge of the content of trace elements Fe, Cu, Mn, Zn in plants used as food additives in piglets diets from experiments presented in the thesis, it is necessary to calculate their level of inclusion in diets (**Untea, 2009; Untea, 2010b; Untea, 2010c; Untea, 2012d**). Seven laboratories from 6 Romanian research institutes participated at the study of.

To establish the robust average mean, the robust standard deviation and the uncertainty of datasets, it was applied algorithm A (Thompson, 2006). Assumed values, standard deviations and uncertainties associated to the data strings are shown in Table 4.44.

Table 4.44. The results obtained using the algorithm A for data strings corresponding to the concentrations of Fe, Cu, Mn and Zn in plant samples collected from wild flora

Element	Measurement	Blueberry		Oregano		Caltrop	
		Average mean mg/kg	Assumed value mg/kg	Average mean mg/kg	Assumed value mg/kg	Average mean mg/kg	Assumed value mg/kg
Fe	Average mean	81,32	**81,11**	1886,47	**1868,80**	279,43	**278,25**
	Standard deviation	13,47	**16,57**	250,91	**240,99**	48,89	**51,57**
	Uncertainity		**3,78**		**55,00**		**11,77**
Cu	Average mean	4,68	**4,54**	9,90	**10,87**	7,19	**9,12**
	Standard deviation	1,53	**1,84**	1,78	**1,63**	2,61	**1,46**
	Uncertainity		**0,42**		**0,44**		**0,79**
Mn	Average mean	204,36	**204,10**	76,78	**77,17**	46,07	**46,13**
	Standard deviation	11,35	**11,86**	5,59	**7,13**	4,24	**4,62**
	Uncertainity		**2,71**		**1,63**		**1,05**
Zn	Average mean	53,98	**53,43**	53,40	**53,30**	44,95	**44,73**
	Standard deviation	4,60	**5,68**	3,15	**4,41**	4,34	**4,94**
	Uncertainity		**1,30**		**1,01**		**1,13**

To evaluate the statistical performance of the participants it was used scores Z, Z 'and Zeta.

Statistical parameters calculated for the concentration of trace elements Fe, Cu, Mn, Zn in blueberry samples showed for copper: laboratories 1 and 7 were characterize by zeta values between 2 and 3 or greater than 3. Samples of oregano and caltrop, manganese and zinc determinations showed no differences between participants' performance.

Chapter 5

CHEMICAL - ANALYTICAL STUDY OF CORRELATIONS BETWEEN TRACE ELEMENTS CONTENT OF ANIMAL ORGANISM AND THEIR CONCENTRATIONS FROM FEED DIETS ENRICHED WITH EXTRACTS AND CROP PLANTS

The aim of this study was to evaluate the effect of utilization of plant extracts or crops included in weaned piglets' diets on the correlation between their trace elements content and their retrival in animal organism. Looking for some substances to replace antibiotics for the period of weaning piglets, it was proposed as an alternative the use of plant extracts (Han, 2006).

5.1. CHEMICAL - ANALYTICAL STUDY OF CORRELATIONS BETWEEN TRACE ELEMENTS CONTENT OF ANIMAL ORGANISM AND THEIR CONCENTRATIONS FROM FEED DIETS ENRICHED WITH INULIN

In the international project FP 6 "Feed for Pig Health" it was included an experiment for the evaluation of a natural compound (inulin) used as an alternative to antibiotics. In this project, the role of Research and Development Institute for Animal Biology and Nutrition (IBNA) was to study the bioavailability of trace elements contained in the natural compounds used in experiments and their effect on mineral status of animals.

The experiment took place in two different farms with the aim to assess the influences environmental conditions, by comparison. It was used: a farm that provides strict conditions of hygiene and an environmental conditions specific pig farm.

In this experiment it was assessed how inulin supplement added to feed influenced the trace elements content in the liver of weaned piglets, compared to the group fed only the basic diet (**Untea, 2012b, Untea, 2012c**).

The results obtained in the study of the correlation between the content of microelements in animal organism and their concentration in diets enriched with plant extracts

Liver samples were taken from slaughtered pigs at the beginning of the experiment (day 0) after 5 days and after 11 days (the end of the experiment). Concentrations of trace elements in liver samples collected from slaughtered pigs are shown in Tables 5.3.

Table 5.3. The effects of inulin supplementation on deposits of Cu, Mn and Zn in the liver of weaned piglets (commercial farms)

Concentrations / Samples	Control group			Experimental group		
	Cu ppm	**Zn** ppm	**Mn** ppm	**Cu** ppm	**Zn** ppm	**Mn** ppm
Premix	**0,00**	**50,00**	**35,00**	**0,00**	**50,00**	**35,00**
Raw materials	**6,44**	**34,88**	**2,44**	**6,44**	**34,88**	**2,44**
Compound feed	**6,44**	**84,88**	**37,44**	**6,44**	**84,88**	**37,44**
Liver - 28 days (BL)	**207,14 ± 5,7**	**206,79 ± 8,7**	**10,41 ± 0,8**	**207,14 ± 5,7**	**206,79 ± 8,7**	**10,41 ± 0,8**
Liver - 33 days	**144,12*± 16,5**	**193,43 ± 17,1**	**10,85 ± 0,5**	**143,38* ± 10,8**	**188,57* ± 19,4**	**10,79 ± 0,6**
Liver - 39 days	**26,62* ± 5,5**	**131,95* ± 2,8**	**10,29 ± 1,4**	**71,31* ± 7,6**	**128,15* ± 15,2**	**9,93 ± 1,4**

Note: * - significantly different from LB ($p \leq 0.05$) (BL = baseline)

There were no significant differences ($P \leq 0.05$) between groups in terms of liver Zn concentrations for piglets slaughtered at 33 or 39 days. At the end of the experiment, liver zinc concentrations were significantly lower ($p \leq 0.05$) than those determined in liver samples from pigs slaughtered at 33 days. The explanation is related to the zinc content of the diet. The Zn supplement brought by premix in the diet was only 50 ppm compared to 80 ppm level recommended by the National Research Council, NRC (1998). An objective of the experiment was to reduce the quantities of trace elements with role growth promoter (Cu, Zn) from diets. Consequently, the zinc absorption from feed was reduced, liver Zn concentration was reduced being directly proportional to the level of the Zn in diet, experimental data in concordance with the literature (Carlson, 1999). Inulin in the diet had no effect on liver Zn concentrations.

Experimental results have shown that supplements of inulin in feed did not affect manganese absorption in piglet body.

Concentrations of Cu in the liver of piglets at the end of the experiment were significantly lower than values at baseline. The results obtained for concentrations of Cu in the liver, are in agreement with data published in the literature at the same category of piglets (Jondreville, 2005; Apgar, 1995). The results in Table 5.3. showed no significant differences between groups after 5 days of treatment (33 days old), but concentrations of Cu in the liver were significantly different ($P = 0.0002$) between the groups at the end of the experiment (39 days old). These results show that although the level of copper in the diet did not differ between groups, inulin supplementation improved absorption and utilization of copper in the organisms of weaned piglets. Evolution of Cu concentrations was part of a downward trend during the experiment, the final concentrations of copper in the liver of control group piglets, representing only 12.5% from Cu concentration recorded in the liver of piglets slaughtered at the beginning of the experiment. For the experimental group, the final concentrations of copper represented 34.43% from baseline (table 5.4.).

The level of Cu in diets (6.44 ppm) corresponds to the requirements of piglets, 5 ppm (NRC, 1998), but the diet sources are only raw materials and Cu bioavailability is reduced. A Cu deficient diet causes a weak Cu superoxide dismutase activity in the liver and red blood cells (Iskandar, 2005) which can lead to low levels of Cu in the liver.

In Figure 5.1. it can be observed that deposits of Cu in the liver of piglets from experiment conducted in commercial farms are smaller than the experimental farm for both control and experimental group. In the case of commercial farm, the liver Cu concentrations decreased during the experimental period, while for piglets from experimental farm, after an initial downward trend, the levels of liver Cu increased in the final week. A possible explanation could be an improved intestinal absorption of copper in organisms of animals raised in farm with ideal conditions of hygiene.

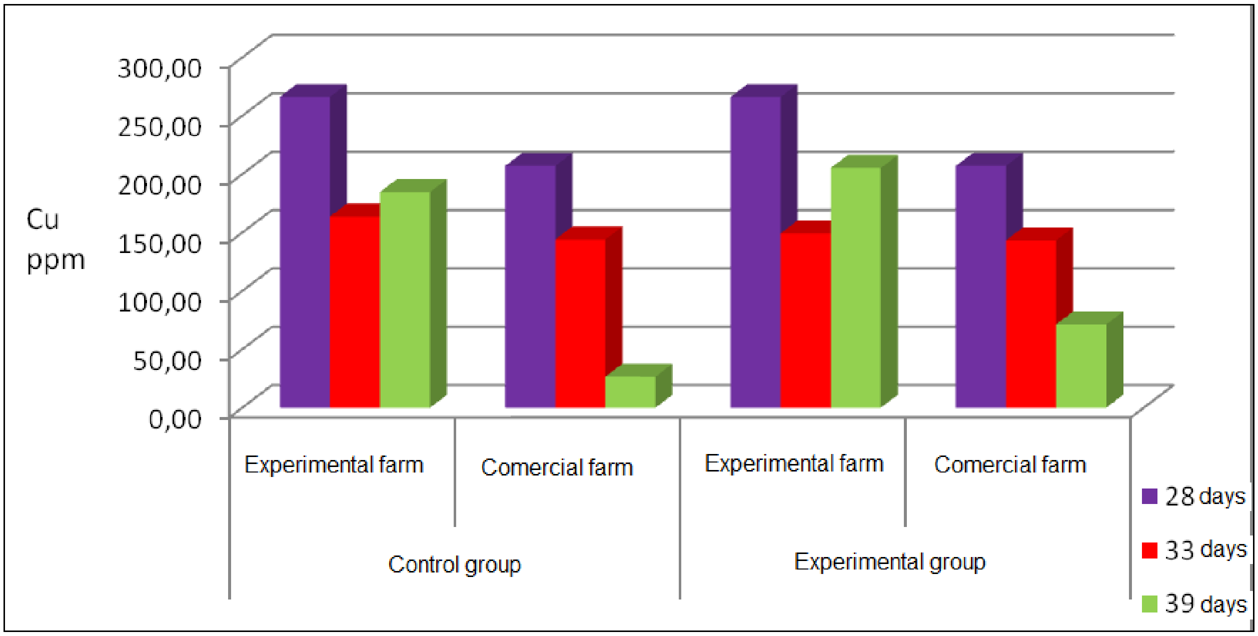


Fig. 5.1. Evolution of liver Cu concentrations in time for the piglets reared in 2 different farms

5.2. CHEMICAL - ANALYTICAL STUDY OF CORRELATIONS BETWEEN TRACE ELEMENTS CONTENT OF ANIMAL ORGANISM AND THEIR CONCENTRATIONS FROM FEED DIETS ENRICHED WITH A MIXTURE WITH A BOTANICAL MIX BASED ON JERUSALEM ARTICHOKES AND BUCKTHORN LEAVES

In order to assess the effects of a botanical mix included in weaned piglets' diets, on animals organism trace elements content, salts of Fe, Cu, Mn, Zn, which are commonly used as mineral additives, were replaced partially with a botanical mix based on jerusalem artichokes and buckthorn leaves (**Untea, 2010d , Untea, 2010; Untea, 2012f**).

The vegetal materials used to produce this botanical mix are by-products from the production of natural feeding supplements. The Jerusalem artichokes are rich in inulin, an oligosaccharide which acts within the digestive tract of the piglets (Breves et al., 2001), favouring the absorption of trace elements (Rumessen et al., 1990; Lopez et al., 2000), while the buckthorn is a true natural vitamin-mineral premix (Beveridge, 1999; Gutzeit et al., 2008).

The botanical mix based on Jerusalem artichokes and buckthorn leaves was tested at a level of 3% in combined fodder in a microtest using individual metabolic cages and in farm conditions in collective pens. Trace elements from raw materials overload the requirements (NRC, 1998). But due to their poor bioavailability, due to the presence of phytate and mineral additives, animals' diets are supplemented with minerals. The experimental diet includede the partial replacement of inorganic salts of Fe, Cu, Mn and Zn with supplements from botanical mix.

In figure 5.4. are presented the trace element concentrations determined in piglets' diets in the 2 types of experiments.

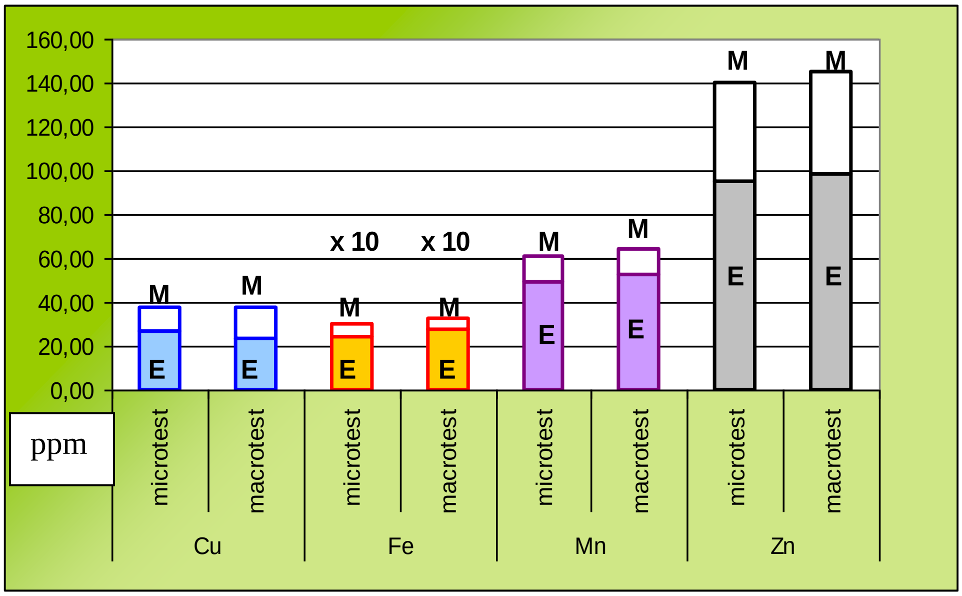


Fig. 5.4. Comparative graphical presentation of trace elements concentrations determined in piglets' diets in microtest vs macrotest

The data presented in Figure 5.4. showed that for all four studied micronutrients Fe, Cu, Mn and Zn, both for microtest and macrotest, their level in experimental diets, E (3% botanical mix) was lower than control diet.

The results obtained in the microtest were compared with those obtained under macrotest, both for control and experimental groups. There were no significant differences between the mean weights of piglets at the beginning of the experiments. At the end of experiment, there were significant differences between groups of microtest and macrotest. There were no significant differences between groups of piglets from the same experiment.

The results obtained for blood parameters showed that in both types of experiment, the values recorded at the end of experiments fit within the normal range for this category of piglets.

For the trace elements concentrations in feces there were recorded higher values for piglets within the groups of macrotest for Cu, Mn and Zn, but comparable to those obtained for microtest, respecting the proportion to the concentrations calculated by reference to the control group, C .

The iron concentrations determined in faeces were significantly ($P \leq 0.05$) higher, considering specific experimental conditions (farm) for the macrotest.

The concentrations of iron, for all three types of organs collected from slaughtered pigs in both groups of macrotest were lower than those found in piglets' organs from the micro test. This observation complies with comparative results obtained with concentrations found in feces. Iron concentrations in animal faeces participating in macrotest were significantly higher than those of microtest. In this context, Fe deposits in the organs studied are higher for the microtest groups.

Concentrations of Cu in the main storage organs were comparable for the two types of experiments. The concentrations were also comparable for the groups C and E, with the exception of kidney Cu concentration in the microtest, where the concentration of Cu in group E was significantly ($P \leq 0.05$) higher than in group C.

Concentrations of Zn determined in organs collected from both groups of macrotest piglets were slightly higher than those determined in organs for piglets from the microtest, but the differences were not significant.

Chapter 6

CHEMICAL – ANALYTICAL STUDY OF CORRELATIONS BETWEEN TRACE ELEMENTS CONTENT OF ANIMAL ORGANISM AND THEIR CONCENTRATIONS FROM FEED DIETS ENRICHED WITH PLANTS HARVESTED FROM WILD FLORA

In this chapter there are described two mineral balance experiments on weaned piglets (10-30 kg) to determine the bioavailability of trace elements Fe, Cu, Zn, Mn from compound feed suplemented with phytoadditives. The used phytoadditives were: oregano, blueberries, caltrop.

6.1. DETERMINATION OF Fe, Cu, Mn, Zn IN OREGANO, BLUEBERRY AND CALTROP HARVESTED FROM WILD FLORA

Evaluation of antioxidant potential of plants harvested from the wild flora

The results obtained for the antioxidant capacity of plants harvested from spontaneous flora shows that alcoholic extracts of oregano, bluberry and caltrop contain polyphenols with antioxidant activity. Polyphenols from plants listed have the ability to chelate transition metal ions and neutralize free radicals (**Untea, 2010f**).

Quantitative determination of Fe content of soil samples

Analysis of soil samples taken from the oregano plant harvesting (area Pausesti) reveal a slightly acid soil (pH = 6.81) and a mobile iron concentration of 75.5 mg / kg. PH is characteristic of moderate soil acidity.

Quantitative determination of the content of Fe, Cu, Mn, Zn from diets' components

In order to assess the effects of a botanical mix suplement on weaned piglets' organism, it were organized 2 experiments for the study of correlation between the content of microelements in animal body and their concentration in diets enriched with plants supplements harvested from wild flora (**Untea, 2010g; Untea, 2011; Untea, 2012**).

In the first experiment conducted on 3 groups of piglets (M and E1, E2) was tested suplement of oregano, in the presence of components that provide different rates of trace elements (Fe, Cu, Mn, Zn) in the premix. Mineral premix used in piglets in group E1 were added only salts of Zn - 0.5% and piglets in group E2 received premix included trace elements Fe, Cu, Mn, Zn at a rate of 0.5% (half of ration inclusion of microelements in piglets in group M).

In the second experiment, conducted on 3 groups of piglets (M, E1, E2) were tested mixtures of oregano (included in the diet at a rate of 3%) and blueberry (included in the diet at a rate of 1%) - for E1 group, and oregano (included in the diet at a rate of 3%) and caltrop (included in the diet at a rate of 1%) - E2 group. Premix administered to experimental groups included trace elements Fe, Cu, Mn, Zn at a rate of 0.5% (half of inclusion of microelements in the ration of lot M).

Experiment 1 - Oregano harvested from spontaneous flora has a high content of Fe. This is shown by analysis, as described in Chapter 4.5. The level of Fe in E2 group is slightly higher than in group M, while the content of iron (II) sulphate of E2 premix was reduced to half of the M premix. By reducing the amount of inorganic salts premix, experimental groups had a Cu content of 68% and 34% lower than the control group, Mn by 46% and 22% lower than the control group. In experimental groups, zinc oxide content of premix was 50% lower than the level of the control group. The two experimental groups were not differed between them by level of zinc in the diet, but the levels of other micronutrients studied: Fe, Cu, Mn.

Experiment 2 - Plants introduced experimental diets substituted part of the iron premix made routinely by these groups so that diets had an Fe content with only 13-16% lower than M. By reducing the amount of sulfate of Cu (II) and Mn sulphate (II) premixes of experimental groups had an intake of 73-75% Cu, Mn respectively with 32-34% lower than those of M. the rations experimental groups the amount of zinc oxide premix was 50% lower than the batch M. the two experimental groups did not differ by level of zinc in the ration, or by the other trace elements studied (Fe, Cu, Mn) and Zn contents were approximately 35% lower than the control group.

6.2. CORRELATION STUDY BETWEEN THE CONTENT OF TRACE ELEMENTS IN ANIMAL ORGANISM AND THEIR CONCENTRATIONS IN THE DIETS ENRICHED WITH OREGANO

The experiment presented in this chapter, is a microtest type experiment conducted in an experimental hall equipped with metabolic cages (**Untea 2010g, Untea 2011, Untea 2012**).

Average daily gain were not significantly different ($P \leq 0.05$), even while reducing inorganic salt content of Fe, Cu, Mn and Zn in premixes diets for experimental groups E1 and E2 compared to group M. The results obtained from measurements on hematological and biochemical parameters of blood shows that piglets in the 3 groups studied, the values recorded at the end of the experiment is within normal limits for species and category (Pârvu, 2003). Despite the biological variation piglets, there were significant differences ($P \leq 0.05$) between groups.

Mineral balance data

To study the correlation between mineral content of piglets' organism and their concentration in diets enriched with oregano, the determinations of trace elements in feed and excreta (faeces and urine) was used to calculate a mineral balance.

The mineral balance results showed that Fe intake in piglets from group E1 was approximately 12% lower than those in group M. For the piglets from group E2, intake was higher by 8% compared to those in group M, due to high concentrations of iron existing in oregano. Under these circumstances, Fe excreted in the faeces was lower in absolute value for piglets from group E1 (about 7%), but no significant differences ($P \leq 0.05$) between this group and the pigs from the other two groups studied. Coefficients of iron absorption and retention in group M were significantly ($P \leq$

0.05) higher than those recorded in group E1, without any significant differences ($P \leq 0.05$) compared with E2. The lack of significant differences between coefficients of iron absorption of piglets from group M and group E2, might be explained by the low levels of Mn and Zn in these diets, competing elements with iron absorption in the digestive tract level (Hill, 1983).

The intake of the experimental group E1 without mineral premix, but oregano supplement of 3%, was 74% smaller than M, and in group E2, which received half of the premix of group M and 3% oregano, with 46% less. Under these conditions, for E1, the amount of copper excreted through the faeces was 61.4% lower than the control group and for E2, it was 35% lower than the control group. As expected, the coefficients of absorption and retention of copper in piglets in group M were significantly ($P \leq 0.05$) higher than the experimental groups. And between experimental groups were significant differences ($P \leq 0.05$) for the values of coefficients of absorption and retention. Mineral balance data showed that the absorption coefficient values fall in the range of values reported in the literature (Veum, 2004 and Carlson, 1999) at about the same group of animals, when the animals received diets containing 16.5 ppm Cu.

The amount of manganese ingested in group E1 was 57% lower than the control group piglets and in group E2, it was 21% lower than M group. Under these conditions, for E1 group, the amount of Mn excreted through the faeces, was 40% lower than for M group, and it was 30% lower than the other experimental group, E2. Absorption and retention coefficients of manganese determined for group M were significantly ($P \leq 0.05$) higher than the experimental groups. And between experimental groups were significant differences in the coefficients of absorption and retention value calculated, resulting in higher values for group E2.

Mineral balance results showed that Zn intake in pigs from experimental groups was 25% lower than the control group piglets. For experimental groups, the amount of Zn excreted in the faeces was 17-18% lower than in the control group piglets. For control group, absorption and retention coefficients for Zn, were only 5-10% higher than the coefficients obtained for experimental groups. It is noteced that the absorption coefficients for piglets from E1 group were lower ($P \leq 0.05$) than the coefficients obtained for piglets from the other two groups, M and E2, although experimental groups had the same level of zinc in the diets. Explanation could be the effect of lack of Cu and Fe added to the E1 diet. Absorption coefficients obtained for Zn are higher than the values reported in the literature in experiments conducted under similar conditions (Veum, 2004 - 32.8% and Carlson, 2004 - 20.9%, Revy, 2002 - 26.9%).

In order to quantify the relationship between the amount of micronutrients ingested and eliminated through the feces and urine were determined regression equations and the following correlation coefficients: Fe: R2 = 0.9918; With: R2 = 0.9918, Mn: R2 = 0.9895, Zn: R2 = 0.9960. The data presented showed that the three groups studied followed the same relationship of proportionality between the intake and excrete for all trace elements studied.

During the experimental period, the mineral balance was conducted weekly, so it could follow the evolution in time trace elements eliminated through faeces. The amount of Fe excreted in group E2 was higher throughout the experiment, than the quantities eliminated by piglets from M group. This fact is considered normal because the level of Fe ingested by E1 group was higher than in M group. An opposite situation occurred for group E1, where a smaller amount of Fe intake, compared to group M, led to lower values of Fe excreted. The elimination of Cu and Mn through faeces for piglets from group M was higher since the beginning of the experiment, which is considered normal, given that the group M quantities of Cu, Mn ingested were significantly ($P \leq 0, 05$) higher than the experimental groups. The lowest values of Cu and Mn excreted in faeces were recorded in group E1 which received feed without added mineral premix.

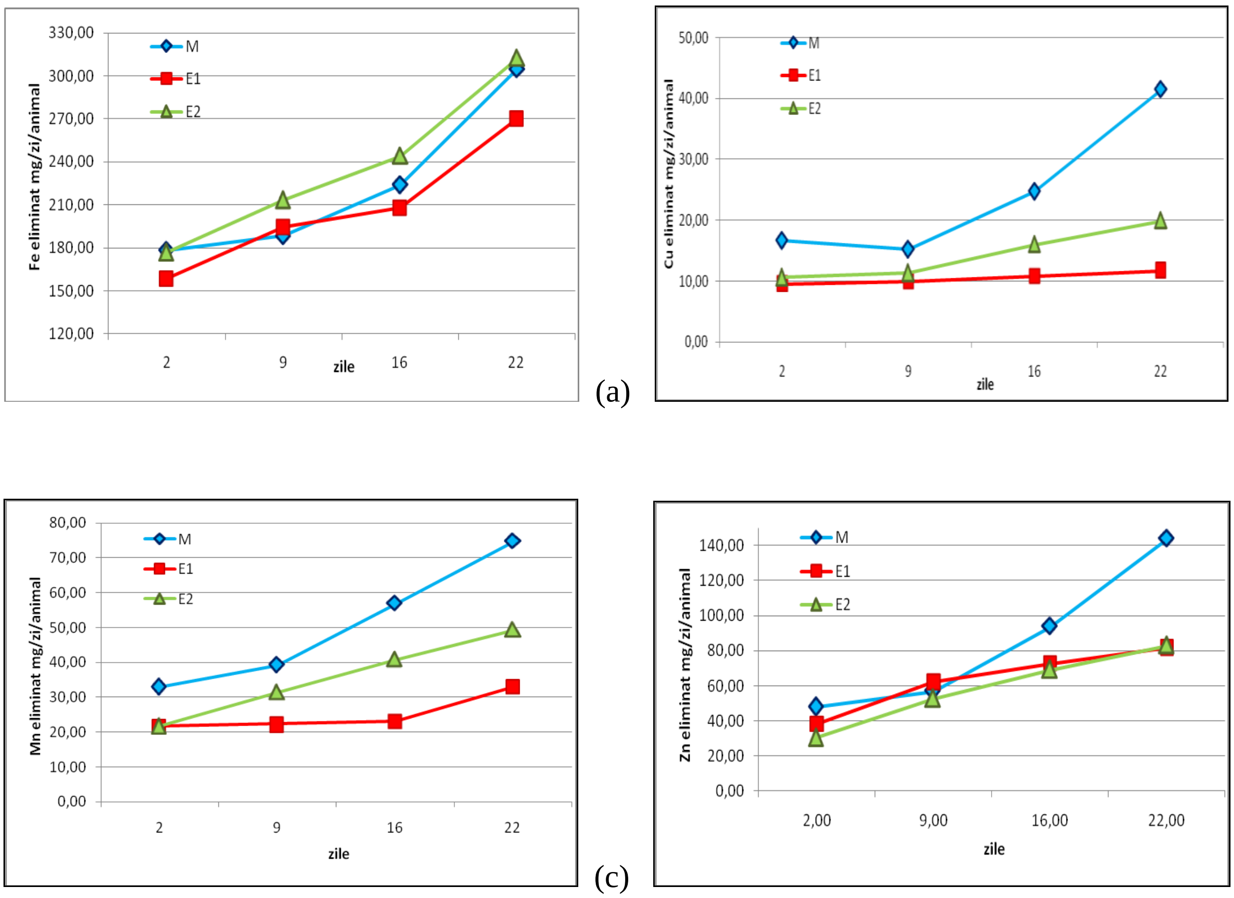
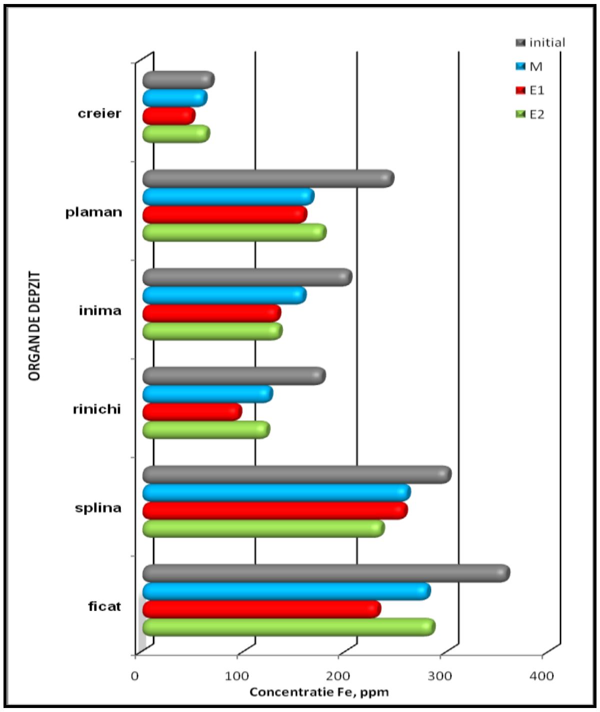
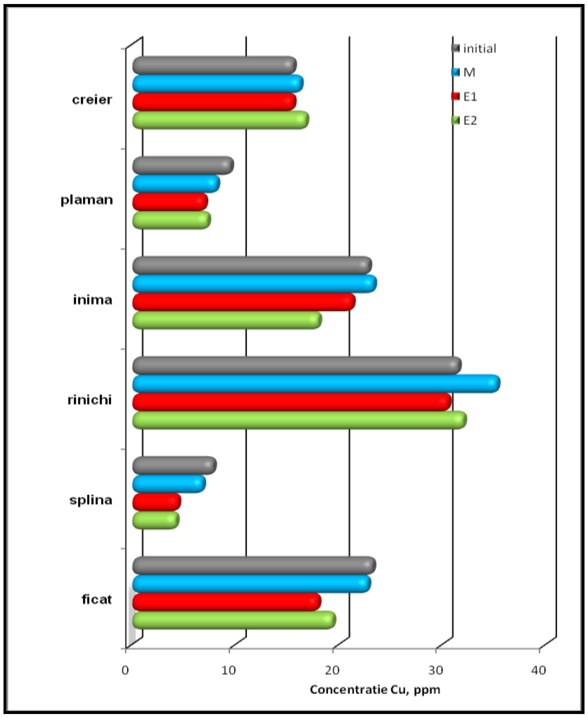


Fig. 6.6. The evolution of trace elements elimination through faeces: (a) iron, (b) copper, (c) manganese, (d) zinc

The values obtained at the beginning of the experiment for iron deposits in the liver, spleen, kidney, heart, lung and brain, were not reached by the values determined at the end of the experiment, for any of the 3 groups studied. This fact could be considered normal, because: micro deposits with piglets born; ability to assimilate large pre-weaning, Fe injection at the 2 nd day of life, in order to prevent anemia (common in all farms). The highest concentrations of Fe were found in the liver and spleen. Liver Fe levels in piglets of group E1 was significantly ($P \leq 0.05$) lower than that recorded for piglets from groups M and E2. Concentrations of Cu in storage organs of piglets at the beginning of experiment have been met by the piglets from group M at the final of experiment. The highest concentrations of Cu were in kidney, heart and liver. Between experimental groups E1, E2, there were no significant differences. Except brain, the Cu concentrations determined in organs of piglets from group M were significantly ($P \leq 0.05$) higher than those determined in groups E1 and E2. Manganese concentrations in organs of piglets at the end of the experiment exceeded the concentration determined at baseline. The highest concentrations of Mn were found in the liver, kidneys and lungs. Between the studied groups there were no significant differences ($P \leq 0.05$). Zinc concentrations in organs of piglets at the end of the experiment did not exceed the level of Zn recorded at baseline. The highest concentrations of Zn were found in the liver where the values determined in group M were significantly different ($p \leq 0.05$) than the experimental groups values determined. In other organs, Zn concentrations were comparable between groups.

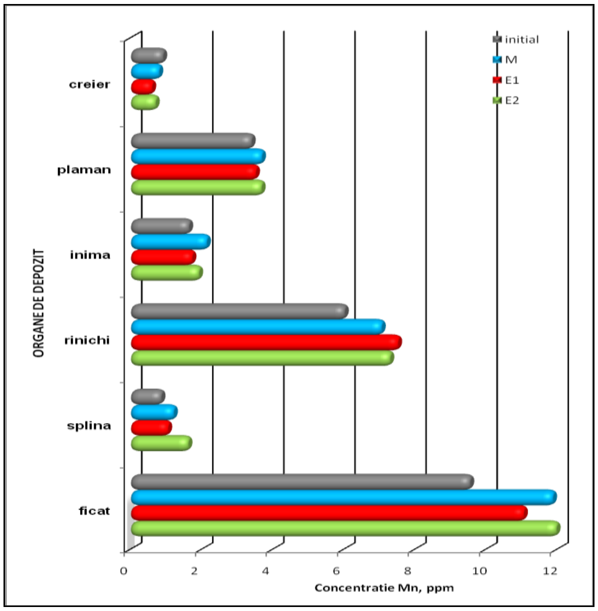


(a)



(b)

Fig. 6.10. Average values of concentrations of Fe, Cu determined in deposit organs



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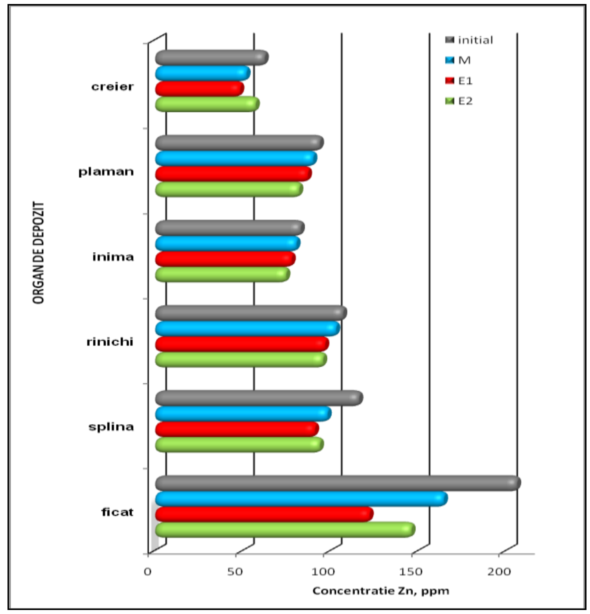
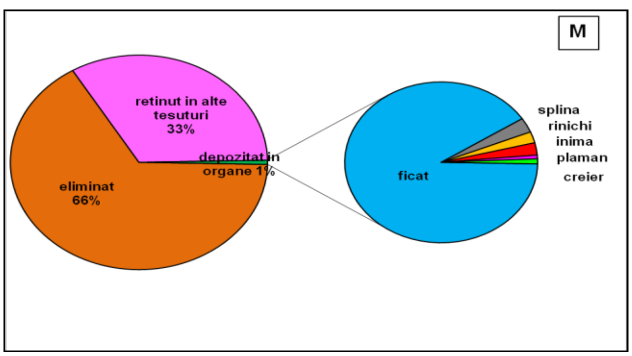


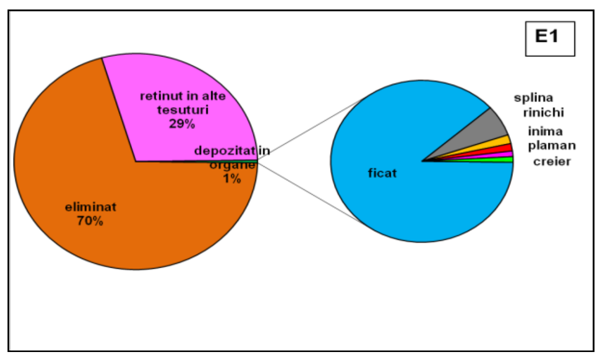
(d)

Fig. 6.10. Average values of concentrations of Mn, Zn determined in deposit organs

Figures 6.11-6.14 present the results of distribution processes of trace elements ingested for all 3 groups studied.



(a)



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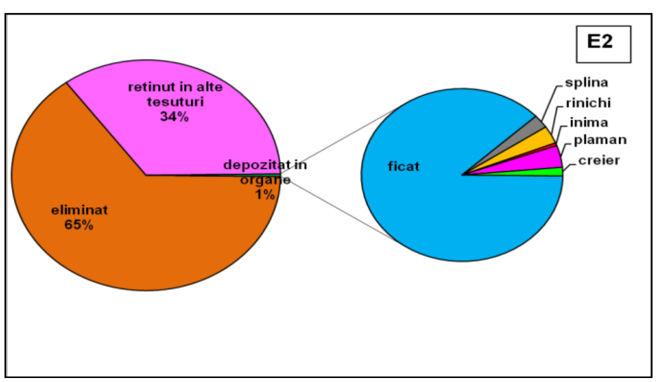


(c)

Fig. 6.11. Graphical representation of experimental results on the distribution of iron ingested in the organism of piglets and the excreted amount: (a) M, (b) E1, (c) E2

The amount of trace elements retained in the animal organism was considered (Pop, 2006) as the difference between the amount of nutrients ingested and the mineral amount excreted in feces and urine. From the amount retained in the organism, it was considered the trace elements amount deposited in organs (liver, spleen, kidney, lung, heart, brain) during the experiment (the difference between the amount calculated at the end of the experiment and the quantities calculated at the beginning of the experiment). By difference, it was calculated the amount of trace elements retained in other tissues.

The highest percentage of Fe eliminated has been recorded for piglets in group E1. In the organs was stored under 1% of iron ingested and this amount is distributed mainly in the liver.

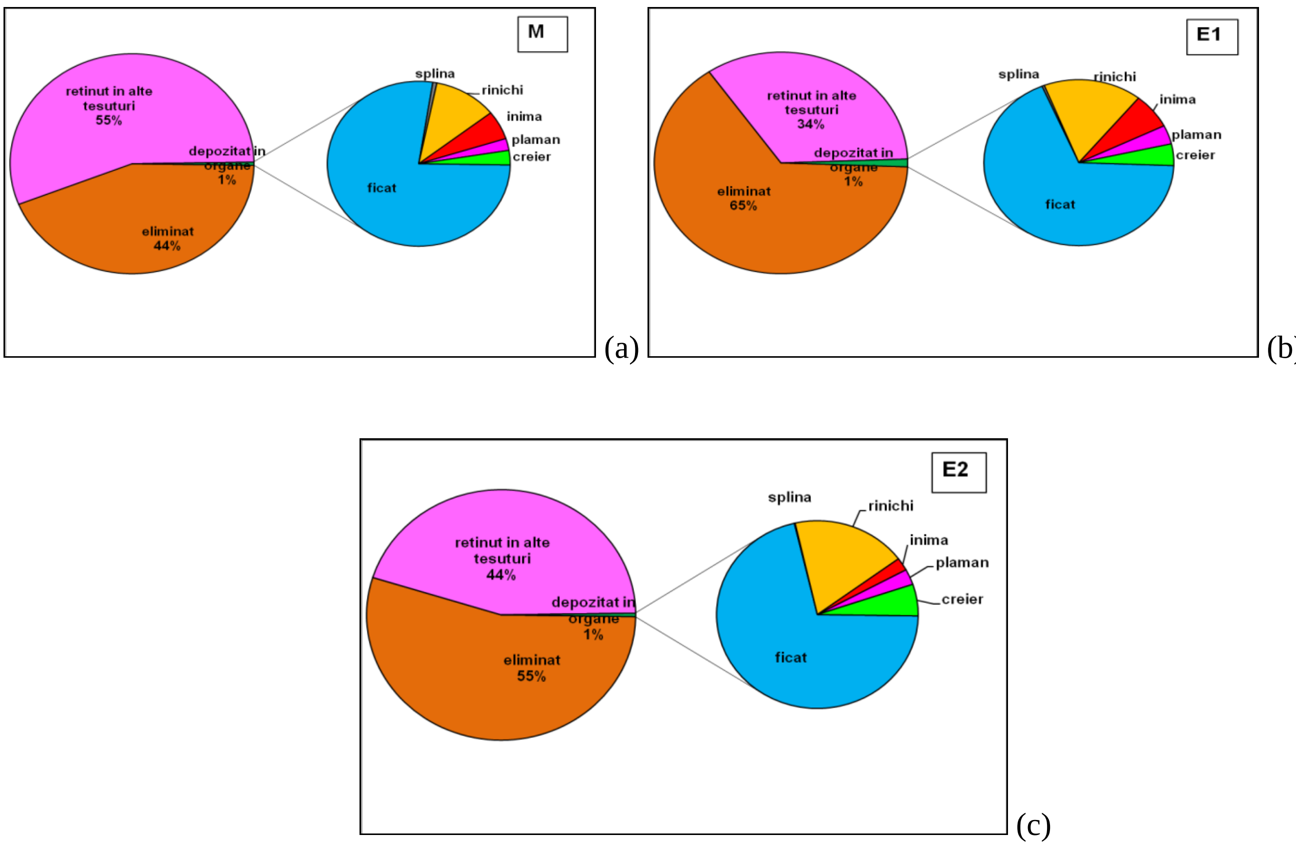
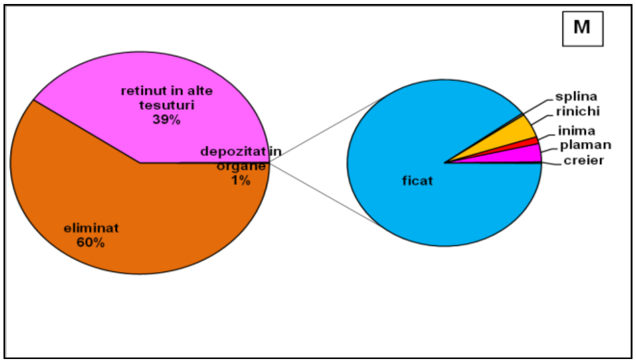
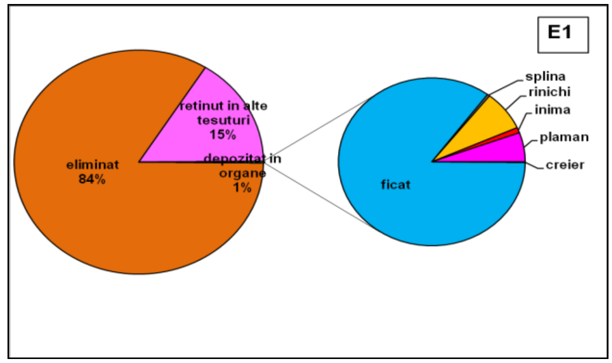


Fig. 6.12. Graphical representation of experimental results on the distribution of copper ingested in the organism of piglets and the excreted amount: (a) M, (b) E1, (c) E2

The largest percentage of copper retained in animal organism was recorded for piglets in group M, where the amount ingested was the most important. The liver has the highest proportion of copper deposited in organs followed by the value of the quantities stored in the kidneys.



(a)



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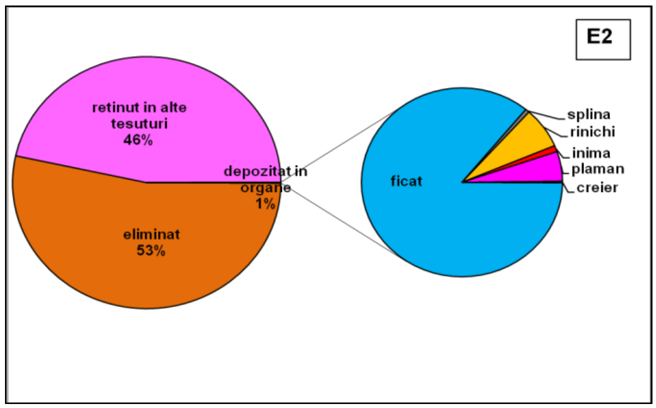
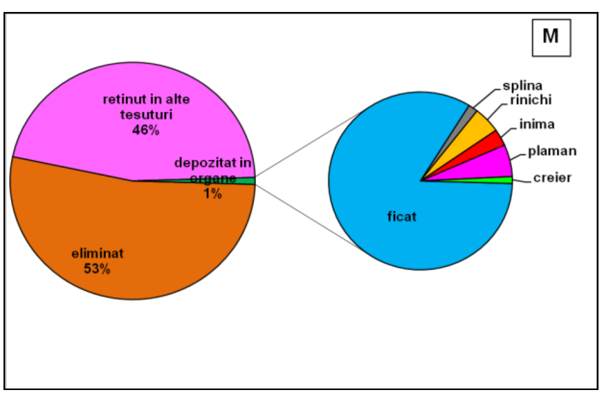


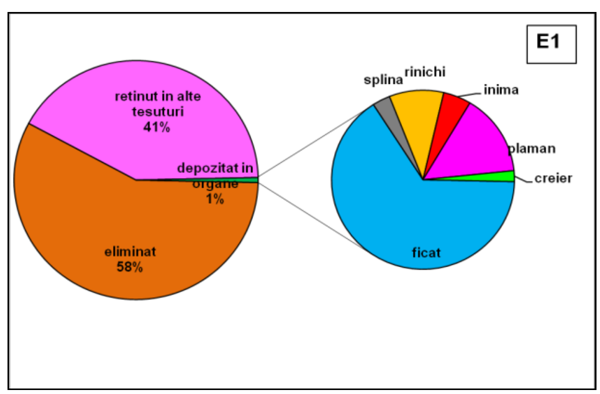
(c)

Fig. 6.13. Graphical representation of experimental results on the distribution of manganese ingested in the organism of piglets and the excreted amount: (a) M, (b) E1, (c) E2

As in the case of iron and copper, the manganese highest percentage eliminated was registered for the group with the smallest amount of Mn ingested. The largest share of distributed Mn in the organs was in liver.



(a)



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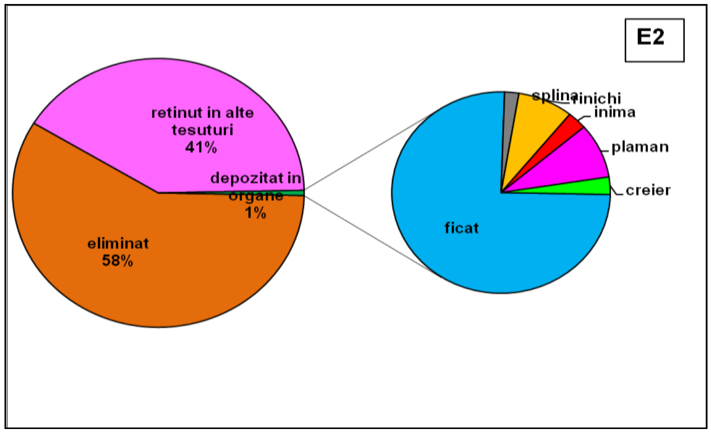

(c)

Fig. 6.14. Graphical representation of experimental results on the distribution of zinc ingested in the organism of piglets and the excreted amount: (a) M, (b) E1, (c) E2

From Figure 6.14. it can be noteced that the highest percentage of Zn has been eliminated for group E1 although the amount of Zn intake did not differ between experimental groups. In terms of trace elements deposited in organs during the experiment, the largest share is occupied by liver.

The highest concentration of trace elements was determined in liver (2-3 times higher than in other organs). After reporting the organic tissue mass, it was confirmed that the liver is the organism's main store of micronutrients.

Iron deposited in organs by piglets from group M was about 1.7 times higher than in the experimental groups. Efficiency of inorganic iron present in feed of group M was higher.

In the case of copper, the percentage ratio of ingested quantity and the quantity stored and between the stored quantity and the eliminated quantity looks like: E1> M> E2. On this basis it can be concluded that a lower level of copper in the diet led to a more efficient use of it.

And for Mn, the numerical values of the same ratio looks like: E1> M> E2. On this basis it can be concluded that a lower level of Mn in the diet resulted in a more efficient use of it.

6.3. CORRELATION STUDY BETWEEN THE CONTENT OF TRACE ELEMENTS IN ANIMAL ORGANISM AND THEIR CONCENTRATIONS IN THE DIETS ENRICHED WITH MIXTURES OF PLANTS HARVESTED FROM WILD FLORA

Average daily gain values calculated for groups M and E2 (group supplemented with oregano and cranberry mixture) were not significantly different ($P \leq 0.05$), even while reducing inorganic salt content of Fe, Cu, Mn and Zn in the diets. Although diet of group E1 (group supplemented with oregano and caltrop) had included the same type of premix as E2 group, growth performance of piglets from this group were significantly ($P \leq 0.05$) lower than those of animals M and E2. These results were caused by feed consumption, significantly ($P \leq 0.05$) lower, registered for this lot. The explanation of the lack of appetite of E1 piglets is that the inclusion of caltrop in the feed, gave a rough consistency of it, irritant for piglets.

Hematological and biochemical parameters are within the normal range of values for pigs from groups M and E2. In the case of group E1, it was determined a number of leukocytes below the lower limit of the normal range which could indicate a state installed infectious in organism or a type of anemia.

Mineral balance data

According to balance results, the iron intake of piglets from group E1 was approximately 60% lower than those from group M due to the low consumption of food. For piglets from group E2, the intake was higher by 18.5% than those in group M due to intake of iron from added plants. Under these conditions, Fe excreted in the faeces was significantly ($P \leq 0.05$) lower in groups E1 (approximately 55%) and E2 (approximately 15%) than M. Iron absorption and retention coefficients calculated for the piglets from group M were significantly ($P \leq 0.05$) higher than the coefficients determined in pigs from groups E1 and E2.

The copper intake for group E2 (group supplemented with oregano and cranberry mixture), was 75% smaller than M, and for group E1 (group supplemented with oregano and caltrop) was 85% lower than M. As iron intake difference between the two experimental groups was due to a lower feed consumption recorded for piglets in group E1. Under these conditions, the amount of Cu excreted in the feces of piglets from group E2 was 56% lower than the piglets in group M. The amount of Cu excreted in the faeces of piglets from group E1 was 75% lower than that of piglets in group M. As expected, the copper absorption and retention coefficients, for group M were significantly ($P \leq 0.05$) higher than the experimental groups. And between experimental groups were significant differences in the coefficients of absorption and retention value calculated.

Mineral balance results show that Mn intake for piglets from group E2 was 55% lower than for the control group. In group E1, Mn intake was 75% lower than in group M. The difference between experimental groups was determined by different feed consumption. The amount of Mn excreted in the faeces of group E2 was 52% lower than for group M and for piglets from E1 were 62% lower than for group M. The coefficients of absorption and retention of manganese for group M were significantly ($P \leq 0.05$) higher than the experimental groups. And between experimental groups were significantly differences ($P \leq 0.05$) in terms of coefficients of absorption and retention values calculated according with the data obtained for the intake and excretion.

Zinc intake for piglets from group E2 was 25% lower than the piglets from group M, and for group E1 was 85% lower than the piglets from group M. Differences between experimental groups was brought by feed consumption. The amount of Zn excreted in the faeces in group E2 was 19% lower than in group M and for piglets in the experimental group E1 was 75% lower than those from group M. Zinc absorption and retention coefficients for piglets from group M were greater than the piglets from group E2, but insignificant. Values calculated for the same coefficients for piglets from group E1 differed significantly from animals from groups M and E2.

With mineral balance data were constructed graphs presented in Figure 6.19. It highlights the very good correlation between the amount of ingested and excreted amounts of trace minerals by animals participating at experiment.

To quantify the relationship between the quantities of micronutrients ingested and eliminated through faeces and urine it were considered regression equations and correlation coefficients: for Fe: $Y= 0,5061X + 54,918$; $R^2 = 0,9964$; for Cu: $Y= 0,3764X + 3,1152$; $R^2 = 0,9956$; for Mn: $Y= 0,5133X + 5,8562$; $R^2 = 0,9889$; for Zn: $Y= 0,4727X + 9,5494$; $R^2 = 0,9970$.

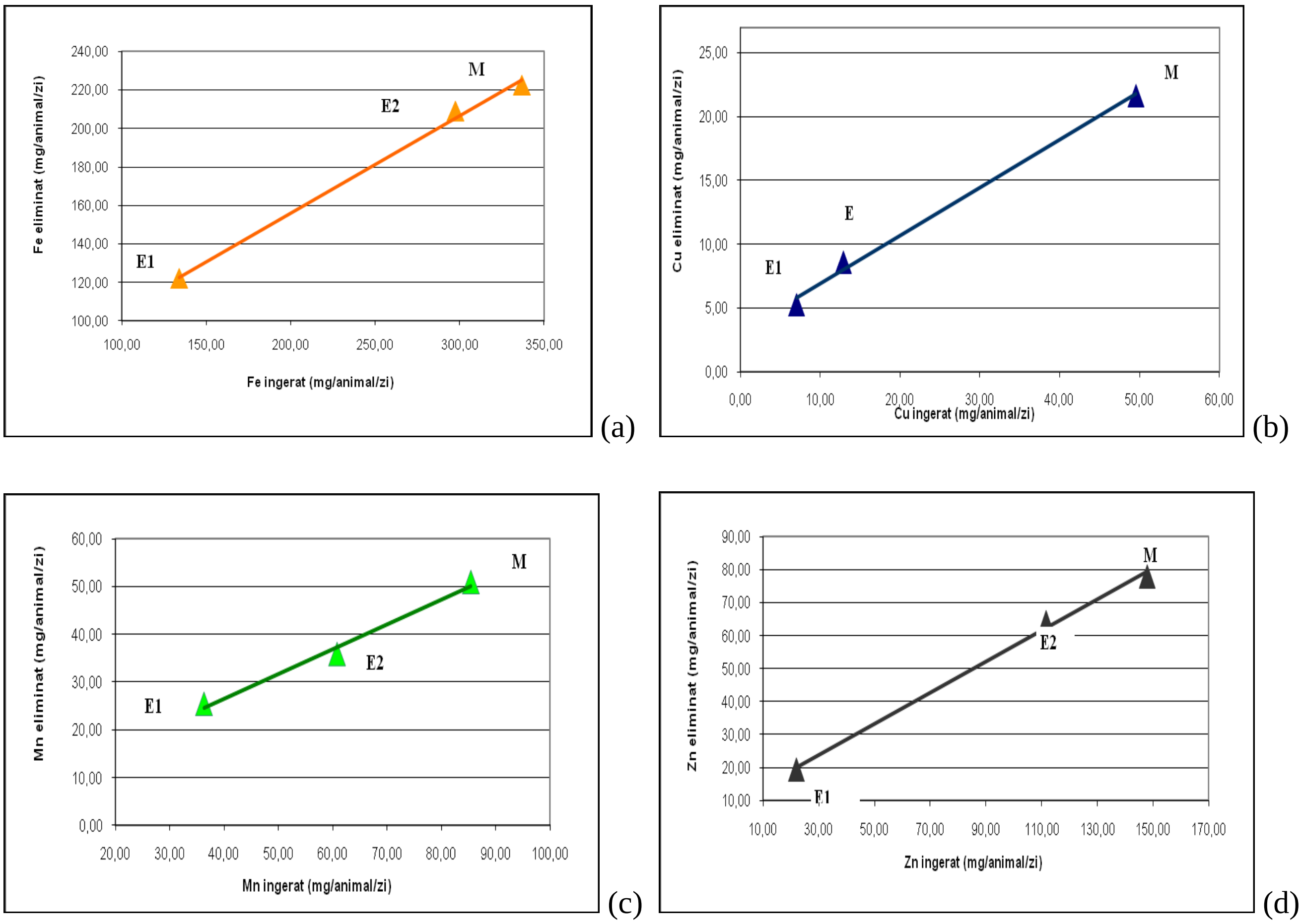

Fig. 6.19. The correlation between the amounts of trace elements ingested and excreted: (a) iron, (b) copper, (c) manganese, (d) zinc

The data presented in Figure 6.19. shows that in the three studied groups observed the same relationship of proportionality between the intake and excrete of all trace elements studied. Small amounts of micronutrients ingested and excreted by animals from group E1 are shown by placing the corresponding point in the lower part of the graph.

The experiment was conducted on weekly mineral balance, it can be seen the evolution of trace elements elimination through faeces (Figure 6.20.).

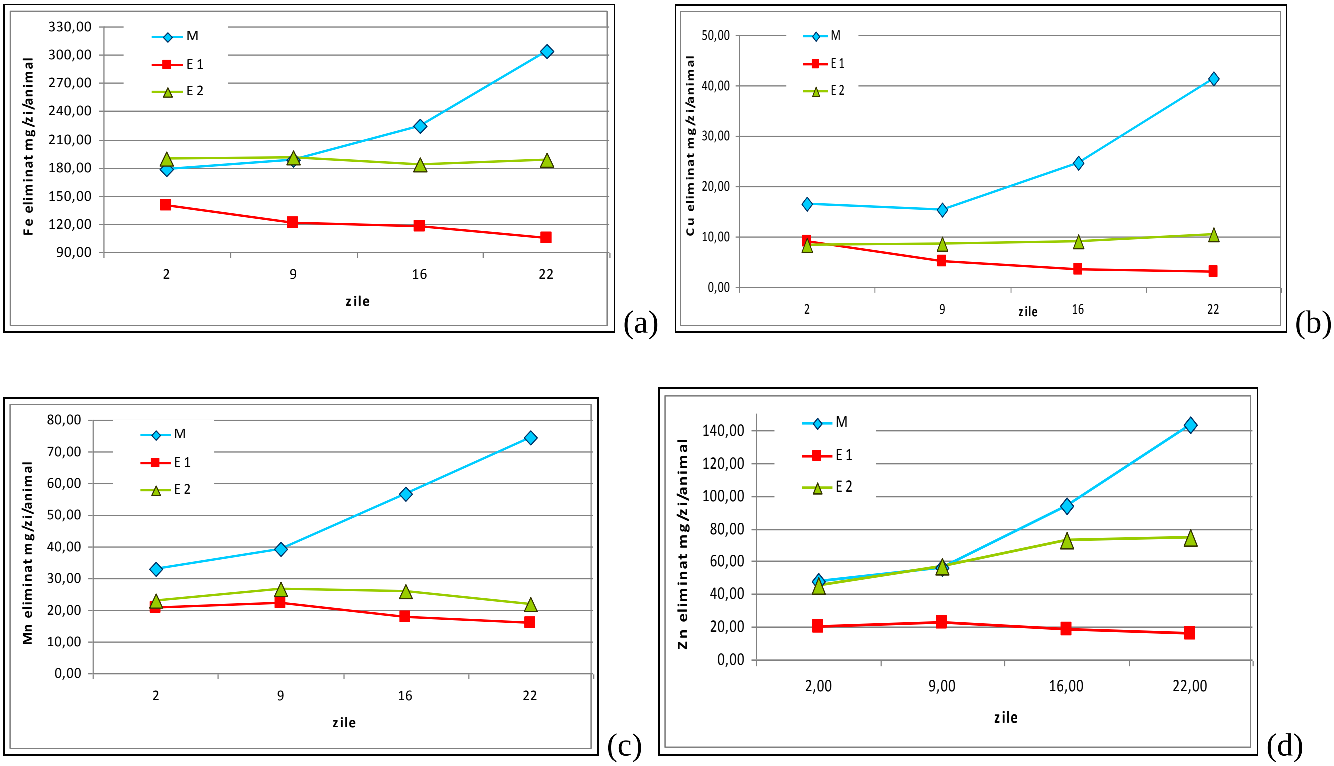

Fig. 6.20. The evolution of trace elements elimination through faeces (a) iron; (b) copper; (c) manganese, (d) zinc

Small amounts of ingested iron are found proportionally in the faeces excreted. The tendency of Fe eliminaton for piglets from group M is upward, while the corresponding grapf for piglets from group E2 is constant in time. The elimination of Cu and Mn in the feces from piglets from group M were higher since the beginning of the experiment, due to higher concentrations of micronutrients in feed. The downward trend of elimination of Cu and Mn in time is similar to observations made in the case of iron. The Zn excretion through faeces of piglets from group M excess of the corresponding quantities in the two experimental groups of animals. The smallest trace elements amounts eliminated throughout the experiment characterized piglets from group E1, according to the mineral quantities ingested.

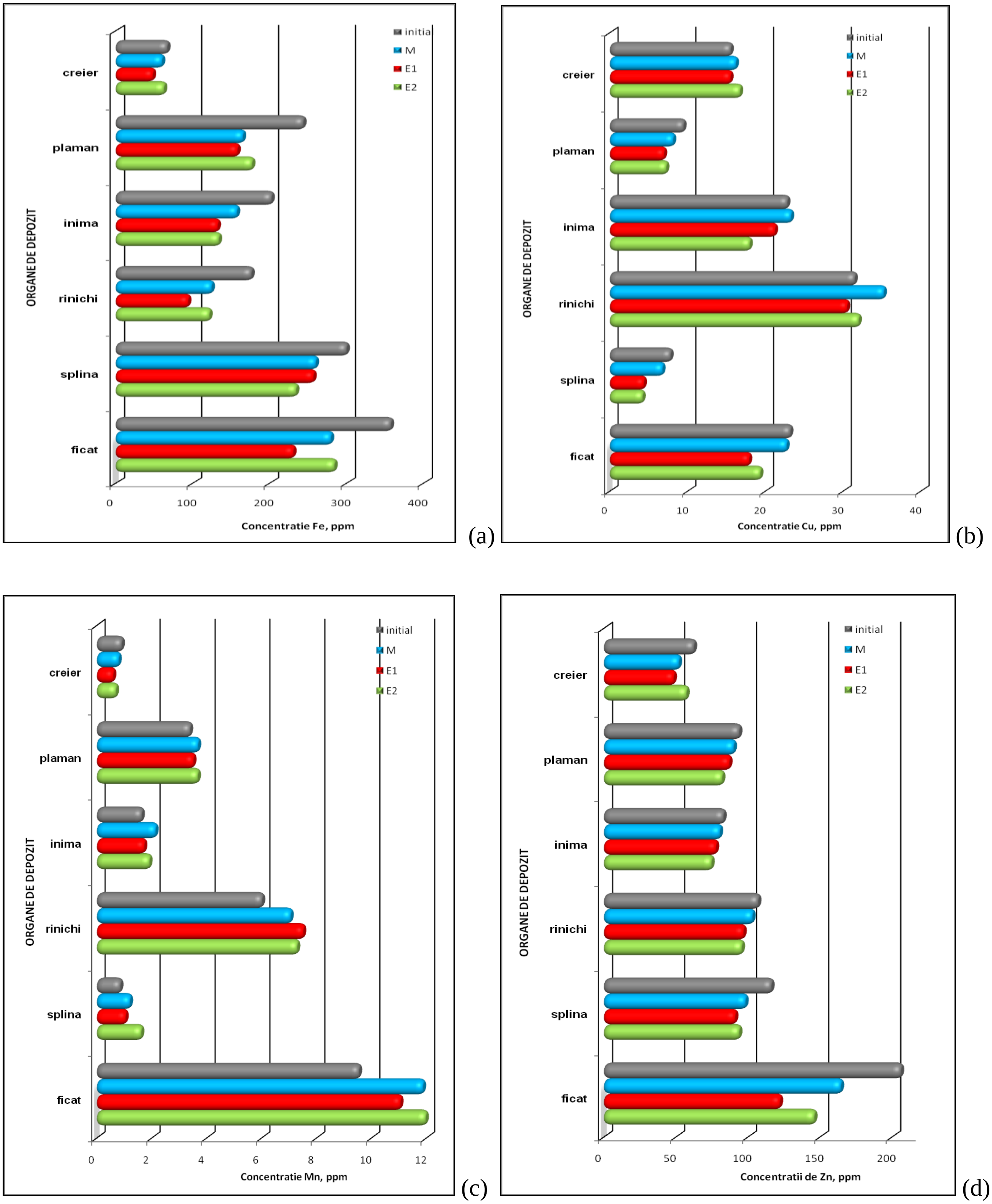

Fig. 6.21. Average values of concentrations of Fe, Cu, Mn, Zn determined in deposit organs

Iron concentrations determined in organs of piglets slaughtered at the beginning of the experiment were not equal at the end of experiment at any of the 3 groups. Iron determined in animal organs of group E1 are in agreement with previously measured parameters, significantly lower than animals of groups M and E2. For all organs, concentrations of Cu determined in organs of piglets from group M were significantly ($P \leq 0.05$) higher than those found in organs of experimental piglets. The concentrations of Cu in organs of animals of group E1 were significantly ($P \leq 0.05$) lower than those recorded in groups M and E2. There were no significant differences between manganese concentrations in organs from animals of group M and E2. The highest concentrations of Zn were found in the liver. Zn concentrations determined in animal organs other than the liver were not significantly different ($P > 0.05$) between groups M and E2.

Iron deposited in organs of piglets from group M was about 2.7 times higher than in the case of group E2. Even if the amount of copper ingested by animals from group M was 4 times higher than the amount ingested by group E2, the amount of Cu deposited in organs of pigs in group M each day of the experiment was only 2.3 times higher. It may be considered that a lower level of Cu in the diet led in a more efficient use of it. The ratio between the amount of ingested Mn for piglets from group M than that ingested by animals from group E2 was 2.2. The ratio of the amount of Mn deposited by the piglets from group M to E2 was 1.7. Reports of the quantity stored and ingested and the quantity stored and retained in the body are favorable to animals from group E2. It may be considered that a lower level of Mn in the diet led to a more efficient use of it. For piglets from group E1, in which was a massive elimination of Mn through the feces, the amount retained in the body was a small amount compared to other groups. Due to the negative value recorded for Zn deposits in organs of piglets in group E1, negative values were obtained for the ratio calculated so that it can be concluded that the diet given was ineffective on zinc storage in animals. Coefficients obtained by the two types of ratios were superior to animals in group M.

CONCLUSIONS

Trace elements are essential for most basic metabolic processes occurring inside the animal organism. The main functions of trace elements are related to participation in blood oxygen transport, hematopoiesis, tissue respiration and functional role in enzyme systems. Piglets' diets are usually supplemented with microelements, which are added to animal feed as organic or inorganic compounds of natural or synthetic sources. Main factors limiting the micronutrients supplementation in the base diets of piglets are related to bioavailability of sources of trace elements and their impact on the environment. In this context, within the development of this thesis, studies have been conducted to replace inorganic salts supplements in feed plants or plant extracts for improving animal product quality and to obtain a better environmental protection.

Originality data presented in the thesis are:

- Characterization of plant resources following:, buckthorn, Jerusalem artichokes, oregano, blueberries and caltrop, in terms of the levels of trace elements Cu, Fe, Mn, Zn. Based on the data obtained, it could be established that many of these plants can be used as sources of micronutrients in diets of recently weaned piglets;

- Evaluation of the effects of proposed plant resources in terms of their use as sources of trace elements on mineral status of weaned piglets. These plants were used and evaluated for the first time, as potential agents for the prevention of diseases associated with mineral imbalances caused by weaning of piglets, such as iron deficiency anemia. Also, by using these plants, it was studied the possibility of reducing the additions of inorganic trace elements in piglet diets (usual practice), thus reducing the amount of microelements eliminated through the faeces, important component of environmental protection strategies.

In order to achieve the objectives, it was started from conducting a bibliographic study on essentiallity of trace elements and the using of spectrophotometric methods for the determination of trace elements in biological samples of vegetable and animal origin.

Further, in the first chapter of the experimental part, practical aspects were highlighted by specifying practical aspects of the study, included the ways of sampling and sample preparation for analisys, and establishing the optimal working conditions for the determination of trace elements Fe, Cu, Mn, Zn from biological samples and animal nutrition experiments organization: microtest and macrotest experiments. This step was followed by validation of the method determination of trace elements Fe, Cu, Mn, Zn in biological samples and present the results obtained in animal nutrition experiments.

From analytical point of view, biological samples are a complex organic matrix containing chemical species whose proportion can exist from trace level till to major component. This fact has required the development of a wide range of methods. However, atomic absorption spectrometry is considered the reference method for analysis of trace elements in biological samples.

Practical aspects of organizing experiments refer to: sampling and sample preparation of biological materials (plant or animal samples) for assessment, establishing optimal working conditions in order to determine the concentration of trace elements, organizing animal nutrition experiments: micro and macro test type experiments.

Study concerning comparation of decomposition methods on biological samples of animal and vegetable, for quantitative determination of trace elements content was performed on reference materials and resulted in the election of microwave decomposition method being considered as the optimum way of bringing into the solution the trace elements originated from liver samples and samples of plant material. This method requires the shortest time of working, an average volum of concentrated reagents and is characterized by accuracy expressed by values very close to 100%.

The protocol of validation microwave decomposition method and determination by FAAS microelements Fe, Cu, Mn and Zn in liver samples and plant material was followed - as reference material. Following validation of the method for the determination of iron, copper, manganese and zinc in biological samples of animal and vegetable origin, by flame atomic absorption spectrometry after microwave digestion and uncertainty calculation, it could be conclude that the analytical method correspond of the proposed aim - a chemical-analytical study of the correlation between the content of microelements in diets supplemented with plants or plant extracts and their retrieval in the animal organism.

Since there is no reference material to validate the method for determining trace elements Fe, Cu, Mn, Zn in samples of plant harvested from spontaneous flora and determining the content of trace elements in such plants, an interlaboratory study was organized. The results provided by participants covered a relatively wide a range of values and arithmetic mean calculated not characterize strings. To establish uniform datasets, average mean and standard deviation it was applied algorithm A. In order to evaluate the analytic capacity of participants, it were calculated the Z, Z 'and Zeta Scores. From all results obtained, there were nine questionable or unsatisfactory zeta values. These results can be attributed to underestimated values of calculation uncertainties reported by participants in the interlaboratory study.

The results obtained for benchmarks as a result of Algorithm A application showed that all 3 analyzed plants contained significant concentrations of trace elements. Determined values justify the use of these plants in animal nutrition studies. Oregano plants, blueberry and caltrop, once analyzed were used as additives in the food rations of weaned piglets in two experiments.

The results of chemical analyses performed in the animal nutrition experiment within this chemical-analytical study of the correlation between the content of trace elements in animal organism and their concentrations in feed diets enriched with inulin showed that the presence of inulin improved the bioavailability of copper in feed rations of piglets weaned. There were no significant differences between groups concerning Mn and Zn deposits from liver. Bioavailability of copper was higher in piglets raised under hygienic conditions than those grown in ideal conditions of commercial farms.

The conducted experiment to study the chemical-analytical correlation between the content of trace elements in animal organism and their concentrations in diets enriched with crop plants demonstrated that using a mixture of crushed Jerusalem artichoke tubers (*Helianthum tuberossum*) and buckthorn leaves (*Hippophae rhamnoides*), dried powder in piglets' diet, the intake of inorganic salts was reduced by half (compared with a commercial premix), it did not adversely affect the performance bio or profile haematological parameters of piglets, which values are entered in specific species and category. The mixture was tested both in microtest and macrotest conditions experiment and the data obtained from microtest were confirmed by the results from macrotest experiment.

To study the correlation between the content of trace elements in animal organism and their concentration in the diets enriched with plants harvested from wild flora or crops were organized two experiments. Phytiadditives used in experiments were included in diets singly or in mixtures. Plants used were oregano (*Origanum vulgare L.*), blueberry (*Vaccinium myrtillus L.*), caltrop (*Tribulus Terestris*) and a mixture of buckthorn and Jerusalem artichoke.

In the experiment, wherein in the feeding of some weaned piglets, oregano supplements were added as organic sources of micronutrients, bio performance was not affected by decreasing the content of trace mineral premix administered. to piglets. Levels of trace elements (Fe, Cu, Mn, Zn) excreted were proportional to the intake, which is important in terms of reducing environmental pollution by heavy metals. Bioavailability of trace elements from oregano, compensated by the low level of mineral element received by premix.

In the experiment, there where in weaned piglets' feeding were added mixtures of plants harvested from spontaneous flora as natural sources of micronutrients, bio performance was not affected by decreasing the content of trace mineral premix in piglets administered, when using a mixture of oregano and cranberries. Levels of micronutrients Fe, Cu, Mn, Zn excreted were proportional to those ingested. Bioavailability of trace elements in oregano and cranberries mixture, compensated by the low levels of nutrients received by premix.

Due to irritating hardness which caltrop supplement imprinted upon piglets that ate this feed, the parameters and bioavailability of trace elements bio recorded were significantly ($P \leq 0.05$) lower compared to the other groups studied. And the quantities of trace elements excreted were reduced to this group, this being in accordance with small amounts of micronutrients ingested. During the experiment were not deposited amounts of trace elements in organic tissues evaluated. Bioavailability of trace elements from mixture of oregano and caltrop was reduced and affected piglets trace elements status, so this mixture can not compensate the trace elements commonly received from the premix by piglets.

The results of chemical-analytical studies on biological samples of plant material and animal origin samples obtained in animal nutrition experiments are presented and discussed in the thesis across four chapters (Chapters 3-6). They are presented in 134 tables and 66 figures. The experimental data were discussed in relation to data from the literature, presented in Chapters 1 and 2.

Based on the experimental results obtained in studies performed for developing this thesis, we can estimate that mineral additions in the form of salts or inorganic oxides can be replaced by certain crop plants, from spontaneous flora or plant extracts. Using these natural sources of minerals has a beneficial effect on animal health, food quality and environmental protection. For this purpose, can be recommended inulin (extracted from chicory), oregano and blueberries (from spontaneous flora) and buckthorn and Jerusalem artichokes grown as medicinal plants.

The thesis is presented over 270 pages and includes 207 references.

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