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Ultrasound-assisted extraction for rapid determination of Zn, Cu, Fe, Mg and Mn in liver of diabetic rats under different antioxidant treatments

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ABSTRACT

The metabolism of several essential elements is altered in diabetes mellitus and these nutrients might have specific roles in the pathogenesis and progress of this disease, nevertheless, the mechanisms are still far from known.

Variations in Zn, Cu, Fe, Mg and Mn in rat liver have been measured both in control and diabetic rats which have been given antioxidants (either synthetic or natural extracts) or a placebo.

Classical contaminant and time-consuming digestion methods for sample pre-treatment have been substituted by ultrasound-assisted liquid extraction (USLE). The effect of several parameters was studied, the best results being obtained for: 0.2 g of sample in 10 mL 10% HNO_3 and 8 min of sonication with 19 kHz frequency. The complete analytical method was validated regarding linearity, precision, accuracy and limits of detection (LOD) and quantification (LOQ). Values for LOD ranged from 0.6 for Mn to 12.5 for Mg, and LOQ ranged from 1.8 for Mn and to 62.5 for Mg expressed as $\mu g\, g^{-1}$ in sample.

Results showed an increase of all the metal assayed in the liver of diabetic rats as compared to controls. Nevertheless, when animals were treated either with antioxidants or *Dunaliella* extracts in a short term assay, Fe increase in liver of diabetic rats was lower than with the other treatments, while *Rosmarinus* (rosemary) extracts were the only treatment producing a decrease in Mn on diabetic rats.

1. Introduction

Ultrasonic solid–liquid extraction (USLE) of metals from biological samples can be considered a new trend in sample pre-treatment, already classical in other areas of analysis. Ultrasound-assisted extraction in tissues is dependent on the cell disruption effects of ultrasonic waves. The possible advantages of ultrasound in extraction are as follows [1]: Intensification in mass transfer; cell disruption; enhanced penetration and capillary effects. Additionally, a diluted acid medium is normally used, thus decreasing blank values and reducing both reagent and time consumption, as compared to traditional wet digestion using conductive or microwave-assisted heating. Moreover, a small sample amount can be used, which makes it interesting for assays where bigger samples are not available.

Sample pre-treatment is the most important bottleneck of flame atomic absorption spectrometry (FAAS), on the other hand a simple and robust technique. Therefore, coupling USLE and FAAS brings up a rapid tool for the analysis of large sets of complex samples. Nevertheless, to avoid loosing reliability with the coupling, as compared to classical digestion methods, parameters affecting ultrasound extraction should be studied and optimized.

Ultrasonic bath and ultrasonic probe systems are the two most common devices used in ultrasound-assisted extraction. Although ultrasonic baths are more widely used, they have two main drawbacks [2] that considerably decrease experimental repeatability and reproducibility: lack of uniformity in distribution of ultrasound energy (only a small fraction of total liquid volume in the immediate vicinity of the ultrasound source experiences cavitation) and decline of power with time, so the energy supplied to bath is wasted. In addition, bath sonication is limited in the amount of energy it transfers to the sample. Due to the higher energy transferred to the sample by probe sonication, it is the selected tool for total metal extraction.

Capelo et al. [3] have reviewed the different variables which affect ultrasonic solid–liquid extraction, namely, the ultrasonic device chosen for ultrasonic extraction (e.g. ultrasonic bath or ultrasonic probe), particle size, acid concentration, sonication time and sonication amplitude, sample mass and analyte–matrix binding. The authors conclude that the failure to consider (not considering) these factors is the cause of controversial data reported by different workers.

Ultrasound-assisted extraction using probe sonication has been applied to determining V and Ni from dried mussel tissues [4];

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to Cd and Pb in biological samples (blood and scalp hair) [5], this method having been validated by using certified materials BCR 397 human hair and BCR 185R bovine liver; to a set of bio-environmental samples (IAEA lichen 336 and mussel tissue NIST 2976) [6], where quantitative recoveries were obtained for most of the elements for which certified concentrations were available; to Cd and Pb from plant leaves [7] extracted prior to determination by electrothermal atomic absorption spectrometry (ETAAS); to Cu and Zn in ten different Mediterranean crops to test their tolerance to polluted soils [8]; and to Zn, Cd, Pb, and Cu, in different portions of the plants at two harvesting times to determine the accumulation and translocation of the environmental pollutants [9]. In addition, applications of ultrasound-assisted extraction to selenium determination in different matrices either the total amount or aiming speciation has been reviewed by Capelo et al. [10].

This type of acid leaching not involving sample destruction has also been employed with microwave-assisted procedures either for speciation purposes [11] or total element leaching such as Al, Ba, Cd, Cr, Cu, Fe, Mn, Pb, Sn, V and Zn determination in mussels [12]. In general microwave ovens for laboratory purposes are expensive and in this last case reported concentrations of acids and R.S.D. values were slightly higher than those obtained with ultrasound-assisted methods.

In recent years the use of plant and algae extracts with potential nutraceutical activity has been growing worldwide but there is a lack of scientific evidence of their activity.

Our group has been interested in developing and studying the effect of antioxidants and different extracts with potential antioxidant activity in a model of oxidative stress such as the rat made diabetic by streptozotocin (STZ) injection [13,14].

Diabetes is a disorder characterised by chronic hyperglycaemia. It is a well-established fact that oxidative stress is a contributory mechanism in many disorders where poor glycaemic control exists. Although altered systemic regulation of trace metals in diabetes has been previously investigated [15], it is still unclear whether changed trace metal metabolism would cause heart disease in common forms of diabetes and whether metal chelation can reverse this condition. The accumulation of redox-active trace metals including Cu and Fe and changes in the corresponding redox couples, may, at least in part, result in the generation of excess reactive oxygen species.

The aim of this work was firstly the development and validation of a method with USLE–FAAS for rapid and reliable sample pre-treatment and secondly, the application to study metal concentrations in the liver of eight groups of animals: control and diabetic receiving either a placebo or a mixture of synthetic antioxidants (vitamin E + C), or extracts of *Dunaliella salina* or *Rosmarinus officinalis* obtained with supercritical fluid extraction.

2. Materials and methods

2.1. Reagents and solutions

Nitric acid 60% (Panreac, Barcelona, Spain), hydrochloride acid 37% (Carlo Erba, Barcelona, Spain). 1000 mg L^{-1} Mg(II) stock solution was prepared from commercial salt, $Mg(NO_3)_2{\cdot}6H_2O$ (99.8%, ACS, Sigma, Madrid, Spain). Stock solutions of 1000 mg L^{-1} Zn(II), Cu(II), Mn(II) and Fe(III), dissolved in HNO_3 0.5 M (Merck, Barcelona, Spain) were used. All the concentrated solutions were stored at 4 °C in the dark and used for the daily preparation of dilute standard solutions.

All solutions were prepared with water purified by a Milli-Qplus 185 water purification system (Millipore Iberica SA, Madrid, Spain).

2.2. Instrumentation

The determination of Zn, Cu, Fe, Mg and Mn carried out using an Atomic Absorption Spectrophotometer (PerkinElmer model 3110) with an acetylene–air flame. Hollow cathode lamps (PerkinElmer) were used as a radiation source for all the studied elements. The instrumental parameters employed were a spectral bandwidth of 0.7 nm, a 30 mA lamp intensity and the following wavelengths: 213.9, 324.8, 248.3, 285.2 and 279.5 nm for Zn, Cu, Fe, Mg and Mn, respectively.

Ultrasonic-processor XL sonicator (Misonix, Farmingdale, NY, USA) with 6 mm of diameter probe and lyophilizer Cryodos 50 (Telstar, Tarrasa, Spain) were employed for sample treatment.

2.3. Samples and sample treatment

Eight groups of animals were employed for the assay and comprised:

- CV and DV: 7 control and 7 diabetic rats receiving a placebo mixture.
- CX and DX: 7 control and 7 diabetic rats receiving an antioxidant mixture.
- CR and DR: 7 control and 7 diabetic rats receiving *R. officinalis* extract.
- CD and DD: 7 control and 7 diabetic rats receiving a *D. salina* extract.

Rats (Sprague–Dawley strain from our animal quarters at San Pablo-CEU University) that received an intraperitoneal dose (50 mg/kg) of STZ and which showed blood glucose levels over 200 mg/dL after 4 days were classified as belonging to the diabetic group. The animals were sacrificed 14 days post-STZ administration and both tissues and plasma were stored at −80 °C for further determinations.

At 72, 64, 48, 40 and 24 h before sacrifice, rats received (by gavage) a dose of either an antioxidant mixture of vitamins C (264 mg) and E (30 mg) dispersed in 1 mL placebo vehicle; a *R. officinalis* extract; a *D. salina* extract; or only the placebo vehicle. Throughout the experiments the animals were kept in appropriate conditions in the animal quarters of our University. The male rats, of age 12 ± 2 weeks, were housed in groups of three in a room maintained at 22 ± 2 °C, 55 ± 10% humidity with 12 h light and dark cycles. The rats were fed with a standard diet (Harlan Global Diet 2014, Harlan Interfauna Iberica, Madrid, Spain) *ad libitum*. They also had free access to tap water. All studies were performed after achieving the necessary approval from the Ethical Committee of the University San Pablo CEU.

After sacrifice, the livers were frozen immediately under liquid N_2, weighed separately, and kept at −80 °C until the analysis.

A sample from each experimental group was composed by weighing 1 g of liver of each animal in the group. The mixture was homogenized, lyophilized and ground to powder in order to have it in the same conditions as the reference material used for the method optimization. Finally, 0.2 g were probe sonicated for 8 min with 10 mL of 10% nitric acid at 19 kHz. Quantification was performed by interpolating results in the corresponding calibration curve obtained for standards.

Certified Reference Material of beef liver (NCS ZC71001) containing 192 ± 12, 8.92 ± 0.84, 91.6 ± 3.8, 668 ± 49, 346 ± 31 μg g^{-1} of Zn, Mn, Cu, Mg and Fe, respectively was employed for method validation.

2.4. Extracts

D. salina is a micro alga from the Chlorophyceae family. *D. salina* can proliferate over a large range of salinities for massive carotenoid accumulation (400 mg/(m^2 day)) which makes cultivation easy and economical [16]. Microalgae extract were processed as described by Jaime et al. [17], and the extract which provided the highest antioxidant activity was employed for the *in vivo* assay. Its complex composition has been shown to provide stronger antioxidant activity than the synthetic β-carotenes.

R. officinalis is a common dense, evergreen, aromatic shrub grown in many parts of the world. The leaves of (rosemary) were subjected to supercritical CO_2 extraction (SFE) [18] and its active constituents were analyzed by HPLC-UV, HPLC–MS, CE and CE–MS [19–20]. Carnosic acid, carnosol and rosmarinic acid are among those more active antioxidant compounds in the extracts.

3. Results and discussion

An ultrasonic probe was applied for solid–liquid extraction because it can produce a higher intensity than the ultrasonic bath and, in these experiments, animal tissues should be disrupted simultaneously to the extraction. Parameters such as ultrasonication time, extractant concentration and extractant volume/sample mass ratio were evaluated by using a certified reference material consisting of beef liver as described in the previous section. It is well known that the analyte–matrix binding can have different effects in the real sample than those when they are studied in samples spiked with the free metal.

3.1. Sonication time

Initially certain parameters were established: sample weight was 0.200 g, 10.0 mL of 10% HNO_3 and the maximum frequency in the sonication equipment which was 19 kHz. Sonication time varied from 2 to 10 min in 2 min intervals and in triplicate samples.

Fig. 1 shows the percentage recovery and the corresponding standard deviation. Recovery slightly increased with increasing sonication time, 4 min being sufficient for most of the metal assayed. As can be observed, Fe is the metal in which it is more difficult to achieve a quantitative recovery. For that reason sonication time was established at 8 min when this metal is fully recovered. Higher sonication times produced an overheating both in the sonication probe and in the solution.

3.2. Acid medium concentration

HNO_3 is the acid more commonly used for metal extraction, acid solutions in probe sonication typically being under 10%. Therefore, 1, 5, 10 and 20% HNO_3 was assayed using 0.200 g of tissue, 10 mL

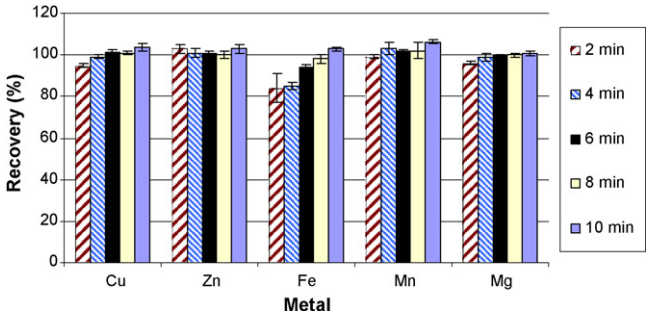

Fig. 1. Influence of sonication time in Cu, Zn, Fe, Mn and Mg extraction from beef liver reference material (NCS DC 71001) using 10 mL of 10% HNO_3 for extraction and FAAS for quantification ($n = 3$).

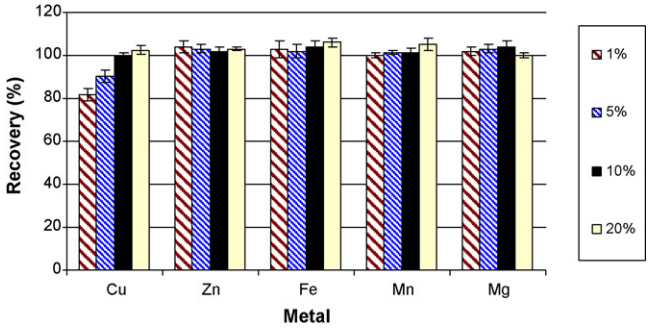

Fig. 2. Influence of HNO_3 concentration in Cu, Zn, Fe, Mn and Mg extraction from beef liver reference material (NCS DC 71001) using 10 mL of HNO_3 during 8 min for extraction and FAAS for quantification ($n = 3$).

volume of acid and 8 min as extraction time. Frequency was always kept at its maximum value (19 kHz).

As can be observed in Fig. 2, all the elements tested, except for Cu, gave recoveries around 100% for all the nitric acid concentrations tested even 1%. Nevertheless, 10% was necessary to achieve a 100% recovery for copper and that was the concentration selected for further work.

HCl was also assayed at 1, 2.5 and 5% concentrations. Again similar results were obtained, with copper needing the highest concentration, and nitric acid was preferred because HCl can attack the probe tip.

3.3. Sample mass/extractant volume

In a first assay 0.200 g of sample were extracted for 8 min with 10% HNO_3 at 19 kHz with volumes of 10 and 25 mL.

Recoveries were quantitative for all the elements assayed with both volumes except for iron, which decreased slightly with increasing volume and therefore, a higher volume provided no improvement in the extraction. In another experiment, the mass of sample was changed from 0.200 to 0.500 g, keeping constant 25 mL of 10% nitric acid as solvent. Results showed that iron recovery was low in both cases. These results contradict those obtained by Amoedo et al. [21]. These authors found that extraction efficiency was independent of the mass, provided that the mass-to-volume ratio was kept constant. 0.5 g of sample in 25 mL was the same ratio as 0.2 g/10 mL, however recovery was lower in the first case. That could be explained with a higher volume of extractant making it more difficult to achieve the same energy transmission.

The final conditions, which resulted, are therefore explained above in Section 2.

3.4. Method validation

Reliability of the results obtained with the optimized method was demonstrated with validation parameters corresponding to linearity, accuracy and precision. Limits of detection and quantification were also calculated.

For linearity assay, five concentrations in the ranges included in Table 1 were prepared by triplicate and measured by FAAS. The Table also includes slope, the intercept with the corresponding confidence intervals ($\alpha = 0.05$) and correlation coefficients. The method can be considered linear for all the analytes tested. Correlation coefficients are over 0.999 in all cases, and slopes are different from zero. A small bias appears in some cases because the intercept with its limits of confidence did not include the zero value, but it had no practical significance, as it could be seen with recoveries which are not different from 100%. It could be justified with the good fit of

Table 1
Main validation parameters for determination of Zn, Mn, Cu, Mg and Fe in beef liver by USLE-AAS method.

Validation parameters		Zn	Mn	Cu	Mg	Fe
Standards linearity	Concentration range, mg L^{-1}	0.2–1.0	0.1–0.5	0.5–2.5	0.05–0.30	1.0–5.0
	$a \pm$ L.C.	0.008 ± 0.002	-0.001 ± 0.001	0.001 ± 0.001	0.028 ± 0.003	0.004 ± 0.002
	$b \pm$ L.C.	0.279 ± 0.003	0.095 ± 0.001	0.073 ± 0.001	0.82 ± 0.02	0.039 ± 0.001
	r	0.9997	0.9994	0.9998	0.9992	0.9994
Instrumental precision ($n = 10$)	Theoretic concentration, mg L^{-1}	0.6	0.3	1.5	0.15	3.0
	Mean ± L.C., mg L^{-1}	0.59 ± 0.01	0.306 ± 0.007	1.52 ± 0.03	0.152 ± 0.004	3.05 ± 0.07
	R.S.D., %	1.02	1.01	0.87	1.16	1.01
Method precision ($n = 6$)	Mean ± L.C., mg g^{-1}	207 ± 10	10.3 ± 0.9	93 ± 5	691 ± 30	354 ± 33
	R.S.D., %	1.69	3.40	2.09	1.69	3.63
Samples accuracy ($n = 6$)	Certified value in beef liver CRM, μg g^{-1}	192 ± 12	8.92 ± 0.84	91.6 ± 3.8	668 ± 49	346 ± 31
	Found value, μg g^{-1}	195 ± 4	10.0 ± 0.3	93 ± 2	654 ± 17	354 ± 14
	Recovery %	102 ± 3	112 ± 3	101 ± 2	98 ± 9	102 ± 9
	R.S.D., %	1.98	2.57	1.98	2.53	3.77
Limit of detection	In solution, mg L^{-1}	0.020	0.012	0.014	0.005	0.034
	In sample, μg g^{-1}	5.0	0.6	0.7	12.5	8.5
Limit of quantification	In solution, mg L^{-1}	0.047	0.035	0.045	0.025	0.091
	In sample, μg g^{-1}	11.8	1.8	2.2	62.5	22.8

a: intercept (u.a.); b: slope (L/mg); L.C.: limits of confidence ($p = 95\%$).

points to the regression line that made limits of confidence very narrow.

Precision was first evaluated for the instrumental system. For that, 10 values of the same standard containing the five elements at the concentrations described in Table 1 were obtained. R.S.D.s for instrumental precision ranged from 0.75 to 1.02%, which confirms that repeatability is a well-known characteristic of FAAS. Method precision for samples was tested simultaneously to accuracy.

Accuracy can be assessed by analyzing a sample of known concentration and comparing the measured value to the true value. The reference material beef liver (NCS ZC71001) was used for this purpose by analyzing 6 samples independently processed. Results in Table 1 showed recoveries close to 100% in all cases except for Mn which was 112%, probably because this element had the lowest concentration in the group. R.S.D.s ranged from 1.98 to 3.72%, showing a good method precision.

Limits of detection were calculated by taking 10 times the value of a blank sample, processed (following) after the complete procedure and calculating the lowest concentration producing a response three times above that value or ten times for the limits of quantification. Values expressed as concentration both in solution and in liver sample are shown in Table 1.

3.5. Metal content in liver of control and diabetic rats after different treatments

Data in Table 2 show the results obtained for the five metals content in liver in the eight experimental groups. In addition each group was compared with the corresponding control and all the experimental groups were compared for each metal with ANOVA and Bonferroni multiple comparison test with $p < 0.05$. Streptozotocin-induced diabetic rats on average had higher content in liver (as μg g^{-1}) of Zn, Cu, Mn, Mg and Fe than the corresponding controls independently of the treatment.

Metal content in the two extracts (Dunaliella and Rosmarinus) was determined but was found not to be significant (about 1000 times lower for the daily amount given to animals) as compared to the content in the pellet.

In a previous study [22] the role of two potential contributory factors, hyperphagia and alterations in fuel metabolism, on the development of tissue trace element accumulation in the experimentally induced diabetic rat were assessed. In this work, diabetic rats were hyperphagic and had lower plasma Mg, and higher liver Zn, Cu, and Mn concentrations than control rats, regardless of dietary mineral intake. By modifying the carbohydrate, fat and protein content in the diets the authors concluded that tissue-specific biochemical needs, such as the need for metals as cofactors for enzymes, rather than hyperphagia per se, may drive the accumulation of trace elements in the diabetic animal.

In another study [23], the concentrations of zinc, copper, and manganese in liver, kidney, duodenum, pancreas, testes, bone, and serum from control and untreated, spontaneously diabetic BB Wistar rats were compared. Chronic insulin deficiency resulted in significant alterations in the concentrations of one or more of these essential micronutrients in several tissues. The amounts of zinc and copper bound to metallothionein in the liver and kidney of untreated spontaneously diabetic rats were also markedly increased. The effects of spontaneous diabetes on tissue trace metal status are quite similar to those reported for chemically induced diabetes. Thus, these results demonstrate that chronic endocrine imbalance is responsible for a series of tissue-specific changes in the transport and metabolism of zinc, copper, and manganese.

Table 2
Metal concentrations in liver of different groups of rats: CV and DV: control and diabetic rats receiving a placebo mixture; CX and DX: control and diabetic rats receiving an antioxidant mixture; CR and DR: control and diabetic rats receiving *Rosmarinus officinalis* extract; CD and DD: control and diabetic rats receiving a *Dunaliella salina* extract.

Metal	Concentration mean ± L.C. (μg/g)							
	CV	DV	CX	DX	CR	DR	CD	DD
Zn	$104^{b} \pm 15$	$120^{c} \pm 5$	$95^{ab} \pm 3$	$132^{d} \pm 2$	$91^{a} \pm 4$	$162^{e} \pm 10$	$96^{ab} \pm 9$	$125^{cd} \pm 9$
Cu	$12^{a} \pm 1$	$14.7^{c} \pm 0.1$	$12^{a} \pm 1$	$16.1^{d} \pm 0.2$	$11.7^{a} \pm 0.2$	$16.99^{e} \pm 0.05$	$12.5^{a} \pm 0.5$	$13.8^{b} \pm 0.2$
Mn	$8.5^{b} \pm 0.5$	$13.06^{d} \pm 0.1$	$7.8^{a} \pm 0.1$	$14.2^{e} \pm 0.2$	$7.32^{a} \pm 0.1$	$12.02^{c} \pm 0.05$	$7.40^{a} \pm 0.2$	$12.90^{d} \pm 0.2$
Mg	$684^{a} \pm 12$	$743^{cd} \pm 17$	$686^{a} \pm 13$	$743^{cd} \pm 37$	$696^{ab} \pm 39$	$789^{e} \pm 30$	$721^{bc} \pm 23$	$765^{de} \pm 18$
Fe	$472^{b} \pm 12$	$652^{e} \pm 52$	$487^{b} \pm 13$	$593^{d} \pm 15$	$446^{b} \pm 60$	$678^{f} \pm 37$	$411^{a} \pm 20$	$545^{c} \pm 22$

L.C: limits of confidence ($\alpha = 0.05$; $n = 3$) ANOVA and Bonferroni multiple comparison test with $p < 0.05$ for each metal. Different letters show significant differences.

Looking at individual metals, the effects of hyperinsulinism/hyperglycemia on metal tissue concentrations are difficult to evaluate. Cordova et al. [24] found increased tissue Zn in liver, muscle and kidney in streptozotocin diabetic rats. These are the tissues that are responsive to insulin in terms of glucose transport.

It is noticeable that rosemary extract treatment provided a higher Zn content in liver of diabetic rats. It did not occur in the corresponding control group, where the Zn concentration decreased. Theoretically, zinc can exert a number of indirect antioxidant functions: protection against vitamin E depletion [25,26]; stabilization of membrane structure [24]; restriction of endogenous free radical production [27,28]; contribution to the structure of the antioxidant enzyme extracellular superoxide dismutase [29,30]; maintenance of tissue concentrations of metallothionein, a possible scavenger of free radicals [31].

Regarding other metals studied and the reported values, there are discrepancies too. One of the parameters related to the development of coronary disease in diabetic patients is the tissular Zn/Cu ratio. Aguilar et al. [32] evaluated the levels of Zn and Cu, and the Zn/Cu ratio in insulin target tissues in diabetic and normoglucemic growing Wistar rats in order to determine the influence of diabetes and the disease evolution period. Diabetes was induced chemically by administration of streptozotocin. The Zn/Cu ratio showed a generalized decrease, except in skeletal muscle. This decrease was dependent on the presence of diabetes mellitus and the duration of the disease ($p < 0.01$).

In our study Zn/Cu ratios were CV: 8.7; CX: 7.9; CR: 7.8; CD: 7.7; DV: 8.1; DX: 8.2; DR:9.5; DD: 9.1. These values were not in accordance with such a decrease, but a significant increase in Zn/Cu ratio after rosemary and Dunaliella extracts treatment in diabetic rats should be pointed out.

Another interesting observation was a significant iron decrease both in control and diabetic rats treated with Dunaliella extracts. Iron depletion by deferoxamine has proved to up-regulate glucose uptake, and increase insulin receptor activity and signaling in hepatocytes *in vitro* and *in vivo* [33]. On the contrary, iron concentration was increased in the group of diabetic animals treated with rosemary extract more than in any other group but this effect did not appear in control animals.

Manganese was deceased in all antioxidant treated control animals as compared to vehicle treated, but in the diabetic group it only decreased when rats received rosemary extract.

Finally magnesium increased both in control and diabetic animals when they were given either rosemary or Dunaliella extracts.

In summary, there is still a long way to go in trying to understand the mechanism under metal activity in diabetes but in the meantime, a simpler, more rapid, accurate and precise method has been developed that will simplify the process.

Metabolic changes characteristic of the type 1 diabetic condition influences trace metal concentrations in liver, but the mechanism is still unclear. Metallothioneines may play a role in the process. Short-term antioxidant treatments in an acute model of diabetic rats have produced different changes depending on the antioxidant or extract that deserve further work. The work described here has focussed mainly on the development of rapid and reliable analytical methodology to facilitate those studies.

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