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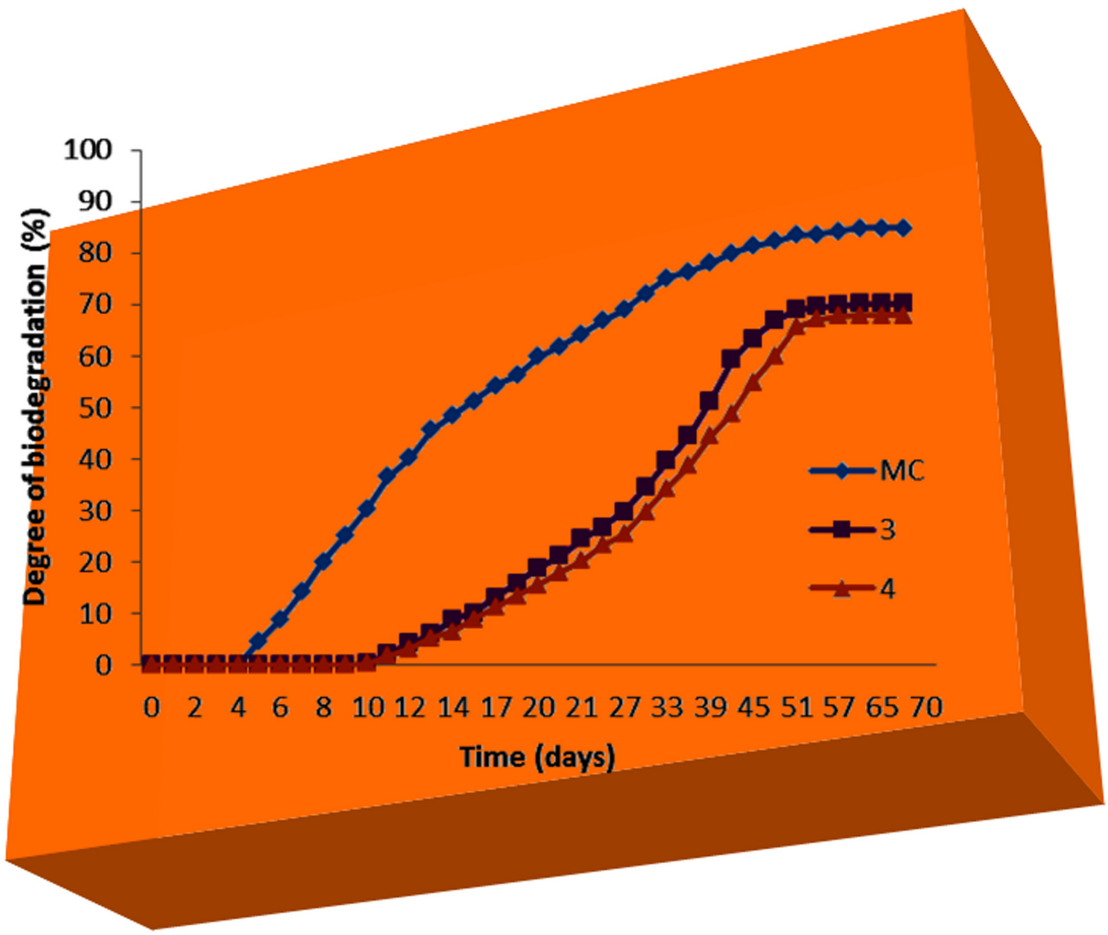


# CHEMIA

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# S T U D I A
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# CHEMIA

## 1

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### CUPRINS – CONTENT – SOMMAIRE – INHALT

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STUDIA UBB CHEMIA, LVII, 1, 2012 (p. 5 - 6)

**Prof. Emil Cordoş PhD** is currently honorific consulting professor at the Babes-Bolyai University, Faculty of Chemistry and Senior Research Fellow at ICIA Cluj. He became a student of the Faculty of Chemistry in 1954 and had graduated in 1959 with a degree of merit, specialty Inorganic Chemistry. In 1960, he obtained, by competition, a preparatory position at the Department of Inorganic and Analytical Chemistry led by Academician Raluca Ripan. In 1961 he was promoted to assistant at the same chair in the discipline of analytical chemistry, led by Professor Candin Liteanu. His university career unfolded, until today, in the same department, by going through the consecrated steps of the academic hierarchy: lecturer in 1968, associate professor in 1976, professor in 1990 and consulting professor in 2005. He defended his PhD in 1969 with a thesis entitled “Contributions to the automation of kinetic methods of analysis”. Professor Cordoş also occupied leadership positions at the Babes-Bolyai University, Faculty of Chemistry: Vice Dean, 1977-1981, Dean, Faculty of Chemistry, 1990-1992 and Head of Analytical Chemistry Chair, 1996-2002. As vice dean of the Faculty of Chemistry has contributed to the establishment of departments of chemical engineering. Professor Cordoş was the first Dean of the Faculty of Chemistry since 1990, in a very tense mandate. In spite of it, he manage to reestablish the traditional departments of the faculty, ensure a beginning of compatibility with top European university curricula and a smooth continuation, without major shocks, of the teaching process. Specialization of Professor Cordoş in Analytical Chemistry is the Instrumental Analysis with emphasis on automated analytical instrumentation and its applications, mainly in spectrometric methods. He made the first steps in this field at the University of Illinois, USA, where he worked for three years as Fulbright fellow and postdoctoral research associate. He understood that good research in this field implies an interdisciplinary approach and proper infrastructure that could not rely only on resources and staff of a Romanian university chair. Therefore, besides the remodeling of the Instrumental Analysis course he formed research teams and collectives within specific projects. In 1990 these collectives merged and, finally, in 1996, they became the Research Institute for Analytical Instrumentation, ICIA, a subsidiary of the National

Institute of Research and Development in Optoelectronics. Those teams and the institute could and did address projects on very broad topics including spectrophotometry systems, from simple devices to complex systems of analysis based on radio frequency generated plasma, sensors based on ceramic semiconductor, robots specialized in analysis and, recently projects related to the environment and alternative fuels. In this institute were formed professionals who become professors, directors of collectives in other institutions in the country or abroad, scientific researchers or business owners in the domain. Professor Cordoş achievements in the domain of instrumental analysis, especially in spectrometric methods, are described in 205 papers, 128 of these being published in ISI indexed journals. He published five books: Electronics for Chemists, Ed. Stiintifica (1978), Atomic Absorption and Fluorescence Spectrometry, Ed. Academiei (1984), Analysis by Atomic Spectrometry, Ed. INOE (1998). Analysis by UV- Vis Molecular Absorption Spectrometry, Ed. INOE (2001), Analytical Atomic Spectrometry with Plasma Sources, Ed. INOE (2007); three chapters in books and a chapter in a megaencyclopedia devoted to analytical chemistry into the third millennium. To these should be added the studies, methods and instrumentation resulted from more than 100 projects and research contracts or grants covered as project director and 10 patents. A special note for the spectrometric systems based on radio frequency generated plasma sources, made before 1990 and continued through a series of projects to date and for the environmental projects in European programs. Under his leadership were presented 21 doctoral thesis and others are under development. Regarding the connection with Studia, Prof. Cordoş, as Dean, was Studia's responsible editor in 1990-92. He published his first papers in Studia in 1962, volume VII. Professor Cordoş is member of many professional societies among that stands: American Chemical Society, Romanian Society of Chemistry, Society for Applied Spectroscopy, International Society of Environmental Epidemiology, EURACHEM Romania (founding member). He was president of the first subsidiary in Cluj-Napoca of the Romanian Society of Chemistry and is president and founder of PROANALITICA XXI.

**Issue Editor,**
**Tiberiu FRENŢIU**

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# VALIDATION OF INDUCTIVELY COUPLED PLASMA ATOMIC EMISSION SPECTROMETRY TECHNIQUE FOR THE DETERMINATION OF TRACE ELEMENTS IN GRANULAR WASTE

**MICHAELA PONTA[a,*], TIBERIU FRENŢIU[a]**

**ABSTRACT.** The study presents the validation protocol for the determination of Cd, Cr, Cu, Pb and Zn by inductively coupled plasma atomic emission spectrometry in leachate of granular waste with size below 4 mm at liquid-to-solid ratio of 2 L/ kg and in aqua regia digest. The assay was found to be linear for a concentration range between limit of quantitation and 25 mg $L^{-1}$ (R=0.999). The limits of detection in leachate allow the determination of elements at concentration levels for the acceptance of non-hazardous wastes, while in aqua regia digest at values ten times lower than the normal levels in soil. Internal repeatability was in the range 0.8-5.4 %, reproducibility between 2.6 – 12 % and recovery 91-109 %, which fully complied with imposed targets in Romanian regulations. The maximum variance of the method was found for Zn (5.65 %), while the maximum relative expanded uncertainty for Cu (21 %).

***Keywords:*** *method validation, trace elements, atomic emission spectrometry, granular waste, leaching test*

## INTRODUCTION

The problem of making rapid and inexpensive determinations in order to evaluate the risk represented by solid wastes is of high concern. Chemical measurements are needed to compare the concentrations of hazardous substances present in solid waste with the limit values imposed by the Romanian legislation complying with that in the European Union. Classification of wastes and their disposal in accordance with the Directive 1999/31/EC (Landfill Directive, Annex II) and Decision 2003/33/EC impose the following types of tests: Level 1. Basic characterization, Level 2. Compliance testing. Level 3. On-site verification.

Romanian legislation establishes criteria and preliminary procedures for waste acceptance in different landfill classes [1]. Certain wastes such as waste glass, concrete, bricks, tiles, ceramics and mixtures thereof free of

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hazardous substances are considered inert and can be landfilled without preliminary testing. All other wastes are subject to testing in order to be categorized as inert/non-hazardous/hazardous. Tests include a range of inorganic determinants in eluate derived from the European standard leaching test ISO EN 12457:2002 and several specific parameters determined on waste it self such as pH, acid neutralization capacity and total organic carbon. Limit values in water leachate for each waste category are given for liquid-to-solid ratios (L/S) of 2 L/kg (2:1), 10 L/kg (10:1) as well as for the percolation test. Indicators refer to the contents of As, some metals (Ba, Cd, total Cr, Cu, Hg, Mo, Ni, Pb, Sb, Se, Zn), anions (chlorides, fluorides, sulphates), phenol index, organic compounds dissolved and total dissolved solids.

Validation is a matter of great importance as it attests the capability of the laboratory to provide reliable results [2,3]. In agreement with Mermet [4], one of the possible research areas in ICP-AES analysis is method validation for intended purposes as the instrumentation is well implemented. The topic related to method validation by ICP-AES is supported by the scarcity of publication in the field [5-7].

The aim of this study was to validate and extend the application of inductively coupled plasma atomic emission spectrometry (ICP-AES) for the characterization of granular wastes from the non-ferrous industry and soil contaminated with heavy metals. The selection of elements (Cd, Cr, Cu, Pb and Zn) took into account their high levels in these materials. Validation refers to analysis of waste leachate at L/S (2/1) and aqua regia digest of granular wastes with size below 4 mm. The validation protocol combines the existing standards elaborated for the determination of elements by ICP-AES in water and soil [8, 9].

## RESULTS AND DISCUSSION

The characteristics considered to be of interest for the procedure proposed for validation were specificity/selectivity, limit of detection, limit of quantification, repeatability, reproducibility, linearity and measurement uncertainty.

**Selectivity/specificity.** These characteristics assessed confidence in measurement in the presence of interference. The reference standard for this proposed procedure [4] reveals possible interferences, such as that of Fe on Cd, Zn, Cu, Cr and interference of Cu and Ni on Zn. The high resolution of the used spectrometer (9 pm) and the low relative intensity of interfering wavelengths reduced the likelihood of interference. Negative controls gave baseline signal for the analyte. One point or two points approaches were used for background correction provided by the software of the ICP spectrometer.

**Limit of detection**. Table 1 presents LODs (3 σ criterion) by ICP-AES for the leaching and aqua regia digestion tests on granular wastes expressed as dry mass based on detection limits in solution and sample preparation protocol.

**Table 1.** Limits of detection (mg $kg^{-1}$) by ICP-AES for the leaching test (L/S 2:1) of granular waste and aqua regia digestion test

| Element | Leaching test | Aqua regia digestion test |
|---|---|---|
| Cd | 0.004 | 0.05 |
| Cr | 0.036 | 1.2 |
| Cu | 0.018 | 1.6 |
| Pb | 0.042 | 0.7 |
| Zn | 0.010 | 1.1 |

LOD was estimated from the calibration function for a signal equal to the net signal of blank and three times its standard deviation. The blank signal included contribution of reagents, microwave digestion system and glass-ware. Standard deviation of blank resulted from the analysis on the same day of 10 independent reagent blank solutions, measured once each.

The performance criteria targeted for the leaching test were the limit values of the selected elements (mg $kg^{-1}$ dry mass) at L/S (2/1) for the acceptance of non-hazardous wastes: Cd - 0.6; Cr- 4; Cu - 25; Pb - 5; Zn – 25 [1]. For the aqua regia digestion test the performance criteria in view were concentrations ten times lower than the normal levels in soil (mg $kg^{-1}$): Cd – 0.1; Cr – 3; Cu – 2; Mn – 90; Pb – 2; Zn – 10 [10]. Data in Table 1 show that performance targets were largely achieved. Quantitation is possible with reasonable accuracy at concentrations 3 times higher than the limits of detection.

**Precision.** The most common assays to evaluate precision are internal repeatability and reproducibility [11]. Results obtained in the internal repeatability assay for the leaching test at L/S (2/1) and the aqua regia digestion test conducted on 6 parallel samples and one blank by a single operator using the same equipment are presented in Table 2. The self-imposed targets meeting provisions in [12,13] were standard deviation of repeatability below 9% and limit of repeatability below 25%.

Table 3 summarizes results from the reproducibility study for the leaching test at L/S (2/1) on 10 replicate waste samples from the non-ferrous industry and in the aqua regia digestion test on 6 parallel samples of certified contaminated soil with heavy metals (CRM 025-050). Protocols were carried out by different operators using the same equipment in different days. Blanks were run in each case. Reproducibility characteristics in Table 3 fully correspond to imposed target of 23 % as maximum standard deviation of repeatability [12,13].

**Table 2**. Results obtained in the repeatability assay by ICP-AES

| Element | Leaching test, L/S (2/1) | | | Aqua regia digestion test | | |
|---|---|---|---|---|---|---|
| | $\overline{A}$ /mg kg$^{-1}$ | $s_r$ / % | r / % | $\overline{c}$ / mg kg$^{-1}$ | $s_r$ / % | r / % |
| Cd | 0.33 | 3.4 | 9.4 | 18.5 | 1.4 | 3.9 |
| Cr | 0.43 | 2.8 | 7.9 | 384 | 3.9 | 10.9 |
| Cu | 22.8 | 5.4 | 14.9 | 592 | 1.1 | 3.0 |
| Pb | 5.9 | 2.3 | 6.5 | 1311 | 0.8 | 2.4 |
| Zn | 111 | 2.7 | 7.6 | 2513 | 4.6 | 12.6 |

$s_r$ - standard deviation of repeatability; r - limit of repeatability ($s_r$x2.8); $\overline{A}$ - average release of constituent in the leaching test; $\overline{c}$ - average concentration in aqua regia digestion test

**Table 3**. Results obtained in the reproducibility assay by ICP-AES

| Element | Leaching test, L/S (2/1) | | | Aqua regia digestion | | | |
|---|---|---|---|---|---|---|---|
| | ($\overline{A}$)/ mg kg$^{-1}$ | $s_R$/ % | R / % | Cert. conc. ±S.D./ mgkg$^{-1}$ | Found conc. ±S.D/ mg kg$^{-1}$ | $s_R$/ % | R / % |
| Cd | 0.39 | 12 | 34 | 369±46.3 | 365±9.3 | 2.6 | 7.2 |
| Cr | 0.47 | 12 | 33 | 441±50.1 | 454±14.9 | 3.3 | 9.2 |
| Cu | 24.43 | 9 | 27 | 7.76±1.68 | 9.18±0.81 | 8.8 | 24.7 |
| Pb | 5.98 | 5 | 15 | 1447±203 | 1523±59 | 4.0 | 10.8 |
| Zn | 112 | 8 | 22 | 51.8±8.29 | 59.1±5.09 | 8.6 | 24.1 |

$s_R$ - standard deviation of reproducibility; R - limit of reproducibility ($s_R$x2.8); $\overline{A}$ - average release of constituent in the leaching test; S.D.- standard deviation

**Recovery.** Comparison of found concentration of elements in CRM 025-050 following aqua regia digestion test with the certified values gave recovery in the range of 97-103 % for Cd and Cr, 91-109 % for Cu and Zn and 96-104 % for Pb. Recovery agreed with provision in [13].

**Linearity of the calibration function and working concentration range.** The target criteria were correlation coefficient of the calibration function (R) above 0.995, homogeneity of dispersion at the limits of the range and variation coefficient of the method (Vox) below 7%. The performance characteristics were estimated according to reference [14].

Linearity of the assay was evaluated from the regression function of calibration using 6 standards, the lowest concentration of which corresponded to the limit of quantitation (3xLOD), while the others were 1; 2; 5; 10; 25 mg L$^{-1}$element.

To check homogeneity of dispersions, the standard deviations ($s_1$) and ($s_2$) were calculated for series of 6 information values for (3xLOD) and 25 mg L$^{-1}$ element respectively, and the ratio ($s_1^2/s_2^2$) was compared with the critical value $F_{5;5;0,99}$=10,97. The values for R, Vox and ($s_1^2/s_2^2$) ratios as

well as the working concentration range for the determination of Cd, Cr, Cu, Pb and Zn by ICP-AES are presented in Table 4. Data reveal R values higher than 0.999 over the considered working concentration range, a random difference between checked variances and a maximum Vox value of 5.65 % (Zn).

**Table 4**. Working concentration ranges, variance ratio at the limits of the range, correlation coefficients (R) and variation coefficients of the method ($V_{ox}$) in the analysis of wastes by ICP-AES

| Element | Concentration range/mg $L^{-1}$ | $s_1^2/s_2^2$ | R | $V_{ox}$ |
|---|---|---|---|---|
| Cd | 0.005 - 25 | 6.53 | 0.9997 | 3.51 |
| Cr | 0.05 - 25 | 6.12 | 0.9996 | 3.95 |
| Cu | 0.03 - 25 | 6.19 | 0.9999 | 1.71 |
| Pb | 0.06 - 25 | 7.35 | 1.0000 | 1.42 |
| Zn | 0.02 - 25 | 6.90 | 0.9993 | 5.65 |

As the target criteria were met, the liquid samples were appropriately diluted before analysis, when necessary, such that the analyte concentrations lie in the linear range.

**Standard uncertainty.** The protocol to estimate uncertainty followed the classical steps of specifying the measurand, identifying the uncertainty sources, quantifying uncertainty components and calculus of combined uncertainty [15]. The potential sources of uncertainty for the leaching test and aqua regia test on granular waste < 4 mm and analysis by ICP-AES are shown in a cause-effect diagram (fishbone diagram) in Fig. 1.

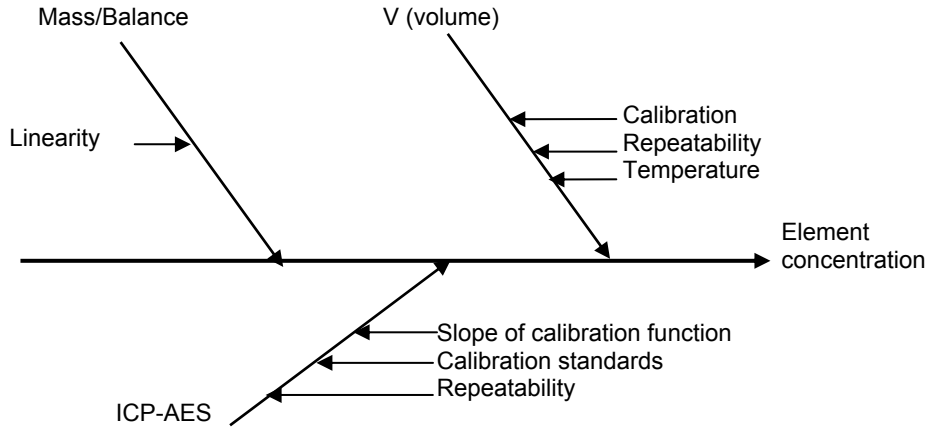


**Figure 1.** Cause and effect diagram showing uncertainty contributions in the measurement of element concentrations by ICP-AES

The combined standard uncertainty ($u_c$) was calculated as square root of squares of individual standard uncertainties, according to the specific protocol. The expanded uncertainty (Uc) resulted by multiplying ($u_c$) by the coverage factor (2) and provided a confidence interval expected to encompass 95% of results attributable to the measurand. The relative expanded uncertainty ($U_{rel}$) was expressed as $U_{rel} = U_c \times 100$. Results from the uncertainty study in the two tests are given in Table 5.

**Table 5.** Uncertainty in the analysis of granular waste leachate at L/S (2/1) and aqua regia digests for 1 mg $kg^{-1}$ contaminant by ICP-AES

| Element | Leaching test | | | Aqua regia digestion test | | |
|---|---|---|---|---|---|---|
| | $u_c$/ mg $kg^{-1}$ | $U_c$/ mg $kg^{-1}$ | $U_{rel}$/ % | $u_c$/ mg $kg^{-1}$ | $U_c$/ mg $kg^{-1}$ | $U_{rel}$/ % |
| Cd | 0.073 | 0.147 | 15 | 0.044 | 0.088 | 9 |
| Cr | 0.070 | 0.140 | 14 | 0.056 | 0.112 | 11 |
| Cu | 0.103 | 0.206 | 21 | 0.073 | 0.146 | 15 |
| Pb | 0.062 | 0.124 | 12 | 0.029 | 0.059 | 6 |
| Zn | 0.076 | 0.151 | 15 | 0.068 | 0.135 | 14 |

$u_c$ – standard uncertainty; $U_c$ –combined standard uncertainty; $U_{rel}$ –relative expanded uncertainty.

The target values for ($U_{rel}$) were 25 % of the measured value for the leaching test and 15 % respectively, for the aqua regia digestion test according to [12,13]. Data in Table 5 demonstrate compliance with aimed limits. The main contribution to uncertainty in the leaching test came from the weighing of waste sample and measurement of leaching agent volume, while in the aqua regia digestion test from the determination by ICP-AES.

## CONCLUSIONS

The study provided a procedure for the determination of Cd, Cr, Cu, Pb and Zn by ICP-AES in leachate of granular waste with particle size below 4 mm at L/S ratio (2:1) and in aqua regia digest. The estimated parameters within the validation protocol (selectivity/specificity, limit of detection, internal repeatability, reproducibility, recovery, linearity of the working concentration range and uncertainty) were found to meet the imposed performance criteria and the procedure was validated for the intended use.

## EXPERIMENTAL SECTION

**Reagents and calibration.** Acids used were of highest purity (analytical grade): 37 % (m/m) HCl and 69 % (m/m) $HNO_3$ (Merck, Germany). Calibration standards were prepared starting from the multielement ICP solution IV 1000 mg $L^{-1}$ (Merck, Germany) by dilution with 5 % (v/v) $HNO_3$. Reagent blanc 1 consisted of 6 mL 37 % HCl + 2 mL 69 % $HNO_3$ to 100 mL water solution, reagent blanc 2 was distilled water, while calibration blanc was 5 % (v/v) $HNO_3$.

Distilled water (< 0.5 mS $m^{-1}$) grade 3 according to EN ISO 3696 was used throughout the experiment. The certified reference material Soil CRM 025-050 (Laramie, New York, USA) was used in the reproducibility assay and recovery assessment.

**Instrumentation.** Instrumentation used for sample preparation included Kern Balances (Kern&Sohns, Germany) of 0.1 g and 0.1 mg accuracy respectively, Memmert oven with adjustable temperature (Memmert GmbH & Co. KG, Germany), Retsch sieving shaker AS200 (Retsch GmbH, Germany) with mesh size of 4 mm, 10 mm and 250 μm; Heidolph Reax 20/8 Overhead Shaker (Heidolph, Germany), Berghof MWS3 + high-pressure microwave digestion system (Berghof, Germany), Sartorius vacuum filtration unit 0.45 μm pore size filters (Sartorius Stedim Biotech SA, Germany) and Hettich Universal 320 centrifuge (Hettich, Germany). The inductively coupled plasma multichannel spectrometer SPECTROCIROS CCD (Spectro Analytical Instruments, Germany) was used for the determination of metals. The operating conditions were 1400 W, 12 L $min^{-1}$ Ar for plasma generation, 1 L $min^{-1}$ Ar for sample nebulization. Working wavelengths (nm) were: Cd 214.438; Cr 283.563; Cu 324.754; Pb 220.351; Zn 213. 856. Argon (5.0 quality) from Gas SRL Cluj-Napoca, Romania, was used.

**Materials.** During sample preparation volumetric flasks, graduated and volumetric pipettes of class A, polyethylene bottles for solid samples and solutions, porcelain mortar, exicator were used.

**Sample pretreatment for the leaching tests.** The test material (waste from non-ferrous industry, contaminated soil) was sieved with a grain size of at least 95% mass below 4 mm. When oversized material exceeded 5 % mass, the entire oversized fraction was crushed. The water content of the samples (MC, %) [16,17] was previously determined and found to be in the range of 0.5 - 2 %. Its determination was necessary in order to correct for the volume of distilled water (L) added for leaching.

**Leaching procedure for L/S 2/1** [12]**.** A test portion of total mass of $M_W$ corresponding to 0.175 ± 0.005 kg dry mass ($M_D$) was weight and put in a 0.5 L volume polyethylene bottle together with a volume of distilled water (L) calculated for L/S=2/1 ratio. The capped bottle was agitated at about 10 rpm for 24 ± 0.5 h at (20 ± 5) $^0$C. After a decantation time of 15 min, the mixture was centrifuged and filtered through 0.45 micron filters. A volume of 0.44 L distilled water as blank was subjected to the same protocol.

**Digestion for subsequent determination of aqua regia soluble portion of elements** [9]. A portion of 0.5 – 1 g dried sample sieved at < 250 μm was digested with 10 mL aqua regia in the high-pressure microwave system following a protocol recommended for such samples and diluted to a final volume of 100 mL. A reagent blank was prepared in the same way.

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# QUANTIFICATION OF CESIUM TRACES IN NATURAL SAMPLES BY FAES TECHNIQUE USING Pt-LOOP AS SAMPLE INTRODUCTION DEVICE INTO THE METHANE-AIR FLAME

**ANDREEA-REBEKA ZSIGMOND[a,*], LADISLAU KÉKEDY-NAGY[b]**

**ABSTRACT.** A rapid and simple FAES technique for the quantification of traces of cesium in natural samples is described. A volume of 3 μL of liquid sample was placed on a Pt-wire, dried, and atomized in the methane-air flame. The flame atomization conditions of cesium were optimized, which are as follows: λ = 852.19 nm, the height of 2 mm over the burner head, gas flow rates of 200 L $h^{-1}$ air and 24 L $h^{-1}$ methane. The effect of Na, K, Mg, Ca on the emission of cesium was studied too. Boric acid, citric acid and acetone were tested, as chemical modifiers. The lowest limit of quantification (6σ) obtained is of 11.9 ± 1.2 pg in the presence of 10 % v/v acetone (P = 0.05). The cesium content of mineralwater and lichen samples has been determined by using the standard addition method. The concentration of cesium varied between 0.016–0.124 mg.$L^{-1}$ in the waters and 0.76–5.36 mg.$L^{-1}$ in the lichens. The results of the two procedures agree within the determination errors.

***Keywords:*** *flame, atomic emission, Pt-loop, cesium, water, lichen*

## INTRODUCTION

Cesium is an alkaline metal; its compounds are in general chemically similar to those of other alkali elements. It resembles most with potassium and rubidium, the chemistry of cesium is more like that of these two elements than that of the lighter alkali ones. Cesium is the rarest of the naturally occurring alkali metals, being widely distributed in the earth's crust at very low concentrations. Granites contain in average 1 ppm cesium, sedimentary rocks 4 ppm, and seawater 0.2 ppm, respectively [1]. The presence of cesium in natural waters as a trace element provides main hydrogeological informations. Lichen is a widely used biomonitor for air pollution, which can easily accumulate different metals.

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Analysis of trace levels is carried out by the most appropriate technique, which may include atomic emission and absorption spectrometry, X-ray fluorescence spectrometry etc. [2–5]. Cesium was quantified in natural waters by FAES technique using methane-air flame, the limit of detection being of 3 $\mu g.L^{-1}$[6]. For low levels of cesium in medical research, the proton induced X-ray emission technique has been developed [7]. Cesium has also been determined in porcine brain by instrumental neutron activation analysis [8]. The most sensitive, but most expensive technique for the cesium quantification is the ETV-ICP-MS, which has limit of detection of tenth of $ng.L^{-1}$ [9].

Cesium ionizes significantly in hot media, as in acetylene flames or in ICP plasma, phenomenon which can be confined by use of an ionization suppressor. So, the cooler flames as, methane-air (M-A), PB-air etc. are advantageous for cesium assay. Using the electron avalanche amplification of the laser enhanced two-step ionization of Cs in hydrogen and propane flames proved to be very sensitive [10]. The sensitivity of the determinations could be further increased by sample atomization from an electrically heated platinum loop, investigated first for three decades by Berndt and Messerschmidt [11]. The main feature of this technique, by comparing with the direct nebulization, is the lower detection limit (about one order of magnitude) and considerably less sample volume necessity for the analysis (few microliters only). The use of low-temperature flame, including the M–A flame, in combination with platinum wire atomization is not reported, the cesium content was not quantified in this way.

The aim of this study is to elaborate and evaluate a sensitive micro-analytical technique for the rapid and simple quantification of traces of cesium from natural mineral water springs and digested lichen samples.

## RESULTS AND DISCUSSION

### Optimization of the operating conditions

First the observation height over the burner head (h) and the flame composition has been optimized. For this purpose 3 µL of 1 $mg.L^{-1}$ Cs solution was atomized into the M-A flame, the burner head being moved downward in 1 mm steps in the 2 – 6 mm height domain. The results show that the intensity of the analytical signal decreases uniformly versus the height with the simultaneous increase of the standard deviations. The maximal value of the S/N was registered at h = 2 mm, so this value was considered as optimal.

Next the influence of the flame composition on the Cs analytical signal was studied, by using 6 different air- methane flow rates ($L.h^{-1}$): 200-22, 200-24, 300-34, 300-36, 400-44 and 400-46. In all cases stable flame was obtained. Five parallel measurements were made, the mean, the standard deviation, the S/N was calculated. The results are presented in the Fig.1.

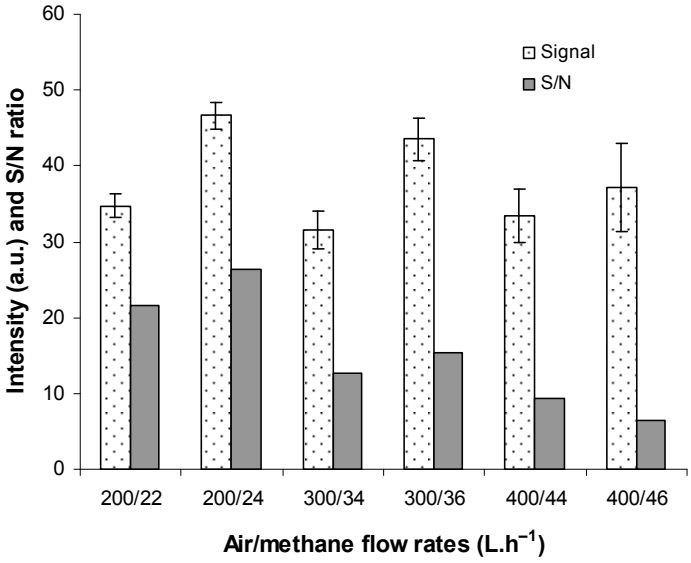


**Figure 1.** Influence of the air/methane flow rates on the analytical signal and the S/N ratio (Cs 1 mg.$L^{-1}$ )

The increase of the air/methane flow rates lead to the decrease and the reproducibility of the analytical signal. The signal intensity is always higher in reducing flame, despite of the air flow rate. The optimum flow rates at which the highest S/N was recorded (26.5) are of 200/24 L.$h^{-1}$, considered as optimal for cesium assay.

In the following step the bandpass of the monochromator was optimized. The spectral bandpass (determined by its width of the slit) as well as the reproducibility of the determinations are simultaneously influenced, but not in the same extent. Therefore the optimal slit width for which the S/N ratio is the highest can be determined. The flame and instrumental parameters used were the optimal ones, determined earlier. The influence of the slit on signal intensity and S/N ratio was studied in the 100-1000 μm domain, in steps of 100 μm. The influence of the slit width (d) of the monochromator on the 1 mg.$L^{-1}$ cesium emission and the background signal is represented in the Fig. 2. The emission signal increases steeply (over 9 times) up to 500 μm then varies insignificantly. The background signal up to 500 μm slit width is close to zero, then increases sharply. The standard deviation of the means is homogeneous up to a width of 500 μm and then they differ significantly. The highest value for the S/N ratio was found at this value of the width of 500 μm, considered optimum.

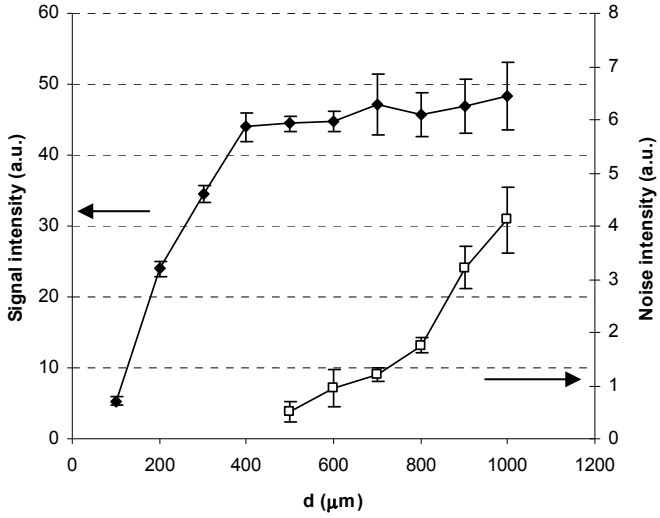


**Figure 2.** Influence of the slit width (d) of the monochromator on the 1 mg.L$^{-1}$ cesium emission and the background signal

## Study the influence of chemical modifiers

Three chemical modifiers have been tested: boric acid, citric acid and acetone. Boric acid is known as an oxide generating agent from inorganic chlorides [12]. At 600 °C it reacts with chlorides and evaporates as volatile $BCl_3$. The dissociation energy of the $Cs_2O$ is considerably lower (297 kJ.mol$^{-1}$), than of the CsCl (439 kJ.mol$^{-1}$). It is expected, that the generation of Cs atoms in the flame should be facilitate by the cesium-oxide rather than by the cesium-chloride. The influence of the citric acid is not well known, although its benefic effects have already been proved [13]. The acetone is known to form complexes with alkali metals in aqueous solutions [14, 15], by yielding a low boiling point organic compound. The concentration of Cs standard in all cases was of 0.5 mg.L$^{-1}$ to which boric and citric acid was added in final concentrations of 5, 10, 20, 50, 100, 200, 400, 800 mg.L$^{-1}$ and 5, 10, 20, 50, 100, 200, 400, 500 mg.L$^{-1}$, respectively. Acetone was added in concentrations of 5, 10, 15 and 20 % (v/v). In all cases five replicate measurements were made, the mean peak height and peak area values have been calculated (Table 1). It was found; that all the chemical modifiers tested enhance the analytical signal, the most efficient proved to be the citric acid in a concentration of 100 mg.L$^{-1}$. The evaporation time of the compound from the platinum surface has been recorded too (Table 1). The cesium compounds evaporate in the shortest time when boric or citric acid is present. Presence of acetone shortens the evaporation time, but less outstanding.

**Table 1.** Influence of chemical modifiers on the emission signal of cesium

| Solution | Peak height (a.u.) | Peak area (a.u.) | Relative signal enhancement | | Evaporation time (s) |
|---|---|---|---|---|---|
| | | | Peak height | Peak area | |
| 0.5 mg.L$^{-1}$ Cs | 12.47 ± 1.07 | 711.6 ± 61 | 1.0 | 1.0 | 2.11 ± 0.05 |
| 0.5 mg.L$^{-1}$ Cs + 10 % (v/v) acetone | 20.80 ± 0.46 | 1029 ± 72 | 1.7 | 1.4 | 1.96 ± 0.04 |
| 0.5 mg.L$^{-1}$ Cs + 100 mg.L$^{-1}$ boric acid | 24.17 ± 0.60 | 851.8 ± 65 | 1.9 | 1.2 | 1.58 ± 0.02 |
| 0.5 mg.L$^{-1}$ Cs + 100 mg.L$^{-1}$ citric acid | 48.93 ± 2.80 | 1709 ± 74 | 3.9 | 2.4 | 1.63 ± 0.03 |

In order to understand better the influence of the chemical modifiers, the change of the analytical signal versus time has been recorded too (Fig. 3). The shape of curve of the analytical signal for cesium alone shows two peaks, corresponding to different cesium compounds with different boiling points as well as dissociation energies. The first, the more volatile one, should be the oxide or hydroxide and the second one, with higher boiling point, should be the chloride (Fig. 3.*a*.). In the presence of 100 mg.L$^{-1}$ boric acid the peak height increases, there appears a single peak, which is likely corresponding to the more volatile and easier dissociating cesium-oxide (Fig. 3.*b*.).

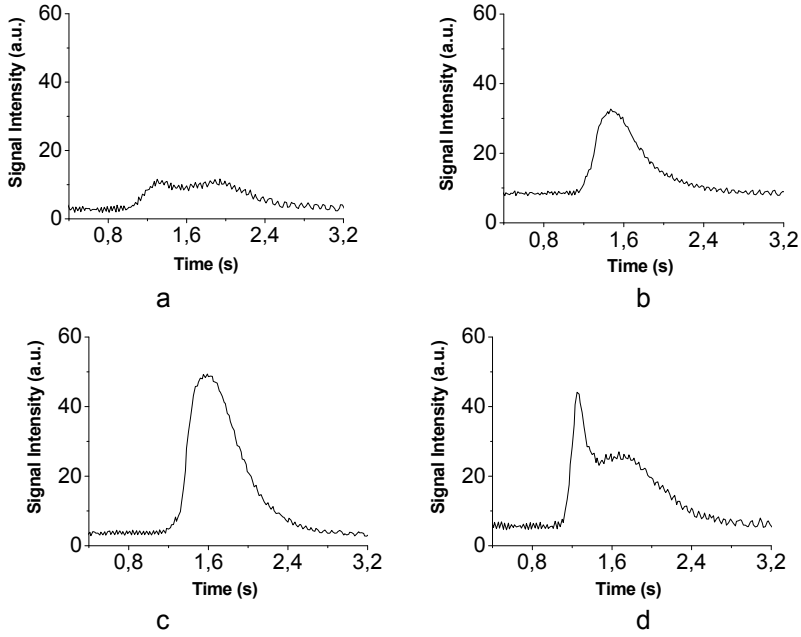


**Figure 3.** Variation of the emission signal of 0.5 mg.L$^{-1}$ Cs versus time: alone (*a*) and in the presence of 100 mg.L$^{-1}$ boric acid (*b*); 100 mg.L$^{-1}$ citric acid (*c*); 10 % (v/v) acetone (*d*)

In the presence of 100 mg.L$^{-1}$ citric acid the form of the analytical signal is similar to that obtained earlier, but the peak is higher presumably due to the complete dissociated Cs compound (Fig. 3.*c.*). In the presence of acetone the shape of the signal resembles the most with the signal of the cesium, with a more pronounced peak of the more volatile and easier dissociated compound (Fig. 3.*d.*). The higher signal peak and larger peak area suggests that acetone enhances the evaporation of cesium, and it generates the formation of the more volatile and easier dissociated cesium-oxide.

## Study of the chemical interferences

The influence of Na, K, Mg and Ca on the emission signal of Cs has been studied. The metals have been added to the 0.5 mg.L$^{-1}$ Cs solution in final concentrations of 5, 10, 20, 50, 100 and 200 mg.L$^{-1}$. Each element enhances the emission of Cs in low concentrations; magnesium is proved to be most efficient (it raises the peak height 4.8 times and the peak area value 3.6 times). Over 100 mg.L$^{-1}$ of the interferent the analytical signal decreases in all cases due to the evaporation inhibition in solid phase.

The spectral interference which could have caused by the presence of K and Ca was tested too, be measuring the emission signal in the vicinity of the Cs line at λ = 849.0 nm. No emission signal was recorded, suggesting the absence of spectral interference.

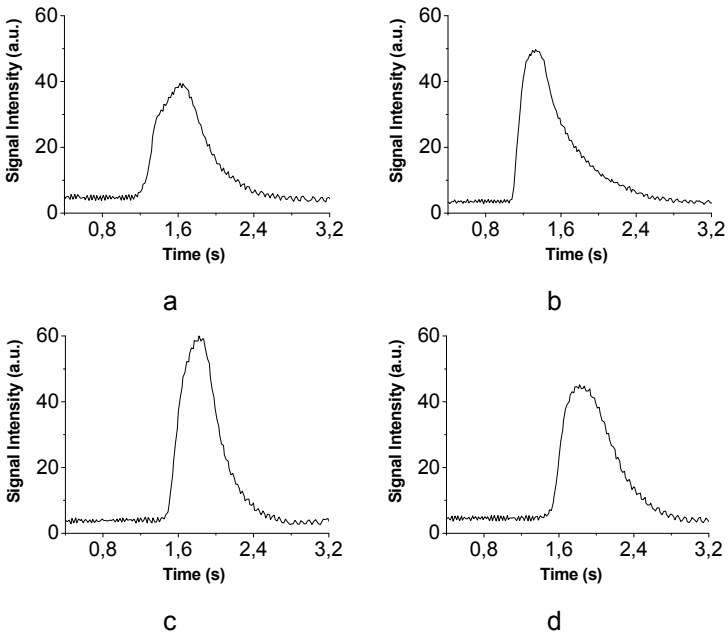


**Figure 4.** Variation of the emission signal of 0.5 mg.L$^{-1}$ Cs in the presence of: 50 mg.L$^{-1}$ Na (*a*); 10 mg.L$^{-1}$ K (*b*); 50 mg.L$^{-1}$ Mg (*c*); 100 mg.L$^{-1}$ Ca (*d*)

The change of the analytical signal vs. time in the presence of Na, K, Mg and Ca has been also recorded (Fig. 4.). In the presence of K, Mg and Ca the second peak disappears, or it is negligibly small comparing with the first one. The peak height increases considerably in the presence of K and Mg. Potassium acts as an ionization suppressor, magnesium-chloride is very likely to form a double salt with the analyte: cesium-carnallite during the heating process. The carnallite exhibits an incongruent melting below 200 °C [16]. In the presence of Na two overlapping peaks can be detected, the second peak being more pronounced.

## Calibration results and figures of merit

The analytical figure of merit of the method was evaluated in the concentration range of 0.01-1 mg.L$^{-1}$ at the optimized instrumental and atomization parameters. It was evaluated the limit of detection (LOD), precision and dynamic linear range of the method. In Table 2 there are listed the calibration data and limits of detection obtained in the presence of different matrixes.

**Table 2.** Calibration data and detection limits

| Calibration conditions | Calculations | Sensitivity of the calibration | R factor | LOD (μg L$^{-1}$) | LOD (pg) |
|---|---|---|---|---|---|
| CsCl | Peak height | 39.37 | 0.9998 | 32 ± 14 | 67 ± 42 |
| | Peak area | – | – | – | – |
| CsCl + 10% acetone | Peak height | 307.6 | 0.9999 | 5 ± 2 | 14 ± 6 |
| | Peak area | 222.07 | 1.0000 | 4.0 ± 0.4 | 11.9 ± 1.2 |
| CsCl + 100 mg.L$^{-1}$ citric acid | Peak height | 83.86 | 0.9995 | 10 ± 20 | 29 ± 47 |
| | Peak area | 348.2 | 0.9995 | 2 ± 28 | 7 ± 83 |
| CsCl + 100 mg.L$^{-1}$ boric acid | Peak height | 44.81 | 0.9987 | 19 ± 65 | 56 ± 195 |
| | Peak area | 198.71 | 0.9995 | 4 ± 41 | 13 ± 123 |
| CsCl + 50 mg.L$^{-1}$ Mg | Peak height | 62.05 | 0.9998 | 15 ± 19 | 44 ± 57 |
| | Peak area | 266.95 | 0.9999 | 3 ± 16 | 10 ± 48 |
| CsCl + 10 mg.L$^{-1}$ K | Peak height* | 97.11 | 1.0000 | 11 ± 8 | 33 ± 24 |
| | Peak area* | 290.08 | 0.9997 | 4 ± 19 | 11 ± 57 |

*calibration data obtained only in the 0.1–1.0 mg.L$^{-1}$ domain

The results show that both the peak and peak area values vary proportional in the whole calibration range with the cesium concentration. Unfortunately calibration in the concentration range of 0.01–0.1 could not be performed due to the significant Cs content of the K standard solution. All

matrixes enhance the emission signal, i.e. the sensitivity, in average about 2 times. The instability of the analytical signal increases disproportionately, so due to the high dispersion values most of the calculated LOD values are meaningless. The best chemical modifier proved to be acetone in 10 % (v/v) by increasing the calibration sensitivity nearly 8 times without the significant increase of the standard deviation (the highest S/N value recorded among all). However, in the followings, the determinations were carried out in the presence of acetone. A limit of detection calculated for this case (6σ at P = 0.05) based on peak area values is the lowest of all, being of 4.0 ± 0.4 $\mu g.L^{-1}$ Cs, 11.9 ± 1.2 pg Cs respectively.

## Quantification of cesium

Cesium has been quantified in optimal instrumental conditions, established earlier. Acetone has been added to the samples in final concentration of 10 % (v/v). Standard addition method has been used; the cesium standard has been added to the sample solutions in three steps. The final concentrations of the standards were of 0.02, 0.04 and 0.06 $mg.L^{-1}$ in the case of water samples, and of 0.05, 0.1 and 0.15 $mg.L^{-1}$ in the case of lichen samples. The results are shown in Table 3 and Table 4.

**Table 3.** The Cs content ($\mu g.L^{-1}$) of the mineral waters

| Water sample | Peak height | Peak area |
|---|---|---|
| 1 | 84 ± 37 | 82 ± 44 |
| 2 | 33 ± 12 | 27 ± 20 |
| 3 | 16 ± 4 | 26 ± 3 |
| 4 | 31 ± 4 | 35 ± 15 |
| 5 | 180 ± 80 | 200 ± 40 |
| 6 | 63 ± 9 | 71 ± 27 |
| 7 | 250 ± 20 | 230 ± 20 |
| 8 | 20 ± 8 | 18 ± 1 |
| 9 | 290 ± 10 | 270 ± 90 |
| 10 | 124 ± 40 | 132 ± 13 |
| 11 | 41 ± 1 | 41 ± 1 |
| 12 | 27 ± 1 | 30 ± 6 |
| 13 | 25 ± 2 | 25 ± 4 |
| 14 | 27 ± 30 | 24 ± 16 |

**Table 4.** The Cs content ($\mu g.g^{-1}$) of the lichens

| Lichen sample | Peak height | Peak area |
|---|---|---|
| 1 | 1.28 ± 0.32 | 1.44 ± 0.16 |
| 2 | 3.08 ± 1.40 | 3.64 ± 0.44 |
| 4 | 0.76 ± 0.32 | 0.96 ± 0.04 |
| 4 | 0.96 ± 0.24 | 1.28 ± 0.24 |
| 5 | 1.40 ± 0.28 | 1.52 ± 0.28 |
| 6 | 1.12 ± 0.44 | 1.44 ± 0.44 |
| 7 | 5.36 ± 0.80 | 6.2 ± 0.04 |
| 8 | 0.88 ± 0.24 | 0.84 ± 0.04 |

## CONCLUSIONS

The FAES method using a Pt-loop sample introducing device proved to be efficient, sensitive for the quantification of traces of cesium in spring waters and lichens. The calibration curve is linear in the concentration range of 0.01–1.0 mg/L in the presence of 10 % (v/v) acetone. The LOD obtained is of 11.9 ± 1.2 pg. Cs also in the presence of 10 % (v/v) acetone (P = 0.05). Acetone, boric- and citric acids are proved to be efficient chemical modifiers by enhancing the analytical signal 1,7-3,4 times. Na, K, Mg and Ca enhance the analytical signal too, the most efficient being K and Mg. For the assay of Cs in natural samples the standard addition method was applied, the peak height and peak area data were calculated. The results between the two methods agree within the error of determination.

## EXPERIMENTAL SECTION

### Chemicals

Stock solutions of Cs, Na, K, Ca, Mg, boric acid and citric acid were of 1000 $mg.L^{-1}$, prepared from CsCl (Merck, Darmstadt, Germany), NaCl, KCl (Reactivul, Bucureşti, Romania), $CaCO_3$, Mg (Specpure, Johnson Matthey Chemicals Limited, England), $H_3BO_3$, $C_6H_8O_7$ (Reactivul, Bucureşti, Romania), respectively. As matrix modifier acetone (Merck, Darmstadt, Germany) was used. The calibration solutions were obtained by diluting a given volume of stock solution with double distilled water. The diluted solutions were prepared just before measurements.

## Sampling and sample handling

The mineral spring waters originating from Harghita County (Romania) were collected during the 2010 summer period in accordance with EPA prescriptions: collected in 500 mL PET bottles, conserved on the spot with 1 mL of conc. $HNO_3$ and kept at 4 °C. The lichen samples have been collected from the Hasmas Mountains. The samples have been dried in oven at 105 ºC and meshed; desegregated by acid digestion at atmospheric pressure using 5 mL concentrated $HNO_3$ and 5 mL $H_2O_2$. The solutions have been diluted with double distilled water to final volume of 50 mL.

## Instrumentation

The measurements were carried out at the atomic line of λ = 852.19 nm using a single-beam HEATH-701 (Heath Co., Benton Harbor, MI, USA) spectrometer equipped with a HEATH EU-700 scanning monochromator, a HEATH EU-700-30 type photomultiplier module and a M12FC51 (NARVA, Germany) photomultiplier (–1000 V). The photomultiplier signal was fed via a homemade I/U converter and a data acquisition card (Decision–Computer International Co. Ltd., USA) into Pentium II PC (120 MHz, 32 Mb RAM) computer. The data were processed with a home written Q-basic software program language using the boxcar averaging method. The fuel gas was the 99% purity methane from pipes; the oxidant was compressed air. The original slot-type burner (for $C_2H_2$-air flame) was replaced with a similar, Mecker-type, developed for atomic absorption/emission measurements in the methane-air flame [17]. The burner was operated at three different airflow rates of 200, 300, 400 L $h^{-1}$ and at corresponding methane rates of 24, 26; 34, 36; 44 and 46 L $h^{-1}$, respectively. In all cases stable premixed flame was obtained. The atomizer, consisted of an Φ = 0.08 mm-diameter 50-mm-long Pt-wire with a 3-mm-diameter loop in the middle, was described in details elsewhere [18,19]. The solutions were injected onto the platinum surface using a 10 µL volume 701N type syringe for gas chromatography (Hamilton Bonaduz AG, Switzerland).

## Procedure

Aliquots of 3 µL solution were injected onto the platinum wire. The sample was dried by electrical heating of the wire to 120 °C for about 40 s and then it was introduced into the flame by manual rotation of the Teflon head. During atomization, electric heating was maintained. The change of the emission signal versus time until it reaches the blank signal level was registered. Five replicate determinations were made in each case, i.e., the mean, the standard deviation, and S/N were calculated. The homogeneity of the means was tested by the F test at a significance level of 0.05. The width of the slit of the monochromator was 0.500 mm (spectral band pass of 0.05 nm). The peak height values and the area under the peak have been considered, using the OriginLab Corporation (Northampton, MA 01060, USA) software package (version 7.0220).

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# STUDY OF RELATIONSHIPS BETWEEN THE METALS CONTENT IN NATIVE VEGETATION AND SOIL USING MULTIVARIATE ANALYSIS

**MARIN ŞENILĂ[a,*], ERIKA LEVEI[a], LĂCRIMIOARA ŞENILĂ[a], OANA CADAR[a], MIRELA MICLEAN[a]**

**ABSTRACT.** The aim of the present study was to assess the metals concentrations in native vegetation from Baia Mare city and to evaluate the relationships between the metals contents in vegetation and the soil chemical properties using multivariate statistical techniques. The metals concentrations in vegetation (mg $kg^{-1}$ dry weight) in the studied areas ranged between: 0.35 – 18.3 for Cu, 2.30 – 120 for Zn, 0.05 – 2.58 for Cd, 0.02 – 15.0 for Pb, 0.21 – 4.09 for Ni, 0.08 – 2.03 for Cr, 1.65 – 389 for Mn, 5.20 – 278 for Fe and 12.0 – 398 for Al. The average content of Cd in vegetation from Ferneziu district exceeded the maximum level (1.0 mg $kg^{-1}$) allowed in animal feed set by the European Directive 2002/32/EC. Higher concentrations of Pb, Zn, Cr, Mn and Cd, were found in vegetation collected from the industrial part of the city, in the vicinity of smelting industrial units presently closed or partially closed, while the concentrations of Ni, Fe and Al were slowly higher in vegetation from the area considered unaffected by mining activities. By applying multivariate analysis it was found that soil pH is negatively correlated with all metals in vegetation and plays an important role in the soil-plant transfer. Generally poor correlations were found between pseudo-total metal contents in soil and metals content in vegetation.

***Keywords:*** *native vegetation, soil pollution, heavy metals, Baia Mare, multivariate analysis*

## INTRODUCTION

One of the main negative effects of mining related activities is the environment contamination with metals. Due to their non-biodegradability and their long biological half-lives, metals persist in soil for many years even after pollution ceases [1]. In addition, high concentrations of essential elements such as Mn, Fe, Cu and Zn along with the non-essential elements such as Cd, Cr, Pb, Al are hazardous for the ecosystems and human health [2, 3].

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Metals contents in vegetation reflect, usually, their concentrations in soil, water and in ambient air [4-8]. Even if vegetation has the capacity to select or limit the uptake of toxic elements, these processes are influenced by the geochemistry of growth media. Consequently, concentrations of metals in vegetation are often correlated with the abundance of these elements in soils [9], despite the fact that metals from soil can be adsorbed or retained by carbonates, organic matter, iron-magnesium oxides, primary or secondary minerals [10].

The city of Baia Mare, NW Romania, was the subject of many studies [11-15] regarding the soil pollution with metals caused by the non-ferrous mining activities developed in this area since ancient times. However, no data was found in the literature regarding the metals content in native vegetation from different parts of the city. The European Directive 2002/32/EC [16] established the maximum concentrations of toxic metals in vegetation used as animal feed in order to avoid its transfer and further accumulation in the higher trophic levels of food chain. The aims of this study were: (1) to evaluate the metals concentrations in native vegetation in the urban and peri-urban areas of Baia Mare, Romania; (2) to study the relationships between metal contents (Fe, Al, Cr, Mn, Cu, Zn, Ni, Pb, Cd) in vegetation and soil properties (pH, Total Organic Carbon (TOC)) using multivariate statistical techniques.

## RESULTS AND DISCUSSIONS

The city of Baia Mare (Fig. 1) is located in the northwest Romania, at an altitude of 228 m above the sea level. The territory surface together with the peri-urban area is 235.7 $km^2$, of which 31.7 $km^2$ agricultural fields [17]. As a consequence of the non-ferrous mining activities developed in this area, Baia Mare was found to be one of the most polluted industrial centres with metals.

A total of 29 sampling points were randomly chosen from Ferneziu (A1), Săsar (A2), Baia Mare center (A3), and Dura (A4) districts. Surface soil samples (0-20 cm) and native vegetation (*Agrostis, Agropirum, Trifolium repens, Urtica dioica*) grown on these soils were sampled from each selected point. The locations of sampling points are presented in Figure 1.

### Soil characteristics

The pH values were generally slightly acidic to neutral in the all studied areas, while the average TOC values were generally at higher levels in Ferneziu and Sasar than in Baia Mare center and Dura.

The *aqua regia* extractible metals (Fe, Al, Cr, Mn, Cu, Zn, Ni, Pb, Cd) concentrations, expressed in mg $kg^{-1}$ dry weight, in soils sampled from the four studied areas from Baia Mare are presented in Table 1.

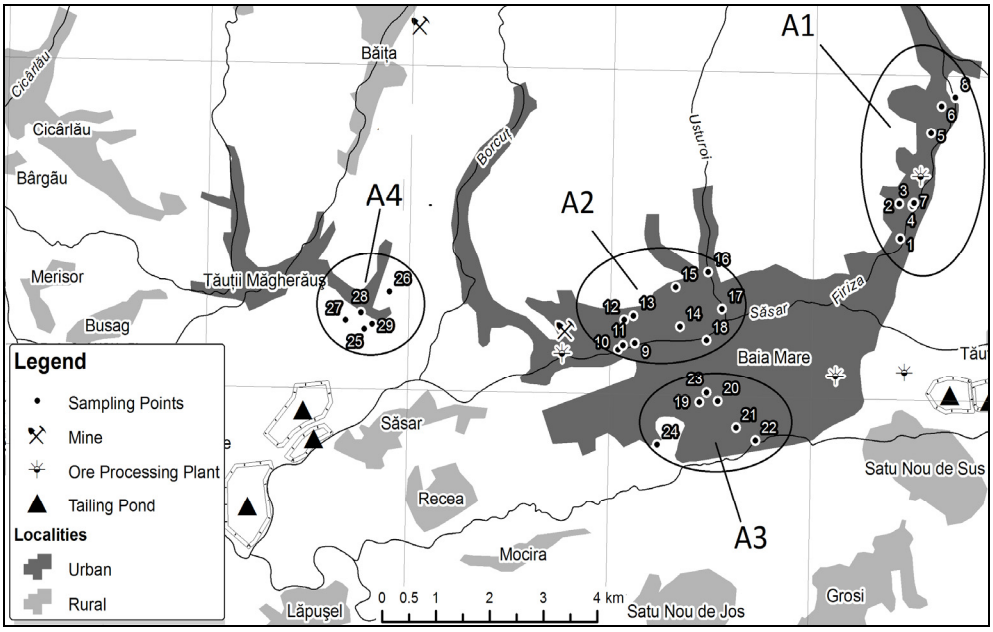


**Figure 1.** Sampling points and the main pollution sources from the four districts of Baia Mare: Ferneziu (A1), Săsar (A2), Baia Mare center (A3) and Dura (A4)

High concentrations of Pb, Cd, Cu and Zn were measured in soils sampled in the vicinity of Pb smelting plant (A1), with values that exceed the corresponding alert or intervention levels established by Romanian legislation [18]. The average concentration of Pb from this area exceeded of more than 30 times the intervention level. In area A2, the average concentrations of Cu and Pb exceeded 2.5 and 7 times respectively the intervention levels. The average concentration of Zn exceeded the alert level but was below the intervention level. The average concentrations of Cd and Zn in areas A3 and A4 were below the alert levels, whereas the average concentrations of Pb and Cu exceeded the corresponding alert levels. The concentrations of Ni and Cr did not exceed the corresponding alert or intervention levels. The concentration of Mn exceed the alert level only in three sampling points from A1, while for Fe and Al there are not established maximum admitted levels for soil.

The pollution with heavy metals as Pb, Cd, Cu and Zn are in accordance with the results obtained in previous studies conducted in Baia Mare area [19]. Also, in a previous study, it was showed that the concentrations of As exceeded the maximum admitted levels in areas from the industrial part of the Baia Mare [20].

**Table 1.** *Aqua regia* extractible metals concentrations (mg $kg^{-1}$) in soils sampled from Baia Mare urban area

| *Sampling point* | *Cu* | *Zn* | *Cd* | *Pb* | *Ni* | *Cr* | *Mn* | *Fe* | *Al* |
|---|---|---|---|---|---|---|---|---|---|
| **A1** | | | | | | | | | |
| 1 | 823 | 1530 | 35.1 | 5520 | 27.6 | 47.9 | 2540 | 46200 | 33900 |
| 2 | 620 | 1570 | 56.5 | 5150 | 12.4 | 21.5 | 1100 | 55600 | 36600 |
| 3 | 295 | 1160 | 20.2 | 1860 | 8.50 | 19.6 | 652 | 41100 | 35100 |
| 4 | 15.3 | 247 | 0.50 | 105 | 1.20 | 0.75 | 14.2 | 11100 | 17200 |
| 5 | 12.9 | 105 | 0.53 | 154 | 1.55 | 0.70 | 3.70 | 11200 | 17400 |
| 6 | 10.0 | 147 | 0.49 | 166 | 1.20 | 0.95 | 14.7 | 9800 | 12800 |
| 7 | 1740 | 1180 | 30.2 | 5390 | 28.8 | 50.1 | 1800 | 9340 | 13200 |
| 8 | 660 | 426 | 17.9 | 3540 | 21.6 | 32.1 | 1620 | 25700 | 21100 |
| **A2** | | | | | | | | | |
| 9 | 137 | 431 | 1.45 | 916 | 0.60 | 7.10 | 1000 | 32000 | 32700 |
| 10 | 269 | 681 | 3.25 | 1330 | 5.05 | 15.8 | 1040 | 35700 | 35600 |
| 11 | 107 | 336 | 1.30 | 333 | 6.75 | 15.2 | 1350 | 31800 | 34200 |
| 12 | 105 | 247 | 1.15 | 230 | 7.45 | 17.9 | 748 | 29400 | 35500 |
| 13 | 59 | 271 | 0.40 | 125 | 8.25 | 17.4 | 509 | 23100 | 23500 |
| 14 | 1660 | 1200 | 7.45 | 778 | 12.4 | 14.8 | 1290 | 45300 | 19200 |
| 15 | 168 | 628 | 0.90 | 469 | 2.85 | 9.05 | 843 | 22800 | 20500 |
| 16 | 652 | 512 | 2.12 | 1130 | 2.66 | 8.65 | 584 | 21500 | 17600 |
| 17 | 73.5 | 241 | 1.00 | 326 | 3.20 | 7.25 | 730 | 36500 | 37700 |
| 18 | 189 | 428 | 1.50 | 532 | 3.80 | 17.4 | 1150 | 31800 | 28700 |
| 19 | 140 | 148 | 0.30 | 90.0 | 8.25 | 11.3 | 361 | 15000 | 11600 |
| 20 | 206 | 335 | 1.00 | 482 | 7.05 | 50.6 | 281 | 20300 | 30400 |
| **A3** | | | | | | | | | |
| 21 | 309 | 428 | 0.90 | 532 | 10.2 | 39.8 | 511 | 25200 | 28800 |
| 22 | 214 | 239 | 0.40 | 244 | 11.9 | 29.8 | 576 | 29900 | 37680 |
| 23 | 161 | 443 | 0.45 | 315 | 7.35 | 8.30 | 855 | 23200 | 29000 |
| 24 | 73.3 | 192 | 0.10 | 129 | 15.4 | 39.2 | 749 | 31700 | 39200 |
| 25 | 102 | 178 | 0.15 | 147 | 11.7 | 20.0 | 831 | 18100 | 20800 |
| **A4** | | | | | | | | | |
| 26 | 50.1 | 69.9 | 0.05 | 34.0 | 12.1 | 28.4 | 441 | 28200 | 25600 |
| 27 | 47.8 | 80.8 | 0.05 | 61.2 | 7.75 | 25.3 | 774 | 26500 | 24200 |
| 28 | 24.4 | 68.1 | 0.05 | 38.1 | 9.85 | 31.0 | 545 | 29900 | 28600 |
| 29 | 19.8 | 61.1 | 0.05 | 38.7 | 9.50 | 30.2 | 450 | 28100 | 28600 |
| **AL*** | 100 | 300 | 3 | 50 | 75 | 100 | 1500 | - | - |
| **IL**** | 200 | 600 | 5 | 100 | 150 | 300 | 2500 | - | - |

*Alert level for soil from sensitive areas, according to Romanian legislation [18]
**Intervention level for soil from sensitive areas [18]

## Concentrations of metals in native vegetation

The metals (Fe, Al, Cr, Mn, Cu, Zn, Ni, Pb, Cd) concentrations, expressed in mg $kg^{-1}$ dry weight, in native vegetation (*Agrostis, Agropirum, Trifolium repens, Urtica dioica*) sampled from Baia Mare urban area are presented in Table 2.

**Table 2.** Metals concentrations (mg $kg^{-1}$) in native vegetation sampled from Baia Mare urban area

| *Sampling point* | *Cu* | *Zn* | *Cd* | *Pb* | *Ni* | *Cr* | *Mn* | *Fe* | *Al* |
|---|---|---|---|---|---|---|---|---|---|
| **A1** | | | | | | | | | |
| 1 | 5.49 | 47.1 | 0.89 | 6.52 | 0.42 | 0.55 | 5.65 | 79.1 | 22.4 |
| 2 | 18.3 | 59.1 | 1.81 | 15.0 | 3.22 | 2.54 | 10.5 | 89.0 | 20.7 |
| 3 | 0.75 | 120 | 1.22 | 6.42 | 0.41 | 1.85 | 7.65 | 18.6 | 19.1 |
| 4 | 0.45 | 75.8 | 0.58 | 3.93 | 0.22 | 0.17 | 2.80 | 7.12 | 28.9 |
| 5 | 0.35 | 90.5 | 1.03 | 1.04 | 0.23 | 0.19 | 7.95 | 23.9 | 39.5 |
| 6 | 1.12 | 65.5 | 1.12 | 1.12 | 0.21 | 0.15 | 5.50 | 26.6 | 49.1 |
| 7 | 3.50 | 70.2 | 1.11 | 1.09 | 0.24 | 0.19 | 6.31 | 29.9 | 57.0 |
| 8 | 2.30 | 30.1 | 1.12 | 2.96 | 0.81 | 0.25 | 11.2 | 68.3 | 65.1 |
| **A2** | | | | | | | | | |
| 9 | 10.0 | 87.9 | 0.25 | 1.45 | 1.11 | 1.3 | 26.6 | 86.7 | 50.2 |
| 10 | 16.1 | 70.5 | 0.44 | 2.11 | 1.81 | 2.63 | 22.4 | 97.0 | 63.1 |
| 11 | 6.71 | 44.4 | 0.25 | 1.14 | 1.62 | 10.2 | 60.0 | 101 | 74.0 |
| 12 | 4.93 | 19.5 | 0.10 | 0.05 | 0.28 | 0.08 | 6.50 | 22.3 | 12.0 |
| 13 | 14.6 | 105 | 0.11 | 2.24 | 1.30 | 3.55 | 35.1 | 119 | 77.1 |
| 14 | 9.30 | 109 | 0.41 | 2.05 | 2.32 | 2.35 | 59.4 | 92.1 | 44.3 |
| 15 | 7.95 | 115 | 0.72 | 1.05 | 1.91 | 1.03 | 86.0 | 68.2 | 32.2 |
| 16 | 15.9 | 93.1 | 2.58 | 11.7 | 1.84 | 6.75 | 202 | 110 | 94.1 |
| 17 | 10.8 | 94.5 | 0.89 | 1.32 | 2.27 | 5.60 | 110 | 119 | 47.4 |
| 18 | 16.7 | 104 | 1.05 | 1.85 | 3.02 | 3.04 | 107 | 164 | 75.0 |
| 19 | 6.11 | 38.5 | 0.15 | 1.45 | 1.85 | 6.05 | 21.4 | 171 | 54.1 |
| 20 | 6.60 | 57.1 | 0.21 | 5.10 | 1.33 | 1.75 | 13.8 | 117 | 120 |
| **A3** | | | | | | | | | |
| 21 | 2.95 | 23.3 | 0.11 | 2.34 | 0.61 | 0.50 | 19.2 | 93.9 | 68.0 |
| 22 | 1.05 | 2.30 | 0.10 | 0.02 | 0.65 | 0.85 | 1.65 | 5.20 | 37.9 |
| 23 | 2.45 | 26.5 | 0.15 | 0.75 | 1.68 | 0.72 | 18.3 | 12.4 | 31.8 |
| 24 | 2.95 | 13.6 | 0.05 | 1.52 | 0.78 | 0.59 | 29.5 | 118 | 93.0 |
| 25 | 1.06 | 9.21 | 0.08 | 1.13 | 0.46 | 1.37 | 10.8 | 83.0 | 76.0 |
| **A4** | | | | | | | | | |
| 26 | 2.50 | 18.7 | 0.09 | 1.73 | 3.36 | 10.3 | 106 | 278 | 398 |
| 27 | 1.45 | 21.4 | 0.08 | 1.51 | 3.16 | 10.3 | 227 | 206 | 255 |
| 28 | 0.45 | 25.1 | 0.22 | 2.12 | 3.35 | 5.95 | 389 | 136 | 101 |
| 29 | 9.45 | 13.6 | 0.24 | 1.48 | 4.09 | 8.55 | 92.8 | 168 | 162 |

The metals concentrations in vegetation (mg $kg^{-1}$ dw) in the studied areas ranged between: 0.35 – 18.3 for Cu, 2.30 – 120 for Zn, 0.05 – 2.58 for Cd, 0.02 – 15.0 for Pb, 0.21 – 4.09 for Ni, 0.08 – 2.03 for Cr, 1.65 – 389 for Mn, 5.20 – 278 for Fe and 12.0 – 398 for Al.

The average content of Cd in vegetation from Ferneziu exceeded the maximum level (1.0 mg $kg^{-1}$) established by the European Directive 2002/32/EC allowed in animal feed. Also the maximum level for Cd was exceeded in several samples from Sasar district. The maximum admitted level for Pb (10 mg $kg^{-1}$) was exceeded in only one vegetation sample from Ferneziu district.

According to the data from the literature [9], the critical concentrations of metals in plant tissues, that affects the vegetation growth (especially for sensitive vegetation species) are in the range of 5-10 mg $kg^{-1}$ for Cd, 1-2 mg $kg^{-1}$ for Cr, 15-20 mg $kg^{-1}$ for Cu, 20-30 mg $kg^{-1}$ for Ni and 150-200 mg $kg^{-1}$ for Zn. As presented in the Table 2, the found values in the analysed vegetation were generally below the critical concentrations, except for Cr and Cd, in several samples from Sasar and Ferneziu districts. Although the vegetation growth is not affected, metal concentrations are high enough to consider the vegetation contaminated, and unsuitable to be used as animal feed.

Generally, higher concentrations of metals such as Pb, Cd, Zn and Cu were found in vegetation collected from industrial part of the city. Thus, the average Pb concentration in A1 was approximately 1.7 times higher than in A2 and 2.5 times higher than in A3 and in A4, explained by the presence of a Pb smelter in that area. Also, the average of Cd concentration was found to be higher in A1 (2 times higher than in A2 and 10 times than in A3 and in A4), while the averages of Zn concentration in vegetation from industrial districts A1 and A2 were approximately 3 times higher than in A3 and in A4. The Cu concentrations were found to be higher in vegetation collected from the vicinity of the Cu smelter, A2 (2 times higher than in A1 and 3 times higher than in A3 and A4). The highest concentrations of Cr were found in the analysed vegetation from A2, while the concentrations of Ni, Mn, Fe and Al were higher in the vegetation from the area A4, situated far from the industrial smelting facilities.

**Multivariate statistics**

The Pearson's correlation matrix revealed strong positive correlations between the pseudo-total (*aqua regia* extractable) concentrations of Cu, Zn, Cd and Pb in soil, metals that can be attributed to the pollution generated by the mining activities. Also, strong positive correlation between the pseudo-total concentrations of major element in soil, Al and Fe, was found, while moderate correlations between Fe and Zn, Cd and Cr concentrations in soil were observed. In case of metals contents in soil and vegetation, low correlations were found for Zn, Cd and Pb, while for Cu, Ni, Cr, Mn, Fe and Al no significant correlations were observed. The poor correlations between pseudo-total contents of metals in soil and in vegetation revealed that the accumulation in vegetation is not directly influenced by the total metal contents of soil, but mainly by the metals mobility and plants uptake mechanisms.

The varimax rotated factor loadings of principal components (PCs) for metal concentrations in vegetation and soil and chemical properties of soil are presented in Table 3. The loadings in bold face correspond to variables that dominantly influence the selected latent factor. Four PC's with eigenvalues higher than 1 explains about 76% of the total variance of the system. The first component (PC1) exhibits 30% of the total variance with positive loadings

on pseudo-total (*aqua regia* extractable) content of Cu, Zn, Cd, Pb, Ni, Cr and Mn, and reflects the influence of anthropogenic sources on metal contents in vegetation. Generally, poor correlations were found between total metal content in soil and metal content in vegetation since many factors control the availability of metals from soil [21].

The second component (PC2) explains about 20% of the variability and contains the metals (Ni, Cr, Mn, Fe, Al) accumulated in vegetation and negatively correlated with soil pH. The elements from this group are metals that can be transferred to plants as free ions resulted from dissolution of soluble species, such as carbonates, under influence of soil pH. Additionally, at the soil-roots interface, the pH may vary by up to 2.5 pH units from that of the bulk soil solution as a result of roots exudates of organic acids with influence on the availability of metals [22].

**Table 3.** Factor loadings after Varimax rotation

| | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| Cu soil | **0.771** | -0.111 | 0.281 | -0.186 |
| Zn soil | **0.736** | -0.270 | 0.399 | 0.302 |
| Cd soil | **0.790** | -0.154 | 0.276 | 0.279 |
| Pb soil | **0.884** | -0.183 | 0.240 | 0.109 |
| Ni soil | **0.896** | 0.004 | -0.324 | -0.070 |
| Cr soil | **0.677** | 0.181 | -0.467 | 0.053 |
| Mn soil | **0.776** | -0.033 | 0.033 | 0.238 |
| Fe soil | 0.334 | 0.151 | 0.152 | **0.835** |
| Al soil | 0.013 | -0.003 | -0.219 | **0.871** |
| TOC | 0.084 | -0.002 | **0.756** | -0.215 |
| pH | 0.102 | **-0.554** | **-0.495** | -0.148 |
| Cu vegetation | 0.028 | 0.217 | **0.616** | 0.453 |
| Zn vegetation | -0.101 | -0.220 | **0.735** | 0.010 |
| Cd vegetation | 0.284 | -0.064 | **0.830** | -0.097 |
| Pb vegetation | 0.375 | 0.082 | **0.620** | 0.260 |
| Ni vegetation | -0.074 | **0.821** | 0.108 | 0.299 |
| Cr vegetation | -0.158 | **0.858** | 0.004 | 0.045 |
| Mn vegetation | -0.147 | **0.725** | 0.133 | -0.064 |
| Fe vegetation | -0.038 | **0.887** | -0.123 | 0.068 |
| Al vegetation | -0.045 | **0.807** | -0.277 | -0.180 |

The values in bold face show the highest loadings

The third component (PC3) explains approximately 17% of the total variability, and contains the metals (Cu, Zn, Cd and Pb) negatively correlated with soil pH and positively correlated with soil TOC. As expected, soil pH plays

an important role in the mobilization of metals, while TOC has a different effect on the metals mobility and their transfer to plants. The different metal mobilization patterns are explained by the type of complexes that are formed between each metal and organic matter. It is recognized that elements such as Cu, Zn and Pb forms strong complexes with dissolved organic matter that facilitates metal transport through soils in the form of organic complexes [23]. These complexes are not dissolved even at low pH, but are absorbed by vegetation particularly if they have low molecular weight. Previous studies showed that the addition of the fertilizers can increase the metals uptake by vegetation [24]. For the elements attributed mainly to the natural background such as Al, Mn, Fe, Cr and Ni, no correlation was found between soil TOC and metals contents in vegetation, explained by theirs initial complexation by organic matter with low molecular weight which, in time, are converted into compounds with higher molecular weight (humic substances) [25].

The fourth factor (PC4) contains the major elements (Al and Fe) related to the natural conditions from soil. This factor explains only 9% of the total variability.

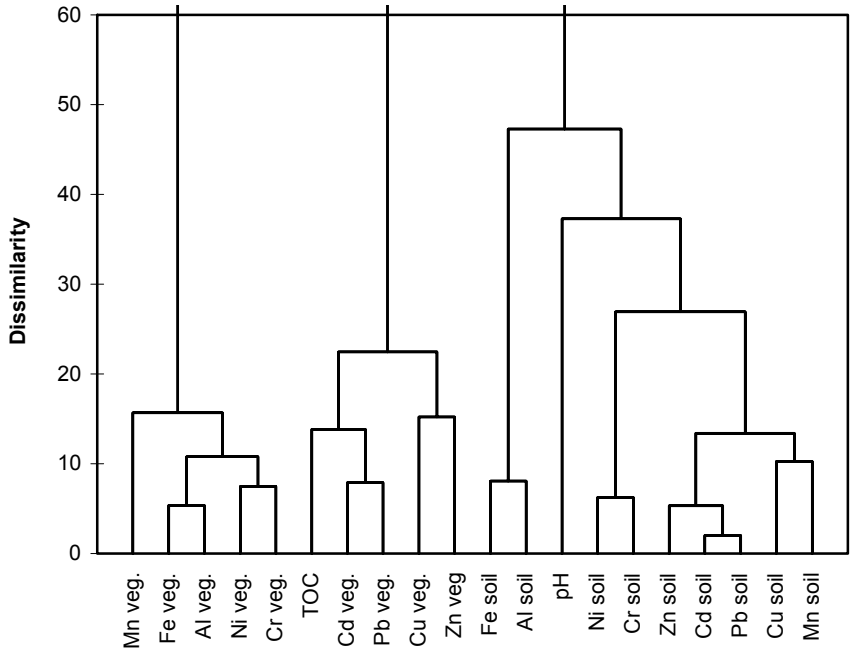


**Figure 2**. Dendogram showing the clustering of metals in vegetation, soil and chemical soil properties

The dendrogram resulting from AHC (Agglomerative Hierarchical Clustering), created by using Ward method and the Euclidian distance for dissimilarity, presented in Figure 2, shows 3 major clusters: cluster 1 (Mn, Fe, Al, Ni, Cr from vegetation), cluster 2 (soil TOC and Pb, Cd, Cu and Zn accumulated in vegetation), cluster 3 (soil pH and aqua regia extractable metal concentrations), and confirm the results obtained by the PCA.

## CONCLUSIONS

The obtained results showed high concentrations of some metals (Pb, Cd, Cu and Zn) in soil, exceeding the corresponding maximum admitted levels, established by the Romanian legislation. In vegetation, higher concentrations of Pb, Zn, Cr, Mn and Cd, were found in the industrial parts of the city, while the concentrations of Ni, Fe and Al were slowly higher in the vegetation from the area situated far from smelting facilities. Multivariate statistical analysis showed poor correlations between metals concentration in soil and in vegetation. The study revealed that pH plays an important role in the metals mobilization, with influence in the soil-plant transfer, while TOC from soil is a lower indicator for metals transfer to vegetables, in the studied area.

## EXPERIMENTAL PART

### Site description and Sampling

Soil samples at 0-20 cm depths and vegetation from the spontaneous flora (*Agrostis, Agropirum, Trifolium repens, Urtica dioica*) grown on these soils were sampled from each selected point (presented in Fig. 1). The coordinates of sampling points recorded with a 310 Magellan GPS.

### Materials and methods
### Instrumentation

The content of all metals in soil and Al, Fe, Mn, Zn and Cu in vegetation extracts were measured by inductively coupled plasma optical emission spectrometer (ICP-OES) Optima 5300 DV (Perkin Elmer, USA), while Cd, Pb and Ni in vegetation extracts were measured by inductively coupled plasma mass spectrometer (ICP-MS) ELAN DRCII (Perkin Elmer, USA). Total Organic Carbon content was measured by using the Multi N/C 2100S Analyser (Analytic Jena, Germany). The pH was measured with Consort 2000 pH-meter equipped with a combined pH electrode. A mortar grinder PM 100 (Retsch, Germany) and a sieve shaker Analysette 3 Spartan (Fritsch, Germany) were used for samples grinding and sieving.

## Reagents, Standard Solutions and CRMs

Stock multielemental standard solutions containing all the analysed elements (1000 mg $L^{-1}$) Merck (Darmstadt, Germany) were used for ICP-OES calibrations. For all dilutions ultrapure water (18 MΩ $cm^{-1}$) obtained from a Direct Q3 (Millipore, France) system was used. A soil certified reference material SRM 2709 San Joaquin Soil (New York, USA) and two vegetable certified reference materials NCS ZC85006 Tomato (Beijing, China) and IAEA-359 Cabbage (Vienna, Austria) were used for the internal quality control of metals determination.

## Laboratory Analysis

The pre-treatment of soil samples were made according to SR ISO 11466:1999 [26]. Samples were ground to a fine powder and sieved through 100 μm sieve. The vegetables were intensely rinsed with tap water and ultrapure water, to eliminate soil and dust from the roots and shoots, and then dried at 40°C, grounded and the sieved through the 100 μm sieve. All the soil and plant samples were kept in sealed plastic bags until analysis.

The total metal concentrations of soils were determined after *aqua regia* digestion according to SR ISO 11466:1999 [26]. An amount of 1 g soil was heated with 21 mL of 12 M HCl and 7 mL of 15.8 M $HNO_3$, then filtered and diluted to 100 mL with 0.5 M $HNO_3$. The vegetables were digested using a microwave digestion system. Approximately 1.0 g of sample was digested with 6 mL of $HNO_3$ and 2 mL of $H_2O_2$ and then diluted to 50 mL with ultrapure water.

Quality assurance included CRMs analyses and replicate analyses. The recovery degrees (%) of Fe, Al, Cr, Mn, Cu, Zn, Ni and Pb in soil CRM, SRM 2709, calculated using the average of five replicates and relative standard deviation at a 0.05 significance level, were 96±11%, 90±12%, 102±10%, 109±9%, 95±9%, 99±12%, 93±11% and 92±12%, respectively. The recovery degrees (%) of Al, Mn, Cu, Zn, Ni, Cd and Pb in NCS ZC85006 tomato CRM, calculated using the average of five replicates and relative standard deviations at a 0.05 significance level, were 105±14%, 100±12%, 92±15%, 88±13%, 96±16%, 93±13% and 107±16%, respectively. For IAEA-359 cabbage CRM, the recovery degrees (%) of Fe, Mn, Cu and Zn, calculated using the average of five replicates and relative standard deviations at a 0.05 significance level, were 108±10%, 96±14%, 103±15% and 86±17%, respectively.

### Statistical Analyses

The XLStat Microsoft Excel plug-in (Addinsoft) was used for the statistical processing of the data.

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# PREPARATION AND CHARACTERIZATION OF QUASI-ONE DIMENSIONAL ATO FILMS DEPOSITED ON GLASS SUBSTRATE

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**ABSTRACT.** The antimony doped tin-dioxide (ATO) thin nanostructured films are known for their wide range of applications. The paper presents the preparation and characterization of quasi-one dimensional ATO films deposited on glass substrate. Films have been prepared by using the sol-gel one step multipledip-coating and spray pyrolysis techniques. Sb concentration in the precursor solution was 5 and 15 mol%, respectively. The crystalline structure of the ATO films was studied by X-ray Diffraction (XRD) which showed their polycrystalline structure with peaks belonging to the tetragonal rutil structure of $SnO_2$. Atomic Force Microscopy (AFM) revealed the films nanostructured morphology and different geometrical shapes of the nanoparticles depending on the route of preparation and doping degree. X-ray Photoelectron Microscopy (XPS) measurements showed that the main components at the surface of the films are: Sn, Sb, O, Cl and also that Sn and Sb are present in their oxidated state $Sn^{4+}$ and $Sb^{5+}$, respectively.

***Keywords:*** *ATO film, sol-gel technique, spray pyrolysis, crystalline structure, morphology.*

## INTRODUCTION

ATO nanocrystalline thin films are well known for their use as gas sensors, transparent conductive electrode materials, catalysts, protective layers, solar cells, memory cells for fast computers [1-8]. The wide range of applications is due to the excellent mechanical, electrical and optical properties.

Literature presents several techniques for obtaining undoped and doped tin dioxide thin films on glass substrate such as: sol-gel, RF reactive magnetron sputtering, chemical vapor deposition (CVD), pulsed laser deposition

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and spray pyrolysis. Depending on the experimental conditions nanostructured films with different thicknesses, shapes (wire, ring, sphere) and dimensions of particles (20-200 nm), were obtained [9-16].

The paper reports on the investigation of structural characteristics and composition of ATO quasi-one dimensional films deposited on glass substrate prepared by using sol-gel one step multipledip-coating and spray pyrolysis techniques.

The microstructure, crystalline structure, and composition of the films were investigated by AFM, XRD and XPS.

## RESULTS AND DISCUSSION

Literature shows that the thickness of ATO films obtained by sol-gel and spray pyrolysis techniques depend on number of dipping or sprayings, respectively [10, 15]. The films obtained following the routes described in the experimental section were found to have comparable thicknesses ranging in the 100.0-128.0nm domain which allows to be considered as quasi-one dimensional.

ATO films surface morphology and microstructure are affected by several experimental parameters according to the route of preparation: doping degree, annealing temperature, volume of the sprayed solution, nozzle to substrate distance, substrate temperature [11, 15].

The surface morphology and microstructure of the ATO films prepared according the described experimental conditions were investigated by AFM measurements. These revealed the films continuous character and the nanostructured morphology, as well. However, the dimensions and shapes of the nanoparticles were different depending on the route of preparation and doping degree.

The films obtained by spray pyrolysis, showed particles with spherical shape with an average diameter of 120 nm regardless the doping degree (figure 1).

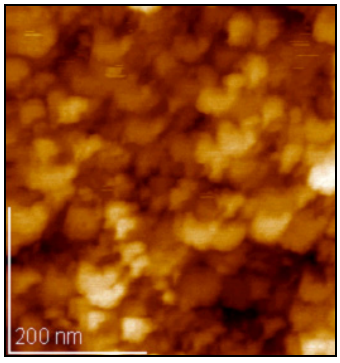



**Figure 1.** Typical AFM image obtained for ATO films prepared by spray pyrolysis

Films obtained by the sol-gel route, showed wire shaped (figure 2) and ring shaped particles (figure 3, a and b) depending on the doping degree. The wire shaped particles were found in films obtained by using the precursor with low content of dopand (5 mol% Sb).

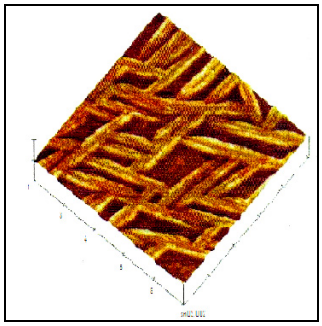
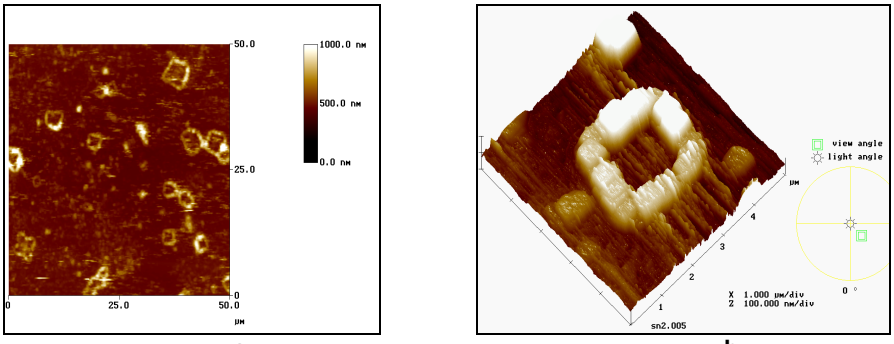


**Figure 2**. AFM image of films obtained by using the precursor with 5 mol% Sb:$SnO_2$-wire shaped particles




**a** **b**

**Figure 3.** a) 2D micrograph of films obtained by using the precursor with 15 mol%Sb:$SnO_2$ - ring shaped particles; b) 3D micrograph of films obtained by using the precursor with 15 mol%Sb: $SnO_2$ - ring shaped particle

The diameter of the ring shaped particles was found to be 850 nm, while the diameter of the wire shaped particles was 120 nm.

Literature data show that similar geometrical shapes were obtained in different experimental conditions but the dimensions of the nanoparticles were smaller [12-16]. The wide diameter of the nanorings in our samples could be assigned to a process of smaller crystallites self assembling during annealing.

The XRD patterns showed in all cases, rutile $SnO_2$ crystallites with tetragonal structure with a highly preferred orientation along the plane (200).

No other peaks assignable to antimony compounds were detected which means that antimony incorporation in the $SnO_2$ lattice has not affected the lattice structure. A typical XRD spectrum is shown in fig. 4.

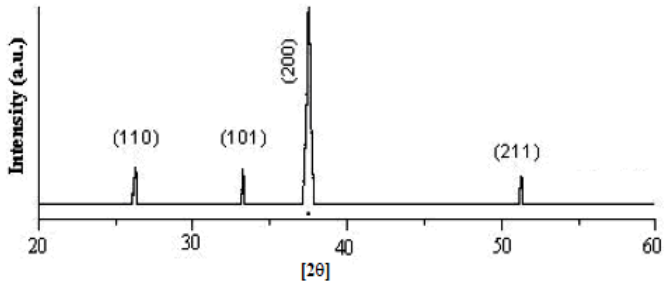



**Figure 4.** XRD spectrum for films obtained by using the precursor with 15 mol% Sb:$SnO_2$

XPS measurements have shown that the major components at the films surface are Sn, O, Sb, C and Cl. Tables 1 and 2 summarize the surface compositions of the obtained ATO films.

By flushing the film surface with a flux of Ar ions (2keV) the content of carbon decreased drastically which indicates that the presence of carbon is due to adsorption from environment. The surface presence of chlorine is due to the composition of the precursor, all salts being chlorides.

**Table 1**. Chemical composition of films by using the precursor with 5 mol% Sb:$SnO_2$

| Ar flush duration (min) | (w%) | | | | |
|---|---|---|---|---|---|
| | Sb | Sn | O | C | Cl |
| 0 | 1.0 | 20 | 52 | 12 | 3.0 |
| 5 | 1.2 | 28 | 55 | 6.0 | 2.0 |
| 25 | 2.0 | 43 | 43 | 1.0 | 1.0 |

**Table 2. C**hemical composition of films obtained by using the precursor with 15 mol% Sb:$SnO_2$

| Ar flush duration (min) | (w%) | | | | |
|---|---|---|---|---|---|
| | Sb | Sn | O | C | Cl |
| 0 | 2.5 | 20 | 50 | 12 | 2.0 |
| 5 | 3.0 | 24 | 48 | 8.0 | 1.5 |
| 25 | 3.5 | 40 | 52 | 1.0 | 1.0 |

The XPS investigation also revealed that on a depth of 5 nm the composition of the films is identical.

The $Sn3d_{5/2}$ and $Sn3d_{3/2}$ spin-orbital splitting photoelectrons in all samples were located at binding energies 487.5 eV and 495.3 eV (figure 5), respectively.

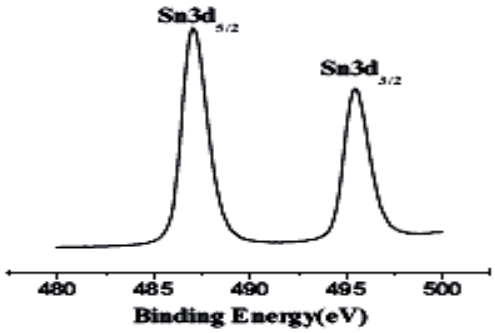



**Figure 5.** XPS spectrum for Sn3d peaks

The $Sb3d_{5/2}$ and $Sb3d_{3/2}$ spin-orbital splitting photoelectrons were located at the binding energies 529.5 eV and 539.5 eV, respectively. The large binding energies allow to conclude that Sn and Sb are present in their oxidate state, $Sn^{4+}$ and $Sb^{5+}$, respectively.

## CONCLUSIONS

Quasi-one dimensional ATO films were prepared by sol-gel one step multipledip-coating and the spray pyrolysis techniques using precursors with Sb concentration of 5 and 15 mol%. The films were characterized by using film thickness, AFM, XRD and XPS measurements. Films thicknesses ranged between 100.0-128.0nm which allows to consider them quasi-one dimensional. AFM measurements showed that the obtained films are continuous and nanostructurated, with spherical, wire or ring shaped particles depending on the route of preparation and dopand level in the precursor. The diameter of the ring shaped nanoparticles in our films was found to be higher than other reported in literature. XRD measurements revealed the tetragonal rutile crystalline structure belonging to $SnO_2$ with no other diffraction peaks assignable to the dopand. XPS measurements showed that the main components at the films surface are Sn, Sb, O, and Cl and that the oxidation state of Sn and Sb are $Sn^{4+}$ and $Sb^{5+}$, respectively.

## EXPERIMENTAL SECTION

### 1. Materials

$SnCl_4$ fumans (Fluka), $SnCl_4 \cdot 5H_2O$, (Sigma), $SbCl_3$ (Fluka), $C_2H_5$-OH, n-propanol, ammonia (Merck), reagent grade. In all cases, the substrate was a flat soda-lime-silica glass. The substrate (20x10mm) was cleaned with a neutral detergent solution, followed by rinsing with de-ionized water and acetone.

## 2. ATO films preparation

**2.1.** ***Sol-gel technique****:* the precursors were prepared as follows: $SbCl_3$ and $SnCl_4 \times 5H_2O$ mixtures with $Sb:SnO_2$ 5 and 15 mol%, respectively, were dissolved in 50 ml n-propanol under continuous stirring at $25^0$C. 10 ml concentrate aqueous ammonia solution was next added drop by drop to accomplish the hydrolysis of precursor. The solution was heated to 70 C° in a water bath and stirred for four hours.

The films were obtained by one step multipledip-coating using a Nima Tensiometer. The glass slides were immersed perpendicularly into the sol with a constant speed of 5 mm/min and then withdrawn under the same conditions. The process was repeated 3 times. Immediate heating up to 500C° for one hour with a constant rate of $20^0$C/min and annealing for 1 hour followed.

**2.2.** ***Spray pyrolysis technique****:* The glass substrate was heated in a furnace up to 500C° and kept at this temperature for 20 min. 30 ml of the precursor solution was sprayed on the substrate from 30 cm nozzle to substrate distance. The process was repeated three times at time intervals of one minute. Compressed air was used as carrier gas. The ATO layer was annealed at 360C° for 45 minutes.

## 3. ATO films characterization

Film thickness was measured by using Filmetrics F20.

Surface morphology and microstructure of samples were investigated by AFM in contact mode (Nanoscope type Atomic Force Microscope).

The crystalline structure was examined by XRD (Philips PW 3710 diffractometer- with CuKα source). The scanning range was $20\text{-}60^0$.

The composition of the films was investigated by using XPS (EA 125 Omicron type X-ray Photoelectron Spectrometer).

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STUDIA UBB CHEMIA, LVII, 1, 2012 (p. 47 - 56)

# ESSENTIAL AND TOXIC ELEMENTS IN DIETARY SUPPLEMENTS DETERMINED BY ICP – MS

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**ABSTRACT.** Samples of widely used dietary supplements distributed on the Romanian market were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) in order to assess the levels of oligoelements Co, Cr, Cu, Mn, Ni, and Zn as well as potentially toxic heavy metals Cd and Pb, and compare them with the maximum allowable levels (MAL). The following concentration ranges were obtained (in $\mu g\ g^{-1}$): Cd 0.01-0.09; Co 0.03-0.6; Cr 1.1-11.9; Cu 5.5-14.7; Mn 1.8-39.1; Ni 0.4-4.7; Pb 0.8-3.1; Zn 18.8-12119.4. Several analyzed products had metals levels above the maximum allowable limits (Pb: one plant based product and one multivitamin product; Zn: two multivitamin multimineral products). The estimated cumulative daily intakes of only one formulation was higher than the oral permitted daily exposures set by the United States Pharmacopeia (USP) Advisory Panel on metal impurities (Pb: one plant based product). Such products present a significant additional source of metals in the human diet, and therefore could be harmful for human health.

***Keywords:*** *dietary supplements, inductively couple plasma mass spectrometry, heavy metal levels, cumulative daily intakes*

## INTRODUCTION

According to the Romanian legislation, dietary supplements are defined as food supplies which have the purpose to complete the normal diet and are concentrated sources of nutrients or other substances with nutritional or physiological effect, individually or in combinations [1].

The industry of dietary supplements has recorded a growth on a global level [2-5], mainly because of the increase in their consumption as alternative medicines, which is based on the consumers' belief that these products are natural, safe and without any adverse effects [2, 6-8]. According

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to the Office of Dietary Supplements (2005), in 2004, 55% of Unites States adults took a dietary supplement and 35% of these users took multivitamins and multiminerals supplements.

Essential trace elements, also known as oligoelements, have the specific and irreplaceable function of ensuring the optimal performance of the entire organism as well as activating the catalytic sites of enzymes. However, those elements can determine illness by their deficit, disequilibrium and poisoning if the limits are exceeded [9]. The clinical manifestations of metal poisoning have been characterized [10]. Heavy metal poisoning has decreased because of improved industrial hygiene and environmental controls so that the signs and the symptoms of such poisoning are likely to go unrecognized [11]. If metal poisoning is identified, the true source may be wrongly associated with environmental occupational exposures, not medicaments [12]. Failure to establish the true cause of exposure means that the subject continues taking the metal-containing product. Thus, the screening of traditional remedies and dietary supplements has been recommended to protect public health [13].

The objective of this study was to evaluate the metal levels in dietary supplements available on Romanian market, produced by both domestic and foreign manufacturers. The levels of oligolements, Co, Cr, Cu, Mn, Ni, and Zn, and as well of potentially toxic heavy metals Cd, Pb, were determined and compared with maximum allowable levels (MAL) [14]. Since metal bioaccumulation could cause toxic effects and ultimately result in health disorders or diseases if contaminated dietary supplements are used on a long term basis, a further objective of the present study was to estimate metal cumulative daily intakes (CDI) from analyzed dietary supplements. These were compared with the oral permitted daily exposures (PDE) for dosage forms, as stated by United States Pharmacopeia (USP) Advisory Panel on Metal Impurities [14], to deduce whether the analyzed products could present a potential threat to human health.

## RESULTS AND DISCUSSION

Parallel to the metal determination in selected dietary supplements, the concentrations of metals were also determined in a certified reference material (CRM), INCT-MPH-2, for the quality control. This CRM represents a mixture of nine varieties of herbs grown in Poland, collected in a non-contaminated rural area and used for drugs production. The obtained results (Table 1) were in good agreement with the certified values, and the variation coefficient for the percent recovery means was below 5%.

The obtained levels of metals found in the analyzed samples are summarized in Table 2. It must be mentioned that the obtained results were compared to the MALs proposed by the experts in the USP Advisory Panel on Inorganic Impurities and Heavy Metals included in the revised document “Draft Metals and Limits Table” [14].

**Table 1.** Defined and measured concentration and recovery grade of metals in the INCT-MPH-2 certified reference material

| Metal | Defined concentration [a] μg g$^{-1}$ | Measured concentration [b] μg g$^{-1}$ | Recovery grade [c] % |
|---|---|---|---|
| Cd | 0.19 ± 0.02 | 0.2 ± 0.02 | 104 ± 3 |
| Co | 0.21 ± 0.03 | 0.21 ± 0.02 | 102 ± 2 |
| Cr | 1.69 ± 0.13 | 1.62 ± 0.18 | 96 ± 4 |
| Cu | 7.77 ± 0.53 | 7.67 ± 0.8 | 99 ± 1 |
| Mn | 191 ± 12 | 194.1 ± 7.1 | 102 ± 2 |
| Ni | 1.57 ± 0.16 | 1.53 ± 0.08 | 98 ± 2 |
| Pb | 2.16 ± 0.23 | 2.11 ± 0.12 | 98 ± 3 |
| Zn | 33.5 ± 2.1 | 33.4 ± 0.6 | 100 ± 1 |

[a] results as the average ± the expanded uncertainty (U) which is obtained by multiplying the combined standard uncertainty ($u_c$) by a coverage factor k = 2 corresponding to 95 % confidence level, where $u_c$ is defined as square root of the sum of the quadrats of the standard deviation of the overall mean ($u_i$) and the standard uncertainty estimated from the long term stability studies ($u_s$).

[b] results as the average ± the expanded uncertainty (U), obtained by multiplying the standard deviation (s) by a coverage factor k = 2 corresponding to 95 % confidence level.

[c] ratios of measured to defined concentrations ± the variation coefficient.

Table 2 presents the results expressed as medians, with the minimum and maximum values given in the brackets. Number of samples above the maximum allowable level (>MAL) as well as the ratio between measured metal levels and MALs (median, minimum and maximum) are indicated in the table.

**Table 2.** Metal levels in the analyzed dietary supplements

| Metal | Metal level μg g$^{-1}$ | MAL μg g$^{-1}$ | Metal level / MAL % | >MAL |
|---|---|---|---|---|
| Cd | 0.02 (0.01 – 0.09) | 2.5 | 0.7 (0.2 – 3.5) | 0 |
| Co | 0.2 (0.03 – 0.6) | 100 | 0.2 (0.03 – 0.6) | 0 |
| Cr | 1.5 (1.1 – 11.9) | 25 | 5.8 (4.6 – 47.7) | 0 |
| Cu | 8.9 (5.5 – 14.7) | 250 | 3.6 (2.2 – 5.9) | 0 |
| Mn | 2.2 (1.8 – 39.1) | 250 | 0.9 (0.7 – 15.6) | 0 |
| Ni | 0.5 (0.4 – 4.7) | 25 | 2.1 (1.4 – 18.9) | 0 |
| Pb | 1.1 (0.8 – 3.1) | 1 | 98.3 (79.1 – 312.1) | 2 |
| Zn | 74.7 (18.8 – 12119.4) | 1500 | 5 (1.3 – 807.9) | 2 |

Four analyzed dietary supplements formulations marketed in Romania contained higher levels of some metals compared to the MALs. For samples 2 and 5 which include Zn on their list of active substances (Table 5) and therefore could not be considered contaminated, the measured levels of Zn reached 178.6% and 807.9% respectively, compared to MAL (Table 2). The found levels are lower than those reported by Garcia-Rico et al., 2007 [15] and similar to those reported by Obi et al., 2006 [11] and Tumir et al., 2010 [16].

Zinc poisoning manifestations include nausea, vomiting, diarrhea, fever and lethargy. Long-term exposure to high excess zinc intakes could interfere with the metabolism of other trace elements [17].

Above MAL levels for lead were found in two dietary supplements, samples 3 and 7 (Table 5). The measured Pb levels for the contaminated formulations reached 127.5% (sample 3) and 312.1% (sample 7) compared to MAL (Table 2). These levels are in concordance with those reported by Dolan et al., 2003 [18] and Timur et al., 2010 [16]. However, the found concentrations were lower than the results reported by Garcia-Rico et al., 2007 [15], Obi et al., 2006 [11] and Garvey et al., 2001 [19]. Lead interferes with a variety of body processes and is toxic to many organs and tissues including the heart, bones, intestines, kidneys, and reproductive and nervous systems [20].

The low levels of the other determined metals (Cd, Co, Cr, Cu, Mn, Ni), found in the analyzed formulations, none above de MALs, indicate that the exposure to these contaminants through the tested dietary supplements is not expected to affect human health.

An interesting fact is that the maximum measured levels for the determined metals, were found in the same dietary supplement, namely sample 7, a plant based supplement produced by a local company. Lower levels of metals have been reported in herbal teas with fewer ingredients than in polyherbal teas [20], this leads to a hypothesis that the large number, origin and type of ingredient may be the reason of the maximum levels for Cd, Co, Cr, Cu, Mn, Ni and Pb. However, this hypothesis should be further investigated.

In order to conclude whether the metal levels determined in this study could be considered harmful for human health, the cumulative daily intakes (CDI) of metals from dietary supplements, expressed as μg per day, were estimated based on the recommended daily doses (RDD) of dietary supplements. The CDIs for each metal tested in this study were then compared with oral permitted daily exposures for dosage forms, PDEs, as recommended by the USP [14]. Table 3 presents the CDIs of the determined metals as medians with minimum and maximum in the brackets, the number of samples for which the ingestion would lead to CDI of analyzed metals above the PDE for dosage forms, as well as the ratio between calculated CDIs and PDEs (median, minimum and maximum).

The CDIs for the products included in the study were lower than permitted and could not be considered harmful, except for sample 7 which had a CDI for Pb of 109.2% of the allowed PDE (Table 3). However, it should be taken into account that the use of the tested dietary supplements is just one of all possible yields that contribute to CDI, besides for instance, ingestion of food and water that can also contain metals, as well as inhalation of air [22].

**Table 3.** The cumulative daily intake (CDI) of metals for the analyzed dietary supplements

| Metal | CDI μg/day | PDE μg/day | CDI / PDE % | >PDE |
|---|---|---|---|---|
| Cd | 0.02 (0.01 – 0.31) | 25 | 0.08 (0.03 – 1.2) | 0 |
| Co | 0.2 (0.1 – 2.2) | 1000 | 0.19 (0.01 – 0.2) | 0 |
| Cr | 1.6 (1.3 – 41.7) | 250 | 0.659 (0.5 – 16.7) | 0 |
| Cu | 13.4 (6 – 85.4) | 2500 | 0.54 (0.2 – 3.4) | 0 |
| Mn | 3.7 (2 – 136.9) | 2500 | 0.15 (0.1 – 5.5) | 0 |
| Ni | 0.65 (0.5 – 16.6) | 250 | 0.3 (0.2 – 6.6) | 0 |
| Pb | 1.15 (0.89 – 10.92) | 10 | 11.5 (8.9 – 109.2) | 1 |
| Zn | 746.96 (24.38 – 12143.6) | 15000 | 4.9 (0.2 – 80.9) | 0 |

In most cases the levels of possible metal bioaccumulation were lower than permitted and therefore do not causes negative effects to human health.

## CONCLUSIONS

From the seven dietary supplements chosen for analysis two were found exceeding the maximum level for Pb and two for Zn, even though they had Zn on their list of active substances and therefor it can't be considered contamination. The cumulative daily intake was exceeded for Pb in only one sample.

The present study emphasizes the need for an international regulatory framework to introduce the obligatory testing of metals in dietary supplements including the starting materials, the in-process and the finished product control, leading to the elimination of low quality products on the market and to assure a higher safety profile of dietary supplements.

## EXPERIMENTAL SECTION

### Samples

Samples of 7 dietary supplements marketed in Romania were selected for this study based on their popularity and frequent usage. The scope of their intended use was very broad (e.g. general improvement of health, cognitive enhancement and better brain blood supply, stress reduction, recovery after intense physical effort, immune system strengthening, support for the intestinal flora, cholesterol regulation, neutralization of free radicals, dietary vitamin and mineral supplementation, detoxification, body mass reduction). Detailed informations about the analyzed dietary supplements are presented in Table 5.

## Sample preparation

Solid samples were homogenized by trituration, whereas the powder samples were used directly in the next step. Three replicates, for each sample and the CRM, were weighed (average sample weight: 0.304 ± 0.002 g) and put into the PTFE vessels for microwave digestion, followed by addition of 8 mL of Lunge mixture (2 mL HCl 37% and 6 mL $HNO_3$ 65%). The digestion process took place in the closed PTFE containers of the microwave system according to the digestion program presented in Table 4. The procedure and settings are those recommended by the equipment producer for this type of samples.

**Table 4.** Operating conditions for the microwave digestion system

| Parameter | Stage | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Temperature / ºC | 145 | 170 | 210 | 100 | 100 |
| Pressure / bar | 30 | 30 | 30 | 0 | 0 |
| Time / min. | 5 | 10 | 15 | 10 | 10 |
| Slope / min. | 2 | 2 | 2 | 1 | 1 |
| Power* / % | 80 | 80 | 80 | 0 | 0 |

** 100 % power refers to 1450 W*

## Reagents and Standard Solutions

Calibration standards in the range of 0 – 100 $\mu g\ L^{-1}$ were prepared by serial dilution of the Instrument Calibration Standard 3, from Perkin Elmer (Perkin Elmer, MA, USA) with 5% $HNO_3$ (v/v) and high purity water (18.2 MΩ cm-1) from a Milli Q system (Millipore, Milford, MA, USA).

Analytical grade nitric acid 65% and hydrochloric acid 37% purchased from Merck (Darmastadt, Germany), were used for sample digestion. Argon (5.0 quality) from Linde Gas SRL Cluj – Napoca, Romania was used as working gas.

## Instruments

During sample preparation a mortar grinder Restch RM 100 (Haan, Germany) and a closed PTFE vessel microwave digestion system, Berghof MWS-3+ with temperature and pressure control (Eningen, Germany), were used.

The quantification of metals was performed using an inductively coupled plasma mass spectrometer, ICP-MS without reaction chamber, (SCIEX – Perkin Elmer ELAN DRC II, ON, Canada). The operating parameters of the employed ICP-MS system are presented in Table 6.

**Table 5.** Code and label ingredients of dietary supplements used in this study

| Code | Type of dietary supplement | Recommended daily dose [a] g/day | Principal label ingredients |
|---|---|---|---|
| 1 | Multivitamin; multiminerals for children | 1.16 | Inulin, vitamin A, vitamin B complex, vitamin C, vitamin D, vitamin E, Folic acid, Biotin, Iodine, Zinc, Iron, Selenium. |
| 2 | Multivitamin; multiminerals for children | 1.13 | Beta glucan, bioflavonoids complex, vitamin C, vitamin A, vitamin B complex, vitamin E, Zinc, Biotin, Folic acid, Iodine, Rosehips, Lysine. |
| 3 | Multivitamin | 1.5 | Vitamin A, vitamin B complex, vitamin C, vitamin D, vitamin E, Folic acid, Iodine. |
| 4 | Antioxidant; multivitamin with selenium | 1.1 | Vitamin A, vitamin C, vitamin E, Selenium. |
| 5 | Antioxidant; multivitamin and multiminerals | 1 | Vitamin A, vitamin $B_6$ (pyridoxine), vitamin C, vitamin E, Zinc, Selenium. |
| 6 | Hydro soluble multivitamins and multiminerals | 10 | Vitamin C, vitamin B complex, vitamin E, vitamin D, Potassium, Phosphorus, Calcium, Magnesium, Zinc, Iron, Chromium. |
| 7 | Plant based supplement for detoxifying and body mass reduction | 3.5 | Medicinal herbs powder (*Plantago*, *Cynara scolymus*, *Hypericum perforatum*, *Rosmarinus officinalis*, *Satureja hortensis*) blended with *Linum*, *Cuminum cyminum*, *Foeniculum vulgare* seed powder, *Hippophaë rhamnoides L.* flour, grey clay and lactic ferments of the Lactobacillus (*casei*, *plantarum*) and Bifidus genus (*longum*, *breve*). Excipients: isomalt and maltodextrin. |

[a] the RDD was estimated by multiplying the dose weight with the number of doses recommended by the producer of the dietary supplement.

**Table 6.** Operating parameters for the ICP-MS system

| Parameter | Value |
|---|---|
| Plasma | |
| Power / W | 1250 |
| Plasma gas flow / L $min^{-1}$ | 15.00 |
| Auxiliary gas flow / L $min^{-1}$ | 1.10 |
| Nebulizer gas flow / L $min^{-1}$ | 0.82 |
| Sample introduction rate / mL $min^{-1}$ | 0.4 |
| Quadrupole | |
| Quadrupole rod offset (QRO) / V | 0.00 |
| Chamber rod offset (CRO) / V | - 8.00 |
| Cell path voltage (CPV) / V | - 20.00 |
| Measuring mode | Peak hopping |
| Dwell time / ms | 200 |
| Detector dead time / ms | 55 |
| Readings per point | 20 |
| Readings per replicate | 1 |
| Measurements per replicate | 5 |
| DRC | |
| Module DRC QRO / V | - 6.00 |
| Module DRC CRO / V | - 1.00 |
| Module DRC CPV / V | - 15.00 |
| Tension on start lens / V | 4.00 |
| Tension on end lens / V | 12.50 |

## Limits of detection

The limits of detection, LOD, were based on the 3σ criterion: standard deviations of ten consecutive metal measurements in the blank sample were multiplied by 3. The estimated LODs are given in Table 7.

**Table 7.** Limits of detection for the used method

| Element | LOD $\mu g\ g^{-1}$ |
|---|---|
| Cd | 0.01 |
| Co | 0.01 |
| Cr | 0.13 |
| Cu | 0.2 |
| Mn | 0.37 |
| Ni | 0.01 |
| Pb | 0.01 |
| Zn | 0.42 |

The LODs for this method are more than 10 times lower than the maximum allowable limits, MAL, imposed by the USP [14], meaning that the used analysis method is suitable for the determination of metals from dietary supplements [22].

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# AIR QUALITY MODELLING OF $SO_2$ EMISSIONS ASSOCIATED TO METALLURGICAL PROCESSES

**NICOLAE AJTAI[a,*], ZOLTÁN TÖRÖK[a], ALEXANDRU OZUNU[a]**

**ABSTRACT.** Metallurgical plants are one of the major industrial pollutants emitting mainly gases ($SO_2$, $NO_x$ and $CO_2$) and particulate matters containing heavy metals. The objective of this study is to compare two situations, regarding the $SO_2$ emissions before and after the installation of the desulphurization system at a metallurgical plant. Two different sets of simulations were performed, considering a three days period for which meteorological data was available. The simulation results were compared to national $SO_2$ air quality limits. The results show a significant decrease of $SO_2$ concentrations after the installation of the desulphurization system, situated well within legal limits. Another objective of this study is to compare qualitatively the concentration data obtained from modelling with data measured on-site by a point-monitor.

***Keywords:*** *air quality, metallurgical industry, $SO_2$ emissions, ISCST3 model*

## INTRODUCTION

The magnitude of the effect humanity and its activities have on environmental change is one of the most pressing issues currently debated by all members of society. Air quality and climate change are issues that have complex socio-political implications. The potential impact of atmospheric processes and climate change on society is of crucial importance and therefore requires advanced scientific research into the causes, consequences and mitigation of such changes, in order to develop efficient strategies.

At European and global level there is a growing preoccupation on strategies and optimal actions for reduction of environmental pollution. The Directive 2008/1/EC of the European Parliament and of the Council of 15 January 2008 [1] concerning integrated pollution prevention and control (IPPC Directive) is the main Directive which create the legal framework for reduction of environmental pollution.

The IPPC Directive aims at a high level of protection for the environment as a whole. The Directive requires industrial activities with a high pollution potential to use 'best available techniques' (BAT). 'Best available techniques'

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are defined by the IPPC Directive as the most effective and advanced stage in the development of activities and their methods of operation which indicate the practical suitability of particular techniques for providing in principle the basis for emission limit values designed to prevent and, where that is not practicable, generally to reduce emissions and the impact on the environment as a whole.

In Romania, the IPPC Directive was transposed by G.E.O. no. 34/2002 on prevention, reduction and integrated pollution control, approved by Law no. 645/2002 [2].

For reduction of pollutant emissions, Best Available Techniques (BAT) are mainly considered: the use of fuel with a low polluting level; improvement of technology and combustion equipment according to fuel type, plant technical characteristics and local environmental conditions; the implementation of reduction techniques for sulphur dioxide ($SO_2$ < 350 mg/$N^3$) and particulate matter emissions (PM < 15 mg/$N^3$).

European policies have resulted in some decrease in $SO_2$ emissions levels. Still, metallurgical plants are one of the major industrial pollutants emitting mainly gases ($SO_2$, $NO_x$ and $CO_2$) and particulate matters containing heavy metals.

These emissions also depend on the technology used in the metallurgical process. “In addition to the primary emissions, the gas to particle conversion processes, which depend on the meteorological conditions (mainly insolation and humidity), also give rise to considerable volumes of secondary particulate pollutants after the oxidation of sulphur and nitrogen oxides” [3].

The atmospheric pollutants emitted by metallurgical plants are then dispersed and carried over large distances (several to hundreds of kilometres) and finally deposited on the ground. The travel distances depend on a number of factors, such as stack height, grain size, and density of the individual particles [3]. Also, the deposition of pollutants is influenced by regional climatic conditions and physiographic features.

Atmospheric modelling is used to aid policy making on environmental issues and pollution control. Several models have been developed for the estimation of atmospheric impact, on a wide spectrum from easy to use regulatory models to more complex ones applicable to specific scenarios.[4].

The description of dispersion processes using models offers the possibility to study the atmosphere from mathematical and engineering point of view. Simulations offer the possibility to test the functionality and the dynamic behaviour of the model.

A complex modelling study requires various sets of data about source terms, emissions, imissions, vulnerability, local meteorology, terrain data etc.. Most of the air dispersion models have been developed for the prediction of downwind concentration of air pollutants and for the estimation of short-term and medium-term effects of these pollutants. The quality of results obtained using these modelling systems depend mostly on the versatility and quality of input data and the right choice of the model [5].

## RESULTS AND DISCUSSION

The case study is built on a non-ferrous metallurgic plant which has zinc and lead as its main products. These products, indispensable to other economical sectors, are obtained in a pyro-metallurgical way, in a process that allows both zinc and lead to be obtained simultaneously form collective or selective sulphide concentrates.

In the first phase of the process these sulphides are oxidized, resulting in the zinc-lead agglomerate, which will be later processed in the furnace, and gases with high concentrations of $SO_2$ and PM containing heavy metals sent to a dry purification process using hose filters. In the past the gases cleaned of PM's were released into the atmosphere through the 250 m stack. This stack height assures a high level of dispersion.

The levels imposed by G.E.O. No. 34/2002 [2] were reached by running these gases through a desulphurization plant which uses the limestone-gypsum wet process [6]. This type of gas desulphurization has a series of advantages in comparison with desulphurization through a sulphuric acid producing process. By installing a desulphurization process the problems concerning the metallurgic plant's $SO_2$ pollution are solved.

For the comparison of the situations, regarding the $SO_2$ emissions before and after the installation of the desulphurization system, two different sets of simulations were performed, considering a three days period for which meteorological and point monitoring imission data was available.

The simulation results regarding to the $SO_2$ dispersion before the installation of the desulphurization system show maximum ground level imission concentrations much higher than the limits established in the G.E.O. No. 592/2002 [7] for the protection of human health for 1, 3 and 24 hr averages. The maximum concentrations are shown in table 1.

**Table 1.** Maximum imission concentrations obtained before desulphurization

| | Maximum concentration [μg/m$^3$] | Limit value for protection of human health (G.E.O. No. 592/2002) [μg/m$^3$] |
|---|---|---|
| 1 hr average | 2880 | 350 |
| 3 hr average | 1136 | 125 |
| 24 hr average | 337 | 20 |

These maximum concentrations were obtained in stable (Pasquill class 6) atmospheric conditions in different imission points. Figure 1 shows the areas where concentrations are higher than the 1 hr average limit of concern established (C > 350 μg/m$^3$).

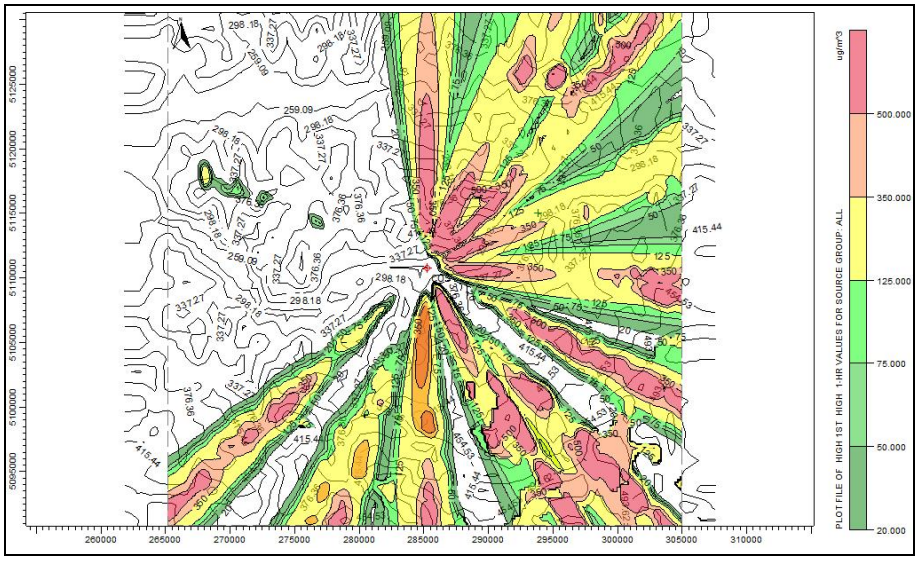


**Figure 1.** Maximum 1 hr average concentrations obtained before desulphurization

Figure 2 shows the areas where concentrations are higher than the 3 hr average limit of concern established (C > 125 μg/m$^3$).

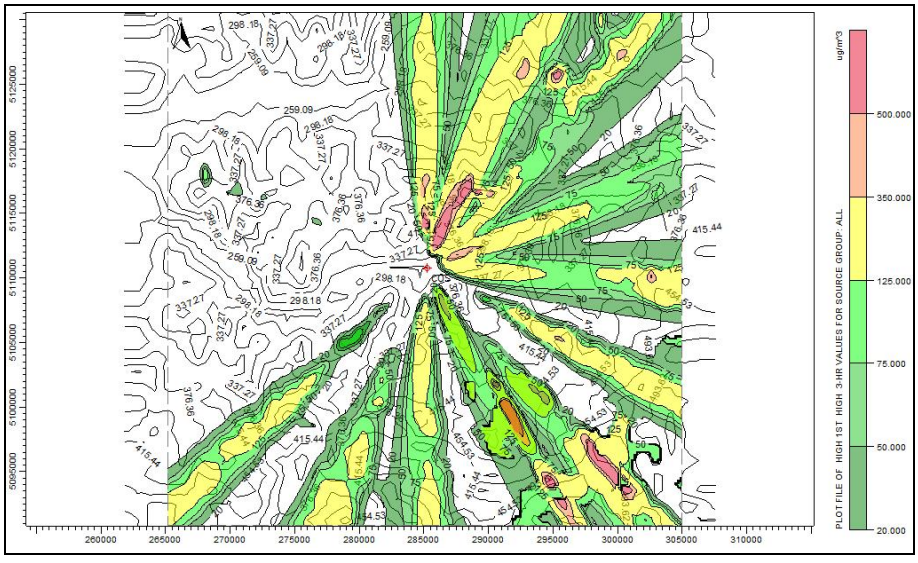


**Figure 2.** Maximum 3 hr average concentrations obtained before desulphurization

Figure 3 shows the areas where concentrations are higher than the 24 hr average limit of concern established (C > 20 μg/m$^3$).

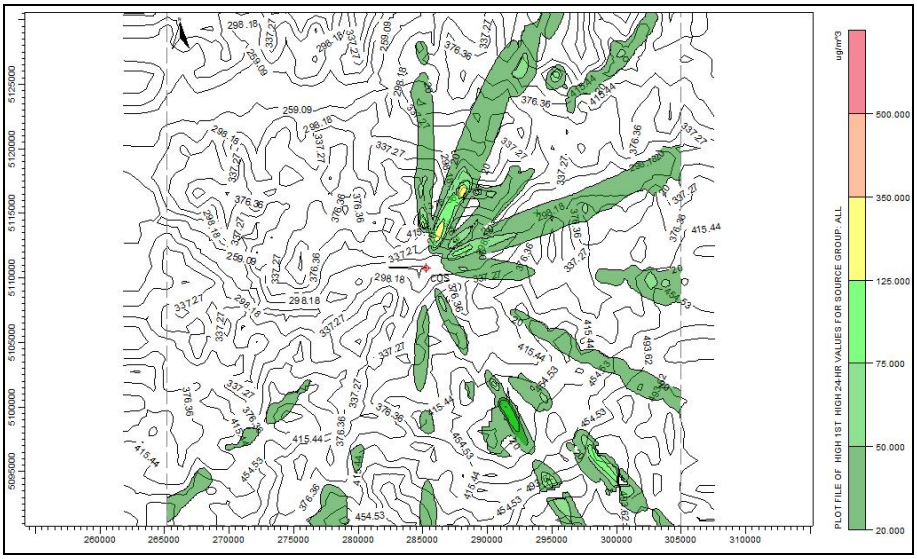


**Figure 3.** Maximum 24 hr average concentrations obtained before desulphurization

Simulation results were qualitatively correlated with imission concentration data measured at an automatic point monitor situated at 8 km from the emission source. The maximum 1hr average concentration obtained in simulation for the monitoring receptor point is 253 μg/m$^3$. It represents the maximum concentration that was reached using a 1 hour averaging period. The values of 1 hr concentrations are shown is figure 4.

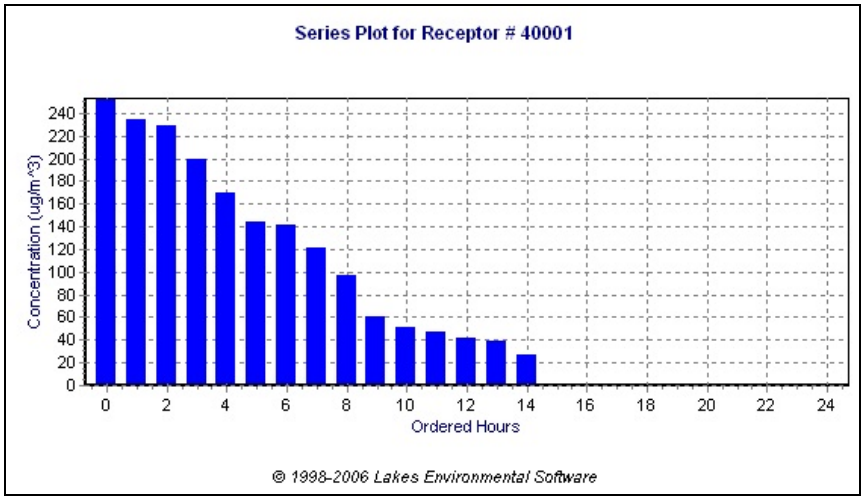


**Figure 4.** Maximum 1 hr average concentrations in monitoring receptor point

A qualitative comparison can be made between the concentration results obtained with simulations and the ones measured by the point monitor, presented in table 2.

**Table 2.** 1 hr average concentration measured at point monitor

| **1 hr average concentrations (µg/m³)** | | | |
|---|---|---|---|
| **Hour** | **Day 1** | **Day 2** | **Day 3** |
| 1:00 | 113.96 | 8.65 | 36.02 |
| 2:00 | 288.04 | 16.49 | 27.16 |
| 3:00 | 349.13 | 17.29 | 24.25 |
| 4:00 | 134.41 | 16.64 | 26.96 |
| 5:00 | N/A | 37.74 | 24.58 |
| 6:00 | 74.55 | 84.87 | 34.29 |
| 7:00 | 79.16 | 99.68 | 31.97 |
| 8:00 | 143.96 | 58.72 | 63.56 |
| 9:00 | 505.24 | 43.16 | 77.93 |
| 10:00 | 105.14 | 136.15 | 64.51 |
| 11:00 | 361.7 | 161.61 | N/A |
| 12:00 | 337.59 | 209.12 | 51.46 |
| 13:00 | 107.23 | 106.47 | 57.51 |
| 14:00 | 204.92 | 101.15 | 36.67 |
| 15:00 | 62.8 | 55.26 | 40.16 |
| 16:00 | 44.69 | 48.51 | 22.11 |
| 17:00 | 88.29 | 195.03 | 19.96 |
| 18:00 | 36.47 | 34.35 | 17.83 |
| 19:00 | 15.61 | 19.46 | 16 |
| 20:00 | 12.5 | 100.78 | 14.22 |
| 21:00 | 10.7 | 163.03 | 13.19 |
| 22:00 | 9.34 | 176.94 | 15.59 |
| 23:00 | 8.66 | 95.83 | 37.8 |
| 24:00 | 8.4 | 52.88 | 32.28 |
| ***Daily average*** | ***134.89*** | ***84.99*** | ***34.990*** |

Comparing the simulation results with the measured ones, it can be observed that the values have the same order of magnitude, but the simulation results show slightly lower values.

The simulation results regarding to the $SO_2$ dispersion after the installation of the desulphurization system show maximum ground level imission concentrations approx. 20 times lower than the limits established in the G.E.O. No. 592/2002 [7] for the protection of human health for 1, 3 and 24 hr averages. The maximum imission concentrations are shown in table 3.

**Table 3.** Maximum imission concentrations obtained after desulphurization process

| | Maximum concentration [µg/m$^3$] | Limit value for protection of human health (G.E.O. No. 592/2002) [µg/m$^3$] |
|---|---|---|
| 1 hr average | 16 | 350 |
| 3 hr average | 6 | 125 |
| 24 hr average | 1 | 20 |

In figure 5 the situation of 1 hr average imission concentrations after the installation of the desulphurization system are represented.

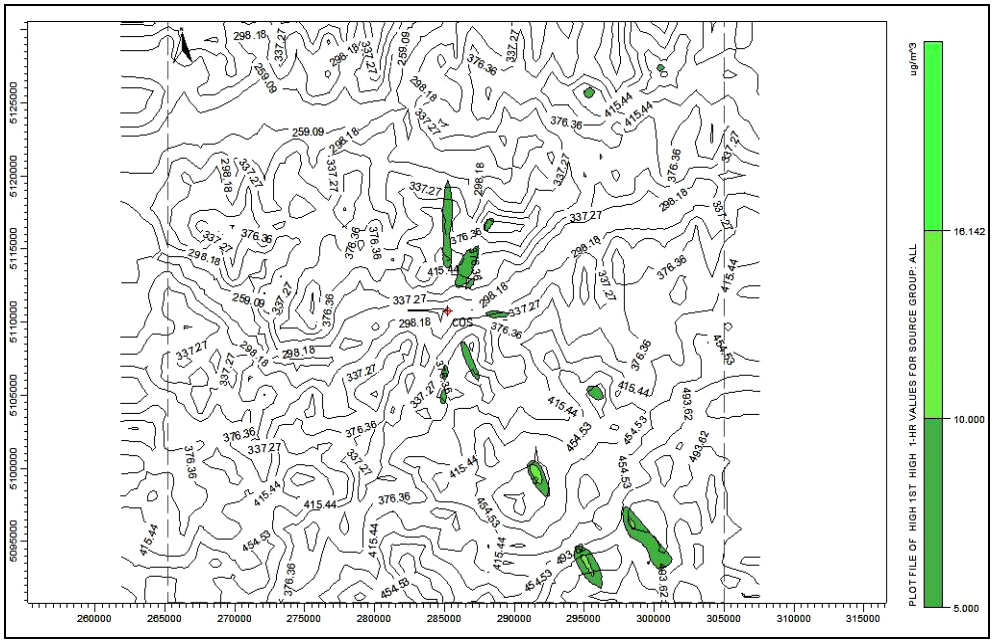


**Figure 5.** Maximum 1 hr average concentrations obtained after desulphurization process

It can be concluded that the installation of desulphurization system reduced the $SO_2$ emissions and imissions significantly and the obtained concentration values are well below the limits imposed by national and EU legislation.

## CONCLUSIONS

The introduction of G.E.O. no. 34/2002 on prevention, reduction and integrated pollution control, approved by Law no. 645/2002, brought a significant contribution to the reduction of atmospheric pollutants such as $SO_2$, increasing air quality near industrial sites.

The objective of the case study was to determine the contribution of the desulphurization system in the reduction of the $SO_2$ emissions and imissions.

The simulation results, obtained for the situation before the installation of the desulphurization system, were compared with point monitoring data, showing a good qualitative correlation.

The simulations performed for the conditions after the installation of the desulphurization system show a significant reduction in the ground level concentrations of $SO_2$, well within the range imposed by national and European legislation.

In the future, imission monitoring measurements will be made and correlated with the simulations performed for the current process conditions.

## EXPERIMENTAL SECTION

### Air quality modelling of $SO_2$ emissions at a metallurgical plant

The case study focuses on the evolution of the air quality in the metallurgical plant area from the point of view of $SO_2$ emissions and imissions before and after the installation of the desulphurization system.

The dispersion simulations were performed using the ISC AERMOD View software package, using the Industrial Source Complex Short Term (ISCST3) model which is a Gaussian plume dispersion model that predicts air concentrations around point or area sources using emission rates (flux) and meteorological conditions as model inputs. ISCST3 is applicable for estimating short-term ambient impacts from point, area, and volume sources out to a distance of about 50 kilometres.

The meteorological data was obtained from the local station near the studied plant for a three days period and was processed using Rammet View, which is part of the ISC AERMOD View software package. Based on this data, Rammet View estimates the atmospheric stability class and mixing layer height for every hour taken into study.

The input data required by ISCST3 model includes: type of dispersion (dry/wet), data regarding the substance (type, dispersion coefficient), complex terrain data (computed by AERMAP, using GTOPO30 topographic data), source data (number of sources, location, height, release rate, release temperature and velocity), receptor data (defined grid).

ISC AERMOD View outputs results in the graphical form of dispersion maps superimposed on topographic maps. 10 maximum concentration scenarios can be computed simultaneously averaged on 1, 2, 3, 4, 6, 8, 12, 24 hours, one month, a specified period up to a year [8].

The in-situ monitoring data was collected by an automated device for the continuous monitoring of atmospheric $SO_2$ using UV fluorescence. The device employs a proprietary, internal dry-method sampling. The dry-method, due to its low maintenance requirements, continuous monitoring and instantaneous analysis of gas, is a preferred method for monitoring atmospheric $SO_2$ [9].

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# BIODEGRADATION BEHAVIOR OF LACTIC ACID, ETHYLENE GLYCOL AND TEREPHTHALIC ACID COPOLYMERS UNDER CONTROLLED COMPOSTING CONDITIONS

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**ABSTRACT.** The copolymers were synthesized by the microwave-assisted polycondensation of L-lactic acid (LA), ethylene glycol (EG) and terephthalic acid (TPA) (different ratio of monomers). Biodegradation tests of the obtained copolymers and microcrystalline cellulose (MC) were performed under controlled composting conditions using a standard test method (ISO 14855-1, 2005). It was observed that the biodegradation of copolymer with higher quantity of lactic acid was faster and more effective than the biodegradation of copolymer with smaller quantity of lactic acid.

***Keywords:*** *L-lactic acid, copolymer, microwave-assisted polycondensation, compost, biodegradation*

## INTRODUCTION

Recently, an amplified contamination of the environment has been observed due to increasing volume of plastic wastes that persist for many years after disposal. The use of biodegradable polymers, namely in applications with a short life cycle of the products (packaging) would be an ecologically viable alternative for reducing the solid plastics waste [1]. The biodegradability of polymers is predetermined by their chemical and/or physical structure. In developing biodegradable polymers, it is very important to synthetize polymers having both satisfactory mechanical properties and biodegradability [2]. Aiming this target, in the last few years, polyesters have been considered the best candidates to replace traditional polymers because of their potential biodegradability given by their hydrolysable ester bonds. However, the biodegradability of aliphatic and aromatic polyesters is completely different, aliphatic polyesters are easily susceptible to microbial attack while aromatic polyesters such as poly(ethylene terephthalate, (PET) are not significantly influenced by the hydrolytic degradation [3-5].

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Recently, in order to decrease the average sequence length of PET and acquire good degradability, copolymerization with biodegradable aliphatic polyesters such as poly(butylene succinate) (PBS), poly(ε-caprolactone) (PCL) and poly(lactic acid) (PLA) was carried out [6]. PET/PEG, poly(ethylene glycol) copolymers and poly(butylene terephthalate)/PEG copolymers were synthesized by polycondensation between ethylene diols, dimethyl (terephthalate) and PEG monomers or by the macromolecular transesterification method, starting from PET and PEG macromers [7, 8]. Due to their low cost and good biodegradability, these copolymers may have different biomedical and ecological applications [9].

The aim of this work was to synthetize medium molecular weight copolymers of L-lactic acid, terephthalic acid and ethyleneglycol using microwave radiation. The obtained copolymers were characterized for acid value, hydroxyl value and number average molecular weight. The obtained copolymers were biodegraded under controlled composting conditions according to ISO 14855-1 [10].

## RESULTS AND DISCUSSION

### Synthesis of copolymers

Copolymers of lactic acid, ethylene glycol and terephthalic acid were obtained by microwave-assisted polycondensation. Copolymers containing high amounts of L-lactic acid (**1** and **2**) were isolated as yellow solids while copolymers containing less amounts of L-lactic acid (**3** and **4**) were isolated as light brown solids. Details regarding the synthesis and characterization of the obtained copolymers were summarized in Table 1. The obtained copolymers average molecular weight ranged from 9500 to 11000. The main advantages of microwave-assisted polymerization are: short reaction time, low power consumption and no need for nitrogen protection or vacuum.

**Table 1.** Physical-chemical properties of the compost before (a) and after (b) biodegradability testing.

| Parameters | MC | | 1 | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | a | b | a | b | a | b | a | b | a | b |
| pH | 7.91 | 8.24 | 7.91 | 7.10 | 7.91 | 7.23 | 7.91 | 7.98 | 7.91 | 8.04 |
| Total dry solids (%) | 50.9 | 48.9 | 50.9 | 48.2 | 50.9 | 48.5 | 50.9 | 49.3 | 50.9 | 49.3 |
| Volatile solids (%) | 28.1 | 25.0 | 28.1 | 18.4 | 28.1 | 20.3 | 28.1 | 24.1 | 28.1 | 25.0 |
| Moisture (%) | 50.8 | 51.9 | 50.8 | 52.3 | 50.8 | 52.0 | 50.8 | 51.4 | 50.8 | 51.8 |
| C/N ratio | 14.0 | 13.9 | 14.0 | 10.3 | 14.0 | 10.5 | 14.0 | 11.2 | 14.0 | 13.1 |

## Biodegradation studies

The biodegradability of microcrystalline cellulose (MC) and lactic acid, ethylene glycol and terephthalic acid copolymers was determined under controlled aerobic composting conditions in the laboratory. In accordance with biodegradability testing, physical-chemical properties were determined both before (a) and after biodegradation testing (b) (Table 1). All physical-chemical parameters, of the inoculums gave suitable composting conditions according to ISO 14855-1: pH (7-9), volatile solid (less than 30%), moisture content (50-55%) and C/N ratio (10-40) [10].

Figure 1 (a and b) display the biodegradation curves of copolymers **1**-**4** and MC under controlled composting condition at 58°C according to ISO 14855-1. It can be seen that MC started to degrade after 5 days of induction, while copolymers **1**-**4** only after 10 days of induction. Copolymer **1** had the fastest speed of biodegradation. The biodegradation curves of all the samples tended to level off after 60 days. The ultimate degrees of biodegradation of **1**, **2**, **3** and **4** were 92.0%, 84.1%, 70.2% and 67.9%, respectively, after 60 days of biodegradation according to ISO 14855-1.

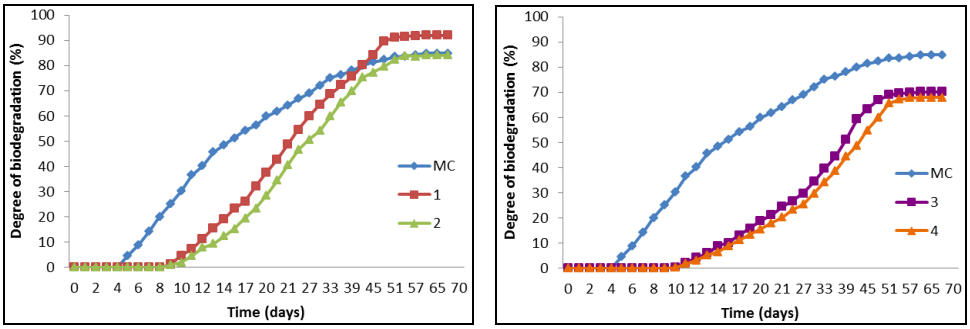


**Figure 1.** Biodegradation curve of (a) copolymers PLA-EG-TPA (**1** and **2**) and (b) copolymers PLA-EG-TPA (**3** and **4**) under controlled composting conditions.

The aerobic biodegradation experiments were valid because the degree of biodegradation of reference material (MC) was over 70% after 45 days. The relative degree of biodegradation of a really biodegradable material, in less than 6 moths, under controlled composting conditions should not be less than 90% [11]. The relative degrees of biodegradation of **1**, **2**, **3** and **4** were 108%, 99.1%, 82.7% and 80%, respectively, relative to microcrystalline cellulose as a reference material. According to the above results, copolymers **1** and **2** were biodegradable materials. The order of biodegradation degree was

**1** > **2** > **3** > **4**. As observed, the biodegradation of copolymer with high amount of L-lactic acid was faster than the biodegradation of copolymer with low quantity of L-lactic acid. Similar results were reported by Iovino *et al.* [12].

Consequently, it can be concluded that the biodegradation degree of tested copolymers (**1**-**4**) was closely related to their composition. This result is in good agreement with data reported by Soni *et al.* [13]. Copolymers **1** and **2** were completely biodegraded under controlled composting conditions, whereas the copolymers **3** and **4** containing lesser amount of L-lactic acid were not fully biodegraded within that time period.

## CONCLUSIONS

PLA-EG-TPA copolymers were synthesized by the microwave-assisted polycondensation of L-lactic acid, terephthalic acid and ethylene glycol (different monomer ratio). The obtained copolymers were characterized using acid value, number average molecular weight and biodegradation behavior under controlled composting conditions (ISO 14855-1). The inoculum parameters (pH, volatile solid, moisture content, and C/N ratio) determined before and after biodegradation tests indicated good composting conditions during experiments. The biodegradability of plastics is a complex process and is influenced by the nature of each material. According to this work, the degree of biodegradation, **1** > **2** > **3** > **4**, was correlated with the copolymer composition (the biodegradation of copolymers containing high amount of lactic acid was faster than the biodegradation of copolymers containing reduced amount of lactic acid).

## EXPERIMENTAL SECTION

### Materials

Thin-layer chromatography grade cellulose powder with particle size <20 μm, all chemicals and materials were purchased from Merck. 85-90 % (w/w) aqueous solution of monomer L-lactic acid was 99% optically pure according to the manufacturer. All chemicals were used as received without further purification or processing. Deionized water was prepared using a Milli-Q system (Millipore, Watford, Hertfordshire, UK). The compost medium was made of organic fraction of approximately 3-months old mature compost obtained from organic agriculture waste (39% vegetable waste, 35% fruits pells, 12% wood chips and 14% old compost).

### Apparatus

The apparatus used for polycondensation was a Panasonic domestic microwave oven (2450 MHz, 1300 W) without any modifications. All reactions were carried out in a hood with strong ventilation. The total organic carbon content was determined by a Multi N/C 2100S Analyzer (Analytic Jena, Germany).

## Preparation of PLA-EG-TPA

The microwave-assisted polycondensation of L-lactic acid (LA), terephthalic acid (TPA) and ethylene glycol (EG) was carried out simply by heating different ratio of monomers in a domestic microwave oven according to values from Table 2. After the addition of $SnCl_2$ $2H_2O$ (0.2 wt.-%) as catalyst, at 10 min, the reaction was continued for 10 min (total, 20 min). After slow cooling of the reaction mixture at room temperature, the obtained mixture was dissolved in approximately 15 ml chloroform. A volume of 20 ml deionized water was added and the obtained precipitated polymer was collected, then dried at room temperature under reduced pressure (1300 Pa) obtaining a white powder. The obtained copolymers were further used for biodegradability testing.

**Table 2.** Summary of the synthesis and characterization of the PLA-EG-TPA copolymers (**1**-**4**).

| Copolymer | LA (mol) | TPA (mol) | EG (mol) | Acid Value | Hydroxyl value | $M_n$ |
|---|---|---|---|---|---|---|
| **1** | 20 | 1 | 1 | 0.021 | 0.020 | 9524 |
| **2** | 2 | 1 | 1 | 0.020 | 0.022 | 10000 |
| **3** | 1 | 10 | 10 | 0.019 | 0.024 | 10526 |
| **4** | 1 | 20 | 20 | 0.018 | 0.027 | 11111 |

## Characterization

The obtained copolymers were characterized for acid value, number average molecular weight and biodegradability.

### *Acid Value (C)*

The acid value was determined according ASTM D 1639, by dissolving a known amount of polymeric material in ethanol and titrated against 0.1 N KOH using phenolphthalein as indicator. The acid number was calculated by the following expression:

$$C = \frac{56.1 \times V \times N}{m} \tag{1}$$

where V - volume of KOH solution, ml; N – normality of KOH, mol/l; m – weight of polymeric sample, g [14].

### *Hydroxyl value*

Hydroxyl value was determined according ASTM D 2849 (method A) by dissolving approximately 0.50 g of sample in 50 ml phthoylating mixture and hydrolyzed by adding 100 ml of cooled distilled water in another flask. 20 ml benzene was added under vigorous stirring and the resulting solution was

titrated against 0.5 N standardized KOH, using phenolphthalein as an indicator. A blank sample was also carried out. The hydroxyl value was calculated by following equation:

$$\text{Hydroxyl value} = \frac{56.1 \times (V_1 - V_2) \times N}{m} \quad (2)$$

where $V_1$ - volume of 0.5 KOH titrated for the blank, $V_2$ - volume of 0.5 KOH titrated for the sample; N, normality of KOH; m - weight of the polymer sample, g [15].

***Number average molecular weight (Mn)***

The number average molecular weight was calculated according to the following relationship:

$$M_n = \frac{n \times 100}{C} \quad (3)$$

where n - functionality of polymer; C - acid number.

***Biodegradation tests***

This study investigated the degradability potential of PLA-EG-TPA copolymers obtained by microwave-assisted polycondensation, under simulated aerobic degradation conditions. The synthetized samples were used as obtained, without further purification. The aerobic degradation testing conditions were performed in a laboratory scale installation in accordance to ISO 14855-1 [10]. This standard specify a general method to determine the ultimate aerobic biodegradability of polymeric materials, under controlled composting conditions by measurement of the amount of evolved carbon dioxide and the degree of biodegradation of the test materials at the end of the test. The test material is mixed with the inoculum and composted under monitored and controlled temperature, aeration and humidity during the 70 days of experiments. The used inoculums consist of 3-months old mature compost obtained from organic agriculture waste. The compost was sieved using a 4 mm sieve and its physical-chemical properties were determined: pH, total dry solids, volatile solids, moisture content and C/N ratio [16-21]. All the properties of the compost are in accordance with ISO 14855-1 requirements. This inoculum was stored at 5ºC for 7 days. The experimental study was performed, in triplicates, in glass flasks of approximately 2 L internal volume containing: (*i*) test material (compost + copolymer), (*ii*) blank (compost) and (*iii*) reference material (compost + MC). In all cases the compost and test material were mixed in the ratio of 60-360 g (dry mass), transferred into a glass flasks and introduced in a water bath at 58ºC and purged with 50 mL/min compressed air (carbon dioxide - free) flow rate. The carbon dioxide from the compressed air was removed by passing the air through a solution of 0.05 mol/l NaOH. During the experiment, the moisture

content of the mixture was maintained at around 45-55% and the compost was regularly mixed to ensure maximal homogeneity. The activated compost used in this study produced 55 mg $CO_2$/gram of volatile solids over the first 10 days of the test [10].

The carbon dioxide produced during the biodegradation process was trapped in 0.05 mol/l NaOH connected with the composting glass flasks by polyethylene tubes. The carbon dioxide traps were changed from daily to three times a week depending on the degradation rate. The total organic carbon content from each trapping solution was determined by Multi N/C 2100S Analyzer, Analytic Jena, Germany [22].

According to ISO 14855-1, the theoretical amount of $CO_2$ which can be produced by the polymeric material ($ThCO_2$, g/flask) was calculated using the following equation:

$$\mathrm{ThCO}_2 = M_{TOT} \times C_{TOT} \times \frac{44}{12} \tag{4}$$

where $M_{TOT}$ – total dry solids in the polymeric material added into the composting flask at the start of the experiment, g; $C_{TOT}$ – proportion of total organic carbon in the total dry solids in the polymeric sample; 44 and 12 – the molecular mass of $CO_2$ and atomic mass of C, respectively.

The degree of biodegradation of test materials for each measurement interval determined from the released cumulative amounts of $CO_2$ was calculated according to the following equation:

$$(\%)\mathrm{Biodegradation} = \frac{(CO_2)_S - (CO_2)_B}{\mathrm{ThCO}_2} \times 100 \tag{5}$$

where $(CO_2)_S$ – the amount of $CO_2$ evolved in each composting flask containing polymer sample, g/flask; $(CO_2)_B$ – the amount of $CO_2$ evolved in blank flask, g/flask [23].

## ACKNOWLEDGMENTS

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STUDIA UBB CHEMIA, LVII, 1, 2012 (p. 75 - 82)

# ANALYSIS OF SOME NUTRITIONAL SUPPLEMENTS DERIVED FROM *SEA BUCKTHORN* AND *BLACK CURRANT*

**ANITTA PUSCAS[a], MIHAI INCEU[a], VIRGIL DANCIU[a], ANAMARIA HOSU[a], CLAUDIA CIMPOIU[a,*], DESPINA GOMOIESCU[b]**

**ABSTRACT.** The interest in the consumption of fresh fruits, products and nutritional supplements made from them is due to their content of bioactive nutrients and their importance as dietary antioxidants. *Sea Buckthorn* and *Black Currant* were used in both Europe and Asia for centuries. In this context, the aims of this work are to obtain a TLC fingerprinting of some nutritional supplements derived from *Sea Buckthorn* and *Black Currant* in order to establish their origin and conditioning type and, also, to correlate their antioxidant capacity with their content of biologically active compounds.

***Keywords:*** *Antioxidant capacity, Total polyphenolic content, Nutritional Supplements, TLC, Fingerprinting, Sea Buckthorn, Black Currant.*

## INTRODUCTION

Many human diseases including different neurodegenerative disorders and cancers are considered to be due to the oxidative damages caused by the free radicals and reactive oxygen species. Antioxidants, produced by the human body or available through the diet, can protect human cells against these oxidative damages.

Studies have shown that polyphenols significantly contribute to the total antioxidant activity of many fruits and vegetables, with high flavonoid content [1]. Polyphenols are one of the most common classes of phytochemical compounds extremely important in terms of morphological and physiological characteristics [2]. The biological importance of polyphenolic compounds is closely related to their antioxidant, anti-allergic, anti-inflammatory and anti-microbial properties, as well as to their cardio-protective and vasodilator effects [3, 4]. All these led interest in discovery and identification of new such compounds, as well as on their exploitation. These results in the increased

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consumption of fruits, vegetables and products obtained from them and in the appearance on market of a high number of nutritional supplements with high contents of polyphenols [5]. Even though many plants are containing polyphenolic compounds with important health effects, their consumption is still seldom.

*Sea Buckthorn* (*Hippophae rhamnoides L.*) is a perennial shrub belonging to the *Elaeagnacaea* family. Over the world, there are known 6 species and 12 subspecies. Commercially available *Sea Buckthorn* is a hardy, multipurpose plant with orange, red or yellow berries with a strong ability to fix atmospheric nitrogen. Their importance is related to the prevention ability against cardiovascular diseases and cancer, as well as in treatment of skin problems, burns, digestive tract disorders, senility, inflammation, radiation and to improve the capacity of the immune system [6]. *Sea Buckthorn* fruits are used in food industry, pharmacy and cosmetics due to their high content in vitamin C (over 400-800 mg/g of fresh juice), and content of A, B1, B2, B6, B9, E, K, PP and F vitamins. *Sea Buckthorn* fruits also contain bioactive compounds such as cellulose, β- carotene, Ca, Mg, K, Na, proteins and different complex oils [7].

*Black Currant* (*Ribes Nigrum*) belongs to the *Grossulariaceae* family being widespread in the temperate areas of Europe and Western Asia. It grows spontaneously in shrubs, in alpine forests, but it is also cultivated for its fruits and leaves, which are used in medicine because of their content in biologically active compounds. *Black Currant*'s leaves contain tannins, vitamin C and traces of green essential oils. Their fruits contain about 150mg/100 g of vitamin C, B vitamins complex, organic acids (citric acid, malic acid), fat oils, sugars, anthocyanins, flavonoids, Ca, etc [8].

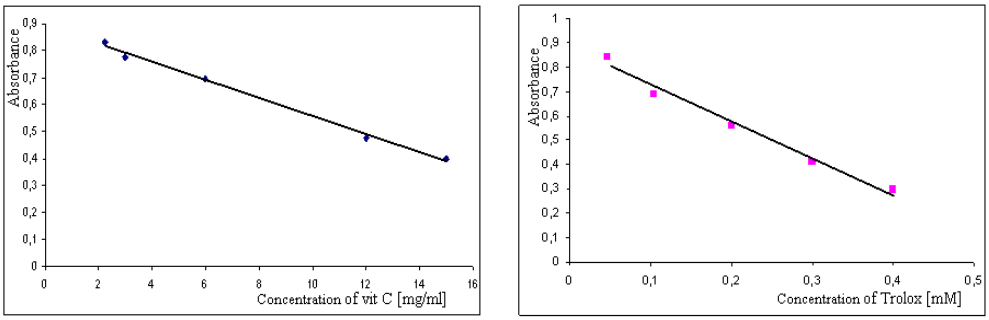
Recently, due to the modern analytical techniques more and more studies were made on different fruits and nutritional supplements obtained from them because of their high content in different biological active compounds. There is an increasing interest in the use of chromatographic methods for the analysis of chemical compounds from Sea Buckthorn and Black Currant [9], thin layer chromatography (TLC) being frequently used for the separation and the determination of natural constituents [10].

The aim of this study is the evaluation of the antioxidant activity and of the total polyphenolic content and the TLC fingerprinting of some nutritional supplements obtained from *Sea Buckthorn* and *Black Currant,* in order to establish their origin and the conditioning type.

## RESULTS AND DISCUSSION

The analyzed nutritional supplements were: alcoholic tincture of *Sea Buckthorn* and *Black Currant* (15% in 30% ethanol), dried fruits, iso-maltose impregnated with juice of *Sea Buckthorn* and *Black Currant*, and *Sea Buckthorn* and *Black Currant* balsamic vinegar.

In the first step, two complementary methods, namely DPPH [11] and ABTS [12] were used for determination of the antioxidant activities of these nutritional supplements. The antioxidant capacities were expressed as vitamin C or trolox equivalents on the basis of calibration curves (figure 1). The results are presented in Table1.




**a** **b**

**Figure 1.** The calibration curves for: a - DPPH method (y=-0,0336x+0,8924; r=0,9973); b - ABTS method (y=-1,5124x+0,8784; r=0,9818).

The experimental results (Table 1) show that DPPH and ABTS methods provide similar results concluding that both methods could be used for the determination of the antioxidant activity of nutritional supplements. Also, it can be observed that in both cases the *Black Currant* nutritional supplements possess a higher antioxidant activity than those obtained from *Sea Buckthorn*. It can be remarked that all the iso-maltose impregnated with fruit juice supplements have the lowest antioxidants activity.

The total polyphenolic contents of the nutritional supplements were determined using the Folin-Ciocalteu method and were expressed in μg gallic acid/mL extract (Table 1), using the calibration curve (Figure 2). The experimental results show that the nutritional supplements derived from *Black Currant* contain the highest level of polyphenols, while the *Sea Buckthorn* nutritional supplements contain a lower quantity of polyphenols. Also, iso-maltose impregnated with fruit juice supplements contains the lowest quantity of polyphenols.

The total phenolic contents are correlated with the antioxidant activities determined both by DPPH and ABTS assays, considering each analyzed nutritional supplements.

In the second step, the TLC analysis of same nutritional supplements derived from *Sea Buckthorn* and *Black Currant* were used in order to obtain their fingerprint. The TLC fingerprints offer valuable information regarding the active compounds present in the samples and the semi-quantitative estimation of their composition.

**Table 1.** The antioxidant activity and total polyphenolic content of analysed nutritional supplements

| No. | Samples | Antioxidant activity | | Total polyphenolic content μg gallic acid /mL |
|---|---|---|---|---|
| | | mg vit C/mL | μmol trolox/mL | |
| 1. | Balsamic vinegar with Sea Buckthorn | 135.6 | 1.050 | 681 |
| 2. | Alcoholic extract of Sea Buckthorn | 110.0 | 0.649 | 590 |
| 3. | Dry Sea Buckthorn | 86.6 | 0.281 | 584 |
| 4. | Lyophilized Sea Buckthorn extract | 34.8 | 0.328 | 153 |
| 5. | Isomalt + Sea Buckthorn juice | 3.5 | 0.039 | 28 |
| 6. | Balsamic vinegar with Black Currants | 627.4 | 6.261 | 2203 |
| 7. | Alcoholic extract of Black Currants | 319.8 | 4.578 | 1951 |
| 8. | Dry Black Currants | 708.8 | 1.393 | 1175 |
| 9. | Dry Black Currant extract | 549.0 | 4.549 | 1832 |
| 10. | Isomalt + Black Currant juice | 0.9 | 0.010 | 27 |
| 11. | Isomalt + Sea Buckthorn juice + Black Currant juice | 0.2 | 0.001 | 14 |

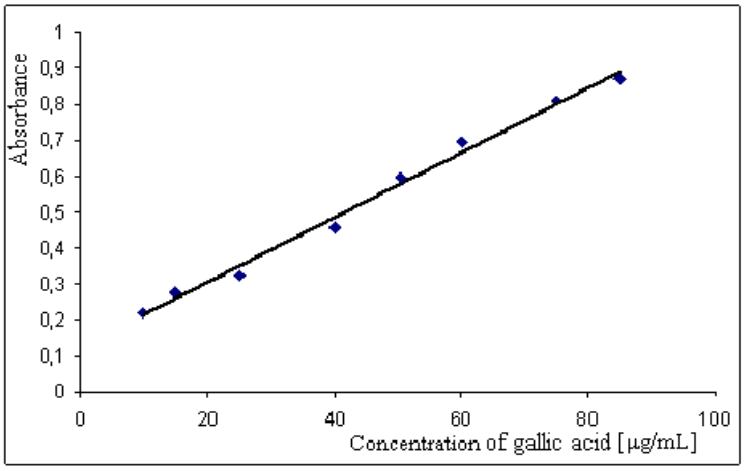



**Figure 2**.The calibration curve for determination of total polyphenolic content ($y=0.009x+0.1247$; $r^2=0.9854$).

The TLC separation of analyzed supplements (Figure 3) showed that even some components occurred in every sample, there are significant differences related to the composition of the samples and to the concentration of bioactive compounds.

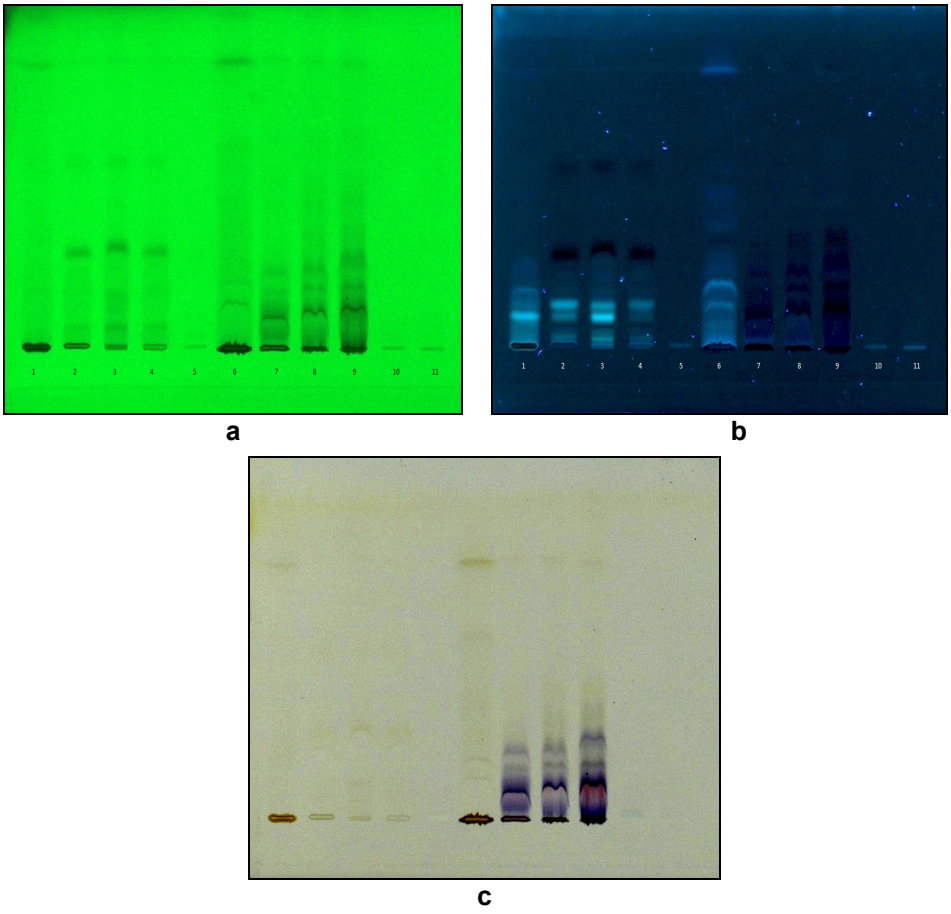



**Figure 3.**The fingerprint of samples (according to table1) in:
UV light at a - 254nm and b – 366nm; c- VIS light.

The comparison between the fingerprints of supplements obtained from the same fruit revealed that the composition depends on their conditioning type. TLC fingerprinting method allows the analysis of bioactive compounds in different nutritional supplements and could be applied not only to their chemotaxonomic classification but also in qualitative and semi-quantitative determinations.

## CONCLUSIONS

DPPH and ABTS methods can be applied for the determination of the antioxidant activity of nutritional supplements derived from *Sea Buckthorn* and *Black Currant*, the obtained results being similar. The results showed that the antioxidant activity and the total polyphenolic content of the supplements obtained from *Black Currant* are higher than those derived from *Sea Buckthorn*. The supplements obtained by impregnation of iso-maltose with fruit juice have the lowest antioxidant activity and polyphenolic content irrespective of fruits. These results confirm that the antioxidant activity and polyphenolic content depend on conditioning type of supplements.

The chromatographic fingerprintings show differences between the nutritional supplements derived from *Sea Buckthorn* and those derived from *Black Currant.* The analyzed supplements contain different quantities of bioactive compounds and their concentration depends on their conditioning type. The nutritional supplements based on iso-maltose contain the smallest amount of active compounds, while those based on *Black Currant* contain the highest concentration of active compounds.

## EXPERIMENTAL SECTION

### Materials and apparatus

All chemicals and reagents were analytical grade and were purchased from Merck Germany. Chromatographic plates were purchased from Merck (Darmstadt, Germany).

Fresh fruits and nutritional supplements derived from *Sea Buckthorn* and *Black Currant* (alcoholic tincture of *Sea Buckthorn* and *Black Currant*, dried fruits, iso-maltose impregnated with juices of *Sea Buckthorn* and *Black Currant*, and *Sea Buckthorn* and *Black Currant* balsamic vinegars) were purchased from Proplanta SRL Cluj-Napoca.

All spectrophotometric measurements were performed in triplicate using a T80+ spectrophotometer (PG Instruments). Lyophilizations were performed on freeze dry system - Freezone 25 PLUS (LABCONCO).

The samples were applied on chromatographic plate using an automatic device LINOMAT 5 (Camag) and the photo-documentation of the developed plates was done using a documentation device REPROSTAR3 (Camag).

### Sample preparation

Fresh fruits were dried either by drying in oven at $80^0$C or by lyophilization at 0.2mbar and -80°C. Alcoholic extracts were prepared by maceration for ten days of 1g dried fruits with 10mL of ethanol-water 8:2 (v/v). The supplements

conditioned on *iso*-maltose were obtained by the impregnation of 500 g *iso*-maltose with 100 mL of fruit extract (15% in ethanol 30%) followed by solvent evaporation. 1g of each supplements were extracted with 5mL ethanol-water 8:2 (v/v) mixture by maceration for 10 days. The liquid supplements were used without any preparation.

## Methods

### *Determination of the antioxidant activity and the total phenolic content*

The DPPH and $ABTS^{+}$ spectrophotometric assays were used to determine the antioxidant activity of the analyzed samples.

*Determination of antioxidant activity using DPPH:* 0.15mL of sample were added to 3mL DPPH solution (0.09 mg/mL) and after 15 minutes the absorbance of mixtures was read at 517 nm. The blank sample was prepared from 0.15mL sample solution and 3mL distilled water. Standard solutions of vitamin C (2–15mg/mL) were used to obtain the calibration curve [11]. The results were expressed as mg of ascorbic acid/ per mL of extract.

*Determination of antioxidant activity using ABTS:* the $ABTS^{\bullet+}$ was obtained from the reaction of 1:1 (v/v) solution of ABTS diammonium salt (7mmol/L) with $K_2S_2O_8$ solution (2.45mmol/L). The reaction mixture was incubated for 24h at room temperature, in the dark. 0.1mL of sample were added to 3mL $ABTS^{\bullet+}$ solution and the absorbance was read at 734nm after 15 minutes against a blank solution containing 0.1 mL sample and 3 mL distilled water. Standard solutions of Trolox (0.05–0.4μmol/mL) were used to obtain the calibration curve [12]. The results were expressed as μmol of Trolox per mL of extract.

*Total polyphenolic content:* 0.3mL sample were mixed with 1.5mL Folin–Ciocalteu reagent (0.2N) for 5min and then 1.2mL $Na_2CO_3$ solution (0.7M) were added. All samples were incubated at room temperature in the dark for 2h and their absorbance was read at 760nm against blank solution containing 0.3mL of sample solution and 2.7mL distilled water. Standard solutions of gallic acid (0–100μg/mL) were used for calibration curve. The results were expressed as μg of gallic acid per mL of extract.

### *Chromatographic analysis*

The TLC analyses of the nutritional supplements were performed on silica gel $60F_{254}$ plate (20x10cm) using a mixture of ethyl acetate: methanol: formic acid: acetic acid: water 80:10:1:1:8 (v/v/v/v/v) as mobile phase. Samples (10μL) were applied as 6 mm bands at 1.5cm from the low edge with a rate of 80nL/s. The chromatographic plate was developed on a distance of 85 mm in N chromatographic chamber pre-saturated for 30 min. The detection was done in UV light (254nm and 366nm) and in VIS light.

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STUDIA UBB CHEMIA, LVII, 1, 2012 (p. 83 - 92)

# TLC-DENSITOMETRIC DETERMINATION OF SYNTHETIC FOOD COLORANTS FROM PHARMACEUTICAL POWDERS

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**ABSTRACT.** A thin-layer chromatographic method combined with a sample preparation procedure has been developed for determination of Sunset Yellow (E110) and Amaranth (E123) synthetic dyes in different pharmaceutical powders. For an accurate separation of investigated dyes, different mobile phases were tested using silica gel G chromatographic plates. The dyes were analyzed using slit scanning densitometry with specific wavelength selection (λ=485 nm for E110 and λ=520nm for E123). Experimental results showed a very good linear correlation ($R^2$ > 0.9940) between area of colored spots and concentration of dyes in range of 4-20 µg/mL. In addition, low values of LOQ (0.29 µg/mL for E123 and 0.46 µg/mL for E110) and LOD (0.58 µg/mL for E123 and 0.92 µg/mL for E110) were obtained. The sample preparation step was focused on the quantitative desorption of dyes from starch based matrix and sugars removing. In the case of non soluble matrix, different extraction solvents and techniques were tested. The best results were obtained using ultrasounds assisted extraction (UAE) using the mixture of MeOH-$NH_3$ (9:1 v/v) as extraction agent. In the case of water soluble samples, a purification step by ion-pair – solid phase extraction (IP-SPE) on C18 cartridges using hexadecyltrimethyl-ammonium bromide (CTAB) was developed. High recovery values (R%) and a good reproducibility (RSD) was obtained for E110 and E123 (99.27±3.73% and 98.84±1.17% respectively). The applicability of the developed method was assessed for Coldrex and Daleron Junior pharmaceutical powders. The obtained results (42.64±3.29mg/kg E110 and 218.86±10.73mg/kg E123 in Coldrex and 54.21±3.95mg/kg E110 in Daleron) showed that the proposed method is suitable for rapid routine analysis of synthetic dyes in pharmaceutical powders.

***Keywords:*** *pharmaceutical powders, food synthetic colorants, sample preparation, TLC-densitometry*

## INTRODUCTION

The first notable characteristic of foods is its colour and often predetermines our expectation of flavour and taste. Natural colorants (pigments) were the first compounds used for colouring. Synthetic colorants (dyes) were discovered in 1856 by William Henry and are organic compounds derived from coal tar. Since then, more and more substances of every colour and tint

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of the rainbow were synthesized, many of them being used in food industry after few testing of their safety. Despite their risk upon the human health, synthetic dyes remain the most popular type of food colors, as they are brighter, more uniform, better characterized, higher tinctorial strength, and less expensive than colors derived from natural sources. The currently used dyes are classified into four chemical groups: azo, xanthene, triphenylmethane, and indigoid [1].

Some of this dyes belonging to the group of azo dyes, such as tartrazine (E 102), cochineal red A (E 124), and sunset yellow (E 110), can cause allergic or pseudo-allergic reactions (PARs), particularly to the people allergic to aspirin and other non-steroidal anti-inflammatory agents, or those affected by urticaria or asthma [2]. The presence of azo dyes, even at low doses, is also correlated with body-weight gain, changes in lipidic profile, blood glucose content, and biomarkers of oxidative stress in tissue and different toxic effects on renal and hepatic function [3].

In European Union, according to "Food Colour Directive" 94/36/EC, 30 June 1994 and EC Regulation No 1333/2008 of the European Parliament the following synthetic food dyes are allowed to be used (if the purity criteria are fulfill): Tartrazine (E102); Quinoline Yellow (E104); Sunset Yellow (E110); Carmoisine (E122); Amaranth (E123); Ponceau 4R (E124); Erythrosine (E127); Allura Red (E129); Patent Blue V (E131); Indigo Carmine (E132); Brilliant Blue FCF (E133); Brown HT (E155) [4, 5]. Due to regulatory restrictions regarding the use of dyes in foodstuff, the control of colorants in pharmaceuticals by their qualitative and quantitative analysis seems to be also necessary. According to the US regulations, FDA has classified the dyes in three categories: FD&C, D&C and Ext. D&C. The acronym FD&C indicates that the dyes are approved to be used for coloring of foods, drugs, and cosmetics. Colors in a more-limited category, D&C, are considered safe to use in drugs and c.osmetics. Colors in a third category, Ext. D&C, are certified only for external use in drugs and cosmetics.

Colorants are used in the pharmaceutical practice in order to ensure the same colour for all the batches of a given product. Adding a colour makes the medicinal product more attractive, easier to recognise and, in some cases, is stabilizing the sensitive ingredients by deposition as a continuous film [6].

First step of dyes analysis is sample preparation, which sometimes can be difficult. Usually, two important steps, namely the extraction from the matrix and the purification of the extract are involved. The used methodology depends on the nature and complexity of the matrix and the presence of other compound that may interfere during the analyses. The sample preparation is strongly linked with the analytical technique that will be used for compounds determination [7]. The solid samples insoluble in water are subjected to extraction with aqueous solution of acetone, methanol, or alkalized alcohols [8]. The composition of the extraction system decisively influences the dyes recovery. Before chromatographic analysis the extracts must be purified of sugars, fats and other substances that may disturb the dyes separation. The purification step is

carried out by colorant adsorption on wool fibre, polyamide [9], cellulose, alumina [6], and on anion resins exchangers or by liquid-liquid extraction of the ion pair compounds formed with different reagents [10, 11]. The solid phase extraction (SPE) on C18 sorbents is being used lately on a large scale [12]. The retention of the anionic colorant is carried out either as unionised species (low pH value), or as ion pairs (IP). Improved solid phase extraction systems which can prevent the development of preferential flowing channels have been also used [13].

The analysis of food dyes involves their identification as well as their quantitative determination. The UV-Vis spectrophotometric absorption methods can be used for the analysis of liquid matrices, which contains one, maximum two food dyes from different colour classes. Complex spectrophotometric methods combined with chemometric processing and interpretation of data must be applied in the case of the sample containing more colorants [14-17]. Currently, both the qualitative and the quantitative analysis are achieved using mainly chromatographic techniques. The dyes have low retention time in RP-HPLC due to the presence of sulphonic and carboxylic groups in their structure [18]. In order to solve this inconvenient the gradient elution [19], the adjustment of the pH value with different buffers [20] or the adding of ion pairs reagents (IPR) [21, 22] can be used. Also, the use of triethylamine as a mobile phase component has been studied [23]. The ion chromatography on an anion exchanger with low hydrophobicity as stationary phase and gradient elution can be applied for food dyes analysis due to their ionic character. [24]. Capillary electrophoresis (CE) was allso used as an alternative technique to HPLC [25-28]. The dyes from pharmaceutical products were determined by micellar electrokinetic capillary chromatography (MEKC) [6].

The dyes began to be analysed since the 1950s, using paper chromatography [29]. Currently, normal phase TLC on silica gel plates developed with a mixture of isopropyl alcohol-aqueous ammonia [30] and reversed phase TLC and HPTLC on C18 and CN [31, 32] are used. HPTLC - densitometry has proven to be an accurate quantitative method but, digital processing of plates image obtained by scanning using special software were also used [33, 34].

In view of the aforementioned considerations, the aim of the present work is to develop a simple, fast, precise and economical method for simultaneous determination of food dyes from pharmaceutical powders using TLC-densitometry. Special attention is paid to sample preparation, which is a quite difficult step due to the requirements that must to be fulfilled.

## RESULTS AND DISCUSSION

### Coldrex HotRem Blackcurrant pharmaceutical powder analysis

Coldrex powder contains paracetamol, phenilephrine chlorhydrate, ascorbic acid, sucrose (4 g), flawors and Amarant (E123), Sunset Yellow (E110) and Black PN (E151) as colorants. The high sucrose content will disturb

the TLC analysis leading to tailing spots and lower $R_f$ values. A simple dissolution of the powder in water followed by IP-SPE should be sufficient for obtaining an extract suitable for TLC analysis.

TLC determinations were performed on silica plates. A new mobile phase isopropyl alcohol–$NH_3$conc–CTAB 0.1M, (7:3:2, v/v) was developed for separation of the dyes E110 and E123. The determined values of the retention factor $R_f$ (±RSD) for E123 and E110 were 0.653(±0.014%) and 0.871(±0.016%) respectively. Good separation was achieved with this system obtaining an $R_s$ value of 4.59. Both dyes declared on the label: Sunset Yellow (E110) and Amaranth (E123) were identified by comparing their $R_f$ values with those of standards. Also, trace amounts of Black PN (E151,) were observed as a pail spot with intermediate $R_f$ value (fig 1a).

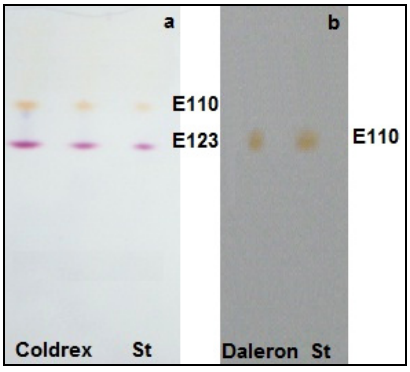


**Figure 1**. Dyes Identification in pharmaceutical samples.
(a) Coldrex powder using isopropyl alcohol–$NH_3$conc–CTAB 0.1M;
(b) Daleron powder using isopropyl alcohol–$NH_3$conc as mobile phase

Quantitative determinations for E110 and E123 were performed by scanning the developed plate at specific wavelengths 485 nm for E110 and 520 nm for E123 (fig 2).

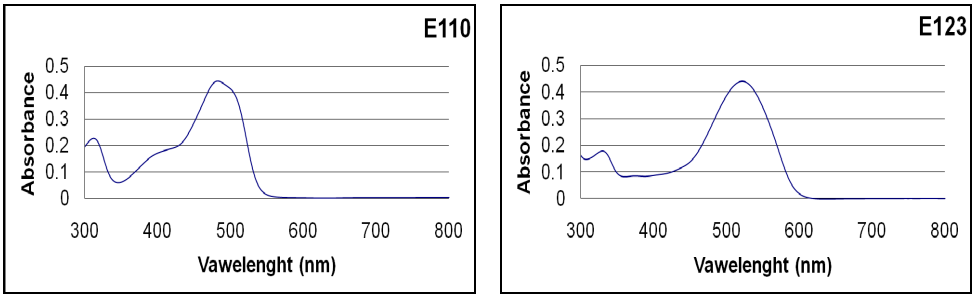


**Figure 2.** UV-Vis spectra of the studied dyes.

The linear domain of quantitative determination was determined by using seven different concentrations of standards. The standards were applied in duplicate and analyzed using the previously mentioned TLC conditions. In all cases linear relationships (Y=bX+a) were observed by plotting peak areas against dye concentrations. The correlation coefficients ($R^2$) corresponding to linear regression equations were at least 0.9940. The limit of detection (LOD) and limit of quantification (LOQ), calculated on the basis of the confidence bands of calibration curve using ordinary least squares method, were found to be between 0.29-0.45µg/mL and 0.58-0.88µg/mL respectively (Table 1).

**Table 1.** Linearity range and linear regression parameters for the investigated dyes (probability of error 0.05, $t_s$=2.57)

| Dye | Linearity range (µg/mL) | b (±SD) | a (±SD) | $R^2$ | LOD (µg/mL) | LOQ (µg/mL) |
|---|---|---|---|---|---|---|
| E110 | 4 - 20 | 723449 ±1501.8 | 7631.8 ±758.4 | 0.9940 | 0.45 | 0.88 |
| E123 | | 558341 ±6907.2 | 1003.6 ±388.9 | 0.9975 | 0.29 | 0.58 |

The recovery of the dyes after sample preparation by IP-SPE was calculated as ratio between the peak area of the processed sample and the peak area of the aqueous dye solution (E110+E123, 0.1mg/mL each). High values of recovery were obtained 99.27% for E110 and 98.84% for E123. Moreover, the proposed method shows good precision, the RSD value being 3.73% and 1.17% respectively.

The analytes content in Coldrex powder was calculated on the basis of the regression equation and spot area. The amounts of the dyes determined in Coldrex powder were 42.64±3.29 mg/kg for E110 and 218.86±10.73 mg/kg for E123 (SD, for 95%).

## Daleron Junior powder analysis

Daleron Junior pharmaceutical product is a powder containing paracetamol, ascorbic and citric acid, maize starch, orange flavor, sucrose and Sunset Yellow (E110) as coloring agent. Based on these informations (provided by the label), sample preparation must to be focused on two problems: colorant desorption from starch matrix and sucrose removing. The synthetic dyes are strongly adsorbed on starch due to the sulphonic moieties, so when desorption is desired the active adsorption sites must be deactivated or the sulphonic groups blocked by reaction with an ion-pair reagent. The problems posed by sucrose presence can be over passed by using extraction solvents with low water content or by performing SPE on C18. When sample preparation fulfills its objectives the determination step do not presents difficulties especially in this particular case when only one component must be determined.

TLC analysis was performed on silica plates using isopropyl alcohol–$NH_3$conc (7:3, v/v) as mobile phase [13]. The Sunset Yellow spot was identified in the upper part of the developed plate (fig.1b), at the $R_f$ value of 0.667 (RSD=0.012%). The chromatogram obtained for Daleron confirms the presence of E110.

The quantitative determination was performed at the optimum wavelength of $\lambda_{opt}$=485nm as indicated by the adsorption spectra (fig. 2b).

The linear regression equation Y=127005(±1396.9)X+3087.3(±139.5) was obtained on the working range 4-20 µg/mL and the correlation coefficient ($R^2$) was 0.9981. The limit of detection (LOD) and limit of quantification (LOQ), calculated on the basis of the confidence bands of calibration curve using ordinary least squares method, were 0.466 µg/mL and 0.92 µg/mL respectively.

Daleron Junior product required a complex sample preparation due to its matrix. Three procedures for sample preparation were applied. The first extraction method was focussed on E110 desorption from starch by optimizing the composition of the extraction system. In a previous paper [35] it was find out that $NH_3$ can block the active adsorption sites of starch. Different extraction solvent systems: $H_2O$ (S1), MeOH (S2); MeOH-$H_2O$ (9:1, v/v) (S3); MeOH-$NH_3$conc. (9:1, v/v) (S4); MeOH-$NH_3$conc-$H_2O$ (8:1:1, v/v) (S5); MeOH-$NH_3$conc (1:1, v/v) (S6); and MeOH-$NH_3$conc (2:1, v/v) (S7) were tested. The obtained extracts were directly analyzed by TLC. Tailed spots and lower $R_f$ values were observed for extracts obtained with systems S1, S6 and S7 and in consequence they were eliminated from further determinations. These anomalies were correlated with a high content of extracted sucrose. The systems S2, S3, S4, and S5 were found to be appropriate for extraction, but their compositions have a major influence upon the desorption process of dye from starch (Table 2). The S4 and S5 systems show best extraction efficiency providing extracts with high amounts of dye.

**Table 2.** TLC determination of E110 from Daleron pharmaceutical powder using different sample preparation procedures

| Sample preparation technique | Solvent system | E110 content (mg/kg) | |
|---|---|---|---|
| | | Average | SD |
| Magnetic stirring | $H_2O$ (S1) | - | - |
| | MeOH (S2) | 29.415 | 4.509 |
| | MeOH-$H_2O$(9:1, v/v) (S3) | 40.847 | 4.639 |
| | MeOH-$NH_3$conc. (9:1, v/v) (S4) | 45.276 | 3.044 |
| | MeOH-$NH_3$conc-$H_2O$ (8:1:1, v/v) (S5) | 45.635 | 2.633 |
| | MeOH-$NH_3$conc (1:1, v/v) (S6) | - | - |
| | MeOH-$NH_3$conc (2:1, v/v) (S7) | - | - |
| Ultrasound assisted extraction | MeOH-$NH_3$conc. (9:1, v/v) S4 | 54.187 | 3.949 |
| IP-SPE | | 53.999 | 3.563 |

Further improving of extraction efficiency can be achieved by using ultrasound assisted extraction (UAE) instead magnetic stirring to break analyte-starch bonds. At the first sight S5 is the best system that can be used, but there are two considerations that lead to S4 selecting: (i) the water content of extraction system must to be as lower as possible in order to obtain an extract with low content of sucrose; (ii) comparing the results obtained with S4 and S5 using Student's t test no statistical significant differences were revealed $t_{exp}(0.218816)<t_{crit}(2.23)$. A higher content of dye 54.216mg/kg with a good precision (RSD=±3.949) was determined when UAE and S4 as extraction system were used (table 2).

The third method based on an advanced desorption of the dye from the starch followed by SPE purification. The advanced desorption was achieved by using CTAB as extraction agent. The ion-pair reagent interacts with the dye destroying its charge and forming a compound which weakly interacts with the starch. On the other hand the surfactant inactivates the adsorption sites. The content of E110 determined by this method was 53.999±3.563) mg/kg and do not differ from that obtained by UAE (statisticaly proofed by Student's t test: $t_{exp}(0.093854)<t_{crit}(2.23)$. Based on these results we can assume that both methods are accurate and precise.

## CONCLUSIONS

TLC analysis of the synthetic food colorants can be performed in good conditions on silica gel plates using isopropyl alcohol–$NH_3$conc–CTAB 0.1M (7:3:2, v/v) or isopropyl alcohol–$NH_3$conc (7:3, v/v) as mobile phases. Linear calibration curve for both dyes ($R^2>0.9940$) on the range 4-20 µg/mL and low values for LOD (0.29 µg/mL for E123 and 0.46 µg/mL for E110) and LOQ (0.58 µg/mL for E123 and 0.92 µg/mL for E110) were obtained. Similar values of LOD (2.7 µg/mL) and LOQ (8.1 µg/mL) for Sunset Yellow were obtained by TLC [12], LOD 0.68 µg/mL for E110 and 0.38 µg/mL for E123 by micellar electrokinetic capillary chromatography [6].

Sample preparation method of the pharmaceutical powders must to be selected according with the sample composition. Water soluble matrices can be processed by IP-SPE for removing the interferences such as sucrose. The procedure using CATB as ion-pair reagent provide good recoveries (R>98%) and high precision (RSD<3.73%). Complex matrices containing soluble interferences as well as insoluble compounds which adsorb the analytes need a more laborious sample preparation. Two methods were found to be efficient UAE using MeOH-$NH_3$conc (9:1, v/v) and solid-liquid extraction with CTAB solution followed by IP-SPE. The content of dye E110 determined by these methods do not present significant statistical differences.

The Daleron pharmaceutical powder contains only Sunset Yellow (54mg/kg) while the Coldrex product contains along Sunset Yellow (42mg/kg) also Amaranth (218mg/kg) in the context of a maximum level of dyes in foods is 100-150 mg/kg E110 and 30-100 mg/kg E123 as indicated by EC Regulation No 1333/2008 of the European Parliament.

## EXPERIMENTAL SECTION

### Chemicals

The solvents used for sample preparation were purchased from Chemical Company (Iasi, Romania). The mobile phases for TLC analysis were prepared using methanol, isopropyl alcohol (HPLC grade), ammonia (concentrated solution), and hexadecyltrimethylammonium-bromide (CTAB) and were purchased from Merck (Darmstad, Germany). The TLC Sil G 60 plates (20x10cm) and LiChrolut® RP-18 SPE cartridges (200 mg) were also purchased from Merck (Germany). The Sunset Yellow (E110) and Amaranth (E123) of analytical purity were purchased from Fluka (Switzerland). The Daleron Junior and Coldrex Hotrem Blackurrant pharmaceutical powders were purchased from local pharmacy. Aqueous solution of, CTAB (0.01M), sucrose (5%), E110 (0.1mg/mL) and E110+E123 (0.1mg/mL each) were prepared. Methanolic dyes standard solution for quantitative analysis were prepared by appropriate dilution of aqueous solution in the concentration range 2.0 - 40.0 μg/mL) were also prepared.

### Instrumentation

Sample preparation was carried out by using an ultrasound bath Transsonic TS3100 (900W, 35 kHz), an Eba 20 centrifuge, and a VacMaster SPE system. The samples were applied with an applicator device - TLC Camag Linomat 5 and the dyes were quantified by scanning densitometry using the Shimadzu CS-9000 dual wavelength flying-spot scanner.

### Experimental

Dyes determination was carried out in two steps: sample preparation and TLC-densitometric analysis. Different approaches were tested for sample preparation due to different composition of the pharmaceutical powders.

Coldrex pharmaceutical powder (0.5g) was dissolved in 45 mL water and 5 mL CTAB solution and purified by IP-SPE on C18. The cartridge was conditioned with 5 mL MeOH and washed with 5 mL CTAB. The dyes retention was performed at a low flow rate (5 mL/min) of the sample solution. After that the sorbent was washed with 5 mL CTAB solution and then dried for 10 min in air current. A mixture of MeOH-conc.$NH_3$ (9:1, v/v) was used for elution of dyes from cartridge. The final volume was brought up to 10 mL in a volumetric flask. The SPE recovery was assessed by processing 50 mL sample that contains 5 mL aqueous dyes solution (E110+E123 0.1mg/mL each), 5 mL CTAB (0.01 M) and 2 mL sucrose solution (5%) in the same manner as described above but, the final volume was 5 mL instead of 10 mL. TLC analysis was performed on Sil G 60 plates developed with the mixture of isopropyl alcohol-$NH_3$conc.-CTAB 0.01 M (7:3:2, v/v) as mobile phase, in normal chromatographic chamber previously saturated for 30 min. The

methanolic standards of E110+E123 and the samples were applied as 5 µL spots with 1 cm distance between spots, at 1.5 cm from lower edge of the plate. The speed application was 50 nL/s. The developed plates (8 cm migration distance) were scanned in reflection mode at $\lambda_{max}$ = 485nm for E110 and $\lambda_{max}$=520nm for E123.

Daleron Junior pharmaceutical powder was processed in three different ways. The first one involves the dyes extraction from 3g powder with 5 mL extraction solvent, 3 times for 5 min under magnetic stirring at room temperature. Between the extraction steps the samples were separated by 5 min centrifugation at 3500 rpm. Due to the presence of starch, which strongly retains the dyes, seven different extraction systems: $H_2O$ (S1); MeOH (S2); MeOH-$H_2O$ (9:1, v/v) (S3); MeOH-$NH_3$conc.(9:1, v/v) (S4); MeOH-$NH_3$conc-$H_2O$ (8:1:1, v/v) (S5); MeOH-$NH_3$conc (1:1, v/v) (S6); and MeOH-$NH_3$conc (2:1, v/v) (S7) were tested. The extracts were evaporated to dryness and the residues were dissolved in 10 mL MeOH-$H_2O$ (9:1, v/v). The second procedure used ultrasounds instead magnetic stirring. The extraction was carried out in the same condition as mentioned above with the mixture MeOH-$NH_3$conc (9:1, v/v). In the third procedure the powder (3g) was mixed with 45 mL $H_2O$ and 5 mL CTAB (0.01 M). The suspension was centrifugated and the supernatant was further processed by IP-SPE on C18 cartridge. The sorbent was then washed with 5 mL CTAB solution, dried for 10 min, and then the compounds were eluted with MeOH-$NH_3$conc (9:1, v/v). The final volume was adjusted to 10 mL with MeOH in a volumetric flask. TLC analyse was performed on on Sil G 60 plates using a mixture of isopropyl alcohol-$NH_3$conc (7:3, v/v) as mobile phase in normal saturated (30 min) chromatographic chamber. The methanolic standards of E110 and the samples were applied as 5 µL spots, 1 cm distance between spots, at 1.5 cm from the lower edge. The application speed was 50 nL/s. The photodensitograms were obtained by scanning the plates in reflection mode at $\lambda_{max}$=485nm.

Each determination was performed on six replicates.

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# METHOD VALIDATION AND UNCERTAINTY ESTIMATION FOR TOTAL PHOSPHORUS DETERMINATION IN WASTEWATER SLUDGE SAMPLES

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**ABSTRACT.** Analytical method and uncertainty estimation for the measurement of total phosphorus in aquatic media was performed in two different laboratories. Method performance criteria used for validation process were investigated on standard phosphorus samples: concentration linearity domain (by calibration curve), limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy and robustness. In order to announce reliable results, uncertainties sources were identified, and different types of uncertainty were estimated. The validated method was further applied for total phosphorus determination from the sludge resulted at one municipal wastewaters treatment plant. Samples were collected from wastewater treatment sludge, before and after the dehydration process. The results show that the method is suitable and gives trustful results for phosphorus determination from wastewater sludge.

***Keywords:*** *wastewaters, sludge, total phosphorus, method validation, uncertainty estimation.*

## INTRODUCTION

Nitrogen and phosphorus containing compounds obtained in the wastewater sludge are nutrients and can be further valorized as fertilizer for agricultural purpose. Consequently, the quality control of the wastewater and wastewater sludge is of great interest for any wastewater treatment plant laboratory. The research group showed interest for analytical procedures for wastewater quality control and previous studies were reported: nutrients and detergents [1], phenolic compounds [2], heavy metals [3], aromatic volatile compounds [4].

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Available phosphorus determination methods for monitoring the effluent quality of wastewater plants are automatic on-line titration unit sensor based [5], colorimetry based on the coloring reagent formation between phosphorus and molybdate [6], inductively coupled plasma atomic emission spectroscopy (ICP-AES) [7] or ion chromatography [8].

This study aims to give the method validation results as well as the uncertainty estimation for total phosphorus ($P_t$) determination in standard solutions and wastewater sludge samples, which is new and not yet imposed in the Romanian laboratory practices. The validation procedure was carried out in the Wastewater Laboratory (WL) while the reproducibility and uncertainty estimation were studied together with the Chemistry Laboratory (CL).

## RESULTS AND DISCUSSION

The study followed specific stages: methods validation for $P_t$ determination, uncertainty estimation for $P_t$ determination in both standard solutions and in wastewaters sludge samples. The validated method was used to determine the total phosphorus content in the sludge produced in two different stages of the wastewater treatment process.

### Methods validation for total phosphorous determination

The investigated performance criteria were concentration linearity domain (by calibration curve), limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy and robustness.

### Calibration curve

The calibration curve is given in Figure 1.

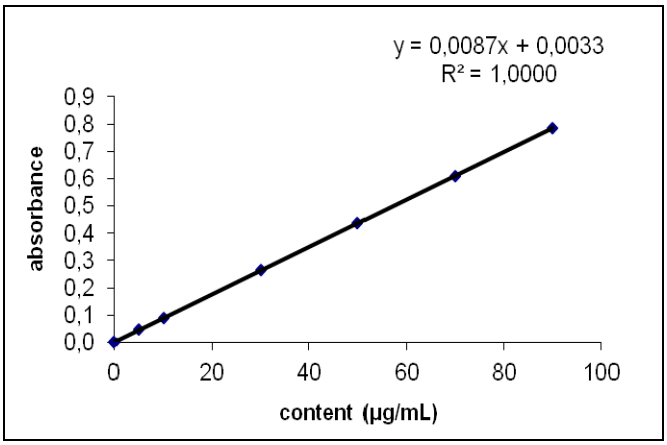


**Figure 1**. Calibration curves for the $P_t$ determination in RM solutions, in WL.

Calibration curve was plotted with 6 solutions of reference material (RM) of different concentrations, that were spectrometrically determined (at 830 nm, blue phosphomolybdenic complex), for the imposed concentration domain of 10–90 µg P/mL. Very good regression coefficient was obtained.

## Limit of detection and limit of quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to the equations (1-2) [9,10] using the measured absorbance values for 10 replicates of blank solution, then transformed in concentration values.

$$LOD = \overline{x_{blanck}} + 3 \cdot s_{blank} \quad (1)$$

$$LOQ = \overline{x_{blanck}} + 6 \cdot s_{blank} \quad (2)$$

were: $\overline{x_{blank}}$ – mean absorbance obtained with the blank solutions; $s_{blank}$ – standard deviations of the blank solutions.

The results are: LOD = 0.32 µg/mL and LOQ = 0.49 µg/mL, both lower than the lowest concentration for total phosphorus determination (10–90 µg P/mL), imposed by the Romanian standard method [11].

## Precision

Standard solutions of phosphorus RM of 40 µg/mL were prepared ($[P]_{theor}$). Repeatability ($RSD_r$) was tested with 7 replicates, intermediate precision ($RSD_{ip}$) with 7 replicates determined by three operators each, and interlaboratory reproducibility ($RDS_R$) with 7 replicates determined by three operators, in each laboratory (WL and CL).

Precision was evaluated by three criteria: relative standard deviation (RSD%), mean deviation ($s_x$) and confidence interval (t-distribution test) of the $P_t$ measured concentration ($[P]_{measured}$). The results are given in Table 1.

**Table 1.** Precision evaluation

| $[P]_{theor}$ (µg/mL) | Precision characteristics | n | RSD (%) | $s_x$ | $[P]_{measured}$ ± confidence interval (µg/mL)* |
|---|---|---|---|---|---|
| 40 | repeatability ($RSD_r$) (WL) | 7 | 0.70 | 0.1054 | 39.55 ± 0.2108 |
| 40 | interim precision ($RSD_{ip}$) (WL) | 21 | 0.87 | 0.0745 | 39.12 ± 0.1489 |
| 40 | reproducibility ($RSD_R$) (WL+CL) | 42 | 7.26 | 0.4498 | 40.13 ± 0.8996 |

*Calculated for a tolerance (t-distribution) of t=2, 95% confidence interval.

The obtained results show good results for repeatability and interim precision, as expected, the RSD% for repeatability being lower than the RSD% for interim precision. As expected, reproducibility values are higher than the repeatability and interim precision ones, RSD% = 7.26 is an acceptable result for the reproducibility test. The final result may be expressed as measured concentration together with the confidence interval. To be noticed that, as expected, $RSD_r < RSD_{ip} < RSD_R$.

## Accuracy

Accuracy was evaluated by the recovery test (R%), applied on 7 replicates of the standard solutions, according to equation (3).

$$R\% = \frac{C_F - C_U}{C_A} 100 \tag{3}$$

where: $C_U$ – concentration determined in the unfortified sample; $C_A$ – concentration of fortification (added solution); $C_F$ – concentration determined in the fortified sample.

Acceptable recovery percentages is a function of the analyte concentration [12]. For the studied analyte concentration (µg/mL) the method is considered accurate if the recovery test gives values in the range of 80% ≤ R ≤ 110%. The results are presented in Table 2.

**Table 2.** Accuracy evaluation

| Volume (mL) | $C_U$ (µg/mL) | $C_A$ (µg/mL) | $C_F$ (µg/mL) | Recovery (%) |
|---|---|---|---|---|
| 2 | 5 | 5 | 10.267 | 104.30% |
| 4 | 5 | 25 | 29.977 | 99.91 |
| 8 | 5 | 85 | 89.573 | 99.50 |

The obtained R% values lower than the accepted upper limit (110%) shows that the method is accurate for all the volumes of tested solutions.

## Robustness

Standard solutions of phosphorus RM of 40 µg/mL were prepared ($[P]_{theor}$). Robustness was tested related to: (a) the volume of the sulfuric acid solution (10%) used for the complex formation, (8 mL is the volume required by the standard); (b) the stability of the molybdenic acid; the use of the reagent was tested at 1, 3, 6 days after its preparation (according to the standard it should be freshly prepared and immediately used). The results are given in Table 3 and Table 4, respectively.

**Table 3**. Robustness evaluation related to the volume of the sulphuric acid solution used for the complex formation.

| $V_{acid}$ (mL)* | $[P_t]_{theor}$ (µg/mL) | $[P_t]_{measured}$ (µg/mL) | Colour of the complex solution |
|---|---|---|---|
| 0 | 40 | 29.73 | blue-grey |
| 8 | 40 | 39.85 | blue |
| 16 | 40 | 38.48 | colorless |
| Mean $[P_t]_{measured}$ (µg/mL) | | 36.02 | |
| RSD% | | 13.39 | |

* three replicates for each volume

Total phosphorus determination is influenced by the volume of the sulfuric acid solution used for the complex formation. Not only that the color of the complex solution is not obtained (blue), but also the relative standard deviation of the measurements is high (13.39%), meaning that the method is not robust relative to the volume of the sulfuric acid solution. The molybdenic acid is stable in aqueous solution. Not only that the color of the complex solution is obtained (blue), but also the relative standard deviation of the measurements is low (0.48%), meaning that the method is robust relative to the stability of the molybdenic acid solution.

**Table 4.** Robustness evaluation related to the stability of the molybdenic acid.

| Day* | $[P_t]_{theor}$ (µg/mL) | $[P_t]_{measured}$ (µg/mL) | Colour of the complex solution |
|---|---|---|---|
| 1 | 40 | 39.58 | blue |
| 3 | 40 | 39.65 | blue |
| 6 | 40 | 39.88 | blue |
| Mean $[P_t]_{measured}$ (µg/mL) | | 39.70 | |
| RSD% | | 0.48 | |

* three replicates for each day

## Uncertainty estimation for the total phosphorus determination

In order to estimate the uncertainty, the measurand was specified as being the content of total phosphorus in standard solutions and in the mineralized solution obtained from the wastewater sludge, respectively. The uncertainty sources were identified, Ishikawa diagrams were developed and different types of uncertainty were calculated according to equations (4-7): standard uncertainty ($u_x$); relative standard uncertainty ($u_r$); combined relative

standard uncertainty ($u_c$); expanded standard uncertainty (U). The final result is announced as the average concentration (C) and the uncertainty contribution (R=C±U).

**Uncertainty sources identification**

For the estimation of the uncertainty arising from the determinations in the standard solutions, the calibration curve, the concentration repeatability and the equipment, were found to be the uncertainty sources. Ishikawa diagram is presented in Figure 2.

$$u_x = \sqrt{\frac{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)^2}{n-1}} \tag{4}$$

$$u_r = \frac{u_x}{x} \tag{5}$$

$$u_c = \sqrt{\sum u_r^2} \tag{6}$$

$$U = u_c \cdot k \cdot 100 \tag{7}$$

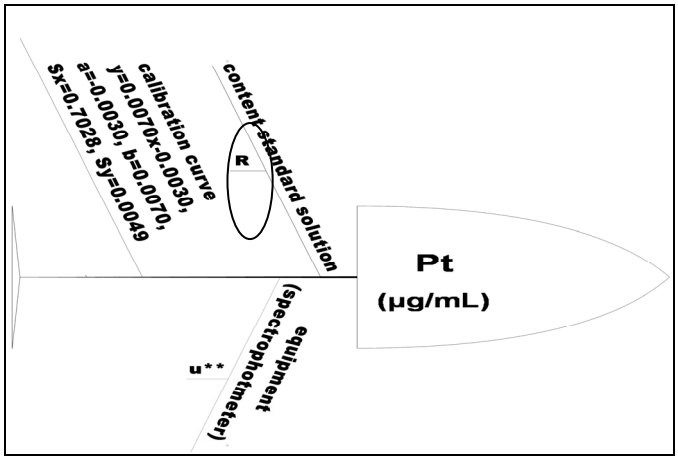


**Figure 2**. Ishikawa diagram for the $P_t$ determination in RM solutions.

For the estimation of the uncertainty arising from the determinations in the mineralized solutions, the equipments (analytical balance and spectrometer), the calibration curve, the concentration repeatability as well as the different glassware used for volumes measurements were found to be the uncertainty sources. For this estimation Ishikawa diagram is presented in Figure 3.

Using RM solutions of 40 µg/mL, expended standard uncertainty were calculated with k=2 (equation 11), with a confidence level of P=95%. For the standard solutions, 7 replicates were measured in both laboratories and the mean concentrations values were considered. For the mineralized solution 7 replicates were measured in the wastewater laboratory. The final result report will be accordingly with the Table 5 and Table 6, respectively.

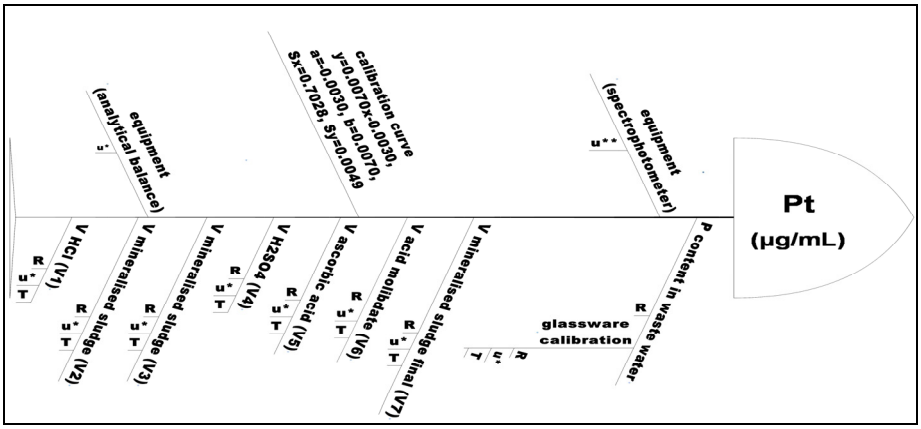


**Figure 3**. Ishikawa diagram for the $P_t$ determination in mineralized solutions.

**Table 5.** Uncertainty estimation for the $P_t$ determination in RM solutions.

| Lab | U source | $u_x$ | $u_r$ | $u_c$ | U | U (%) | Result (µg/mL) |
|---|---|---|---|---|---|---|---|
| WL | calibration curve | 0.2210 | 0.0056 | 0.35 | 0.71 | 1.81 | 39.49 ± 0.71 |
| | repeatability | 0.2789 | 0.0071 | | | | |
| | equipment | 0.0225 | 0.0006 | | | | |
| CL | calibration curve | 0.1480 | 0.0037 | 1.27 | 2.54 | 6.39 | 39.78 ± 2.54 |
| | repeatability | 1.2629 | 0.0317 | | | | |
| | equipment | 0.0152 | 0.0004 | | | | |

To be noticed that $U_{standard,(WL)}<U_{standard,(CL)}$; $U_{standard,(WL)}<U_{mineralized,(WL)}$. For the expanded uncertainty estimations of the $P_t$ determined in standard solution (WL, CL), as well as in the mineralized solution obtained from the wastewater sludge (WL), the higher contribution to the uncertainty has the repeatability. The fact that the extended standard uncertainty obtained for the standard solutions are lower than those for mineralized samples can be explained by the contribution brought to the uncertainty estimation by the sample preparation from the sludge.

## Total phosphorus content determination in the wastewater sludge

According to the European Union regulation related to the environment protection, especially of the soil quality, when sewage sludge is used in agriculture, soil and sludge analysis should include among other parameters phosphorus determinations too [13].

Therefore the phosphorus content in the sludge was of great interest for our study. Using the validated method, the total phosphorus content in the wastewater sludge was determined at WL, in two different stages of the treatment: before and after the dehydration process.

**Table 6.** Uncertainty estimation for the $P_t$ determination in mineralized solutions.

| U source | x (unit) | $u_x$ | $u_r$ | $u_c$ | U | U (%) | Result (µg/mL) |
|---|---|---|---|---|---|---|---|
| analytical balance | 200 mg | 0.2 | 0.0010 | 0.68 | 1.37 | 2.60 | 52.70 ± 1.37 |
| spectrometer | 52.70 µg/mL | 0.0225 | 0.0004 | | | | |
| repeatability | 52.70 µg/mL | 0.5314 | 0.0101 | | | | |
| calibration curve | 52.70 µg/mL | 0.2210 | 0.0042 | | | | |
| $V_1$ | 25 mL | 0.0437 | 0.0017 | | | | |
| $V_2$ | 100 mL | 0.1488 | 0.0015 | | | | |
| $V_3$ | 5 mL | 0.0086 | 0.0017 | | | | |
| $V_4$ | 8 mL | 0.0377 | 0.0047 | | | | |
| $V_5$ | 1 mL | 0.0033 | 0.0033 | | | | |
| $V_6$ | 2 mL | 0.0046 | 0.0023 | | | | |
| $V_7$ | 100 mL | 0.1488 | 0.0015 | | | | |

The measurements were done in three replicates and the results are given in Table 7., as R=C±U.

**Table 7.** Total phosphorus content in the wastewater sludge

| Wastewater treatment stage | $P_t$ content (µg/mL) |
|---|---|
| before dehydration the sludge | 58.04 ± 2.58 |
| after dehydration the sludge | 52.70 ± 1.37 |

The expanded standard uncertainties are increasing with the increase of $P_t$ content in the mineralized sample. The results demonstrate that a part of the phosphorus is lost during the dehydration process.

## CONCLUSIONS

The total phosphorus determination method [11] was validated to the following performances criteria: calibration curve, LOD, LOQ, precision (repeatability, intermediate precision, and reproducibility), accuracy (recovery test) and robustness. The method is sensitive, precise, accurate, not robust against the volume of the sulfuric acid solution, but is robust against the molybdenic acid, both used for the colored complex formation. Based on the identified sources of uncertainty, for both standard and mineralized solutions, the Ishikawa diagrams were designed, and different types of uncertainties were estimated showing that reliable results are to be announced. The higher contribution to the expanded uncertainty had the repeatability. Using the validated method, the total phosphorus content in the sludge produced during the wastewater treatment, before and after dehydration, was determined. Further work will be done for more comparative studies, both for proficiency testing (PT) and inter-laboratory comparisons (ILC).

## EXPERIMENTAL SECTION

The Romanian standard method [11] was used, based on the spectrometric determination of the blue phosphomolybdenic complex. Sludge samples are mineralized with concentrated sulfuric acid and perchloric acid in order to obtain orthophosphate from all different forms of phosphorus content in the sludge. Orthophosphate ions are further transformed by ammonium molybdate in phosphomolibdenic complex, than reduced by ascorbic acid and suphuric acid to a blue complex, that absorbs at 830 nm.

All the reagents were of analytical grade, and were purchased as follows: ascorbic acid, ammonium molybdate tetrahydrate and the phosphorus reference material (RM), monopotassium phosphate, all from Merck, Germany.

The two laboratories used the following equipments: a molecular absorption UV-VIS spectrometer Jasco 550 with double beam optical system (CL) and a molecular absorption UV-VIS spectrometer Secomam 750I (WL). Nabertherm L9/C6 oven was used for sludge mineralization. The determination domain imposed by the standard is 10–90 µg P/mL in the solution obtained after mineralization. Data processing was carried out using specific procedures [9-10, 12], while the uncertainty estimation was based on the EURACHEM guides [14-16].

## ACKNOWLEDGMENTS

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# COMPARISON OF HEATING TECHNIQUES USED IN WET ACID DIGESTION FOR THE DETERMINATION OF METALS FROM SOIL AND PLANTS

**ERIKA-ANDREA LEVEI[a,*], MIRELA MICLEAN[a], MARIN ŞENILĂ[a]**

**ABSTRACT.** Two wet acid digestion procedures using different heating techniques were tested on soil and plant certified reference materials for the determination of metals by inductively coupled plasma optical emission spectrometry (ICP-OES). The advantages and disadvantages of the two digestion methods were compared. The results obtained after conductive heating open vessel digestion were similar to those obtained by microwave assisted digestion both for soil and plant samples. The obtained recoveries (93–104%) showed that the procedures were precise and accurate for all elements, thus both digestion methods can be used for soil and plant sample dissolution prior to routine determination of metals by ICP-OES.

***Keywords:*** *metal determination, sample digestion, soil, plants, certified reference materials, inductively coupled plasma optical emission spectrometry*

## INTRODUCTION

Metal pollution has grown into one of the most important environmental problems all over the world [1]. Due to their high toxicity, persistence and bioaccumulation potential, metals can pose significant risks for water, soil, vegetation or fauna [2]. Thus, the measurement of metals concentrations in environmental matrices is the first step in the assessment of their potential health or ecological hazard. Moreover in case of water and soil the maximum allowable total contents for numerous metals have been legislated.

There are several methods that allow the metals determination directly from solid matrices, but most of the analytical methods require a sample preparation step in order to transform solid samples in solution [3, 4]. Sample preparation is the most time consuming step and represents an important source of errors and contamination. The hot plate, the block digester and the microwave oven are the most used heating devices in solid sample digestion [5]. Since the amounts of solubilized metals depend on the used digestion

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method and on the sample matrix, to achieve reproducible and accurate results, the selection of appropriate digestion method is mandatory. Moreover, problems such as incomplete dissolution, precipitation of insoluble analyte, sample contamination or loss of volatile elements can occur [6].

There are a wide range of digestion methods for solid samples that use different reagents and heating methods, but the most appropriate procedure is still under debate [7-10]. However, the majority of digestion procedures heat the sample with strong acid solutions in conventional conductive heating or microwave-heating systems [11-14]. The most commonly used digestion reagents are nitric acid, hydrochloric acid, sulfuric acid, hydrofluoric acid, perchloric acid and hydrogen peroxide. The advantages of digestion in closed systems consist in the higher working temperatures. While in open systems the operating temperatures are limited by the boiling point of the acid mixtures, in closed systems higher temperatures can be reached. Microwave-assisted acid digestion techniques have become popular and are widely used, due to their suitability for the digestion of complex matrices, low reagent and sample usage, short digestion times, good recoveries and enhanced operator safety [15]. Moreover, microwaves heat the sample to high temperatures very rapidly while the closed vessel helps in preventing losses due to volatilization of elements [16].

Aqua regia is a mixture of conc. HCl and conc. $HNO_3$ in 3/1 (v/v) ratio, and is one of the most used wet digestion methods for the estimation of maximum element availability for plants [17]. By its strong oxidizing effect completely solubilize the soil organic components and partially the elements bound to the siliceous matrix. Its dissolution efficiency depends on the sample grain size, type of matrix, energy input and reaction time. For the digestion of plant samples, generally $HNO_3$ or a mixture of $HNO_3$ and $H_2O_2$ are used. The addition of $H_2O_2$ in oxidizing mixtures increases the oxidation efficiency without to dissolve the siliceous matrix [18, 19].

The objective of the study was to compare the main analytical parameters of metals routine determination by inductively coupled plasma optical emission spectrometry (ICP-OES) after wet acid digestions by classical conductive and microwave heating. For the study two soil and two plant certified reference materials (CRMs) were used.

## RESULTS AND DISCUSSION

The digestion efficiency of the two heating procedures using a mixture of $HCl/HNO_3$ for soils and $HNO_3/H_2O_2$ for plants was evaluated using two soil and two plant CRMs. The obtained results are presented in Table 1-4. The comparison using the T-test showed no significant differences between the two digestion methods.

**Table 1.** Certified (median value) and determined (average± SD) metal concentrations in CRM SRM 2709 San Joaquin Soil after aqua regia digestion

| Element | Certified value (mg kg$^{-1}$) | Obtained value | | | |
|---|---|---|---|---|---|
| | | Conductive heating (mg kg$^{-1}$) | Recovery (%) | Microwave heating (mg kg$^{-1}$) | Recovery (%) |
| Al | 26000 | 26800±2100 | 103 | 25800±3000 | 99 |
| Ca | 15000 | 15600±330 | 104 | 15400±780 | 103 |
| Co | 12 | 11.6±1 | 97 | 12.2±0.8 | 102 |
| Cr | 79 | 76±3 | 96 | 81±5 | 103 |
| Cu | 32 | 33±2 | 103 | 31±2 | 97 |
| Fe | 30000 | 31000±1600 | 103 | 29500±3300 | 98 |
| K | 3200 | 3050±710 | 95 | 3300±850 | 103 |
| Mg | 14000 | 13400±600 | 96 | 14100±740 | 101 |
| Mn | 470 | 457±40 | 97 | 477±38 | 101 |
| Na | 680 | 710±26 | 104 | 650±46 | 96 |
| Ni | 78 | 75±5 | 96 | 80±4 | 103 |
| Pb | 13 | 12.4±1 | 95 | 13.2±0.8 | 102 |
| Zn | 100 | 96±4 | 96 | 101±3 | 101 |

**Table 2.** Certified (average± U) and determined (average± SD) metal concentrations in CRM LGC 6135 Hackney Brick Works Soil after aqua regia digestion

| Element | Certified value (mg kg$^{-1}$) | Obtained value | | | |
|---|---|---|---|---|---|
| | | Conductive heating (mg kg$^{-1}$) | Recovery (%) | Microwave heating (mg kg$^{-1}$) | Recovery (%) |
| Al | 22700±4600 | 23400±3100 | 103 | 22800±2900 | 100 |
| Ca | 21900±520 | 22200±490 | 101 | 21600±610 | 99 |
| Co | 20±4 | 19±3 | 95 | 20±2 | 100 |
| Cr | 336±28 | 327±50 | 97 | 346±22 | 103 |
| Cu | 105±5 | 103±6 | 98 | 108±9 | 103 |
| Fe | 40900±2700 | 42400±1970 | 104 | 42200±2400 | 103 |
| K | 5100±920 | 5250±850 | 103 | 5140±690 | 101 |
| Mg | 7000±580 | 6750±430 | 96 | 7200±730 | 103 |
| Mn | 348±18 | 359±12 | 103 | 351±21 | 101 |
| Na | 362±44 | 376±27 | 104 | 361±32 | 100 |
| Ni | 277±13 | 282±24 | 102 | 271±18 | 98 |
| Pb | 391±16 | 382±30 | 98 | 380±28 | 97 |
| Zn | 316±41 | 305±36 | 97 | 329±27 | 104 |

In the selection of the sample digestion method, besides the method precision, accuracy and dissolution efficiency, the sample homogeneity, reagent consumption and equipment cost should be also considered. The microwave assisted procedure is preferred, in case of small to medium sample number due to reduction of operating time, accurate results and good recoveries. In case of large number of samples, due to the fact that microwaves have 8-20 posts, the saved time decreases and the classical conductive heating digestion method can be used with good results.

**Table 3.** Certified (average) and determined (average±SD) metal concentrations in CRM IAEA 359 Cabbage after $HNO_3/H_2O_2$ digestion

| Element | Certified value (mg $kg^{-1}$) | Obtained value | | | |
|---|---|---|---|---|---|
| | | Conductive heating (mg $kg^{-1}$) | Recovery (%) | Microwave heating (mg $kg^{-1}$) | Recovery (%) |
| Ca* | 18500 | 17800±160 | 96 | 18900±120 | 102 |
| Cr* | 1.3 | 1.21±0.3 | 93 | 1.26±0.2 | 97 |
| Cu | 5.67 | 5.5±0.5 | 97 | 5.7±0.4 | 101 |
| Fe | 148 | 140±11 | 95 | 150±12 | 101 |
| K | 32500 | 33000±2800 | 102 | 32000±2400 | 98 |
| Mg | 2160 | 2250±210 | 104 | 2100±180 | 97 |
| Mn | 31.9 | 31.5±3.0 | 99 | 31.4±2.6 | 98 |
| Na* | 580 | 592±46 | 102 | 572±48 | 99 |
| Zn | 38.6 | 39.6±3.2 | 103 | 39.2±2.6 | 102 |

*Information values

**Table 4.** Certified (average± U) and determined (average± SD) metal concentrations in CRM NCS ZC 85006 Tomato after $HNO_3/H_2O_2$ digestion

| Element | Certified value (mg $kg^{-1}$) | Obtained value | | | |
|---|---|---|---|---|---|
| | | Conductive heating (mg $kg^{-1}$) | Recovery (%) | Microwave heating (mg $kg^{-1}$) | Recovery (%) |
| Al | 2950±430 | 2800±320 | 95 | 3000±340 | 102 |
| Ca | 53100±1900 | 49800±1730 | 94 | 53000±1800 | 100 |
| Cu | 21.1±2.5 | 19.8±1.8 | 94 | 20.8±2.2 | 99 |
| Fe | 1380±150 | 1300±90 | 94 | 1400±120 | 101 |
| K | 5790±520 | 5840±480 | 101 | 5900±500 | 102 |
| Mg | 7360±570 | 6766±740 | 92 | 7200±640 | 98 |
| Mn | 87.1±5.6 | 87.7±4.2 | 101 | 86.8±6.2 | 100 |
| Pb | 4.97±0.54 | 4.78±0.31 | 96 | 4.80±0.62 | 97 |
| Zn | 36.2±3.1 | 34.9±2.8 | 96 | 35.4±3.0 | 98 |

Our results are in agreement with those of Chen [17] who found that the precision and accuracy of soil metal determination by ICP-OES using microwave aqua regia and hotplate aqua regia digestion methods were comparable, except for the silicate-binding metals, such as Al, Ba, K, whose dissolution was slightly greater using microwave digestion. Senila et al. [20] found no significant differences between metal contents in perennial plants determined by ICP-OES after hot plate and microwave digestion using a mixture of $HNO_3/H_2O_2$, while Demirel et al. [21], reported better recoveries and more accurate results after microwave digestion than after dry and wet digestion, for trace element determination in food materials by atomic absorption spectrometry.

## CONCLUSIONS

Both the conductive heating open vessel digestion and microwave assisted digestion can be used for soil and plant sample dissolution prior to metals determination by ICP-OES. In case of a small sample number the microwave assisted digestion is faster, but in case of large number of samples, the saved time decreases.

## EXPERIMENTAL SECTION

### Reagents and materials

Analytical grade reagents (65% $HNO_3$, 37% HCl, 30% $H_2O_2$) and 1000 mg/l multi-element stock solutions were purchased from Merck (Darmstadt, Germany). High purity deionized water obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used. To compare the digestion procedures the following CRMs were used: SRM 2709 San Joaquin Soil (National Institute of Standards and Technology, USA), 6135 Hackney Brick Works Soil (LGC, UK), IAEA 359 Cabbage (International Atomic Energy Agency, Austria), and NCS ZC 85006 Tomato (National Analysis Center for Iron and Steel, China). All the PTFE and glass vessels were cleaned by soaking in 10% $HNO_3$ and rinsed with ultrapure water before use.

### Digestion procedure

Three replicates of approximately 1 g CRM were subjected to open vessels digestion on sand bath. The soil CRMs, were treated with 15 ml conc. HCl and 5 ml conc. $HNO_3$, while the plant CRMs with 10 ml conc. $HNO_3$ and 2.5 ml $H_2O_2$.The digestion was conducted for 16 h at room temperature for slow oxidation of the organic matter then the temperature of the reaction mixture was slowly raised until reflux conditions and maintained for 2h. After cooling to room temperature, the slurry was diluted to 100 ml with distilled water and then filtered through a 0.45 μm cellulose acetate membrane filter.

Three replicates of 0.5 g CRMs were digested with 7.5 ml conc. HCl and 2.5 ml conc. $HNO_3$ (soils) and 6 ml conc. $HNO_3$ and 2 ml $H_2O_2$ (plants). Sampled were left overnight at room temperature for pre-digestion. The microwave assisted digestion program is presented in Table 5. After cooling to room temperature, the slurry was diluted to 50 ml with distilled water and then filtered through a 0.45 μm cellulose acetate membrane filter. For each procedure blank samples were prepared.

### Instruments

The determinations were carried out using the 2100 Optima DV ICP-OES (Perkin Elmer Optima). Details about operating conditions are summarized in Table 6, while the wavelengths and detection limits (DL), calculated according

to 3s criterion, are given in Table 7. A Berghoff MWS-3+ closed vessel microwave system (Eningen, Germany) and a SD8 Sand Bath (Gestigkeit, Germany) were used for the sample heating.

**Table 5.** Operating conditions for the microwave digestion system

| | Stage | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** |
| Temperature ($^oC$) | 145 | 170 | 190 | 100 | 100 |
| Pressure (bar) | 30 | 30 | 30 | 0 | 0 |
| Ramp time (min) | 5 | 1 | 1 | 1 | 1 |
| Hold time (min) | 25 | 10 | 15 | 10 | 10 |
| Power (%*) | 80 | 80 | 80 | 0 | 0 |

*100 % power corresponds to 1400 W

**Table 6.** Instrumental parameters and operation conditions for ICP-OES

| **Generator** | **Free-running, 40.68 MHz, operated at 1300 W** |
|---|---|
| Plasma torch | Inductively coupled plasma, dual viewing<br>Outer flow 15 L $min^{-1}$, Intermediate flow 0.5 L $min^{-1}$<br>Nebulizer flow 0.8 L $min^{-1}$ |
| Sample introduction | 3 channel peristaltic pump, concentric nebulizer, Scott type spray chamber, sample uptake rate:2 mL $min^{-1}$, flushing time:20s, delay time:40s |
| Optics | multichannel spectrometer with Echelle grating 165 – 780 nm, chamber filled with Ar |
| Detector | CCD detector |
| Data processing | WinLab 32 Software<br>two points linear background correction, integration time 10 s,<br>3 successive measurements for each sample |

**Table 7.** Wavelengths and detection limits for metals determination by ICP-OES

| **Metal** | **λ (nm)** | **DL (mg $kg^{-1}$)** | **Metal** | **λ (nm)** | **DL (mg $kg^{-1}$)** |
|---|---|---|---|---|---|
| Al | 396.153 | 1.6 | K | 766.497 | 2.5 |
| Ca | 317.935 | 0.8 | Mg | 285.215 | 0.4 |
| Cd | 228.805 | 0.5 | Mn | 257.611 | 0.5 |
| Co | 228.618 | 1.0 | Na | 589.593 | 4.3 |
| Cr | 267.713 | 0.9 | Ni | 231.606 | 1.5 |
| Cu | 327.398 | 1.1 | Pb | 220.355 | 3.0 |
| Fe | 238.205 | 0.9 | Zn | 213.859 | 0.4 |

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STUDIA UBB CHEMIA, LVII, 1, 2012 (p. 111 - 117)

# OIL EXTRACTION AND FATTY ACID CHARACTERIZATION OF *NANNOCHLOROPSIS OCULATA* MICROALGAE FOR BIODIESEL APPLICATIONS

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**ABSTRACT.** Microalgae, the third generation biodiesel feedstock, have emerged as one of the most promising alternative sources of lipids that can be used in the production of biodiesel due to their advantages over conventional crops. The aim of this study was to obtain *Nannochloropsis oculata* microalgae oil using hexane extraction methods together with fatty acid characterization for biodiesel application. The chemical composition of microalgae showed a high total lipid content, *N. oculata* microalgae being a potential feedstock for biodiesel production. For algal oil extraction, dynamic extraction with Soxhlet apparatus proved more efficient, with a lipid yield of 0.190 g/g dried microalgae comparatively to only 0.136 g lipid/g dried microalgae for static hexane extraction. The main fatty acids for *N. oculata* microalgae are palmitic acid, palmitoleic acid, eicosatrienoic acid and eicosapentaenoic acid, the latest being also the major constituent, with a value of 48.86% (w/w).

***Keywords:*** *microalgae, biodiesel, oil extraction, lipids, fatty acids*

## INTRODUCTION

Microalgae, the third generation biodiesel feedstock, have emerged as one of the most promising alternative sources of lipids that can be used in the production of biodiesel due to their advantages: photosynthetic efficiency, high biomass production, higher growth rates and productivity when compared to conventional crops [1]. Microalgae use photosynthesis to convert solar energy into chemical energy in the form of oil, carbohydrates and proteins.

Biodiesel is a mixture of monoalkyl esters of long-chain fatty acids obtained in the transesterification reaction of vegetable oils or animal fats with short chain alcohols [2]. The cost of biodiesel production remains the

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major obstacle to its use at industrial scale, primarily because of the high cost of vegetable oils used as feedstock [3]. Another important reason is the inefficiency and unsustainability of first and second generation biodiesel [4].

The production of biodiesel from microalgal biomass consists in three steps: oil extraction, transesterification and separation/purification of biodiesel.

Lipid extraction from microalgal is the most important step because this process is one of the more costly processes which can determine the sustainability of algae-based biodiesel. Fatty acid methyl esters obtained from oil are known as biodiesel. Various methods for extracting oil from microalgae were developed, including mechanical pressing, homogenization, milling, solvent extraction, supercritical fluid extraction, enzymatic extractions, ultrasonic-assisted extraction and osmotic shock. Organic solvent extraction with hexane is the most common method for extracting oil from microalgae by repeated washing or percolation.

Total lipid extraction can be achieved by using appropriate methods of cell disruption to release cellular contents into the extraction medium. Numerous methods have been used for cell disruption, such as microwaves, ultrasonic, mechanical crushing [5]. For example, the use of microwaves proved to be an effective method for the extraction of vegetable oils [6], while ultrasonic disruption is widely used for microbial cells [7].

The purpose of this paper was to obtain *Nannochloropsis oculata* microalgal oil and to determine the fatty acid profile for further biodiesel production.

## RESULTS AND DISCUSSIONS

*N. oculata* microalgae have been used for this study due to their commercial availability and for high lipid content. Rodolfi et al. reported that *Nannochloropsis* marine microalgae specie is one of the best candidates for algal oil production due to high biomass productivity and lipid content [8].

The microalgae were first analysed to determine its constituents. Microalgae contain lipids, proteins, carbohydrates and nucleic acids. Table 1 shows the average chemical composition of *N. oculata* microalgae.

**Table 1.** Average chemical composition of *Nannochloropsis oculata* microalgae on a dry matter basis. Values given are the mean of three replicates ± standard deviation.

| Parameter | Content %(w/w) |
|---|---|
| Proteins | 42.5 ± 1.8 |
| Carbohydrates | 18.0 ± 0.9 |
| Lipids | 32.8 ± 1.2 |

According to Brown et al. [9], microalgal composition varies by species in the proportion of protein (6 to 52%), lipid (7 to 23%) and carbohydrate (2 to 23%). In this study, the protein and carbohydrate content in *N. oculata* microalgae were within the ranges reported by Brown et al. (1997), while the lipid content was higher, but in accordance with other studies [8,10]. The high content of total lipids suggests that *N. oculata* microalgae used in this study is a potential feedstock for biodiesel production.

For microalgal biodiesel production the ideal lipid extraction method should not only be lipid-specific – in order to minimize co-extraction of non-lipid contaminants, but also selective – towards only a few lipid fractions, e.g. neutral lipids like triacylglycerols [11]. Even though the classic chloroform-based lipid extraction protocol is particularly suitable for most microalgal lipid analyses, alternative organic solvents that are less toxic are preferred prior to scale-up. Hexane is less efficient than chloroform for extraction of oils from microalgae, but is also less toxic – and has a marginal affinity for non-lipid contaminants, and an apparently higher selectivity for neutral lipid fractions [11]. This is why hexane was chosen for oil extraction from *N. oculata* microalgae.

In this paper two methods have been tested for oil extraction from *N. oculata* microalgae: static hexane extraction and dynamic hexane extraction (Soxhlet). The lipid yield obtained for each method was compared to total lipid content determined using the combination of microwaves with chloroform-methanol extraction. Soxhlet extraction with hexane was found to be significantly more efficient than static hexane extraction, with a lipid yield of 0.190 g/g dried microalgae comparatively to only 0.136 g lipid/g dried microalgae. This improvement was expected since Soxhlet operation, through solvent refluxing, constantly exposed a fresh batch of hexane to the microalgae biomass and enabled continuous re-establishment of mass transfer equilibrium [12].

Microalgae synthesize fatty acids as building blocks for the formation of various types of lipids. The most commonly synthesized fatty acids have chain lengths that range from C16 to C20, similar to those of higher plants [13]. Saturated fats come from animal products such as meat and dairy while most vegetable oils are unsaturated. Fatty acids are either saturated or unsaturated, and unsaturated fatty acids may vary in the number and position of double bonds on the carbon chain backbone. Polyunsaturated fatty acids (PUFAs) contain two or more double bonds.

Fatty acid composition is the most important parameter for a biodiesel feedstock because the properties of the corresponding fatty esters that comprise biodiesel determine the overall fuel properties of biodiesel. Fatty acid composition of *N. oculata* algal oil was determined using a gas chromatograph with flame ionization detector, the chromatogram being presented in Figure 1. The results are given in Table 2, data for each fatty acid being expressed as percentage of the total fatty acids in the microalgal oil.

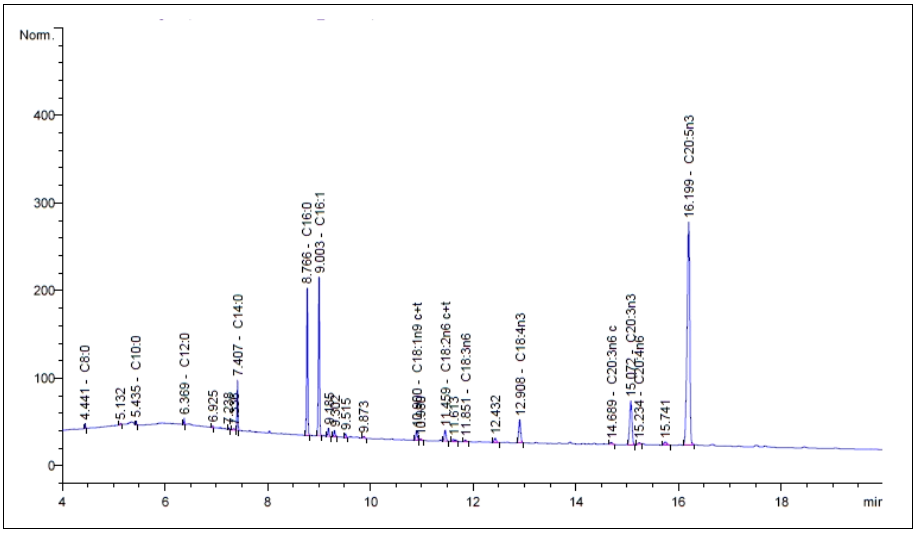


**Figure 1**. The GC chromatogram obtained for the fatty acid profile of *Nannochloropsis oculata* microalgae.

**Table 2** Fatty acid composition of *Nannochloropsis oculata* microalgae on a dry matter basis (%).Values given are the mean of three replicates ± standard deviation.

| Systematic name | Common name | Abbr. | Content % (w/w) |
|---|---|---|---|
| Octanoic acid | Caprylic acid | C8:0 | 0.30 ±0.02 |
| Decanoic acid | Capric acid | C10:0 | 0.19 ± 0.02 |
| Dodecanoic acid | Lauric acid | C12:0 | 0.35 ±0.01 |
| Tetradecanoic acid | Myristic acid | C14:0 | 3.87 ±0.04 |
| Hexadecanoic acid | Palmitic acid | C16:0 | 14.14 ±1.09 |
| 9-cis-Hexadecenoic acid | Palmitoleic acid | C16:1 | 17.02 ± 2.50 |
| cis-9-Octadecenoic acid | Oleic acid | C18:1n9 | 1.20 ± 0.04 |
| cis,cis-9,12-octadecadienoic acid | Linoleic acid | C18:2n6 | 1.47 ± 0.03 |
| 6,9,12-octadecatrienoic acid | γ-Linolenic acid | C18:3n6 | 0.25 ± 0.02 |
| (6Z,9Z,12Z,15Z)-6,9,12,15-octadecatetraenoic acid | Stearidonic acid | C18:4n3 | 3.88 ± 0.04 |
| *all*-cis-8,11,14 -eicosatrienoic acid | Dihomo-gamma-linolenic acid (DGLA) | C20:3n6 | 0.35 ± 0.02 |
| *all*-cis-11,14,17-eicosatrienoic acid | Eicosatrienoic acid (ETE) | C20:3n3 | 7.74 ± 0.03 |
| *all*-cis-8,11,14,17-eicosatetraenoic acid | Eicosatetraenoic acid (ETA) | C20:4n6 | 0.27 ± 0.02 |
| *all*-cis-5,8,11,14,17-eicosapentaenoic acid | Eicosapentaenoic acid (EPA) | C20:5n3 | 48.86 ± 1.77 |

As seen in Table 2, the main fatty acids for *Nannochloropsis oculata* are palmitic acid, palmitoleic acid, eicosatrienoic acid and eicosapentaenoic acid EPA. From these acids the major PUFAs for *N. oculata* microalgae are: eicosatrienoic acid (20:3*n*3), stearidonic acid (18:4*n*3) and eicosapentaenoic acid EPA (C20:5*n*3), the latest being also the major constituent of *N. oculata* microalgae, with a value of 48.86% (w/w). The high level of polyunsaturated acids in *N. oculata* microalgae may cause stability problems, the biodiesel resulted being more susceptible to oxidation process. However, polyunsaturated acids also have much lower melting points than monounsaturated or saturated acid, thus, algal biodiesel should have much better cold weather properties than many other biodiesels.

## CONCLUSIONS

*Nannochloropsis oculata* microalgae have been used for this study to obtain microalgal oil and to determine the fatty acid profile. The chemical composition showed a high total lipid content that proved *N. oculata* microalgae is a potential feedstock for biodiesel production. The fatty acid composition of *N. oculata* microalgae showed a high level of polyunsaturated acids which may cause stability problems, but, in the same time, the cold weather properties should be improved comparatively to other biodiesels.

## EXPERIMENTAL SECTION

### Materials

All chemicals were analytical reagent grade. *Nannochloropsis oculata* microalgae were purchased from Astaxa GmbH (Germany Milz Gerbergrasse). Hexane, chloroform, methanol, acetic acid, sodium hydroxide, sulphuric acid, boric acid and copper sulphate were purchased from Merck (Darmstadt, Germany). Sodium chlorite (80%) was purchased from Alfa Aesar (Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Methyl ester standards were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were chromatographically pure.

### Microalgal lipid extraction

#### *Static hexane lipid extraction*

For static hexane lipid extraction, a quantity of 4 g microalgal powder was used. A volume of 300 mL n-hexane was added to the microalgal powder in a 500 mL Erlenmeyer flask. In order to reduce solvent evaporation, the flask was sealed with a ground joint. The extraction mixture was agitated at 800 rpm

at ambient conditions for 8 h. After extraction cell residues were removed by filtering through Whatman GF/C paper. The hexane phase was collected in a pre-weighed flask and then submitted to vacuum evaporation using a rotational evaporator to enable gravimetric quantification of the lipid extract.

### *Dynamic hexane lipid extraction*

The performance of static hexane extraction with dynamic hexane extraction was compared using a Soxhlet apparatus. A quantity of 4 g microalgal powder was packed in a cellulose thimble inside the extraction chamber of the Soxhlet unit. A volume of 300 mL *n*-hexane was used to extract the lipid and the extraction was performed for 8 h at the rate of approximately 10 refluxes per hour. The extracted lipid collected in a pre-weighed flask and then submitted to vacuum evaporation using a rotational evaporator to enable gravimetric quantification of the lipid extract**.**

## Analytical methods

### *Determination of carbohydrates content*

Composition of carbohydrates from microalgae was determined according to Teramoto method, method used for determination of carbohydrates from wood [14]. Carbohydrates content was determined by treated microalgae with sodium chlorite in acetic acid solution (10%): 5 g sample was treated with 5 g $NaClO_2$ in 375 ml glacial acetic acid. The sample was mixed at 75 °C for 1h (repeated for three times). The product was filtrated, washed with water and acetone, dried at 105 °C for 24 h in vacuo, and weighed. The dried solid was treated with 17.5% NaOH at 20 °C for 40 min, and 25 ml water was added it. The residue was filtrated, washed with 40 ml 10% glacial acetic acid and 1L boiling water. The carbohydrates residue was filtrated, dried at 105 °C for 48 h in vacuo, and weighed.

### *Determination of protein content*

The protein content of *Nannochloropsis oculata* microalgae was determined using Kjeldahl destruction method, all proteins being degraded to $NH_3$ that was quantified titrimetrically with sulphuric acid. An amount of 1 g microalgae biomass was destructed at 390°C with 10 mL 96% $H_2SO_4$ in the presence of $CuSO_4$ as catalyst for approximately 2 hours, until the residue became white and the supernatant clear. After cooling down, the sample was filled up to 100 mL with distilled water, distilled and the resulted $NH_3$ was captured with boric acid ($H_3BO_3$) and quantified titrimetrically with $H_2SO_4$. The amount of protein was calculated by multiplying the measured nitrogen concentration with 6.25.

### *Determination of total lipid content*

Total lipid content was determined using the combination of microwave and liquid-liquid extraction. 0.5 g dry microalgae biomass was mixed with 20 ml distilled water. The mixture was further subjected to cell disintegration using a Speedwave MWS Berghof microwave digester (2450MHz) at 100 °C for 5 min. Total lipids were extracted from microalgae biomass using a modified version of Bligh and Dyer method [15]. Lipids were extracted with a mixture of chloroform-methanol 2:1 (v/v). The volume ratio of biomass subjected to the extraction and the organic solvent mixture is 1:1. The mixture was subjected to stirring for 5 minutes in a separating funnel. After extraction the mixture was introduced over a further 10 ml of methanol to separate the two phases: aqueous and organic. The chloroform organic phase was washed with 10 ml 5% sodium chloride and then submitted to vacuum evaporation using a rotational evaporator to enable gravimetric quantification of the lipid extract**.**

### *Determination of Fatty Acid Composition using GC-FID*

The fatty acid profile was determined using an Agilent 7890A GC gas chromatograph equipped with a DB-WAX capillary column (30m × 0.25mm × 0.25μm) and a flame ionization detector. The column oven temperature was kept at 50°C for 1 min, heated to 200°C at 25°C/min, then to 230°C at 3°C/min and finally maintained for 18 min. The injector and detector temperatures were set to 250 and 280°C, respectively. Helium was utilized as a carrier gas. The gas chromatography calibration was conducted via the analysis of standard solutions of methyl esters and heptadecanoate methyl ester was used as internal standard.

## ACKNOWLEDGMENTS

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STUDIA UBB CHEMIA, LVII, 1, 2012 (p. 119 - 126)

# THE HEAVY METALS IMPACT ON SURFACE WATER AND SOIL IN THE NON-SANITARY MUNICIPAL LANDFILL "PATA RÂT"– CLUJ-NAPOCA

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**ABSTRACT.** This study proposes to investigate the environmental impact caused by heavy metal contamination on soil and surface water from the non-sanitary waste municipal landfill "Pata Rat" from Cluj-Napoca Romania. The samples were analyzed with atomic absorption spectrometer (AAS). The evaluation of the analysis results proves that there is high pollution in soil with some heavy metals (Pb, Cu). The heavy metals content was investigated also in water samples from Zapodie stream that flows near the landfill and in leachate collected from the same landfill.

The overall view of the study presents a recent evaluation of the pollution with heavy metals attributed to the exploitation of the landfill for over 37 years, and establishes the influence and the transfer mode on environmental contamination.

***Keywords:*** *waste storage, landfill, heavy metals, AAS, pollution, environmental impact*

## INTRODUCTION

From the many environmental problems is no doubt that waste is a major problem. The quantities of the generated waste are increasing in an alert way from year to year and the impact on the environment and communities is growing larger. The municipal waste management is a big responsibility primarily for government authorities and for the population.

The municipal waste depositing on the ground is not the most acceptable solution because of the adverse environmental impacts, however it was and is still used. In the EU the amount of the municipal waste stored in landfills has decreased with more than 25% since 1995. The negative effects of the landfills are the formation of methane gas emissions and the production of a leachate

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(150 m3/day) [1]. As a comparison, in the United States, the waste storage remains the predominant method of disposal of the municipal solid waste (MSW), approximately 50% of MSW generated is stored in landfills [2].

In Romania, almost all the municipal waste is discharged in landfills more or less suitable in terms of the environmental protection. The simple storage of the waste was a widespread practice in the past and the municipal waste was discharged in natural pits or simply lies on land, without taking any measurements to protect the environment. This simple storage system is dangerous for public hygiene, unsightly and no environmentally friendly.

The old waste landfills built in Romania in the '70 and '80 do not comply with new regulations imposed by the European Commission. Most of these landfills have been closed or are closing. But the impact on the environment caused by them still exists. This fact had determined the Romanian governmental authorities to provide legislative measures that need to be taken post closure for at least 30 years and actions to reduce their pollution. Notwithstanding, by derogation, the application of the Directive 1999/31/EC, the conditions for water control and leaching management, soil and water, gas control and stability shall not apply in Romania until 16 July 2017, to a number of 101 of existing municipal landfills of waste. Beginning with 2006, Romania has reduced step by step the waste quantity from 101 non-sanitary landfills. In this context in "Pata Rat" landfill the storage activity ceased on 16 July 2010, in accordance with the Accession Treaty and GD 349/2005 [3].

"Pata Rat", the biggest municipal landfill of the Cluj County, located on the road linking the village Pata and Cluj-Napoca city was put into the operation in 1973 and was designed to a capacity of 3.5 million tons on an area of approximately 9 ha and 30 years of operation [4].

At the present it covers about 18 ha of land at a distance varying between 2-10 m from Zapodie stream.

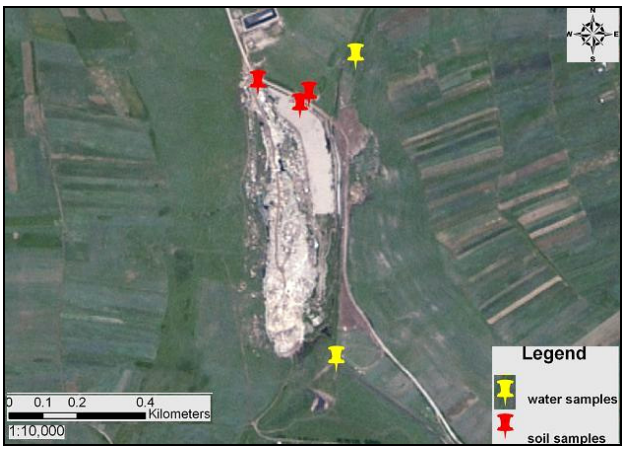


**Figure 1.** The non-sanitary landfill „Pata Rat" of Cluj-Napoca city location

According to the monitoring data, during 37 years of operation, about 10 million of tons of municipal and industrial waste were stored without prior separation or treatment. The waste storage in these conditions has led to changes in the environmen and since 2005 exceeded the designed capacity [5].

## RESULTS AND DISCUSSION

The environmental impact assessment of the heavy metals from ”Pata Rat” landfill was made on the basis of measurements of the parameters from the leachate, water and soil, which revealed changes of the physical and chemical parameters, compared with normal and threshold values [6].

The heavy metals concentrations found in the soil samples are as follows in the Table 1.

**Table 1.** Heavy metals concentration in the soil samples

| Sample | Geografical coordinates | Cd mg/kg | Cr mg/kg | Cu mg/kg | Ni mg/kg | Pb mg/kg | Zn mg/kg |
|---|---|---|---|---|---|---|---|
| $Ss_1$ | N 46°46'07,7"; E 23°41'12,4" | 0.46 | 15.3 | 19.9 | 20.0 | 54.7 | 33.4 |
| $Ss_2$ | N 46°46’05,6"; E 23°41'18,3" | 0.26 | 8.62 | 14.9 | 15.4 | 54.3 | 25.2 |
| $Ss_3$ | N 46°46'06,7"; E 23°41'19,5" | 0.27 | 8.98 | 32.3 | 15.7 | 96.7 | 47.8 |
| **NV** | | **1** | **30** | **20** | **20** | **20** | **100** |
| **AT** | | **3** | **100** | **100** | **75** | **50** | **300** |
| **IT** | | **5** | **300** | **200** | **150** | **100** | **600** |

**Legend:** Ss - soil sample, NV - normal value, AT – alert threshold, IT – intervention threshold, according to the Decree No 756 of the Government of November 3 of 1997

The results proved that in the soil, Cu displays higher concentrations, than normal value specified in the national legislation, but lower than the alert threshold, whilst Cd, Zn and Cr, concentrations are below the normal values. The Pb values are higher than the alert thresold indicating its impact on soil. The obtained results in soil increased in the following order: Pb > Cu > Ni > Cr > Zn > Cd regarding the overpass the normal values. The explanation for the high concentration of lead is given by its tendency to link with iron oxides and carbonates and remaining in the upper soil layers [7]. As other studies have shown, it appears that the solubility of heavy metals is low because MSW is a good adsorbent matrix. [8].

The leachate is a liquid waste generated during the storage of the solid waste activities by: the ingress / percolating the rain water into / through the body of the landfill, the separation of the water contained in the waste stored and the decomposition of the stored biodegradable waste [9]. The leachate

can be loaded with organic and inorganic compounds, metals, etc., requiring collection and treatment. The chemical composition of the leachate may be influenced by the nature of the existing waste on the landfill. In the case of the simple storage without waterproofing base of the landfill, such as the Pata Rat the leachate flows into the soil under the landfill, and infiltrate into the deep water.

Table 2 shows the heavy metals concentration in the leachate sample from the Pata Rat landfill in comparison with the limits allowed by the legislation.

**Table 2.** Heavy metals concentration in the leachate sample

| Heavy metals | Concentration mg/dm$^3$ | Admissible limit values according to the Decree no.188/2002 NTPA 001 |
|---|---|---|
| **Cr** | 0.64 | 1.0 |
| **Pb** | 0.10 | 0.2 |
| **Ni** | 0.13 | 0.2 |
| **Cd** | 0.07 | 0.3 |
| **Cu** | 0.14* | 0.1 |
| **Zn** | 0.07 | 0.1 |

**Legend:** * values which are higher than the admissible limit values set by the Romanian legislation

The leachate analysis results show that heavy metal pollution is not an issue, except for Cu. However, it must be considered that the leachate is collected just from the third part of the landfill, while the other part doesn't have collection pipelines. The low concentrations of heavy metals found in leachate is due to the adsorption capability of the landfill layers. It was proved the tendency of Cu to combine in the form of sulphates with high mobility in soil and to be trained by the water storage [10, 11]. The biggest part of the leachate flows under the landfill for non-sanitary landfills. Even it is naturally refined, it is possible that a part of the hazardous substances reach the groundwater layer. The filtration degree of the soil and the retention of the hazardous substances from the leachate depends on the porosity, the ion exchange capacity and the retention capacity for the dissolved substances. The soil which contain clay will blok more the dissolved substances than the soil with big porosity [12].

The heavy metals content was investigated also in water samples from Zapodie stream that flows near the landfill in order to determine its ecological status, according to the Decree No 161 of the Government of 16$^{th}$ February 2006, used for the assessment and classification of the surface waters.

Table 3 presents the concentration of the heavy metals in the samples collected upstream and downstream of the landfill.

**Table 3.** Heavy metals concentration in the water samples

| Sample | Geografical coordinates | Cd µg/l | Cr µg/l | Cu µg/l | Ni µg/l | Pb µg/l | Zn µg/l |
|---|---|---|---|---|---|---|---|
| $Sw_1$ downstream | N 46°46'10,4" ; E 23°41'25.9" | 5.16 | 43.0 | 17.6 | 233 | 3.08 | 257 |
| $Sw_2$ upstream | N 46°48'58" ; E 24°01'43" | 4.71 | 40.8 | 13.8 | 230 | 2.01 | 20.3 |

**Legend:** Sw water sample

Table 4 presents the clasification of the streams regarding the ecological status, from the point of view of the metal contamination.

**Table 4.** Clasification of the stream regarding the ecological status, according to the the analyzed metal content ($S_{w1}$ (■), $S_{w2}$ (●))

| No. | Metal | Class I very good | Class II good | Class III moderate | Class IV weak | Class V bad |
|---|---|---|---|---|---|---|
| 1 | Cd | | | | ● | ■ |
| 2 | Cr | | ■● | | | |
| 3 | Cu | ■● | | | | |
| 4 | Ni | | | | | ■● |
| 5 | Zn | ■● | | | | |
| 6 | Pb | ■● | | | | |

**Legend:** Sw water sample: $Sw_1$ downstream, $Sw_2$ upstream

Water analysis results shows a bad quality for Cd, Ni, which indicate a high pollution of these metals. For Cu, Pb and Zn the results support a quality class I, and for Cr a quality class II. Although according to the assessment of the water quality in the Environmental Status Report 2010 [13], Zapodie stream was classified into the quality class V (bad), being determined by physical and chemical elements: oxygen regime, nutrients and salinity.

The obtained results highlight that in Zapodie stream has not reached a high concentration of heavy metals, which indicate that the transfer of the heavy metals from the landfill body into the nearby stream was made in a small measure. This can be explained by the fulfillment of the requirements settled by the Environmental Authority in the Environmental Notification for the closure, from 2003, by: fitting slope and cover with gravel the slope from the stream side, drainage channels and retention tanks for the leachate arrangement of Zapodie stream.

## CONCLUSIONS

It has been shown that the greatest impact on soil near the landfill is caused by Pb for which must be taken measures to prevent the further soil pollution and monitor the pollution source, according to the Decree No 756

of the Romanian Government. Copper ranks on second place in terms of the concentration in the soil over the normal value, while the concentrations of Cd, Zn, Cr, Ni in the soil did not exceed the normal values. In the leachate were found overruns just for Cu, and in the surface water were found overruns for Cd and for Zn. From these results, we conclude that the source of the water pollution with heavy metals, may belong of other pollution sources and not to the landfill.

The evaluation of heavy metal pollution of the water stream from the landfill area reveals an ecological status, class IV and V for cadmium and for nickel, and for its reducing should to be taken additional measures, such as stricter controls according to the measures specified in European legislation are that should be imposed on pollution sources.

The transfer of heavy metals from the landfill body into the leachate, soil and nearby water course was done in small measure.

Although heavy metals concentrations exceeded the normal values provided by law, the environmental pollution of the studied matrices (soil, water) is below the expectations, considering that the landfill is a non-sanitary one and it has been used for 37 years.

## EXPERIMENTAL SECTION

The metals from the soil can be strongly bounded or complexed. The sum of total forms represents the total metal content in the soil. The forms under which the metal can be found depends on the nature of the soil, pH and on the presence of the humic matter content [14].

The soil is characterized by the existing heavy metals, which depends on soil type and its composition and by soil contamination with these metals provided by human activity [15]. The soil samples were collected in December 2010, the leachate sampling was collected in January 2010 and the surface water samples were collected in March 2010, all in a rainy period.The soil sampling was carried out in plastic bags with a plastic spatula. Before sampling, the vegetal top layer was removed on an area of 15x20 cm. The samples were taken from a depth of 10-15 cm.

In order to determine the concentration of heavy metals, the atomic absorption spectrometer ZeEnit 700 was used. The atomic absorbtion spectrometer ZeEnit 700 is using to microelements determinations from sollution (the flame is using for mg/l determinations and the grafit furnace for μg/l determinations).

The collected soil samples were mineralized according to ISO 11466, by extraction with aqua regia. Water samples collected for heavy metal analysis were filtrated and acidified with $HNO_3$ 65%, at site, to a pH of about 3.5 to prevent the precipitation of the heavy metal ions and the retention of

these on the vessel wall. The leachate was collected in tightly closed glass or plastic bottles from the collection basin existing at the landfill base. For each metal, the device has an other error of measurement, but the maximum error is plus / minus 5%.

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# DETERMINATION OF PHTHALATES IN BOTTLED MILK USING HEADSPACE SOLID-PHASE MICROEXTRACTION COUPLED WITH GAS CHROMATOGRAPHY-MASS SPECTROMETRY

**MIRELA MICLEAN[a,*], OANA CADAR[a], CECILIA ROMAN[a]**

**ABSTRACT.** This study reports the level of contamination with phthalates of some commercial bottled milk collected in Cluj-Napoca, Romania. The investigated compounds were dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-butyl phthalate (DBP), butyl benzyl phthalate (BBP), di-(2-ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (DOP). The method used for the determination of these phthalate esters in commercial bottled milk consists in headspace solid-phase microextraction (HS-SPME) technique (100 μm PDMS fibre) coupled with gas chromatography-mass spectrometry (GC-MS) in single ion monitoring (SIM) mode. The recoveries for spiked samples were over 75% and under 95% (RSD 8-13%). The obtained concentrations ranged between 2.12-3.93 and 36.8-77.1 ng/g for DBP and DEHP, respectively, DMP, DEP, BBP and DOP were not detected in any sample.

***Keywords:*** *phthalates, bottled milk, HS-SPME-GC-MS*

## INTRODUCTION

Phthalates, esters of the phthalic acid, are used as plasticizers that improve the extensibility, flexibility and workability of polymeric materials and they are worldwide produced in high amounts. The most important representative is bis(2-ethylhexyl) phthalate (DEHP) and represents a quarter of the total production of plasticizers [1].

Humans are exposed to phthalates through the food, air, water, cosmetics, pharmaceutical products, etc., but the main exposure occurs through food, due to the use of PVC in wrapping materials and food processing [2, 3].

Phthalates could easily migrate into foods, beverages and drinking water from the packaging or bottling material, being ingested into the body [4]. Thus, food and beverage packaging could contribute significantly to human xenobiotic exposure, in addition to the environmental contaminants [5]. Phthalates being lipophilic compounds tend to be distributed preferentially in fatty foods (milk, meat, fish, olive oil) [4, 6]. Plastic additives, such as bisphenol-A, phthalates, nonylphenol, are suspected to be endocrine disruptors exhibiting mutagenic and carcinogenic action and are considered as important organic

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pollutants in the environment [1]. Recent research has associated phthalates exposure with the abnormal sexual development and birth defects in humans [7], with cardiovascular, liver, urologic diseases [3]. The US Environmental Protection Agency and the European Union classified phthalates in their top priority lists for risk assessment [8, 9].

Due to the ubiquity of phthalates in the environment, bottled milk could be contaminated in many ways: contamination of water, air, soil, also during bottling process, or migration from the packaging material of the bottle to the milk, leaching from PVC tubing into raw milk during milking at dairy farms, etc. [10, 11].

Recently, many efforts have been made for the development of simple and sensitive analytical methods for determination of phthalates in different samples. Conventional extraction methods, such as liquid–liquid solvent extraction (LLE) and solid-phase extraction (SPE) consume high volumes of toxic organic solvents [1, 11].

Solid-phase microextraction (SPME), developed by Arthur and Pawliszyn (1990), is a simple, rapid and effective extraction technique due to the incorporation of sampling, extraction and concentration into a single solventless step, saving preparation time and the risk of secondary pollution of the sample by reagents and vessels is considerably reduced [8, 12, 13]. Also, the use of headspace extraction has the advantage of the elimination of the complex matrices (milk) [11].

Methods for determination of phthalates in milk samples were reported: SPME/GC-MS [11], ultrasound-assisted dispersive liquid–liquid microextraction (UA-DLLME) followed by gas chromatography–flame ionization detection (GC–FID) [14], selective molecularly imprinted solid-phase extraction (MISPE) technique coupled with spectrophotometry [15], LLE [16], automated solid phase extraction (SPE) coupled to isotope dilution–high-performance liquid chromatography (HPLC) [17].

The aim of this study was to investigate the level of contamination with phthalates of commercially available bottled milk samples (PET packaging) collected from the local markets in Cluj-Napoca, Romania, using HS-SPME/GC-MS method. According to our knowledge, there is a lack of information regarding the occurrence of these types of contaminants in bottled milk in the studied area and in Romania.

## RESULTS AND DISCUSSION

The recovery was determined on five replicates of milk with “zero” phthalates spiked with phthalate standard mix at 1.0 µg/L of each analyte, analyzed by HS-SPME/GC-MS. Mean recoveries are ranged from 75 to 99% and the coefficient of variation varied between 8 and 13%, as shown in Table 1.

Good linear correlation coefficients (R2) were found for the compounds, as shown in Table 1.

The limit of detection (LOD) was calculated from the measured value of the milk with "zero" phthalates sample (mean + 3 standard deviation) analyzed using HS-SPME/GC-MS procedure, and the limit of quantification (LOQ) was calculated as three times detection limit.

**Table 1.** Method performance for phthalates in milk

| | DMP | DEP | DBP | BBP | DEHP | DOP |
|---|---|---|---|---|---|---|
| $R^2$ | 0.9975 | 0.9984 | 0.9991 | 0.9982 | 0.9760 | 0.9662 |
| LOD (ng/g) | 0.25 | 0.16 | 0.13 | 0.21 | 1.22 | 0.36 |
| LOQ (ng/g) | 0.75 | 0.48 | 0.39 | 0.63 | 3.66 | 1.08 |
| Recovery (RSD%) | 75 (9) | 80 (11) | 88 (10) | 90 (9) | 93 (8) | 96 (13) |

The investigated milk samples represent 4 different types of commercial milk and were bottled in polyethylene terephthalate (PET) packaging. The results of phthalates from the four investigated bottled milk samples are shown in Table 2. The results were calculated as the arithmetic means of concentrations obtained for the same analyzed milk samples.

**Table 2.** Mean concentration (ng/g) of phthalate esters in the bottled milk samples

| Sample | DMP | DEP | DBP | BBP | DEHP | DOP | %Fat |
|---|---|---|---|---|---|---|---|
| 1 | < LOD | < LOD | 2.81 | < LOD | 36.8 | < LOD | 2.5 |
| 2 | < LOD | < LOD | 3.93 | < LOD | 77.1 | < LOD | 3.0 |
| 3 | < LOD | < LOD | 2.12 | < LOD | 68.2 | < LOD | 2.0 |
| 4 | < LOD | < LOD | 3.88 | < LOD | 42.6 | < LOD | 3.0 |

ND=not detected, concentrations below the detection limit

DMP, DEP, BBP and DOP were not detected in any sample. DBP and DEHP were detected in all milk samples, in the range of 2.12-3.93 ng/g and 36.8-77.1 ng/g, respectively. The results obtained for DBP are comparable with those obtained by Feng et al. (2005) and the obtained concentrations of DEHP are lower than those obtained by the same authors for processed cow milk samples [11]. The obtained concentrations of DEHP fall within the range reported by Sharman et al. (1994) in commercial milk (50-130 ng/g) [19], except for the samples 1 and 4, which are lower (36.8 and 42.6 ng/g, respectively).

## CONCLUSIONS

Due to the high consumption of bottled milk and due to the potential health risk of phthalates, the control of these consuming products is of special concern.

In this study, solid phase microextraction using PDMS-100 µm fiber followed by capillary gas chromatography coupled to mass spectrometry in the SIM mode acquisition was used for analysis of six phthalates from different

bottled milk samples collected from markets in Cluj-Napoca, Romania. The only two phthalates were found in the investigated samples, namely DBP and DEHP with the concentrations in the range of 2.85-6.28 ng/g and 36.84-112.3 ng/g, respectively. Therefore, commercial milk could be considered as a source of human exposure to phthalates.

## EXPERIMENTAL SECTION

### Reagents, materials and apparatus

A standard stock solution containing six phthalate esters, dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-butyl phthalate (DBP), butyl benzyl phthalate (BBP), di-(2-ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (DOP), in iso-octane at a level of 1000 µg/mL per compound was purchased from LGC Standards (Wesel, Germany). The sodium chloride (99.5%) was supplied from Merck (Darmstadt, Germany). Iso-octane (Suprasolv) was purchased from Merck (Darmstadt, Germany) and was used for the preparation of the calibration standard solutions by serial dilutions. The working standard solutions of phthalates at 0.1, 0.5, 2.5, 5, 10, and 20 µg/mL concentrations were prepared by diluting the individual stock solutions in iso-octane. All solutions were stored at 4ºC. The SPME device for manual sampling consisting of a holder assembly and 100 µm polydimethylsiloxane (PDMS) fiber was obtained from Supelco (Bellefonte, PA, USA). The fiber was conditioned prior to use according to the manufacturer's requirements by heating in the injection port of the chromatographic system.

A magnetic stirrer/temperature-controlled water bath IKA RET with IKA ETS-D5 digital thermometer was from IKA Werke Gmbh (Staufen, Germany). The carrier gas used for GC was helium (>99.9999%) supplied by Linde Gas (Cluj-Napoca, Romania).

### SPME procedure

The SPME was performed according to the methods described by Feng [11] and Cao [18]. 5 g NaCl were weighed into a 20 mL vial, then 10g of milk sample and a magnetic stirring bar were added and the vial was tightly closed with the vial cap. The vial was placed on a preheated water bath (90ºC) on the hot plate and the sample was continuously stirred at a constant speed (700 rotations/min). The SPME syringe was introduced by the septum and the fiber was exposed to the headspace for 60 min. After the extraction, the fiber was immediately inserted into the GC injection port and allowed 10 min for desorption. All samples were analyzed in duplicate. Also, a blank analysis was performed using 10 g of milk with "zero" phthalates in the vial and the fiber was exposed under the same conditions as the standards and the samples. The concentration of phthalates in samples was calculated after subtraction of the blank value.

The SPME fiber was checked for its fiber blank after each sample run to avoid any carryover effect from the previous sample run.

In this study, PDMS-100 µm fiber was used, although this fiber had lower extraction efficiency for DMP and DEP, but 100% efficiency for DBP, BBP, DEHP, DOP, since DEHP and DBP are the two major phthalates detected in milk, in former studies [11].

## GC-MS analysis

The analysis was performed on Hewlett-Packard (Agilent Technologies, Palo Alto, CA, USA) HP 6890 series GC, equipped with a split/splitless injector and a HP 5975 mass selective detector system. The MS was operated at the electron impact (EI) mode (70 eV). Desorption of the fiber into the injection port was carried out in the splitless mode at 280ºC for 5 min and then maintained in injection port 30 min with purge gas turned on, before the next extraction. A HP-5MS, 5% diphenyl 95% dimethyl polysiloxane capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) from Agilent Technologies was used. Oven temperature was set at 100ºC, increased at 8ºC/min up to 260ºC, increased at 35ºC/min up to 310ºC and held for 10 min and the running time being 31.43 min. The MSD transfer line heater, ion source and quadrupole analyzer temperatures were set at 320, 230 and 150ºC, respectively.

The qualitative and quantitative analyses were performed by comparison with the external standards. The target and the qualifier ions were determined by injection of standards under the same chromatographic conditions using full-scan with the mass/charge ratio ranging from 100 to 550 *m/z*. A quantitative analysis was made using selected ion-monitoring (SIM) by acquiring the signals of the target ions (as quantifier).

The compounds of interest were identified by comparing the retention time with that of the standard compounds. The retention times, target and qualifying ions of the investigated phthalates are shown in Table 3.

**Table 3.** Retention time and selected ions for the analysis of the phthalates

| Compound | Retention time (min) | Quantification ions | Identification ions |
|---|---|---|---|
| DMP | 9.463 | 163 | 77, 194 |
| DEP | 11.434 | 149 | 177, 104 |
| DBP | 16.189 | 149 | 223, 104 |
| BBP | 20.503 | 149 | 91, 206 |
| DEHP | 21.723 | 149, 167 | 279 |
| DOP | 22.769 | 149 | 279, 104 |

The milk with "zero" phthalates, used as blank, was obtained from a private cow (in Coruşu village, Cluj county, away from any pollution source); the milk was carefully manual milked and transported to the laboratory in a clean glass bottle.

The blank values of the analytical procedure were determined by extracting the milk with "zero" phthalates in which the phthalates standard was not added. Only a small chromatographic peak for DEHP was recorded in the chromatogram of the blank procedure. The concentrations of phthalates in real samples were calculated after subtraction of the blank value.

The calibration was performed using multilevel spiked samples: 10g of milk with "zero" phthalates samples were spiked with 10 µL of the following working standard solutions of phthalate: 0.1, 0.5, 2.5, 5.0, 10 and 20 µg/mL (in iso-octane) in a 20 mL clear glass vials with Teflon-lined silicone rubber septum (Agilent Technologies), then analyzed using HS-SPME/GC-MS procedure. The obtained theoretical concentrations were: 0.1, 0.5, 2.5, 5.0, 10 and 20 ng/g milk.

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# LC/MS/MS METHOD FOR INVESTIGATION OF FIVE USUAL PESTICIDES FROM WATER

**DORINA SIMEDRU[a,*], ANCA NAGHIU[a], OANA CADAR[a]**

**ABSTRACT.** Pesticides are chemical compounds used in agriculture to destroy the pests and weeds but with great potential on environment contamination. An analytical method for screening and confirming the presence of 5 pesticides (acetamiprid, cymoxanil, mefenoxam, thiacloprid, thiametoxam) was developed. LC/MS/MS was proved to be a sensitive technique for these classes of compounds (insecticides and fungicides). Five calibration curves at six levels of concentration were obtained with good correlation coefficients (0.9960-0.9990). The method detection limit (MDL) ranges between 0.4–0.75 ng/ml and the method quantification limit (MQL) ranges between 1.2–2.25 ng/ml. Two methods: a liquid-liquid extraction and a solid phase extraction were tested. The selection criteria was the recovery degree which was prove to be more adequate for liquid-liquid extraction (71-102%) than for solid phase extraction (32-87%).

***Keywords:*** *acetamiprid, cymoxanil, mefenoxam, thiacloprid, thiametoxam, LC-MS-MS, solid phase extraction, SPE*

## INTRODUCTION

In the modern agricultural practices, different synthetic pesticides such as: insecticides, larvacides, miticides, mollucides, nematocides, fumigants, fungicides, herbicides and defoliants are extensively used to improve the agricultural production by destroying pests and weeds [1]. Besides their positive effects, the use of pesticides may lead to contamination of surface and ground water by drift, runoff, drainage and leaching. This contamination may have ecotoxicological effects for aquatic flora and fauna and for human health if used for public consumption [2]. Neonicotinoids are class of neurotoxic insecticides designed in the ’80s, highly systemic with long-term persistence. The neonicotinoids permanently bind to nicotinic receptors of acetylcholine, blocking the receptors and consequently the passage of nerve impulses [3]. The neonicotinoids are used for pome fruits, stone fruits, citrus, grape, horticultural and industrial crops, flower and ornamental plants against insects such as:

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Aphids, whiteflies, planthoppers, scale insects, Lepidoptera, soil insects, Colorado potato beetle [3]. Acetamiprid, thiacloprid, thiametoxam (Table 1) are among the most used neonicotinoids. Their use is limited in fruits and vegetables by the Regulation (EC) No 396/20053 of European Union, which establishes the rules governing the setting of pesticide MRLs (maximum residues limits) [4-6]. Cymoxanil is a fungicide belonging to aliphatic nitrogen compounds. It acts as a foliar fungicide with protective and curative action. It presents contact and local systemic activity, and also inhibits sporulation [7]. Mefenoxam is the systemic phenylamide fungicide, being widely used in phytophthora disease control which causes late blight, downy mildew, wetting and rot of stems and fruits of many plants, including tomato, cantaloupe, cucumber, eggplant, pepper, squash, grape, pimiento, litchi, watermelon and tobacco. It is the R-enantiomer of metalaxyl that has been on the market since 1996 under various formulations and trade names [8].

Due to their assumed ecotoxicological effects, a method for determining these pesticides is mandatory in order to protect the consumers, for environmental assessment and to establish the quality of agricultural products. In the last years, several techniques such as: ELISA [9], HPLC-UV [10-13], GC–NPD [14], GC-NCI-MS [15], GC-MS-MS [16, 17], LC-MS-MS [18-22], LC/TOF-MS [23] were used to determine some neonicotinoids and fungicides from food, water and soil. The purpose of these studies was to develop an efficient, quick and environmental friendly method at low cost.

This study follows to develop a LC/MS/MS method for determining the residuals of five pesticides (see Table 1) from water samples. These pesticides are widely used in modern agriculture and can occur in waters from the proximity of agriculture's areas.

## RESULTS AND DISCUSSION

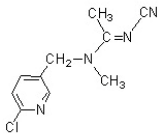
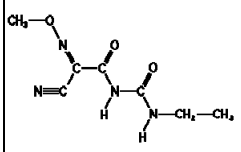
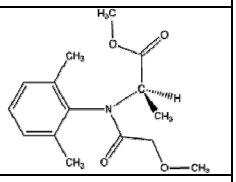
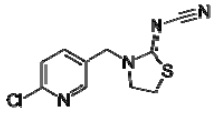
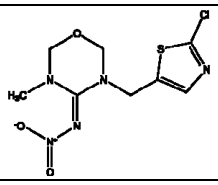
The analytical method developed during this study was intended to investigate several of the most representative pesticides from two classes: fungicides and insecticides. Table 1 contains basic information about the selected pesticides.

### Develoment of ESI-MS profile

The development of ESI-MS profile started with the analysis of all the investigated pesticides both in positive and negative ionization modes. The protonated molecule $[M+H]^+$, representative for positive ionization, was proved to be more abundant in all five cases and therefore was selected for further investigation. Each compound was injected directly in MS, scanned in the first quadrupole (Q1 mode) to select the parent ion, defragmented in the collision cell (Q2) and then scanned in the third quadrupole (Q3 in product

ion mode (PI)) to select the product ion. This process of selecting the parent ion and the product ion was followed by an automatic optimization of the compound dependent parameters (Infusion) for each pesticide. These parameters are: DP (declustering potential), EP (entrance potential), CE (collision energy) and CXP (collision cell exit potential) and the obtained values are presented in Table 2. The automatic optimization of the compound dependent parameters for each precursor ion → product ion transition has optimized the MS/MS conditions in a MRM method (multiple reaction monitoring) characteristic for each compound. An automatic function (Merge MRM) was used to merge all MRM methods in one MRM characteristic to all compounds.

**Table 1.** Basic information of selected pesticides

| Chemical name | Chemical class | Chemical formula | Molecular weight ($g \cdot mol^{-1}$) | Structural formula |
|---|---|---|---|---|
| Acetamiprid | Insecticides (Neonicotinoides) | $C_{10}H_{11}ClN_4$ | 228.68 |  |
| Cymoxanil | Acetimide (Fungicides) | $C_7H_{10}N_4O$ | 198.2 |  |
| Mefenoxam | Phenylamide (Fungicides) | $C_{15}H_{21}NO_4$ | 279.33 |  |
| Thiacloprid | Insecticides (Neonicotinoides) | $C_{10}H_9ClN_4S$ | 252.72 |  |
| Thiametoxam | Insecticides (Neonicotinoides) | $C_8H_{10}ClN_5O_3S$ | 291.71 |  |

## Development of LC/MS/MS profile

The next step in developing the LC/MS/MS method consists is performing FIA (Flow Injection Analysis), an automatic optimization of the source dependent parameters. FIA is accomplished by injecting 100ng/mL standard solution in MeOH:H2O (60/40, v/v) through the LC connected to the MS/MS by a restriction capillary, using the MRM method developed earlier. The source dependent parameters are: CUR (curtain gas), CAD (collision gas), IS (ionspray voltage), TEM (temperature), GS1 (gas 1) and GS2 (gas 2) and their role is to optimize the compounds signal in LC conditions. The obtained values are presented in Table 2.

## Development of chromatographic method

In order to establish the optimum mobile phase, different composition of $H_2O$ (A) and MeOH (B) mixture were tested in isocratic conditions. The tested mobile phase compositions were: A:B (80/20,v/v); A:B (60/40,v/v); A:B (40/60,v/v) and A:B (20/80,v/v). In order to obtain a good separation and well defined shape of the chromatographic peaks the following LC parameters were chosen:

Injection volume: 50µL;

- Flow rate: 500µL/min;
- Column temperature: 28°C;
- Mobile phase: A:$H_2O$+0.1% ammonium acetate; B:MeOH+0.1% ammonium acetate;
- Gradient program:

| Time (min) | Flow rate (µL/min) | A (%) | B (%) |
|---|---|---|---|
| 0.00 | 500 | 20.0 | 80.0 |
| 7.00 | 500 | 50.0 | 50.0 |

**Table 2.** The compound and source dependent parameters obtained by automatic optimizations (Infusion and FIA) for the investigated pesticides

| Compound | Q1 Mass (u) | Q3 Mass (u) | DP (V) | EP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|
| **Acetamiprid** | 223.10 | 126.00 | 41.00 | 7.50 | 27.00 | 4.00 |
| **Mefenoxam** | 280.20 | 220.30 | 26.00 | 6.50 | 17.00 | 4.00 |
| **Cymoxanil** | 199.10 | 128.00 | 11.00 | 12.00 | 11.00 | 4.00 |
| **Thiacloprid** | 253.10 | 126.10 | 36.00 | 4.50 | 27.00 | 4.00 |
| **Thiametoxam** | 292.10 | 211.20 | 26.00 | 9.00 | 17.00 | 4.00 |
| **FIA parameters**<br>CUR:30.00 psi; CAD:Medium; IS:5500.00V; TEM:400°C; GS1:35 psi; GS2:35 psi. | | | | | | |

The 100ng/mL standard solution was injected using the LC/MS/MS method developed earlier with the chosen analytical column. The chromatogram obtained for the investigated pesticides in a single run are presented in Figure 1 and their MRM transitions are presented in Figure 2.

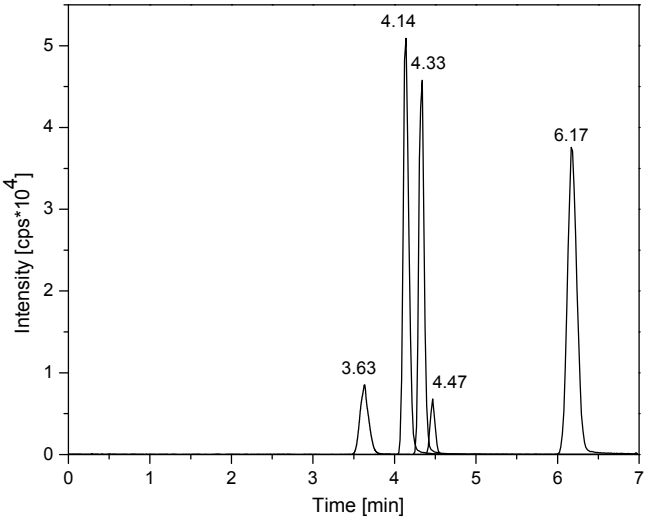



**Figure 1.** Total ion chromatogram of 100 ng/mL standard solution

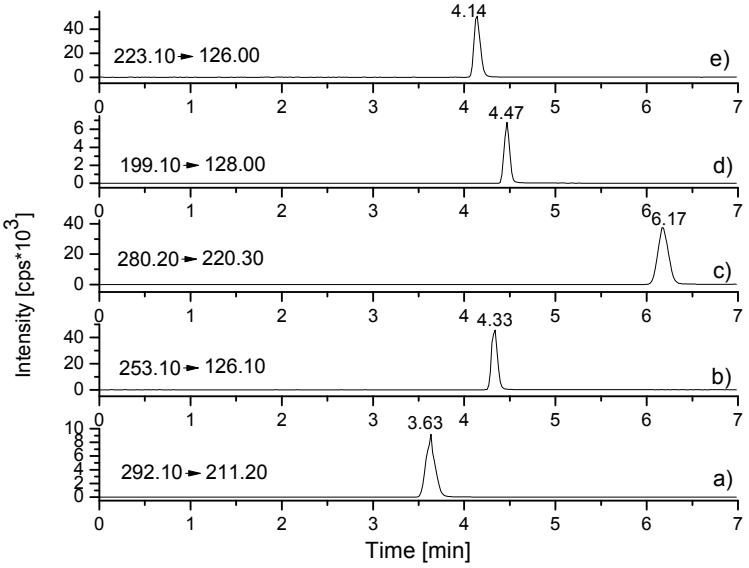



**Figure 2.** MRM transition of a) Thiametoxam; b) Thiacloprid; c) Mefenoxam; d) Cymoxanil and e) Acetamiprid

## Quantitative analysis

Ten concentrations of standard solutions between 0.4 to 100ng/mL were prepared for all investigated pesticides. Five calibration curves at six levels were obtained. The correlation coefficients ($r^2$) were automatically determined using a regression function (1/x*x). Their values were higher than 0.9960 which proves the linearity of the method. The results obtained are presented in Table 3.

For determining the method detection limit (MDL) and the method quantification limit (MQL) the following theory was applied. The method detection limit (MDL) is defined as the smallest amount of an analyte that can be reliably detected or differentiated from the background of a particular matrix (using the specified method). All the matrix interferences must be taken into consideration while determining the MDL. Similarly, the method quantification limit (MQL) is defined as the smallest amount of an analyte that can be reliably quantified with good reliability from a particular matrix (using the specified method) [24]. The real MQL was defined as the lowest validated spike level meeting the requirements of a recovery within the range 70–120% and a RSD≤20% [19]. The instrument detection limit (IDL) and the instrument quantification limit (IQL) were obtained by injecting six time the standard solutions at three lowest detectable concentration levels (0.4ng/mL; 0.75ng/mL; 1ng/mL) [19].

**Table 3.** Reteantion time, linear range, linear equation, $r^2$, MDL, MQL, IDL, IQL and recovery for both extraction methods of the investigated pesticides

| Analyte | Acetamiprid | Mefenoxam | Cymoxanil | Thiacloprid | Thiametoxam |
|---|---|---|---|---|---|
| **Retention time** | 4.14 | 6.17 | 4.47 | 4.33 | 3.63 |
| **Linear range (ng/mL)** | 2.5-75 | 2.5-75 | 2.5-75 | 2.5-75 | 2.5-75 |
| **Linear equation** | $2.95*10^4x+8.29*10^3$ | $2.85*10^4x+7.96*10^3$ | $4.72*10^3x-163$ | $4.14*10^4x+7.25*10^3$ | $6.37*10^3x+807$ |
| **$r^2$** | 0.9960 | 0.9990 | 0.9980 | 0.9970 | 0.9980 |
| **MDL** | 0.4000 | 0.4000 | 0.7500 | 0.4000 | 0.7500 |
| **MQL** | 1.2000 | 1.2000 | 2.2500 | 1.2000 | 2.2500 |
| **IDL** | 0,4335 | 0,5715 | 0,6345 | 0,4245 | 0,6735 |
| **IQL** | 1,4450 | 1,9050 | 2,1150 | 1,4150 | 2,2450 |
| **RSD (%)** | 5,7800 | 7,6200 | 8,4600 | 5,6600 | 8,9800 |
| **Recovery (%) -1st method** | 95 | 73 | 86 | 102 | 68 |
| **Recovery (%) -2th method** | 39 | 32 | 55 | 87 | 41 |

The samples prepared by two extraction methods were analyzed in order to obtain the recovery and the repeatability of each method. Three standard solutions of known concentrations (2.5ng/mL; 5ng/mL; 50ng/mL) were spiked with water samples of known concentration. The samples were analyzed using the LC/MS/MS method developed earlier and the relative standard deviation (RSD) and the recovery obtained for each pesticide were determinate. The values obtained for the method detection limit (MDL), the method quantification limit (MQL), the instrument detection limit (IDL), the instrument quantification limit (IQL), the relative standard deviation (RSD) and the recovery are presented in Table 3.

The LC/MS/MS method was proved to be successful in investigating five pesticides: thiametoxam, thiacloprid; mefenoxam; cymoxanil and acetamiprid from water samples.

## CONCLUSIONS

A LC/MS/MS method was developed for screening and determination of five pesticides (herbicides and fungicides). Five calibration curves at six levels were obtained. The correlation coefficients ($r^2$) range between 0.9960 and 0.9990 depending of the pesticide. MDL (method detection limit), MQL (method quantification limit), IDL (instrument detection limit) and IQL (instrument quantification limit) were determinate. Two methods: liquid-liquid and solid phase extraction were tested. The liquid-liquid extraction was chosen for its superior recovery degree (71-102%) comparing with the one obtained solid phase extraction (32-87%).

The results suggest that the developed method is appropriate for determining the five tested pesticides: acetamiprid, cymoxanil, mefenoxam, thiacloprid, thiametoxam from water samples.

## EXPERIMENTAL SECTION

### Chemicals and reagents

Acetamiprid, Cymoxanil, Mefenoxam, Thiacloprid, Thiametoxam, anhydrous sodium sulphate, (≥99.0%) and hydrochloric acid (37%) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol LC-MS Optigrade (≥99.8%), Ammonium acetate ULC-MS Optigrade (99-100%), Diethyl ether HPLC Optigrade (≥99%), Dichloromethane HPLC Optigrade (≥99.8%) were acquired from LGC Standards. SPE Strata C18-E 200 mg/ 3 mL cartridges were purchased from Phenomenex, USA. Water was purified using a Milli-Q Ultrapure water purification system (Millipore, Bedford, MA, USA).

**Standard solution preparation**

The stock solutions (1mg $mL^{-1}$) were prepared by dissolving 1mg of each pesticide in 1mL of MeOH using a vortex mixer. The samples were stored under refrigeration at 4°C in the dark to avoid the alteration. The working standard solutions of 0.4; 0.75; 1; 2.5; 5; 10; 25; 50 and 75 ng $mL^{-1}$ concentrations were prepared by diluting the stock solution with a mixture of MeOH:H2O (60/40, v/v). These standard solutions were used for method optimization and for the calibration curves.

**Sample extraction**

Two extraction methods, adapted by literature [25, 26], were tested: 1. 0.5L of tap water or ground water, adjusted at pH 2.5 with HCl and spiked with standards, was extracted three times with 50mL of dichloromethane. The residual water was removed from the combined organic extract by addition of anhydrous sodium sulfate. The solvent evaporation was performed in a Laborota 4010 Rotary Evaporator. The solvent was evaporated using a stream of nitrogen gas. 1mL of MeOH:H2O (60/40, v/v) was added to the residue and the solution was then filtered through a 0.45µm membrane before the injection into the chromatograph [25]. 2. The solid phase extraction was performed on a 200mg C18 analytical cartridge. The cartridge was conditioned with 3mL $H_2O$ and 3mL MeOH. 1L of water containing all the investigated pesticides was loaded on the cartridge. After drying the cartridge, the retained analytes were eluted using 3 mL $(C_2H_5)_2O$:MeOH (90/10, v/v) and 3mL MeOH. They were then collected on a conical-bottom centrifuge tube for evaporation to dryness with nitrogen. 1mL of MeOH:H2O (60/40, v/v) was added to the residues [26].

**LC/MS/MS analysis**

The LC/MS/MS analysis were carried out on a HPLC Agilent 1200 Series coupled with an Applied Biosystems API 3200 QTRAP mass spectrometer using a TurboV source. The system was controlled by Analyst 5.1 software. A Zorbax Eclipse XDB-C18 column (4.6 X 150mm, 5µm) purchased from Agilent Technologies was used for the separation. The elution of selected compounds was performed using a gradient mobile phase starting with A:B (80/20,v/v) and finishing with A:B (50/50,v/v), where A is $H_2O$+0.1% ammonium acetate and B is MeOH+0.1% ammonium acetate, in a 7 min run with a flow rate of 0.5 mL $min^{-1}$. The column temperature was set at 28°C.

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# AN ASSESSMENT OF RARE EARTH ELEMENTS COMPOSITION OF ROMANIAN METEORITIC MATERIAL USING AN INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY METHOD

**CLAUDIU TĂNĂSELIA[a], MIRELA MICLEAN[a], CECILIA ROMAN[a], DANA POP[b]**

**ABSTRACT.** Inductively coupled plasma mass spectrometry method was used for assessing rare earth elements (REE) concentrations in two Romanian ordinary chondritic meteorites, Mocs (L5-6) and Pleşcoi (L5-6). The obtained results show that the two rare earth elements compositions are, as expected, similar. The lowest concentration was obtained for Tm in both meteorites: 0.032 ± 0.003 mg/kg for Mocs and 0.034 ± 0.003 mg/kg for Pleșcoi, while the highest values are shared between Ce in the case of Mocs (0.606 ± 0.048), and Nd in the case of Pleşcoi (0.512 ± 0.041). Our results were compared with mean carbonaceous chondrite values found in literature; a scaling factor of 1.7 ± 0.2 was found for our ordinary chondrites.

***Keywords**: inductively coupled plasma mass spectrometry, meteorite, rare earth elements, ultrasonic nebulization*

## INTRODUCTION

Eight meteorite falls were officially recorded within Romania's borders, the most recent one being Pleşcoi, on June 12, 2008. However, the best know and studied Romanian meteorite is also the one with the largest recovered mass (around 300 kg). Its fall in the area of Mocs village (current locality name: Mociu, Cluj County), on February 3, 1882, was witnessed all around Transylvania and partly in the neighbouring regions. Both meteorites are classified as L5-6 type chondrites [1], [2]. Meteorites are rare natural materials, of high scientific and cultural value. Thus, in general, only small samples can be used for investigations using destructive methods, such as inductively coupled plasma spectrometry. In such cases, one has to find methods to optimise the data acquisition efficiency.

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An ultrasonic nebulizer was used in order to achieve higher sensitivity for rare earth elements (REE) determination in the studied meteorites. Information on rare earth elements concentration in chondrites allows performing further geochemical studies involving solar system dynamics [3],[4]. It was investigated if there is any direct relationship between the REE composition of the two ordinary chondrites and carbonaceous chondrites. Carbonaceous chondrites (classified into CI, CM, CV, CO, CK, CR, CH, CB, and C ungrouped) are considered to represent the most primitive and unaltered meteorites. Accordingly, they are compositionally closest to the nebula from which the Solar System formed [6]. This is particularly true for the CI group ("I" originates from the type meteorite in this group, *i.e.*, Ivuna). CI meteorites are thus used as a standard for evaluating the degree of chemical fractionation of materials formed throughout the solar system, including ordinary chondritic meteorites. Rare earth elements concentration was measured in this study and compared with literature values for the same meteorite class, confirming the chondritic nature of both Mocs from Pleşcoi from REE composition point of view and providing new data for the both meteorites.

## RESULTS AND DISCUSSION

Rare earth elements compositions of Mocs and Pleşcoi meteorites are listed in Table 1. The graphical relationship between the two samples is displayed in Figure 1 where 14 points are displayed. The slope for the linear regression is 0.983 ± 0.159 (95% confidence interval) and the interception

**Table 1.** Rare earth elements concentrations measured in Mocs and Pleșcoi meteorites (± one standard deviation), five measurements were performed for each sample. Obtained values are compared with CI carbonaceous chondrites mean concentration (literature data [6]).

| Element | Concentration (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Mocs | | | Pleșcoi | | | CI mean |
| La | 0.470 | ± | 0.038 | 0.470 | ± | 0.038 | 0.237 |
| Ce | 0.606 | ± | 0.048 | 0.512 | ± | 0.041 | 0.613 |
| Pr | 0.086 | ± | 0.007 | 0.085 | ± | 0.007 | 0.093 |
| Nd | 0.532 | ± | 0.043 | 0.567 | ± | 0.045 | 0.457 |
| Sm | 0.256 | ± | 0.020 | 0.216 | ± | 0.017 | 0.148 |
| Eu | 0.118 | ± | 0.009 | 0.061 | ± | 0.005 | 0.056 |
| Gd | 0.367 | ± | 0.029 | 0.375 | ± | 0.030 | 0.199 |
| Tb | 0.054 | ± | 0.004 | 0.055 | ± | 0.004 | 0.036 |
| Dy | 0.429 | ± | 0.034 | 0.460 | ± | 0.037 | 0.246 |
| Ho | 0.090 | ± | 0.007 | 0.097 | ± | 0.008 | 0.055 |
| Er | 0.283 | ± | 0.023 | 0.308 | ± | 0.025 | 0.160 |
| Tm | 0.032 | ± | 0.003 | 0.034 | ± | 0.003 | 0.025 |
| Yb | 0.283 | ± | 0.023 | 0.308 | ± | 0.025 | 0.161 |
| Lu | 0.044 | ± | 0.004 | 0.040 | ± | 0.003 | 0.025 |

plots at 0.0001 ± 0.01707 thus, the data for the two samples show a strong positive correlation. The REE mean values for CI carbonaceous chondrites, as indicated in the literature [6], were considered for comparison. A scaling factor was computed for the Romanian ordinary chondrites for Sm, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu. Other elements (La, Ce, Pr, Nd, Eu) were excluded given their higher concentration or factor variation. The scaling factor was calculated as the corresponding value for the studied ordinary chondrites divided by the CI mean value for each considered element. The obtained value is 1.7 ± 0.2, which is consistent with other studies [7], [8]. The difference in the mean REE concentration of CI and the two ordinary chondrites is caused by the different cosmic history experienced by the two categories of meteorites.

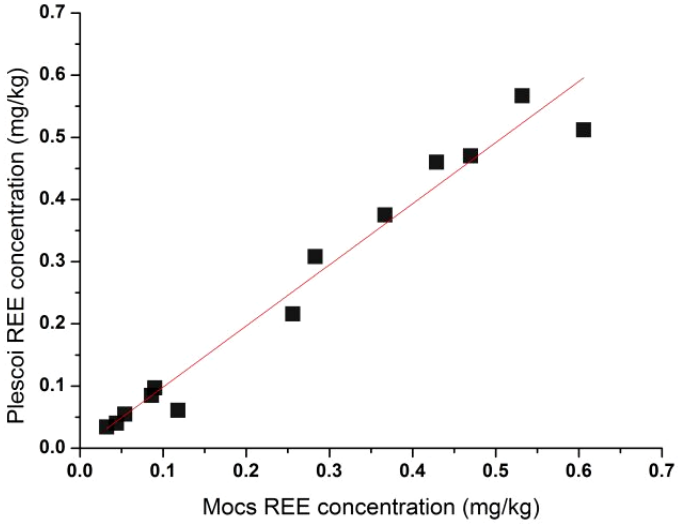


**Figure 1.** Rare earth elements correlation between Mocs and Plești meteorites.

## CONCLUSIONS

Using a simple, four step method, an ICP-MS protocol for determining rare earth concentration in highly valuable samples was developed. In particular, this approach was used for the investigation of two Romanian chondrites, Mocs and Plești - the latest one being a relatively recent fall. For achieving a better sensitivity an ultrasonic nebulizer was used that successfully increased the ion signal up to 20 times, allowing a much better detector response. Rare earth elements concentrations are reported for both meteorites and compared with literature values for CI carbonaceous chondrites.

Our results represent the first REE data for the two meteorites. The consistency of our data suggests that the experimental set up we propose yields reliable results concerning the rare earth elements contents in meteorites, and in similar materials. Also, based on linear regression interpretation of the REE results, both meteorites share the same chondritic class.

## EXPERIMENTAL

A SCIEX Perkin-Elmer DRC II (Ontario, Canada) inductively coupled plasma mass quadrupole spectrometer paired with CETAC UA6000AT ultrasonic nebulizer (Omaha, USA) was used for the measurements. Parameters of the instrument are expressed in Table 2. Ultrapure DI water (18 MΩ $cm^{-1}$) for sample preparation was produced by a Millipore Milli-Q Integral Water Purification System. Solution Standard II containing 16 rare earth elements, also purchased from Perkin Elmer, was used for calibration during the analysis. All other reagents used for sample treatment were purchased from Merck (Darmstadt, Germany).

Calibration was performed by diluting Multi-element Calibration Standard 2 solution (purchased from Perkin Elmer, containing a concentration of 10 mg/L from each of the following elements: Ce, Dy, Er, Eu, Gd, Ho, La, Lu, Nd, Pr, Sm, Sc, Tb, Th, Tm, Y, Yb). The calibration ranged between $1.10^{-4}$ mg/L and $1.10^{-2}$ mg/L. The ICP-MS detector was set in pulse mode since ion counts were less than $10^6$ per second (the highest cps count was obtained for 0.010 mg/L Tm, *i.e.*, $8.2 \cdot 10^5$, while the lowest cps count for $1.10^{-4}$ mg/L Gd, *i.e.*, $1.7 \cdot 10^4$).

**Table 2.** ICP-MS general experimental parameters

| | |
|---|---|
| Plasma power | 1100 W |
| Plasma gas flow | 12 L/min |
| Auxiliary gas Flow | 1.2 L/min |
| Nebuliser gas flow | 0.91 L/min |
| Sample/Skimmer cone | Platinum |
| Quadrupole rod offset | 0 V |
| Cell rod offset | -8 V |
| Cell path Voltage | -16 V |
| Dwell time | 200 V |
| RPq | 0.25 |
| Rpa | 0 |
| Replicates | 5 |
| Lens voltage | 7.1 V |

Before measuring every sample batch, the instrument was optimized for best signal/noise ratio, using a standard set-up solution purchased from Perkin Elmer containing 0.001 mg/L In. This element is used for verifying instrument stability and general performance. Also, Ce and Ba included in the same solution offered a good view on oxides (CeO) and double ionized species ($Ba^{++}$) produced in the plasma region. We acted to keep those levels below 3 % threshold, in order to avoid any significant interference.

Samples were washed with ultrapure water and then processed into a fine powder; 0.2 grams of each were used for measurements [9]. Nitric acid was added in the first step, followed by hydrofluoric and perchloric acid. The mix was heated to 100 °C for a few hours then the temperature was increased to allow the perchloric acid to evaporate. After the sample dried and cooled down, a mixture of nitric acid and deionized water was added to make a total volume of 10 ml. This represented the final solution that was further analysed directly by ICP-MS.

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# INTEGRATED ENVIRONMENTAL IMPACT AND RISK ASSESSMENT OF THE AGRICULTURAL AND RELATED INDUSTRIES IN THE PRUT RIVER BASIN

**CLAUDIA COJOCARIU[a], GEORGE BARJOVEANU[a], BRÎNDUŞA ROBU[a], CARMEN TEODOSIU[a, *]**

**ABSTRACT.** Integrated water resources management is an important challenge in the countries with historic water pollution problems, enhanced by the occurrence of extreme climate phenomena, and by the insufficient stakeholder coordination and co-operation. In Romania, although the main problems that agricultural activities pose to water resources in the Prut river basin are the non-point sources, there are also major water quality issues related to the inefficiency of the municipal/industrial wastewater treatment plants. Environmental Impact Assessment (EIA) and Risk Assessment (RA) procedures identify the possible consequences of a planned/implemented activity, in order to facilitate the process of choosing wisely the best alternative. This study presents the development and implementation of a methodology for the quantitative assessment of the environmental impact and associated risk of the agricultural activities within Prut catchment. The integrated EIA and RA methodology considers environmental impacts determined for one category (*surface water)* by using representative water quality indicators for the case of agricultural pollution: biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), ammonia ($NH^+_4$), total nitrogen (TN) and total phosphorus (TP). The study uses data collected during 2005 - 2008 from the monitored agricultural (and animal farms) in the Prut catchment. The improved EIRA methodology proposes the correlations of the impact magnitude with the wastewater discharges flows, and of the impact gravity with the pollutant concentrations. The results revealed high environmental impacts and associated risks within Prut catchment due to agricultural and related industrial activities. Furthermore, this integrated EIA and RA procedure presents the advantages of rapidity, critical environmental analysis and the potential of improving the decision making processes within sustainable water resources management.

***Keywords:*** *Environmental impact assessment, risk assessment, agriculture, water resources*

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## INTRODUCTION

Sustainable water resources management represents an important challenge for the newest EU member states due to the need to comply with the principles of the European Water Framework Directive (WFD), while solving the historic water pollution problems and ensuring a sustainable pathway for the development perspectives. In Romania, only 52% of the population is connected both to water and sewage services and more than 71% of the wastewater is untreated or insufficiently treated [1,2]. The water "issues" are crucial for the existence of humans and ecosystems and for the economic and social development and thus interconnected also with food assurance and its security, and human health.

Preserving food security involves essential improvement of the agricultural production systems in the imperative direction of higher productivity, low rates of energy consumption and also, low environmental impacts. In order to stabilize output and income, production systems must become more resilient. A resilient and productive agriculture requires changes in the management of environmental components and an efficient use of resources [3].

The Integrated Water Resources Management (IWRM) has been defined by the Technical Committee of the Global Water Partnership (GWP) as, "*a process which promotes the coordinated development and management of water, land and related resources, in order to maximize the resultant economic and social welfare in an equitable manner without compromising the sustainability of vital ecosystems*". This process is emerging as an accepted alternative to the sector-by-sector, top-down management style that has dominated in the past and agrees on the fact that all the uses of water resources are interdependent. High irrigation demands and polluted effluents from agriculture means less freshwater for drinking or industrial use, contaminated municipal and industrial wastewater that pollutes rivers and threatens the natural ecosystems. Agriculture is the main consumer of water in the world, up to 70 % of the water withdrawn from rivers and groundwater goes into irrigation [3], and is becoming a rising concern in an era of water scarcity. On the other hand, it contributes approximately with 60% of nitrates, 25% of phosphorus and 70 % of sediments entering the watercourses [4]. With the increase in food requirement, the sustainability of upland agriculture has posed threats to downstream and coastal areas of river basins [5]. The social, environmental and economic impacts are immense.

These water-related challenges are further enhanced in the new European Union member countries by historic water-related problems in connection to outdated or very often inexistent wastewater treatment infrastructure, as well as major drawbacks in the cooperation and coordination between the relevant stakeholders [1,6], Romania being one of the countries that deals with historic water quality related key issues.

Compliance with the Water Framework Directive (WFD) requires substantial reductions in agricultural wastewater discharges and the diffuse pollution of watercourses from agriculture has become a major environmental concern in Romania due to the impact posed upon the groundwater as well as the surface water by numerous diffuse pollution sources that are difficult to be quantified.

The agriculture pollution point sources should comply with the following requirements:

- 96/61/EC - The Integrated Prevention and Pollution Control Directive – (IPPC Directive) [7],
- 2006/11/EC Directive which improves and replaces the 76/464/EEC Directive, regarding waterbodies pollution with dangerous substances [8],
- 91/676/EEC Directive, regarding nitrates pollution of water bodies from agriculture [9],
- 86/278/EEC Directive (SEVESO Directive), regarding major accident pollution [10].
- the national regulations:
    - 352/2005 Governmental Decision (G.D.) which improves and replaces 188/2002 G.D. regarding wastewater discharging conditions [11];
    - 351/2005 G.D. regarding the gradual discharging and loss of priority substances wastewater loaded [12].

The Romanian agriculture sector is characterized by a significant segment of population - economically and socially vulnerable farmers, who face many impediments in complying with the complex European required set of agriculture regulations. The government's funding support for agriculture is modest; hence the EU financial absorption represents a crucial opportunity to sustain economic growth, and implicitly a sustainable agriculture. So far, Romania's absorption rate of European funds was low. The rate of absorption of structural funds for 2007-2010 is of 13.48 percent [13], due to inadequate management of the funds from the European Union and to the conflicts of interest in managing these funds.

Another opportunity for getting a higher rate of investment in Romanian agriculture seems to be the attraction of foreign investors, due to high potential together with rising prices of agricultural products on the international market. The area of cultivated agriculture land in Romania is around 9.4 million hectares and foreign investors own over 500,000 hectares, according to the [13]. The Italian investors own over 300,000 hectares of agriculture land in Romania and Danish, over 130,000 hectares of forests. Dutch and French are also among the most interested foreign buyers of Romanian agriculture land. But often the foreign investments are hindered by the highly fragmented lands, furthermore, the banks or national programmes are much more focused on large costumers then small ones, precisely, to avoid risk of failure.

All these paradigms are leading to the current situations of Romanian agriculture low development, with high rate of family labour force, small unmonitored farms, inadequate agricultural/zootehnical practices and also

pollutants high loaded wastewaters discharged into natural water bodies. These are also the reasons why, currently, the lack of relevant data and expertise [2] in the Romanian water system for monitoring the environmental indicators, necessary for realistic environmental flows awareness, hinders the sustainable management of the water resources.

Several studies highlight the need for improved and robust management strategies to assess and mitigate the fertilisers, manures, pesticides [14], nitrogen [15] and phosphorus [16,17] losses to water natural resources from agriculture and zootechnics.

Considering all these aspects of both political and water quality related background for Romania, the necessity of scientific reliable management strategies that would help the decision-making processes within IWRM is compulsory. Given these complex particularities of water resources management in Romania, where, according to Barjoveanu et al. [18] an opportune initial step would be the development of an integrated method for environmental impact and associated risk assessment of the major water polluters within a certain river basin. This method quantifies exactly the environmental impacts and associated risks, especially for prioritizing future actions.

This study applies a method for the environmental impact and risk assessment of the main agricultural/zootehnical agents from Prut river basin onto watercourses. Thirteen agricultural/zootechnical activities that discharge wastewater directly into the Prut River were considered for the assessment of the pollution with seven pollutants (BOD, COD, TSS, $NH_4^+$, detergents, TN and TP) specifically selected in accordance with the typically wastewater discharges' composition and the existing available data.

## RESULTS AND DISCUSSIONS

The integrated method was applied to assess the environmental impact and associated risk for thirteen agricultural/zootechnical units from Prut catchment, using the mean concentration values of the 7 quality indicators (BOD, COD, TSS, $NH_4^+$, detergents, TN and TP). The probabilities of occurrence of these impacts were calculated for each indicator, with formula given by Eq. (5), as a frequency of discharge events that overcome 70% of MAC, over a data series that covered three years (2005-2007). Thus, it was possible to compare the impacts and associated risks for each location and for every quality indicator, as presented in the next figures.

Figure 1 presents the results of the integrated quantification of impacts and associated risks induced by the wastewater discharges from agricultural/ zootechnical analyzed units, considering the seven quality indicators. These graphs show a comparison of the impacts and associated risks of these effluents discharged in the Prut River for each of the water quality indicators (BOD, COD, TSS, $NH_4^+$, detergents, TN and TP) and it may be observed that there are 4 most problematic wastewater effluents in terms of BOD and COD discharges in

the natural water bodies. These are the vinery 1, the zootechnics/agricultural unit from Iasi County, the vinery 4 from Vaslui County and the vinery 5 from Galati County.

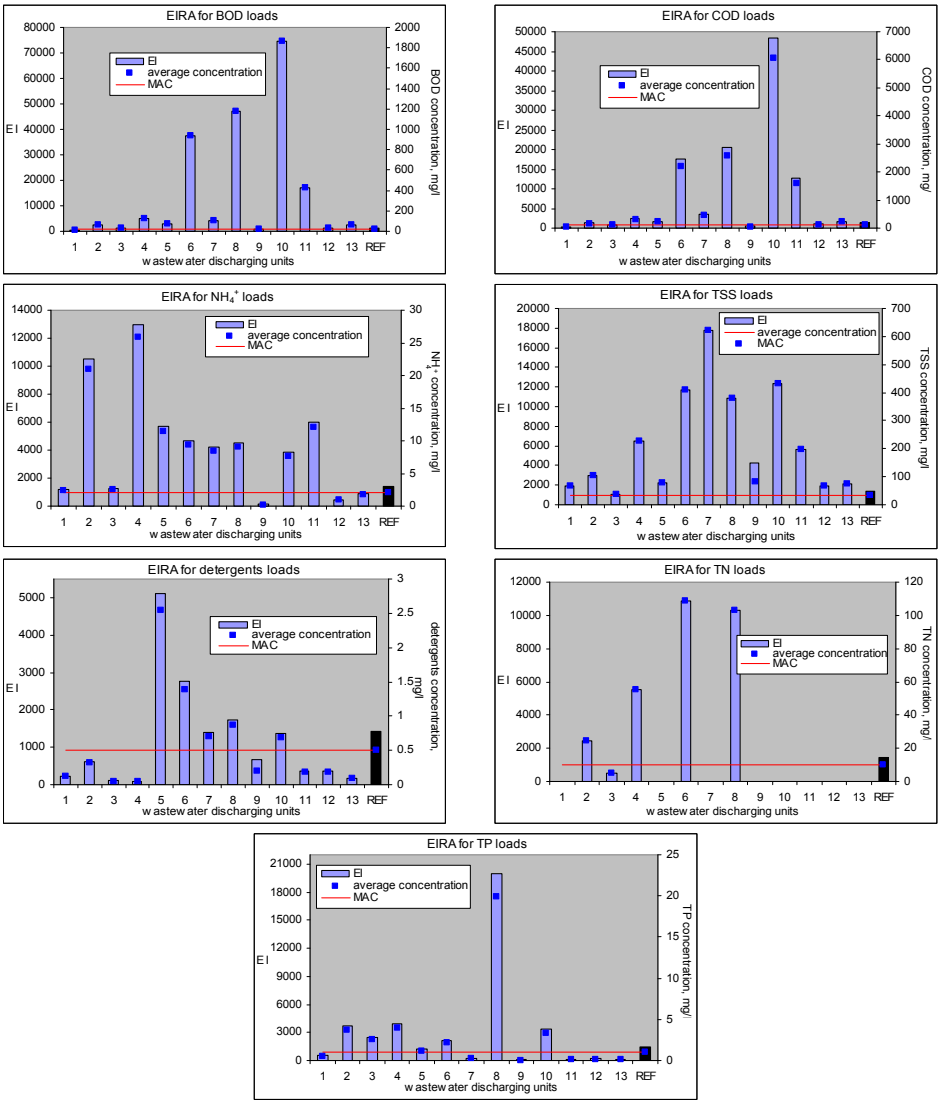


**Figure 1.** Integrated quantification of environmental impacts and associated risks (EIRA) for each of the water quality indicators

***Note***: *The numbers on the X-axis represent the wastewater discharging unit in accordance with Table 3* (1- Zootechnics and food processing 1; 2- Zootechnics and food processing 2; 3-Zootechnics and food Vineyard processing 3; 4- Zootechnics and food processing 4; 5- Poultry production; 6-Vineyard and vinery 1; 7- and vinery 2; 8- Zootechnics and agricultural unit; 9- Vineyard and vinery 3; 10- Vineyard and vinery 4; 11- Vineyard and vinery; 12- Grain crops unit; 13- Sugar production)

Regarding the TSS and $NH_4^+$ quality indicators, it may be observed that the EI magnitude are decreased as compared with the BOD and COD situation, but there are more locations where the MAC value was dramatically exceeded except 5 locations (the zootechnics and food processing 1 and 3 units from Botosani County, the vinery 3 from Vaslui County, the grain crops unit and the sugar production from Galati County). In terms of TN concentration, due to the lack of quality data for this pollutant only five locations were analyzed and four of them present higher values of the EI comparing to the reference. The TP loaded wastewaters discharged from the analyzed agents slightly exceed the reference, except one location (the zootechnics/agricultural unit from Iasi County), where the EI value touch higher magnitude.

The wastewater discharging agents with higher values than reference for EI in terms of detergents concentration are the poultry production, the vinery 1 and 2, the zootechnics and agricultural unit from Iasi County and the vinery 4 from Vaslui County. It may be also observed that in Botosani and Galati counties, the EI of the detergents discharges onto the water bodies are lower then the reference.

The EIRA methodology considers the environmental risk as a function environmental impacts magnitude and their probability of occurrence. The environmental risk was correlated with the probability of occurrence for a pollution event (exceedences of MAC) within the results interpretation (presented in Figure 2). It may be observed how the low probability of occurrence induced low values for the ER, especially in the TP and the detergents concentration cases, where many locations with low values for ER are obtained. The same four locations that were previously highlighted regarding the damages caused on the receiving water bodies by the BOD and COD concentrations, present probabilities of 100% for high magnitude of the MAC exceedences, inducing high values for associated risk.

In Figure 3 for each agricultural/zootechnical pollution source, the global EI and ER were calculated based on the above discussed calculations.

Among all thirteen analyzed discharging points (agricultural/zootechnical units) there are five locations where the global environmental impacts and associated risks values induced on natural water bodies of Prut River (and its tributaries) are in accordance with the reference situation considered by the authors. These locations are the zootechnics and food processing 1 and 3 from Botosani County, the vinery 3 from Vaslui County, the grain crops unit and sugar production agent from Galati county.

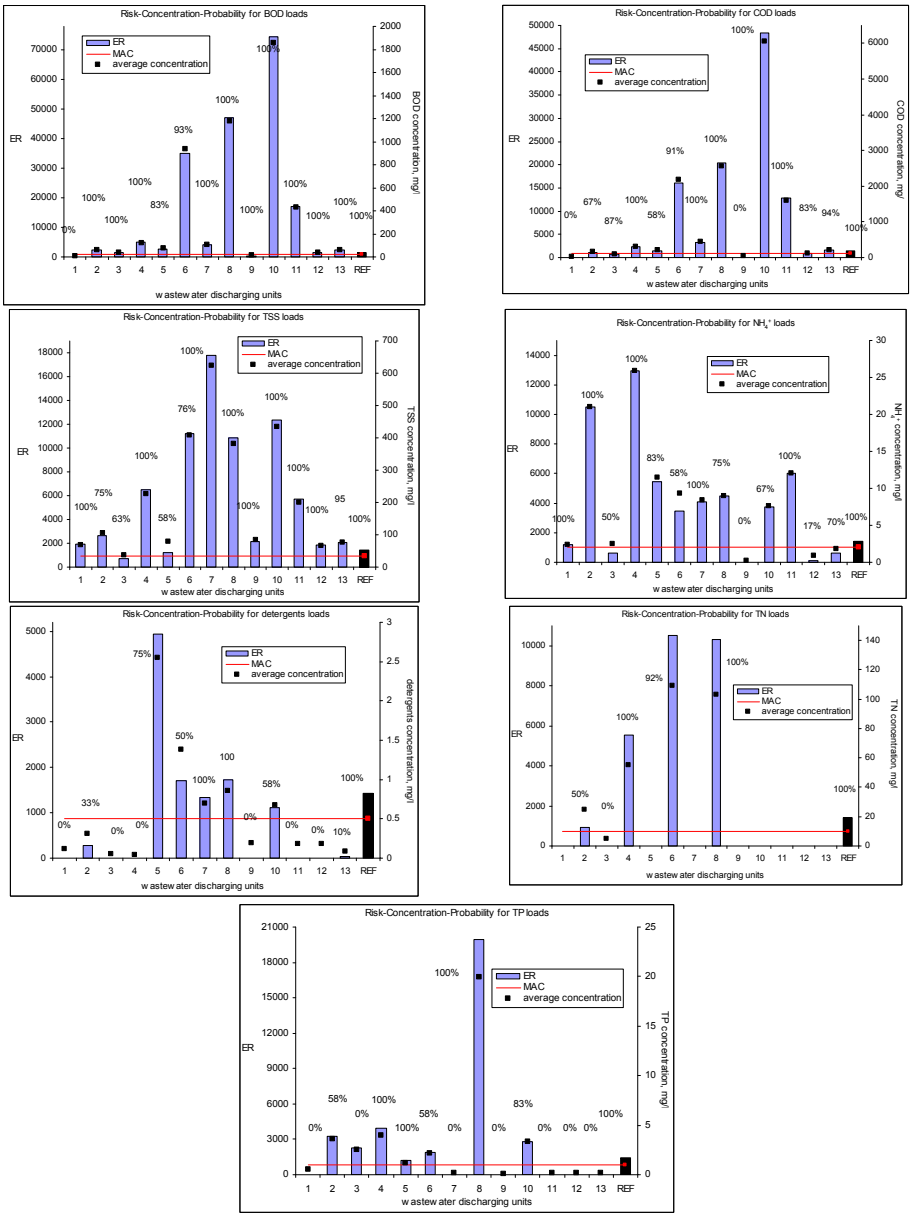


**Figure 2.** The correlation environmental risk-pollutant concentration-event probability

***Note***: *The numbers on the X-axis represent the wastewater discharging unit in accordance with Table 3* (1-Zootechnics and food processing 1; 2-Zootechnics and food processing 2; 3-Zootechnics and food processing 3; 4-Zootechnics and food processing 4; 5-Poultry production; 6-Vineyard and vinery 1; 7-Vineyard and vinery 2; 8-Zootechnics and agricultural unit; 9-Vineyard and vinery 3; 10-Vineyard and vinery 4; 11-Vineyard and vinery; 12-Grain crops unit; 13-Sugar production)

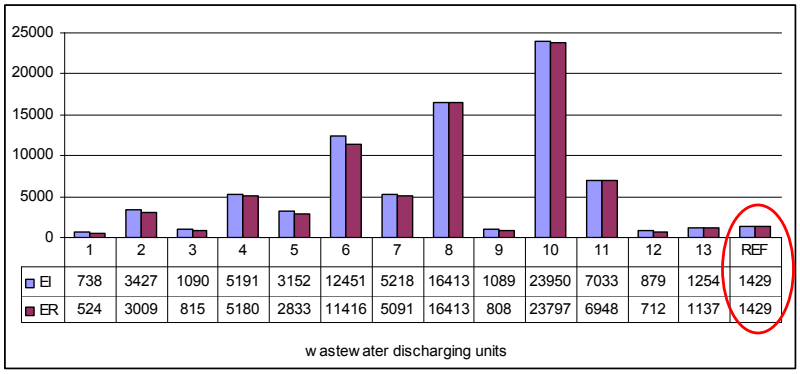


**Figure 3.** Integrated quantification of environmental impacts and associated risks

***Note***: *The numbers on the X-axis represent the wastewater discharging unit in accordance with Table 3* (1-Zootechnics and food processing 1; 2-Zootechnics and food processing 2; 3-Zootechnics and food processing 3; 4-Zootechnics and food processing 4; 5-Poultry production; 6-Vineyard and vinery 1; 7-Vineyard and vinery 2; 8-Zootechnics and agricultural unit; 9-Vineyard and vinery 3; 10-Vineyard and vinery 4; 11-Vineyard and vinery; 12-Grain crops unit; 13-Sugar production)

## CONCLUSIONS

In this case study, the agricultural/zootechnical activities that discharge wastewater into the Prut River and its tributaries were considered for the integrated environmental impact and associated risk assessment of the pollution with seven related water quality indicators that are related to the agricultural pollution (BOD, COD, TSS, $NH_4^+$, detergents, TN and TP).

The integrated assessment is based onto a method that allows the determination of impacts and risks of point-sources on a single environmental component (surface waters) based onto multiple impact components (expressed by the water quality indicators).

The results revealed that all of the analyzed wastewater discharging agents present very high risk values for the surface waters, from both the point of view of the magnitude (environmental impacts), as well as from the probability of occurrence that can confirm the weak points of the existing agricultural production systems and the lack of good agricultural practices.

The EIRA method provide also information on the components and probabilities for every impact category, which in our case demonstrated that for the wastewater treatment plants there are serious problems related especially to BOD, COD and ammonia discharges.

Unless the efficiency of both the agricultural production systems and the wastewater infrastructure do not improve, the environmental risks remain high and the magnitude of the polluting events may also cause dramatically damages to the receiving water bodies. Furthermore, this study has shown that the integrated

impact and risk assessment methodology can be used as a simple and reliable instrument for identifying and analyzing the hot spots in a river basin, which contributes to the decision making processes in sustainable water management.

## EXPERIMENTAL SECTION

### Integrated environmental impact and risk assessment methodology development for applications in water resources management

Environmental Impact Assessment (EIA) is an important procedure used to predict the environmental consequences after or before certain decision is taken. Hence, EIA ensures that the potential effects are foreseen and adequately addressed at an early stage in the project planning, improving prioritization process of the planned actions to facilitate wiser choices/decisions among the alternatives.

EIA is now increasingly being employed within the context of sustainable development objectives to reach better decisions [19, 20, 21, 22, 23], role that was highlighted also at the United Nations Conference on Environment and Development (UNCED) in 1992 by Principle 17 of the *Rio Declaration*.

A number of authors [14, 24, 25] link comprehensive assessments on the expansion of integration addressing both risk assessment (RA) and environmental impact assessment (EIA), as a tool to help decision making process within water resources management. Generally, EIA describes the induced impacts on natural components, meanwhile risk assessment (RA), traditionally refers only to human health (originally referred to occupational health, then public health and safety) and recently, its mean was extended to the environmental level [26,27].

Despite potential benefits of these EIA and RA used to complement each other, revealed by these studies, their application in defining and quantifying the impacts and associated risks related to surface water pollution is rarely used. Developing such an integrative and objective methodology of assessment, that considers both impacts and the associated environmental risks on surface water resources may be a reliable tool for decision making in water resources management.

Two previous studies [18,28] provided a full description of the methodology developed for the integrated environmental and risk assessment (EIRA), as well as the arguments for its application as an instrument for water resources management. The EIRA methodology considers the environmental risk as a function of magnitude of environmental impacts and their probability of occurrence (Eq. 1).

$$ER_j = EI_j \cdot P_j \quad (1)$$

where: $ER_j$ – environmental risk for environmental component j; $EI_j$ – environmental impact on environmental component j; $P_j$ – probability of impact occurrence on environmental component j.

The above mentioned method assesses the environmental impacts ($EI_j$ in Eq. 1) by designating certain importance units for different environmental components (air, surface water, groundwater, human health etc.) and subsequently, by quantifying these impacts through the *environmental quality parameters* (*EQ* in Eq. 2). In this study, only the impacts and risks on surface waters were considered, skipping thus the step of prioritizing among the environmental components and importance units (IU) value of 1000 was used.

$$EI = \frac{IU}{EQ} \tag{2}$$

where: *EQ* – environmental quality parameter; *IU* – importance units.

In Eq. 2, the environmental quality parameter, which measures the magnitude of impacts is calculated by comparing the measured concentrations of the considered pollutants, *i.e.* organic biodegradable compounds expressed by the Biochemical Oxygen Demand (BOD), bio- and non-biodegradable organic compounds expressed by the Chemical Oxygen Demand (COD), total suspended solids (TSS), ammonia ($NH_4^+$), detergents, total phosphorus (TP) and total nitrogen (TN) with their respective maximum allowed concentrations (MAC), leading thus to a non-dimensional measure of the environmental impacts magnitude, as presented in Eq. (3).

$$EQ = \frac{MAC}{MC} \tag{3}$$

where: *MAC* – maximum allowed concentration of quality indicators as regulated by the national legislation, through the Government Decision no. 352/2005 [11] (Table 1); *MC* – measured concentration of quality indicators.

In this study, the EIRA methodology was developed so as to consider the impacts on surface water ($EI_{(sw)i}$) as presented by Eq. (4), for each pollutant separately and furthermore by calculating an average of the global environmental impacts and associated caused by each agriculture/zootechnics activity.

$$EI_{(sw)i} = \frac{IU_{sw}}{EQ_{(sw)i}} \tag{4}$$

where*:* $EQ_{(sw)i}$ – quality of *surface water*, considering the quality indicator i; $IU_{sw}$ – significance units obtained by *surface water*.

The environmental impact assessment of the wastewaters entering the natural system was performed considering seven quality indicators' concentrations of the effluents from thirteen agricultural/zootechnical activities, that discharge wastewaters in the Prut River and that are monitored by the Prut Water Directorate. These indicators were considered based on the fact that they induce significant environmental impact and risks onto the natural water bodies, especially in relation with the agricultural pollution, nutrient inputs, zootechnical and wine associated activities. As soon as the environmental impacts are determined, the quantification of risks is possible by using Eq. 1.

The probability units ($P_j$) of the impact occurrence were calculated using historic data series that allowed for calculating the frequencies of events during which 70% of the maximum allowed concentrations (MAC) was reached, which represents the attention threshold (AT) for a polluting event (Eq. 5).

$$P = \frac{n}{m} \tag{5}$$

where: $n$ – number of attention thresholds reached over the data series; $m$ – total number of measurements of the data series.

Since only one environmental component (surface water) is considered, the scale proposed considers the adaptation of the assessment methodology [28, 29] for quantifying the impacts and associated environmental risks to a reference in accordance with the analyzed context thus bringing objectivity to the evaluation.

An ideal scenario that considers the situation of **reaching the attention threshold** (AT) for each of the 7 analyzed pollutants, with **a maximum probability (**100**%)**, was analyzed. Further more, for each quality indicator was calculated a reference EI and also ER for every wastewater discharging unit (**$EI_{ref}$ = 1428.5** and **$ER_{ref}$ =1428.5**).

## Study area

The Prut River, the second longest tributary of the Danube River, is located in the North-Eastern part of Romania and it forms the border between Romania and Republic of Moldova. With a total area of 27,500 km$^2$, the length of the drainage system totalizes, on the surface of 3 countries (Ucraina, Romania and Moldova), 11,000 km, out of which 3,000 km have permanent water flow (Baseu, Jijia, Bahlui) and almost 8,000 km with intermittent water flow [30].

The Barlad River is the most important left-side tributary of the Siret River. The study area is Prut-Barlad catchment (Figure 4) that lies, almost entirely, on Botosani, Iasi, Galati and Vaslui counties and partially on Neamt, Bacau and Vrancea counties.

Distribution of the river basin by counties in the study area is different depending on the existing drainage system and the establishment of the watershed between catchments of Siret and Prut Rivers, such as counties Botosani, Iasi, Vaslui, Galati have a rate of 90-100% and Bacau, Neamt and Vrancea in smaller proportions (Table 1). In the analyzed catchment a total of 2,277,678 people live, 1,120,160 of them in urban areas and 1,157,518 in rural areas [31].

Land use in the Prut River Basin (Figure 5) is influenced by physical and geographical conditions, and also by human influence factors. Agriculture includes animal farms and the cultivation of arable lands, with mainly maize, potatoes, sunflower, sugar beet. The total agricultural area is about 68.2% (12,406.2 km$^2$) of the Prut River Basin (Management Plan, 2008).

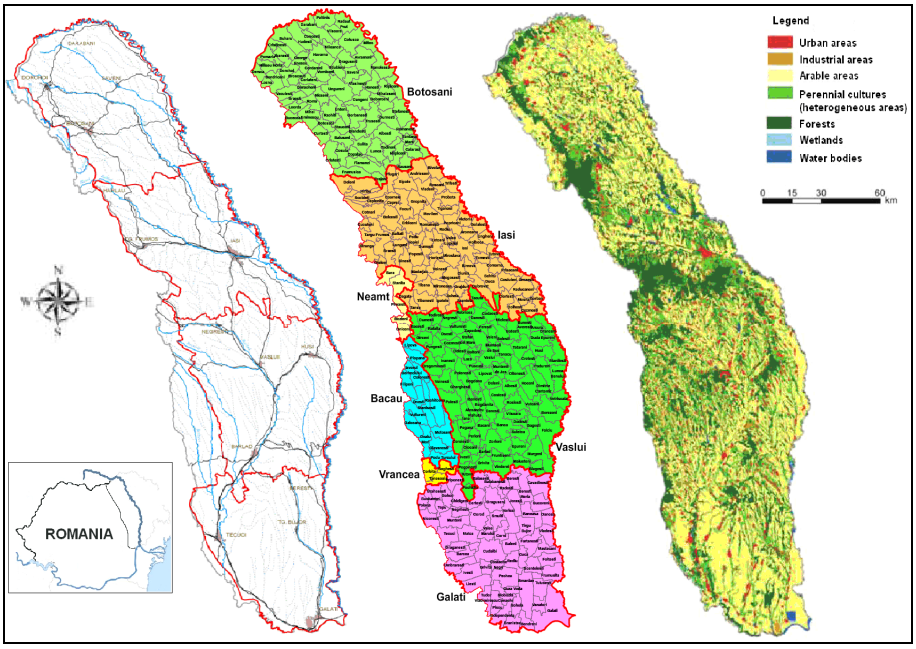


**Figure 4.** The water system, administration allocation and land uses in Prut river basin

**Table 1.** Administrative and demographic characteristics of the Prut River Basin

| No | County | Area ($km^2$) | % from total surface of Prut River Basin (%) | Population (no. inhabitants) | % from total population of Prut River Basin (%) |
|---|---|---|---|---|---|
| 1 | Botosani | 4,782 | 23.60 | 443,558 | 19.47 |
| 2 | Iasi | 4,564 | 22.52 | 680,656 | 29.89 |
| 3 | Vaslui | 5,318 | 26.24 | 452,832 | 19.89 |
| 4 | Galati | 4,328 | 21.35 | 647,455 | 28.43 |
| 5 | Neamt | 172 | 0.85 | 6,533 | 0.28 |
| 6 | Bacau | 946 | 4.67 | 40,372 | 1.77 |
| 7 | Vrancea | 157 | 0.77 | 6,272 | 0.27 |
| Total | | 20,267 | 100 | 2,277,678 | 100 |

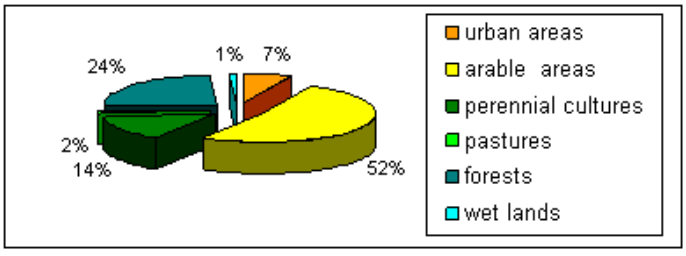


**Figure 5.** Land use in the Prut River Basin

The main agricultural activities developed in the counties included within the Prut river basin are depicted in Table 2, and the major agricultural/ zootechnical/winery related polluters are presented. The average values of the quality indicators concentration that overcome the MAC values are highlighted and the frequency of the measurements are presented in bold. The most common crops related to these activities are the grapes (5 of the analyzed wastewater discharging units have vinery and vineyard activities), sugar beat (1 analyzed production unit), grain (1 analyzed production unit), maize, potatoes and vegetables.

**Table 2.** The agricultural/zootechnical activities evaluated in the EIRA

| No. | Waste-water discharg-ing unit | Mean discharge (frequency) | Mean concentration (range of values) | | | | | | | County |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | detergents | $NH_4^+$ | TSS | BOD | COD | TN | TP | |
| | | ($m^3$/h) | (mg/l) | (mg/l) | (mg/l) | (mg/l) | (mg/l) | (mg/l) | (mg/l) | |
| Reference | | - | 0.5 | 2 | 35 | 25 | 125 | 10 | 1 | |
| 1 | Zootech-nics and food process-ing 1 | 1.8 (3) | 0.11 (0.09÷0.13) | **2.4 (2÷2.8)** | **68 (10÷127)** | 6.4 (0.1÷18.9) | 32.4 (3÷60.8) | - | 0.54 (0.07÷1.55) | Botosani |
| 2 | Zootech-nics and food process-ing 2 | 2.62 (18) | 0.31 (0.05÷1.78) | **21 (2.89÷45.66)** | **104.8 (23-354)** | **60.7 (21.5-202.9)** | 163.6 (64.28-733) | **24.7 (16.24-62.36)** | **3.65 (0.22-12.55)** | |
| 3 | Zootech-nics and food process-ing 3 | 15.12 (7) | 0.053 (0.02÷0.08) | **2.49 (0.43÷8.44)** | **36.3 (15÷73)** | **36 (25÷42.12)** | 99.5 (60.3÷132) | 4.8 (6.3÷77.4) | **2.5 (0.65÷6.25)** | |
| 4 | Zootech-nics and food process-ing 4 | 2.52 (4) | 0.04 (0.126-0.32) | **25.8 (14.82÷46.15)** | **226.3 (74÷423)** | **123.6 (30.4÷170.4)** | **302.3 (112.2÷451.4)** | **55.36** | **3.95 (2.25÷5.08)** | |
| 5 | Poultry production | 1.44 (8) | **2.54 (0.12-6.48)** | **11.4 (0.19-34.3)** | **78.6 (9.7-182)** | **72.5 (9-213)** | **218.1 (25-500)** | - | **1.2** | Iasi |
| 6 | Vineyard and vinery 1 | 9.72 (25) | **1.38 (0.008÷6.4)** | **9.29 (0.05÷70.7)** | **407.8 (4-÷782)** | **937.9 (13÷2400)** | **2191 (37÷4789)** | **108.9 (2.65÷122)** | **2.1 (0.06÷10.8)** | |
| 7 | Vineyard and vinery 2 | 0.54 (3) | **0.7 (0.06÷1.54)** | **8.41 (0.37-28.8)** | **622.5 (354÷891)** | **105.75 (11-256)** | **437.25 (50.5-1045)** | - | 0.192 | |
| 8 | Zootech-nics and agricul-tural unit | 21.6 (4) | **0.86 (0.44÷1.67)** | **8.98 (0.23÷22.5)** | **381 (79÷729)** | **1176.75 (380÷2830)** | **2559.7 (1124-÷5210)** | **102.9 (88.37÷117.5)** | **19.9 (8.19÷26.2)** | |

| No. | Waste-water discharg-ing unit | Mean discharge (frequency) | Mean concentration (range of values) | | | | | | | County |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | detergents | $NH_4^+$ | TSS | BOD | COD | TN | TP | |
| | | ($m^3$/h) | (mg/l) | (mg/l) | (mg/l) | (mg/l) | (mg/l) | (mg/l) | (mg/l) | |
| Reference | | - | 0.5 | 2 | 35 | 25 | 125 | 10 | 1 | |
| 9 | Vineyard and vinery 3 | 1.36 (3) | 0.19 (0.05÷0.34) | 0.18 (0.032÷0.33) | 83.5 (17÷150) | 15.65 (11.8÷19.5) | 56.6 (49÷64.2) | - | 0.05 (0.103÷0.15) | Vaslui |
| 10 | Vineyard and vinery 4 | 0.72 (6) | 0.67 (0.21÷1.67) | 7.66 (4.06÷23.9) | 433.58 (34÷475) | 1860.1 (320.4÷4543.1) | 6043.3 (930.3÷14061) | - | 3.37 (1.37÷9.42) | |
| 11 | Vineyard and vinery 5 | 75.6 (6) | 0.17 (0.11÷0.23) | 12 (10.74-13.26) | 199 (102-296) | 430.3 (398.8-461.9) | 1598.4 (1497.6-1699.2) | - | 0.156 | Galati |
| 12 | Grain crops unit | 7.2 (7) | 0.108 (0.016÷0.237) | 0.88 (0.37÷0.88) | 66.16 (57÷140) | 36.39 (23.01÷177.75) | 119.3 (91.2÷307.2) | - | 0.16 (0.056÷0.32) | |
| 13 | Sugar production | 108 (21) | 0.08 (0.015÷0.45) | 1.77 (0.21÷3.81) | 75.8 (24÷184) | 60.4 (19.36÷144.26) | 217.9 (91÷470) | - | 0.13 (0.042÷0.21) | |

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# HYDROGEN PRODUCTION THROUGH CO-GASIFICATION OF COAL AND BIOMASS WITH CARBON DIOXIDE CAPTURE

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**ABSTRACT.** This paper presents technical aspects of hydrogen production technology through co-gasification of coal and biomass based on modeling and simulation of the process. Three plant configurations containing entrained-flow gasifiers were studied. A performance analysis regarding the energy efficiency of the process, carbon conversion rate and the carbon dioxide capture rate have been carried out. A significant advantage of gasification process described in this paper is the limitations of greenhouse gas emissions through acid gas removal **unit (carbon dioxide capture, hydrogen sulfide processing)**.

***Keywords:*** *hydrogen production, gasification, entrained-flow reactors, energy efficiency, $CO_2$ capture and storage*

## INTRODUCTION

Introducing hydrogen in the energy system as a complimentary energetic vector to electricity represents an issue of most importance in Europe due to the significant advantages it offers [1-3]. Among the advantages of using hydrogen in the energetic system as a complimentary energetic vector to electricity one can mention: low greenhouse gases emission, increased electricity delivery safety, increased economic performance [4]. Hydrogen can be obtained through a variety of technological, chemical, biochemical or electrochemical processes [5]. The choice of the most suitable options to produce hydrogen is influenced by a series of factors such as: raw materials resources, utilities, the possibility of delivering the product, etc.

Through gasification, the energy of solid or liquid fuel (fossil fuel or biomass or industrial or housework waste) is turned into a synthesis fuelling gas (a mixture containing mainly CO and $H_2$) which can be processed to generate hydrogen as well as a large variety of chemical compounds (methanol, ammonia, urea, synthetic fuels, etc.) [6]. The raw material used in the process of gasification described in this paper is a mixture of 80% coal and 20% biomass – in this

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case sawdust. The situation of solid fossil fuel is much better than that of oil and natural gas because they are uniformly spread all over the Earth and due to the sustainable deposits covering a longer period of time in comparison with the annual level of energy consumption, thus resulting better continuity for ensuring raw materials supply [7]. One should stress the fact that this technology is viable only if the carbon dioxide emissions, are captured and stored.

The hydrogen production plant based on gasification of coal and sawdust with carbon dioxide capture and storage has the following sub-systems: (I) Gasification of a mixture of coal and biomass, (II) Catalytic conversion of carbon monoxide ("Water Gas Shift – WGS"), (III) Acid gas removal unit ("Acid Gas Removal - AGR"), (IV) Hydrogen purification („Pressure Swing Adsorption – PSA").

**Coal and biomass gasification.** Currently the majority of gasification processes are based on entrained-flow reactors that operate with feed and blast in co-current flow (oxygen is used as oxidation agent and steam as moderator). The feed is ground to a size of 100 μm or less (that allows transport in the gas and high mass transfer coefficient). The residence time in these gasifiers is in the range of seconds. As a result high operating temperatures are required for a good conversion rate [8]. The outlet gas temperature is between 1250-1600°C, above the ash melting point, which makes all entrained-flow gasifiers operate in the slagging range [9, 10]. High temperatures also lead to high oxygen consumption. Main advantages presented by this type of gasification reactors are: the ability to handle practically any coal as feedstock, to produce a clean and tar-free gas, the ash is produced in the form of an inert slag, a high carbon conversion of over 99%, high quality synthesis gas because of the low methane content [8]. Three plants configuration based on entrained flow reactors were analyzed in this paper.

**Tabel 1.** Gasifiers parameters [8]

| Technology | Stage | Feed | Flow | Reactor wall | Syngas cooling | Oxidant |
|---|---|---|---|---|---|---|
| **Siemens** | 1 | dry | up | Membrane | Water | Oxygen |
| **Shell** | 1 | dry | up | Membrane | Gas | Oxygen |
| **Texaco** | 1 | slurry | down | Refractory | Water | Oxygen |

**Catalytic conversion of carbon monoxide**. The catalytic conversion of carbon monoxide with water vapor ("water-gas shift - WGS") takes place in an installation with two reactors, with intermediary cooling between them, operated in an adiabatic mode. A sulphur tolerant catalyst is used, based on cobalt and molybdenum [5, 11]. The reaction of catalytic conversion of carbon monoxide with water vapor follows the next equation:

$$CO + H_2O \rightarrow H_2 + CO_2 \quad (1)$$

This is an exothermal reaction also called „shift" or „water gas shift - WGS". It is used in the hydrogen production plant with carbon dioxide capture and storage in order to focus the syngas thermal energy (mixture of hydrogen and carbon monoxide) as hydrogen and to focus the chemical species which contain carbon as carbon dioxide [7].

**Acid gas removal unit.** Separation of acid gases (carbon dioxide and hydrogen sulfide) is achieved through the process of gas liquid absorption by using Selexol® as a solvent. This is a physical solvent, a mixture of dimethyl ethers of poly- ethylene glycol whose chemical formula is: $CH_3(CH_2CH_2O)_nCH_3$ where "n" varies between 3 and 9.

**Hydrogen Purification („Pressure Swing Adsorption – PSA").** Hydrogen purification is made through pressure modifications (reduction) – this technique is called „Pressure Swing Adsorption – " [12,13]. High pressure is needed to ensure long distance transport from production sites to end-users with low energy consumption (pressure drop along pipes network). High purity hydrogen is essential to its use in transport sector (99.99% purity is required for compatibility with PEM fuel cell) [5].

## RESULTS AND DISCUSSION

The modeling and simulation of hydrogen production process through co-gasification of coal and biomass, was made using chemical process simulation software (ChemCAD) [14]. The thermodynamic package used in the simulations is based on a modified SRK model.

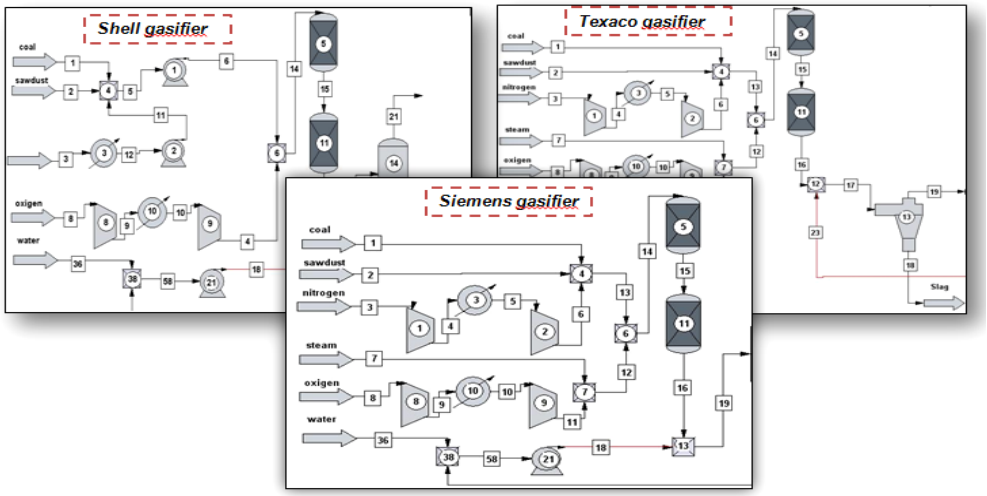



**Figure 1.** Gasification, WGS, PSA units

Three case studies were analyzed: (I) dry feed gasifier with nitrogen as transport gas and water quench for cooling the gas (Siemens); (II) dry feed gasifier with nitrogen as transport gas and gas quench for cooling the resulting syngas (Shell); (III) high pressure slurry feed gasifier with water quench (Texaco).

The main model assumptions used for the modeling and simulation of the gasification process are presented in the Table 3. The necessary data are derived from the literature [15-17].

**Table 2.** Model assumption

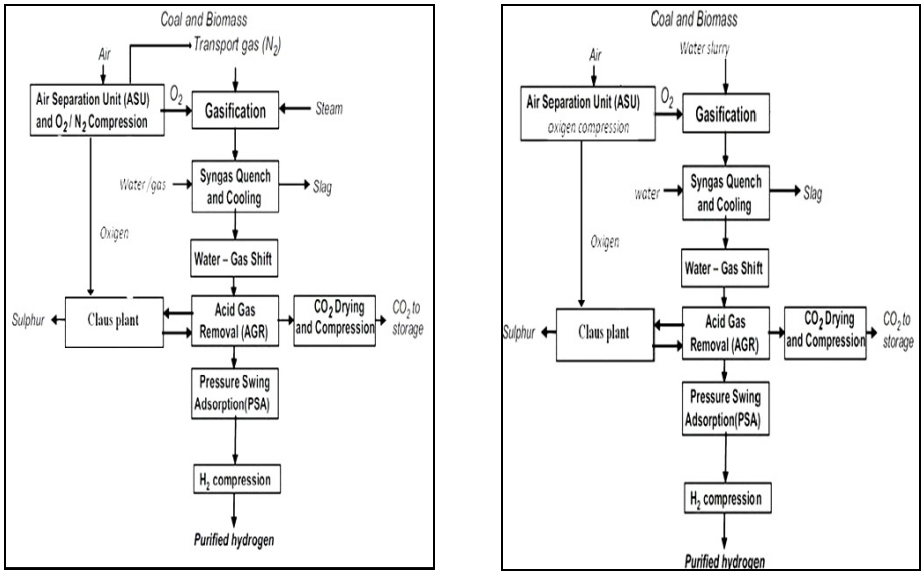
| | |
|---|---|
| **Gasification unit** | P: 40 bar (dry feed)/ 70 bar (slurry feed);<br>T: 1400°C (dry feed)/ 1300°C (slurry feed);<br>Carbon conversion rate: 99.99%;<br>Coal concentration in the water slurry: 62 wt%; |
| **Water gas shift unit** | Catalyst type: sour shift catalyst, temperature range: 250–500°C;<br>Carbon monoxide conversion rate: 96-98%;<br>Two adiabatic catalytic beds; |
| **Acid gas removal unit** | Solvent: Selexol®;<br>Two stages: first $H_2S$ removal, second $CO_2$ removal;<br>First stage: Selexol® flow preloaded with $CO_2$;<br>Solvent refrigeration level: +5°C;<br>Oxygen-blown Claus plant (95% vol.);<br>$H_2S$-rich gas composition to Claus plant: >21% $H_2S$ (vol.);<br>Tail gas is recycled to $H_2S$ absorption stage;<br>Second stage: $CO_2$ removal: absorption tower–flash;<br>Delivery temperature and pressure: 35°C, 110 bar;<br>Carbon dioxide capture rate: 92%-96%; |
| **Hydrogen purification unit** | Hydrogen purity: 99.99% (vol.);<br>Hydrogen purification yield: 85%; |

Feedstock for the gasification reactors consists of coal and biomass (blend mass ratio 80% coal, 20% biomass). Raw material is fed in the gasifiers using nitrogen as transporting gas (dry feed) and water suspension (slurry feed). Oxygen is used as an oxidant agent. The resulting syngas main components are: hydrogen, carbon monoxide, carbon dioxide, nitrogen, water. The highest hydrogen and carbon monoxide content is obtained in the case of the Shell reactor. The slurry feed reactor (Texaco) has the highest carbon dioxide content and the lowest carbon monoxide content (because part of the carbon from the coal must be oxidized totally to carbon dioxide to provide the heat necessary to vaporize the water from the slurry [18]). Carbon conversion rate is 99.99% in all three cases.

The resulting syngas goes to the water gas shift unit. The catalyst used is cobalt–molybdenum based, with an operating temperature range between 250–500°C. Water/carbon monoxide ratio is between 2.08 and 3.1 and the carbon monoxide conversion rate is of 96-98%. The heat that results from the process of carbon monoxide catalytic conversion is used to generate medium and low pressure steam (the steam covers part of the heat requirements of the plant).

After the water gas shift conversion, liquid water is separated, and then the resulting syngas goes to the acid gas removal unit. The AGR unit structure consists in two stages. First, hydrogen sulfide is almost 100% removed with a flow of Selexol® preloaded with carbon dioxide, then $H_2S$ is partially oxidized to sulfur in the Claus plant. In the second stage of the AGR unit carbon dioxide is separated (configuration: absorption tower–flash). The Selexol® solvent is regenerated using a system of four flashing vessels (pressure in the flash vessels: 8, 5, 2, 1 bar). The $CO_2$ is compressed in five stages with inter cooling and sent to storage. The carbon dioxide capture rate is between 92-93% in dry feed configuration, and 96% in slurry feed (because of the high operating pressure). Overall $CO_2$ removal yield is about 96-97%.

The flow free of acid gas is sent to the purification unit (hydrogen purification yield: 85%). The hydrogen flow obtained has 99.99% (vol.) purity and pressure of 27 bar (dry feed)/ 53 bar (slurry feed). Next is compressed at 70 bar to ensure pipeline transportation. The figure below present the scheme of hydrogen production through gasification:




***Dry feed*** ***Slurry feed***

**Figure 2.** Scheme of hydrogen production through gasification

To obtain a better view regarding hydrogen production plant efficiency, with carbon dioxide capture and storage, the performance indicators presented below were calculated. Energy efficiency of the plant (EEP%) was calculated using the formula [7]:

$$\text{EEP}\,(\%) = \frac{(\text{Hydrogen thermal energy} + \text{ electrical power generated})}{\text{Raw material thermal energy}} \quad (2)$$

The thermal energy of hydrogen and raw material was calculated as the product between flows in kg/h and lower heating value in MJ/kg. In all the three cases analyzed, 658.2 MW fuel input was considered. The table below presents the energy balance of all plant configurations assessed in this paper:

**Table 3.** Plants energy eficency

| **Case study** | | **Siemens** | **Shell** | **Texaco** |
|---|---|---|---|---|
| **Parameter** | **Units** | **Value** | **Value** | **Value** |
| **Coal flow** | kg/h | 73520 | 73520 | 73520 |
| **Sawdust flow** | kg/h | 18000 | 18000 | 18000 |
| **Coal heating value** | MW | 567.7 | 567.7 | 567.7 |
| **Sawdust heating value** | MW | 90.5 | 90.5 | 90.5 |
| **Fuel input** | MW conssumed | 658.2 | 658.2 | 658.2 |
| **$H_2$ power** | MW generated | 400 | 390 | 360 |
| **Gas thermal energy** | MW generated | 78 | 73 | 95 |
| **Generated power from PSA tail gas** | MW generated | 43 | 41 | 52.3 |
| **Ancillary power consumption** | MW consumed | 43.7 | 38.88 | 61.35 |
| **Generated power (steam turbine)** | MW generated | 27.2 | 37.97 | 43.08 |
| **Net power output** | MW | 26.4 | 40.11 | 34.03 |
| **Plant efficency** | **%** | **64.8** | **65.4** | **60** |

The slurry feed based plant configuration has a lower efficiency than the dry feed ones, of about 5% points, mainly due to the heat requirement for vaporizing the water in the slurry. But this configuration has the advantage of producing high pressure hydrogen that can be easily transported through pipelines without any additional power consumption for compression.

To evaluate the environmental impact determined by the hydrogen production plant the carbon dioxide capture rate was calculated (%of carbon content of the input fuel that was captured). As can be seen in Table 4, all plant configurations analyzed in this paper have a carbon dioxide capture rate of over 90%. The dry feed gasifier cases present a lower carbon dioxide capture rate (92%, 93%) than the slurry feed gasifier case (96%) due to the lower pressure of the AGR system (27.8 bar compared to 52.8 bar).

**Table 4.** Carbon dioxide capture rate

| Case study | Siemens | Shell | Texaco |
|---|---|---|---|
| **Flowsheet** | $CO_2$ flowsheet (kmol/h) | $CO_2$ flowsheet (kmol/h) | $CO_2$ flowsheet (kmol/h) |
| **Storage** | | | |
| **$CO_2$** | 4698.368 | 4739 | 4928 |
| **CO** | 10.0755 | 9.1 | 7.44 |
| **$CH_4$** | 0.617 | 0.46 | 2.2 |
| **Total storage** | 4709 | 4749 | 4938 |
| **Emissions** | | | |
| **$CO_2$** | 278.58 | 251 | 127 |
| **CO** | 122 | 141 | 77.8 |
| **$CH_4$** | 0.03 | 0.32 | 0.06 |
| **Total emisisions** | 401 | 393 | 205 |
| **Net carbon flow** | 5110 | 5142 | 5143 |
| **$CO_2$ capture rate** | **92%** | **93%** | **96%** |

## CONCLUSIONS

This paper presents technical aspects of ~400 MW hydrogen (99.99% vol. purity) production technology through co-gasification of coal and biomass (blend mass ratio 80% coal, 20% biomass) with carbon dioxide capture and storage. The plant configurations assessed are based on three types of entrained-flow gasifiers (Siemens, Shell, Texaco). A performance analysis regarding the energy efficiency of the process, carbon conversion rate, syngas composition and the carbon dioxide capture rate was carried out. Based on the simulation results, the following conclusions are drawn: i) the slurry feed based plant configuration has a lower energy efficiency than the dry feed one, ii) carbon conversion rate is 99.99% in all the three cases, iii) an important advantage of the gasification process described in this paper is the limitation of greenhouse gas emissions through carbon dioxide capture (carbon dioxide capture rate is over 90%).

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# EFFECTS OF 2-ETHYLHEXYL NITRATE ON AUTO-IGNITION AND COMBUSTION QUALITIES OF RAPESEED OIL

**NICOLAE CORDOŞ[a], PAUL BERE[b], OVIDIU NEMEŞ[c,*]**

**ABSTRACT.** The main objective of the present work was to investigate the influence of additive 2-ethylhexyl-nitrate (2-EHN) on the characteristics of auto-ignition and combustion of rapeseed oil (used as biofuel in diesel engines). The biofuel for diesel engines must meet several goals: to ensure a safe and fast engine start at any environmental temperature, to allow a safe operation of the engine with a yield as high as possible, to burn completely without producing harmful substances for human health, while maintaining the properties. In this sense, the purpose of the experimental research has been to determine: cetane number, ignition delay, the start of the main combustion, the combustion period of crude and filtered rapeseed oil additived in proportions of 1%, 1.5% and 2% with 2-ethylhexyl-nitrate compared with diesel fuel (witness fuel).

***Keywords:*** *rapeseed oil, 2-ethylhexyl-nitrate, cetane number, ignition delay*

## INTRODUCTION

The use of vegetable oils as fuel is not new but dates back in the late 19$^{th}$ century, when Rudolph Diesel, the inventor of the diesel engine [1, 2] at the International Exhibition in Paris in 1900 presented and demonstrated the functionality of a diesel engine, being fed then by peanut oil [3].

Experience demonstrates that reduced combustion and fuel combustion quality, reflected in an increase in ignition delay and a slow or delayed combustion can have significant negative effects on the functioning of compression ignition engines [4].

It is known that diesel engines easy start depends directly on fuel auto-ignition quality and indirectly on fuel's cetane index and viscosity [5]. The high viscosity of vegetable oils as a major cause of poor fuel atomization resulting in operational problems such as engine deposits was recognized early [6, 7].

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The changes made on the refining processes started in the '70s led to an increase of the fuel fractions from a thermal towards a catalytic cracking offered to the heavy oil market. Such fractions have hydrocarbon structures that are not favorable to an efficient use for diesel engines. When these fractions are introduced in the production of fuels, their ability to effectively self-ignite and burn is often irregular [4]. Thus, the direct information on the combustion properties are from this point of view extremely important for insuring a more efficient and fewer of the engine's problems. In addition, even more important is the fact that fuels with good combustion properties reduces the emission of harmful products to the environment. Until now it has been very difficult to determine the properties of combustion heavy fuels. Previously known methods used for determining the quality of these fuels, such as: Calculated Carbon, Aromaticity Index – CCAI and Calculated Ignition Index – CII, have proved to be inappropriate in detecting the fuels' problems. Moreover, such methods cannot detect the effects on the quality of combustion that additives or other contaminants that could be introduced into the fuel might have.

The cetane number (CN) of diesel, specified by ASTM D613 - 10a, is a measure of its ignition delay time. A higher CN, a desirable property in diesel engine, indicates shorter time between the ignition and the initiation of fuel injection into the combustion chamber. The higher CN is correlated with the reduction of nitrogen oxides ($NO_x$) and unburned hydrocarbons (UHC) exhaust emissions, which is important for alleviating air pollution [8].

Biodiesel from vegetable oil sources have been recorded as having a cetane number range of 46 to 52, and animal-fat based biodiesels cetane numbers range from 56 to 60 [9].

A very important suite of additives is cetane improver used in diesel engines to improve the cetane quality of marketed fuel components by reducing the delay between injection and ignition when fuel is sprayed into the combustion chamber. The chemicals most commonly used as cetane improvers are nitrates and certain peroxides [10].

Additives are abundantly manufactured and mixed with IC engine fuels to meet the proper performance of fuel in engine. Additives act like catalyst so that they aid combustion, control emission, control fuel quality during distribution and storage and reduce refiners operating cost [11].

The aim of this paper is to determine and observe the evolution of characteristics of auto-ignition and combustion of rapeseed oil with additives with 2-ethylhexyl-nitrate in different percent.

## RESULTS AND DISCUSSION

The samples that have been used for experiments have been: 100% crude and filtered rapeseed oil (RO), rapeseed oil with additives with 2-ethylhexyl-nitrate (2-EHN) in concentration of 97% in different percent in oil - 1% (RO-EHN-1), 1.5% (RO-EHN-1.5) respectively 2% (RO-EHN-2) and diesel fuel (DF) – witness fuel.

Rapeseed (*Brassica napus*) is widely cultivated throughout the world for the production of animal feed, vegetable oil and biodiesel. Rapeseed oil is one of the preferred oil stocks for biodiesel production, partly because rapeseed produces more oil per unit of land area compared to other oil sources, ranking fourth in the world with respect to production, after soybean, palm and cottonseed [12].

The 2-ethylhexyl-nitrate (2-EHN), the nitric acid ester of 2-ethyl-1-hexanol, is currently added in significant amounts to diesel oil to improve ignition and boost cetane number [13]. Main physico-chemical properties of 2-ethylhexyl-nitrate are [14]: vapor pressure at 20 °C: 27Pa; solubility in water at 20 °C: 12.6 mg l-1; logKo/w: 5.24; liquid density: 0.96.

2-EHN is a large-scale commodity, the worldwide production of which is estimated to be about 100,000 tons per year. It has long been considered as presenting no particular risk to human health. 2-EHN was also nonmutagenic according to the *Ames test*. [15].

The cetane number (or the cetane index) is the numerical value that represents the percentage in volume of cetane (n-hexadecane, $C_{16}H_{34}$) in its mixture to $\alpha$-methylnaphthalene. In order to approximate the cetane number an arbitrary scale has been chosen according to which cetane ($C_{16}H_{34}$) has the value 100, and $\alpha$-methylnaphthalene ($C_{10}H_7$-$CH_3$) has the value 0 (zero). The cetane number shows the tendency to self-ignite of the fuels (e.g.: diesel fuel, etc.) used for diesel engines. The higher a fuel's cetane number is, the easier the fuel ignites.

Cetane number increases with chain length, decreases with number of double bonds and the carbonyl group move toward the center of the chain [16]. The authors have also cited that the cetane number of pure esters of stearic acid was approximately 75, but for esters of linoleic acid with three double bonds, cetane number had dropped to the ~25. Cetane number increased from 47.9 (C12) to 75.6 (C18) for saturated C10 through C18 esters. At and above C12, the cetane numbers were above 60. Knothe has presented that cetane numbers of fatty esters generally increases with: the number of methylene groups (-$CH_2$) in the chain of fatty compound, number of -$CH_2$ groups in the ester moiety and with increasing saturation of the fatty compound [17].

An easy startup of a diesel engine depends of the quality to self-ignite of the diesel, as well as on viscosity and the frosting temperature of diesel (especially for external temperatures) [18].

The determination of the cetane number is being done in a special engine at the speed of 900 rot/min, with an injection advance of 13°, at the injection pressure 10.5 MPa, according to ASTM D 613-10a.

The behavior at auto-ignition of the diesel fuels can be calculated with the help of some easy to determine characteristics. Out of these the cetane index can be mentioned.

The density of diesel fuel decreases with the decrease in the content of aromatic hydrocarbons and the increase of the n-paraffins. Using these comparison indexes, it has been established an appreciation criteria of the sensitivity of auto-ignition for diesel, called the Diesel Index (DI). The Diesel index is being established by knowing the value of the diesel's density, expressed in degrees API (*American Petroleum Institute*) and the aniline point according to the standard test ASTM D976-06.

Table 1 presents the main characteristics of the auto-ignition and burning processes of the experimented fuels, and figures 4-9 present the properties of the used fuels in comparison to the witness fuels, diesel fuel and rapeseed oil.

The apparatus used for these experiments was FIA-100 (*fuel ignition analyzer*).

FIA-100 allows the supplier or fuel user to determine the quality of combustion to the compression-ignition engines based on the measured delay in auto-ignition.

FIA-100 device and its operation mode are presented in the *Experimental section.*

**Table 1.** The results of the main characteristics of the auto-ignition processes of the used fuels

| Fuels | Ignition delay, [ms] | Start of main combustion, [ms] | FIA Cetane number | Combustion period, [ms] |
|---|---|---|---|---|
| **DF** | 8.7 | 8.7 | 57.6 | 11.2 |
| **RO-EHN-1** | 10.71 | 10.65 | 48.3 | 11.5 |
| **RO-EHN-1.5** | 9.21 | 9.2 | 55.6 | 11.5 |
| **RO-EHN-2** | 8.81 | 8.8 | 57.2 | 11.2 |
| **RO** | 9.86 | 10.7 | 48.1 | 12.2 |

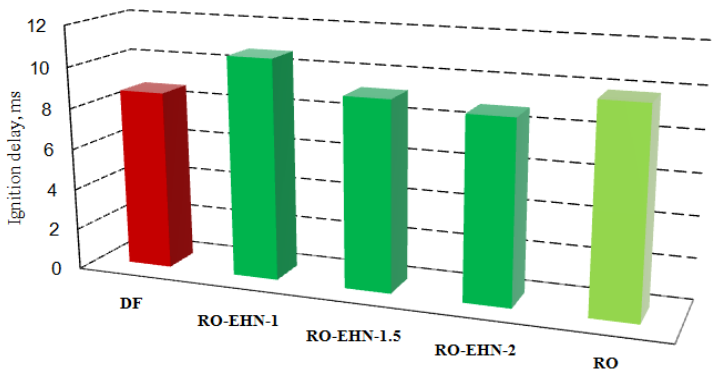


**Figure 1.** Ignition delay of fuels function to the ethylhexyl-nitrate percent in rapeseed oil

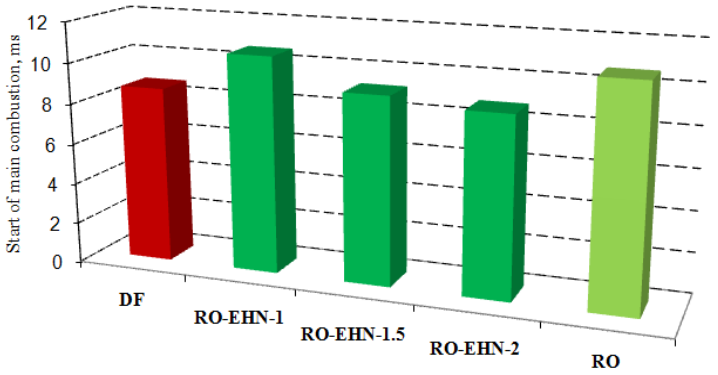


**Figure 2.** Start of main combustion function to the ethylhexyl-nitrate percent in rapeseed oil

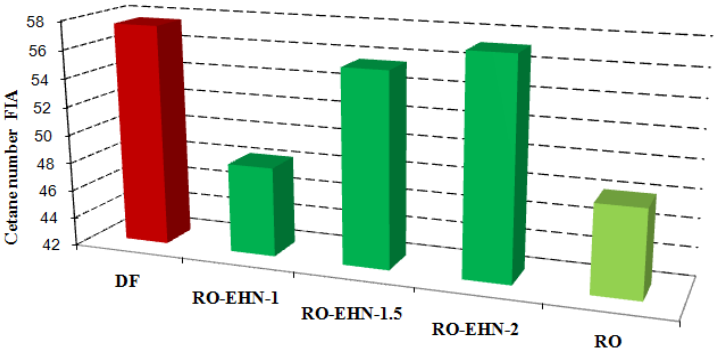


**Figure 3.** Cetane number of fuels functions to the ethylhexyl-nitrate percent in rapeseed oil

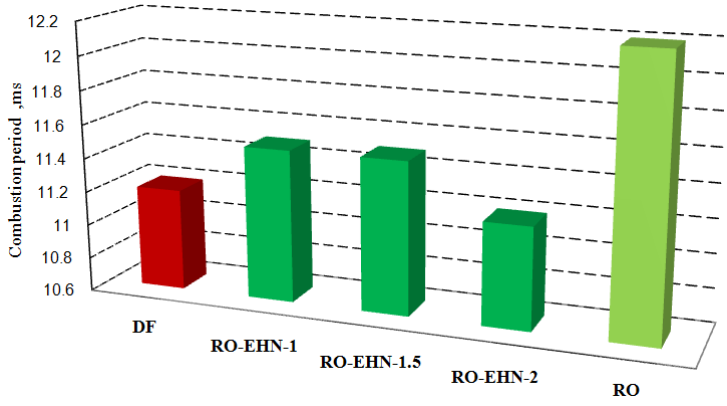


**Figure 4.** Combustion period function to the ethylhexyl-nitrate percent in rapeseed oil

As a result of the experimental research it can be noticed that rapeseed oil with additives with 2-ethylhexyl-nitrate have values which are really close to the auto-ignition and combustion characteristics of diesel fuel. This additive increases the value of cetanic number with 16.5% in comparison without additives rape oil.

The additive 2-ethylhexyl-nitrate used in a percentage of 2% is the closest to diesel values. Thus, the cetane number is 57.2, being with only 0.69% higher than diesel fuel cetane number. The start of main combustion and the average delay at self-combustion also have values close to diesel's, the differences being with only 1.13% higher than diesel' values.

The period of the main combustion is identical as value with the one for diesel, respectively 11.2 ms. The same happens for rape oil with the additive 2-ethylhexyl-nitrate, in 1.5%, where the cetane number is only 3.4% higher than the value of diesel's cetane number.

## CONCLUSIONS

After the experimental results (Figures 1 – 4) it has been noticed that the values of auto-ignition and combustion qualities of rapeseed oil are being improves by additive 2-ethylhexyl-nitrate.

The lowest cetane number belongs to the rapeseed oil (48.1) compared to the cetane number of diesel fuel (57.6). The nearest value for cetane number in comparison to diesel fuel is for RO-EHN-2 (57.2).

For as good combustion of fuel in the combustion chamber, it must burn spontaneously and have a minimum self-ignition delay. According to experimental results, it was found that the nearest value of diesel fuel is for RO-EHN-2 (8.81).

The start of main combustion is closely connected with experienced fuel cetane. When the fuel is injected in the combustion chamber, it does not start to burn immediately. First of all it mixes with the air in the combustion chamber. It is heated until it reaches its self-ignition point, and then begins to burn. All these processes take time. After it starts combustion, the pressure, temperature, turbulences in the combustion chamber and the combustion process accelerates a lot. The diesel fuel- etalon fuel has the value of the start of main combustion 8.7 ms and RO-EHN-2 has value 8.8 ms.

The diesel fuel has the value of the combustion period of 11.2 ms, and rapeseed oil 12.2 ms, with 1 ms more. RO-EHN-2 has the value of combustion period equal to the value of diesel fuel (11.2).

## EXPERIMENTAL SECTION

The positioning of FIA 100 and of the computer is presented in figure 5. The fuel's sample injected in the FIA 100’s combustion chamber self-ignites and burns as in a real engine. The auto-ignition and combustion

conditions in the FIA 100's combustion chamber have been automatically simulated with an external electric source (230 V CA/50 Hz) and compressed air (at 50 bars).

A sample of the investigated fuel has been injected in this combustion chamber by using a high pressure injection pump, mechanically activated, and an injector. During the injection, the fuel jet self-ignited and burned in the combustion chamber with a constant volume. The testing conditions and the combustion process have been carefully monitored, and the measured data have been registered in a separate electronic unit which includes a microprocessor. The data is immediately presented on the computer's monitor, analyzed, stored and saved in the computer.


**Figure 5.** The stand with FIA 100: 1 - the apparatus FIA100;
2 - PC unit for data recording.

The instruments for testing the fuel are based on a combustion chamber with constant volume. The basic idea was to stimulate the conditions of the combustion process of a real combustion-ignition engine. The advantage of this technology is the ability to make repeated, highly precision measurements, in a controlled environment.

The combustion chamber is endowed with temperature and pressure sensors that gather the data of the process during the ignition and combustion phases.

Together with an advanced electronic system and a soft for controlling the process the automatic functioning of the instrument has been possible at a high accuracy and repeatability of measurements.

The data obtained by using this instrument have been represented by a number of parameters that can be used for the quantitative as well as the qualitative analyses of the sample.

Sample based on fuel from rapeseed oil was tested on 100 FIA device according to the procedure established for this instrument for heavy fuels (Figure 6). Thus, the investigation took place at an air pressure of 4.5 MPa and its temperature of 500 °C.

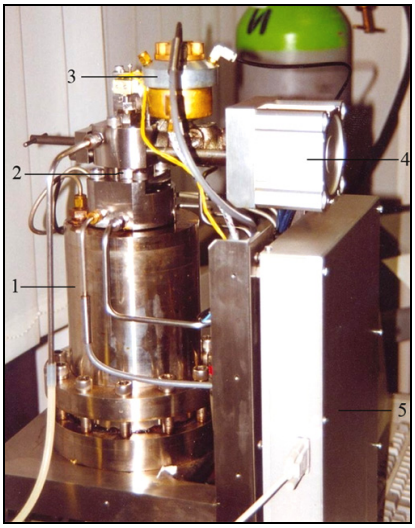


**Figure 6.** The apparatus FIA 100: 1 - cylinder; 2 - injector; 3 - fuel tank; 4 - high pressure pump; 5 - control electronic unit with microprocessor.

Depending on the viscosity of the sample the fuel was preheated to get an injection viscosity of fuel at about 15 $mm^2/s$. The kinematic viscosity of crude rapeseed oils is about an order of magnitude greater than that of conventional, petroleum-derived diesel fuel.

During the fuel's combustion, the increase of the pressure is monitored and transmitted towards a computer for analyses and recording. The reported parameters have been calculated for an average of 14 individual combustion cycles.

On FIA 100, the delay in auto-ignition is defined as the time interval, expressed in milliseconds, from the start of the injection when an increase in pressure with 0.02 MPa over the initial chamber pressure has been noticed.

Moreover, the starting phase of the main combustion is determined as being the time (in ms) in which an increase in pressure can be detected, with 0.3 MPa over the initial chamber pressure.

The delay period for auto-ignition is strongly influenced by the physico-chemical properties of the fuel. The physical component mostly depends on viscosity, superficial tension and fractioned composition, and the chemical component on the molecular structure. Thus the delay includes a phase of physical changes, in which the fuel is sprayed, partially vapored and mixed with the air, and another one of chemical changes, when the oxidation reactions take place and the reach of the auto-ignition temperature. The two time periods are simultaneous [10, 19]**.**

The start of the main combustion has been used for establishing the quality of the fuel's auto-ignition tested as "CC FIA" (cetane number). The base for "FIA Cetane Number" is a reference curve for the instrument, which shows the ignition properties for mixtures that are between the reference fuels *U15* and *T22* (standard fuels) determined by *Phillips Petroleum International* [7]. Thus reference curve establishes the relation between the quality of the ignition registered in milliseconds and the cetane number of different mixtures of the basic fuels. In the case of heavy fuels, the auto-ignition properties are between CC=18.7 up to CC=40.

There are usually at least 12 injections/ignitions at each test. Between each injection there is a pause of approximately 5 minutes until the installation reaches the characteristics for starting the injection (chamber pressure, chamber temperature, the fuel's temperature and the cooling water's temperature).

Based on this data, the average delay period for auto-ignition has been established, as well as the starting point of the main combustion, the cetane number – CC FIA and the speed of the released heat.

The determination of the cetane number for Diesel with FIA 100 has been optimized with respect to the comparability to the results obtained on a conventional testing engine. As an evaluation bases the landmarks for the determination of cetane number according to standard DIN 51773 have been used.

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STUDIA UBB CHEMIA, LVII, 1, 2012 (p. 185 - 194)

# GC-MS BINDING MEDIA STUDY OF TRANSYLVANIAN PAINTED CEILINGS

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**ABSTRACT.** Gas chromatography coupled with mass spectrometry (GC-MS) was used to investigate the organic materials of painted coffered ceilings of five medieval churches in different regions of Transylvania, applying a methodology designed to identify organic binding medium in the same microsample. Results showed a very restrained use of organic materials since only animal glue was identified in most of the samples. The study provided a better understanding of the painting technique and of the decay processes of this specific local heritage, and proved helpful in the planning of suitable conservation strategies.

***Keywords:*** *GC-MS analyses, organic binders, animal glue, painted ceilings, Transylvanian heritage*

## INTRODUCTION

Cultural heritage objects are often decorated with painted surfaces. Paint layers are complex structures consisting of colored material, the pigment, embedded in an organic matrix which enables the application of the pigment on the surface and which is responsible for the cohesion of the resulting layer and its adhesion to the surface. The type of organic materials in the painted surfaces have major contribution to the aspect of the paint; actually they define the painting technique [1,2]. The degree of degradation of these organic materials is mainly responsible for the condition of the paint layers. A flaking or powdery paint has usually an organic binder affected by ageing, complex chemical alteration of the material in time, mainly due to free radical auto-oxidations, ionic or enzymatic hydrolyses and cross-linking reactions [3-5]. Consequently, it is very important to identify by analytical methods the organic constituents of paint layers in order to understand their painting technique, their degradation processes and to properly plan their conservation.

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Up to now, a comprehensive study of the organic materials in paint layers was not performed on Romanian heritage objects. Staining tests [6] were reported to check the protein content of wall painting samples [7]; Fourier Transformed Infrared spectroscopy (FTIR) and direct infusion mass spectrometry (DI-MS) were used to identify classes of organic materials used in some icons [8].

Painted woodworks were chosen as subject of the present study since they are widespread in Transylvania giving a specific character to the built heritage of the region (Figure 1.). In fact, painted ceilings are encountered in about 150 medieval churches [9]. The interior of these churches was decorated with painted woodworks mainly during the 17$^{th}$, 18$^{th}$ century by painter-carpenter workshops. A famous painter-carpenter family of the 18$^{th}$ century was the Umling family, Umling Lőrinc the elder, together with his sons, Lőrinc the younger and János. They were active in Călata (Kalotaszeg), the region around Cluj, living behind a corpus of work that can be found in over 40 churches of the region. Most of the samples studied originate from their works (Figure 2).

The study was carried out using gas chromatography coupled with mass spectrometry (GC-MS), applying a procedure which permits the quantitative characterization of the organic binders encountered in painted surfaces in a single sample [10].

The aim of the research was to characterize the painting technique of the ceilings and woodworks, to evaluate the use of binders on the basis of the pigments applied, and to compare the techniques used by different workshops. Finally, the scientific data could improve understanding of the observed decay processes in the paint layers and help choosing a proper conservation strategy.

## RESULTS AND DISCUSSION

Samples analyzed were collected by the restorer Ferenc Mihály from the painted ceilings of five medieval churches from different regions of Transylvania. Their interior painted woodwork decoration dated from 17$^{th}$ and 18$^{th}$ century. Three of the churches were decorated by the Umling workshop mentioned above. Five samples were taken from different colors of the same ceiling coffer in order to compare their binders. One of the samples belonged to a pew parapet (L7) most probably painted by the same workshop performing the ceiling. This permitted to check if the apparently similar paintings of the ceiling and of the pew were realized with the same technique. Samples from ceilings painted by other workshops, in other periods, were also provided in order to obtain and compare information connected to different workshops and historic periods.

A number of 13 samples were analyzed. Before analyses samples were observed and documented with low magnification optical microscope (Figure 3). Detailed sample description is reported in Table 1.

**Figure 1.** Detail from the painted ceiling of the Reformed church in Crasna (Kraszna, SJ), 1736, painted by Pataki Asztalos János (sample K1)


**Figure 2.** Coffer G13 from the painted ceiling of the Reformed church in Luna de Sus (Magyarlóna, CJ), 1752, Umling Lőrinc, the elder, showing the sampling places for samples L1 to L5

## Working methodology

Ethical aspects of sampling from heritage objects impose minimum sample size which should be used to provide as many information as possible. Thus, the adopted working methodology enabled the identification of all usual organic binders from the same microsample avoiding interferences from inorganic media [10].

The method is based on a multi-step chemical pretreatment of the sample. First proteins and polysaccharide materials were subjected to ammonia extraction in order to separate them from lipid and resinous materials. Proteins

and sugars were separated afterwards by monolithic sorbent tip technology with a C4 stationary phase and purified before hydrolysis. Three fractions were generated analyzed separately by GC-MS. Lipids and resins were subjected to saponification assisted by microwaves and the three fractions were separately derivatised. A detailed description of the procedure has been published elsewhere [10].

**Table 1.** Detailed sample description

| Sample code | Sampled painted ceiling | Weight (mg) | Sample description |
|---|---|---|---|
| A1 | Reformed church in Alunişu (Magyarókereke, CJ), 1746, Umling Lőrinc, the elder | 0.1 | Blue paint layer with white ground layer, possibly with wood fibers |
| A2 | Reformed church in Alunişu (Magyarókereke, CJ) 1786, Umling Lőrinc, the younger | 0.6 | Grayish-blue paint layer fragments with white ground layer |
| L1 | Reformed church in Luna de Sus (Magyarlóna, CJ), 1752, coffer G13, Umling Lőrinc, the elder | 0.1 | White paint layer fragments |
| L2 | Reformed church in Luna de Sus (Magyarlóna, CJ), 1752, coffer G13, Umling Lőrinc, the elder | 0.9 | Black paint layer fragments with white ground layer |
| L3 | Reformed church in Luna de Sus (Magyarlóna, CJ), 1752, coffer G13, Umling Lőrinc, the elder | 0.5 | Light green paint layer fragments with white ground layer |
| L4 | Reformed church in Luna de Sus (Magyarlóna, CJ), 1752, coffer G13, Umling Lőrinc, the elder | 0.8 | Red paint layer fragments |
| L5 | Reformed church in Luna de Sus (Magyarlóna, CJ), 1752, coffer G13, Umling Lőrinc, the elder | 0.2 | Ochre paint layer fragments |
| L6 | Reformed church in Luna de Sus (Magyarlóna, CJ), 1752, Umling Lőrinc, the elder, another coffer | 0.4 | Blue paint layer fragments with white ground layer |
| L7 | Pew parapet, Reformed church in Luna de Sus (Magyarlóna, CJ), 1768, Umling Lőrinc, the younger | 0.4 | Blue and black paint layer fragments with white ground layer |
| G1 | Catholic church in Ghelinţa (Gelence, CV), 1628 | 0.7 | Green paint layer fragments with wood fibers |
| P1 | Reformed church in Petrindu (Nagypetri, SJ), ), 1713, Zilahi Asztalos János | 0.3 | Red paint layer fragments with white ground layer |
| K1 | Reformed church in Crasna (Kraszna, SJ),1736, Pataki Asztalos János | 0.1 | Green paint layer fragments with white ground layer |
| K2 | Reformed church in Crasna (Kraszna, SJ),1736, Pataki Asztalos János | 1.1 | Red paint layer fragments with traces of white ground layer |

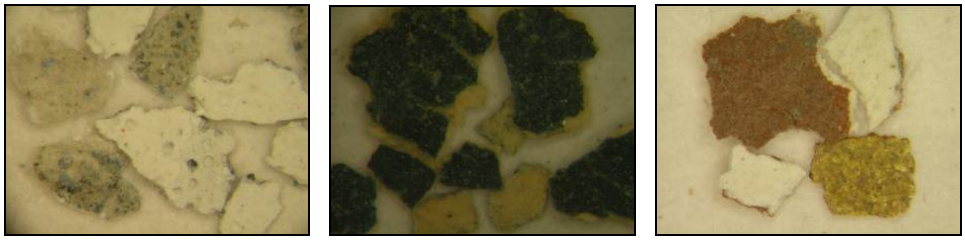

**Figure 3.** Optical microscope image of samples A2, L6 and P1 (40x)

## Data interpretation

Chromatograms were acquired in Synchronous SIM/Scan mode that enabled collection of both SIM (Selected Ion Monitoring) data and full scan data TIC (total ion chromatogram) in a single run. Quantitative determinations of the compounds in each fraction were based on the corresponding SIM chromatograms. Calculations were performed using calibration curves based on standard solutions of amino acids, aldoses and uronic acids or aliphatic mono- and dicarboxylic acids. The individual response of each analyte was evaluated by daily recoveries. Individual derivatizations and injections were controlled by adding internal standards (norleucine, mannitol or tridecanoic acid respectively, and hexadecane). Running blanks of the procedure revealed low levels of contamination. Limit of detection (LOD) and quantification (LOQ) were evaluated for each analyte.

## Proteins

Figure 5 reports the SIM chromatogram of the aminoacidic fraction of sample L4.

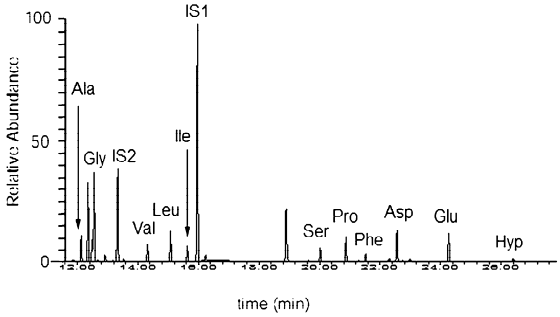


**Figure 5.** SIM chromatogram of the amino acid fraction of sample L4.

The corresponding m/z values for each monitored amino acid are given in parentheses. Ala – alanine (158, 232); Gly – (147, 218, 246, 261); Val – valine (186, 260); Leu – leucine/ Ile – isoleucine/ IS (internal standard: norleucine) (200, 302); Ser – serine (362, 390); Pro – proline (258, 330); Phe – phenylalanine (302, 336); Asp – aspartic acid (302, 418); Glu – glutamic acid (330, 432); Hyp -hydroxiproline (460, 462); IS2 – injection standard (hexadecane)

Proteinaceous materials were identified based on the percentage amino acid content of the corresponding amino acid fraction, reported in Table 2.

The main proteinaceous materials encountered in paint layers - egg, animal glue, and casein - have characteristic amino acid compositions [3, 5]. If used as mixtures, it is more difficult to distinguish between them on the basis of amino acid percentage content. Statistical data treatment by Principal Component Analysis (PCA) enabled an easier and more unambiguous interpretation. Introducing as variables the percentage amino acid content of the 11 amino acids monitored in SIM, two principal components of the correlation matrix have been taken into account. The corresponding score plot position of the samples was checked against 121 protein reference samples (egg, casein and animal glue) analysed in the laboratory in Pisa [11]. Unmixed proteins would integrate in the corresponding cluster; mixtures will be located between the clusters. The score plot of the historical samples compared to the references is presented in Figure 6.

**Table 2**. Percentage amino acid content and protein content of the samples

| Sample | Ala | Gly | Val | Leu | Ile | Ser | Pro | Phe | Asp | Glu | Hyp | Protein content (µg) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A1** | 8.2 | 22.8 | 4.6 | 7.4 | 4.0 | 9.7 | 11.0 | 3.1 | 12.4 | 15.9 | 0.9 | 0.4 |
| **A2** | 9.3 | 23.3 | 4.0 | 5.7 | 3.1 | 5.1 | 15.7 | 3.3 | 9.0 | 12.5 | 8.9 | 1.4 |
| **L1** | 10.8 | 19.3 | 6.8 | 8.5 | 4.7 | 2.9 | 10.9 | 4.1 | 13.3 | 16.7 | 2.0 | 0.3 |
| **L2** | 11.9 | 33.0 | 4.2 | 5.1 | 2.7 | 2.6 | 14.6 | 2.9 | 10.2 | 10.9 | 1.9 | 3.8 |
| **L3** | 11.3 | 22.5 | 5.5 | 5.5 | 2.9 | 6.2 | 10.9 | 3.1 | 9.0 | 10.0 | 13.2 | 3.9 |
| **L4** | 10.1 | 24.2 | 3.1 | 4.5 | 2.0 | 3.5 | 15.4 | 2.7 | 8.5 | 15.6 | 10.3 | 13.8 |
| **L5** | 14.3 | 35.7 | 3.7 | 3.3 | 1.8 | 3.9 | 6.6 | 1.9 | 12.2 | 14.8 | 1.9 | 46,0 |
| **L6** | 11.2 | 21.4 | 6.8 | 8.5 | 4.4 | 3.5 | 14.2 | 4.4 | 13.3 | 11.5 | 0.9 | 1.3 |
| **L7** | 10.3 | 25.4 | 3.4 | 4.1 | 1.9 | 3.2 | 13.5 | 2.6 | 10.1 | 16.2 | 9.4 | 12,9 |
| **G1** | 9.8 | 29.5 | 3.9 | 4.6 | 2.2 | 2.9 | 18.5 | 2.5 | 6.7 | 14.9 | 4.5 | 6,8 |
| **P1** | 9.8 | 26.1 | 2.9 | 4.1 | 2.0 | 3.4 | 14.5 | 2.6 | 8.4 | 14.8 | 11.5 | 1.8 |
| **K1** | 9.2 | 26.3 | 4.2 | 7.4 | 3.6 | 6.1 | 11.6 | 2.7 | 10.1 | 12.3 | 6.5 | 0.6 |
| **K2** | 10.6 | 27.1 | 4.0 | 5.5 | 2.4 | 3.7 | 16.5 | 3.2 | 8.0 | 12.1 | 6.9 | 14,9 |

The statistical data treatment highlights that animal glue is present in almost all the samples. Three samples, A1, L1 and L6, are positioned between the egg and animal cluster, meaning that they could be a mixture of the two. Crosschecking with the sample description (Table 1) it can be noticed that these three samples are from paint layers of Umling the elder; L1 from a white layer, the other two samples from blue layers, suggesting that the elder master would prefer to use egg (if animal glue was the binder of the ground layer of the samples) or egg mixed with animal glue for some colors. From his biography, we know that he was formed in a panel painting workshop, where egg was a usual binder[1]. He might have preserved this usage for some of his colors in his painter-carpenter activity.

[1] Personal communication of Ferenc Mihály, May, 2011.

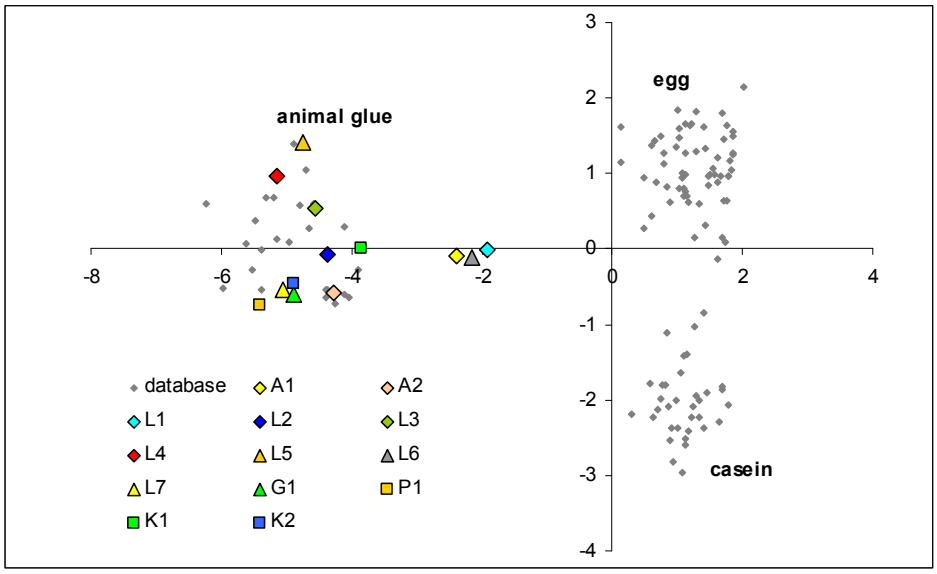


**Figure 6.** PCA score plot of the percentage amino acid content of the samples compared to a database established in Pisa laboratory on reference protein materials

Samples coming from painted ceilings of other masters, working in different periods, contain only animal glue as proteinaceous material, showing that the use of this material was a specific feature of the painter-carpenter painting technique of the 17$^{th}$ and 18$^{th}$ century. Sample L7, coming from a pew parapet has also animal glue in its composition, suggesting that the same decorative technique has been used both for painted ceilings and other painted woodwork in the interior of the church.

## Polysaccarides

Saccharide fractions were analysed for six samples. The sugar content of sample K2 was below detection limit; the sugar content of all other analysed samples is reported in Table 3. The saccharide profile was compared with that reported in the literature [12]. Two samples taken from the wooden support of the ceiling in Alunişu (Magyarókereke, CJ) were also analyzed according to the same procedure to be used as environmental blanks. The obtained average saccharide profile is also reported in Table 3.

Samples A1, A2 and P1 showed a qualitative and quantitative profile that it is not in agreement with any of the reference materials in the literature. The high content of xylose seems to point to a contamination probably due to migration from the wood support. Samples G1 and K1 show the same qualitative and similar quantitative saccharide profiles. The presence of methylpentoses

and uronic acids in significant amounts (>1%) points to the presence of a polysaccharide material which cannot be identified, since the saccharide profile is not in agreement with the references in the literature.

**Table 3.** Saccharide profile (percentage relative monoglucide and uronic acid content) of some of the samples and of the wood from the support (Xyl – xylose; Ara – arabinose; Ram – ramnose; Fuc – fucose; Gal ac – galacturonic acid; Glu ac – glucuronic acid; Glu – glucose; Man – mannose; Gal – galactose)

| Sample | Xyl | Ara | Ram | Fuc | Gal ac | Glu ac | Glu | Man | Gal | Sugar content (µg) |
|---|---|---|---|---|---|---|---|---|---|---|
| **A1** | 59.1 | 14.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 18.6 | 8.0 | 0.7 |
| **A2** | 51.5 | 5.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 25.5 | 17.5 | 1.6 |
| **G1** | 28.7 | 8.0 | 8.6 | 2.3 | 1.0 | 4.8 | 0.0 | 26.2 | 20.4 | 3.1 |
| **P1** | 51.4 | 8.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 12.5 | 27.2 | 1.2 |
| **K1** | 20.0 | 10.6 | 5.0 | 9.2 | 1.0 | 10.0 | 0.0 | 21.9 | 22.3 | 2.0 |
| **wood** | 25.7 | 16.2 | 1,7 | 1 | 0.0 | 0.0 | 32.0 | 17.5 | 6.4 | 2.5 |

## Lipid-resinous materials

Glycerolipid identification was based on the mono- and dicarboxylic aliphatic acid content resulting from the lipid-resinous fraction. Waxes and natural resins were checked from the same fraction looking at specific molecular patterns and/or stable degradation markers [4].

The evaluation of the SIM chromatograms acquired from the lipid-resinous fraction of these samples showed that the lipid content of all samples was below detection limit, chromatograms presenting a typical blank profile. Peaks corresponding to the markers of wax and terpenoid resins could not be identified in the TIC chromatogram.

Moreover, the lack of lipid content in samples A1, L1 and L6, containing egg in their protein fraction suggested that egg could be used as a glair.

## CONCLUSIONS

The study focused on the analysis of the organic materials in painted surfaces, mainly wooden ceilings from different Transylvanian churches. Thirteen samples were analyzed by GC-MS applying a methodology that enables the identification of the natural organic binders from the same microsample avoiding interferences due to inorganic media. The analyses revealed that the paint layers on painted woodwork were mainly applied with animal glue as binder. No lipid or resinous materials were identified in the samples, which is in good agreement with the matte aspect of the paintings. A polysaccharide material could be detected also in some samples though its identification was not straightforward. Painting technique proved to be similar for the studied ceilings

painted by different workshops in the 17th and 18th century. Other painted woodwork in the churches, such as the pew parapet studied also here, seems to be painted in the same manner. Therefore, macroscopic decay of the painted surfaces could not be related to the use of a particular binding media. However, an interesting specific feature was revealed for the binding media used by Umling, the elder, who mixed egg and animal glue to apply some of his colors (white and blue in the present study).

The results give a preliminary view on the painting technique of Transylvanian painted woodwork and its decays, and may help in planning suitable conservation strategies.

## EXPERIMENTAL SECTION

Analyses were performed in the laboratories of the research group "Chemical Science for the Safeguard of Cultural Heritage", within University of Pisa, Department of Chemistry and Industrial Chemistry.

Microphotographs were taken by a Nikon SMZ800 microscope, equipped with Nikon digital camera.

A microwave oven model MLS-1200 MEGA Milestone (FKV, Sorisole, Bergamo, Italy) was used for the hydrolysis of proteins and polysaccharide materials.

The OMIX C4 pipette tips were purchased from Varian (Milan, Italy). Zerolit DMF cation/anion exchange resin was supplied by BDH Chemicals Ltd. (UK).

GC-MS analyses of the samples were performed on a 6890N GC System Gas Chromatograph (Agilent Technologies), coupled with a 5975 Mass Selective Detector (Agilent Technologies) single quadrupole mass spectrometer, equipped with a PTV injector. MS was operating in the electron impact (EI) positive mode (70 eV). The MS transfer line temperature was 280°C; the MS ion source temperature was kept at 230°C; the MS quadrupole temperature was at 150°C. Chromatographic separations were performed on an HP-5MS fused silica capillary column (5% diphen-yl-95% dimethylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Agilent Technologies, Palo Alto, CA) coupled with a deactivated silica precolumn (2 m × 0.32 mm i.d) using a quartz press fit. Detailed working conditions are reported in the literature [10].

Principal Component Analysis was performed using SCAN Release 1.1 (Minitab Inc., USA)

### ACKNOWLEDGMENTS

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STUDIA UBB CHEMIA, LVII, 1, 2012 (p. 195 - 204)

# ANALYSIS IN TERMS OF INTERMOLECULAR FORCES OF THE THERMODYNAMIC PROPERTIES OF THE MIXTURES CONTAINING (1R,4S)-(+)-FENCHONE, METHYL CHAVICOL AND *TRANS*-ANETHOLE

**IOAN BÂTIU***

**ABSTRACT.** The experimental vapor-liquid equilibrium data (VLE) in binary and ternary systems containing (1R,4S)-(+)-fenchone, methyl chavicol and trans-anethole, reported in previous papers, were used to discuss the thermodynamic properties of the mixtures, taking into account the intermolecular forces, intramolecular electronic effects and the effect of the steric hindrance.

***Keywords:*** *Terpenoids, Intermolecular forces, Vapor - liquid equilibria,*

## INTRODUCTION

(1*R*,4*S*)-(+)-Fenchone [(1*R*,4*S*)-(+)-1,3,3-trimethylbicyclo[2.2.1]heptan-2-one], methyl chavicol [4-allyl-1-methoxybenzene] and *trans*-anethole [*trans*-1-methoxy-4(prop-1-en-1-yl) benzene] are the main components of the essential oil from the fruits of bitter fennel (*Foeniculum vulgare Mill, fam. Umbelliferae*). Both the raw essential oils and its isolated pure components are used in perfumery, cosmetics, pharmacy, aromatherapy and food industry [1].

Generally, essential oil components belong to the terpenoid class. Terpenoids are natural products comprising a large number of compounds with complex chemical structures. Many essential oil components are monoterpenoids ($C_{10}$) and sesquiterpenoids ($C_{15}$), acyclic, monocyclic or bicyclic, saturated or unsaturated.

Terpenoids and nonterpenoid compounds, main components of the essential oils, frequently contain oxygenated functional groups, some of them being in mesomeric relationship or being subjected to other electronic effects or proximity effects.

The chemical structures of the main components of the essential oils of fennel are presented in Figure 1. The names of the components referred to in this paper are: (+)-fenchone, methyl chavicol and *trans*-anethole.

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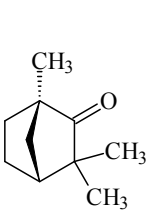
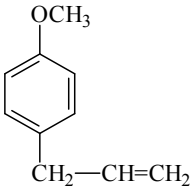
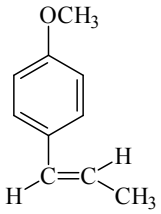



(1*R*,4*S*)-(+)-Fenchone




Methyl chavicol




t*rans*-Anethole

**Figure 1.** Chemical structure of (1*R*,4*S*)-(+)-fenchone, methyl chavicol and *trans*-anethole

(+)-Fenchone is a bicyclic terpenoid ketone while methyl chavicol and *trans*-anethole are two semi-aromatic ethers which do not belong to the terpenoid class.

Thermodynamic properties of any pure substance are determined by intermolecular forces that operate between the molecules of that substance. Thermodynamic properties of the mixtures depend on intermolecular forces that operate between the molecules belonging to the same component, but also to interaction between dissimilar molecules of the mixture. Frequently, the theory of intermolecular forces gives us no more than a qualitative, or perhaps semiquantitative basis for understanding phase behaviour, but even such a limited basis can be useful for understanding and correlating experimental results [2]. A brief discussion of the intermolecular forces in molecular thermodynamics of fluid-phase equilibria [2] and in supramolecular chemistry [3] was done.

In previous papers [4] we have discussed intermolecular interactions in similar binary systems containing terpenoids: (S)-(+)-carvone+(+)-limonene; (-)-beta-pinene+eucalyptol; (R)-(-)-carvone+eucalyptol; *n*-octane+(+)-limonene; (R)-(-)-carvone+*n*-decane; *n*-decane+(-)-menthone and *n*-decane+(+)-fenchone, respectively.

In the present paper we selected and discussed other terpenoids and nonterpenoids, main components of the essential oil of bitter fennel: (+)-fenchone, methyl chavicol and *trans*-anethole. Due to their chemical structure, the compounds present complex intermolecular interactions.

Literature describes different types of intermolecular forces [2], but for our purpose here, only *electrostatic forces* (between permanent dipoles), *induction forces* (between a permanent dipole and an induced dipole – e.g. a dipole induced in a molecule with polarizable electrons), *dispersion forces* (forces of attraction between nonpolar molecules based on *hydrophobic interactions*) and, respectively *specific (chemical) forces* leading to association and solvation, i.e., to the formation of loose chemical bonds; hydrogen bonds and charge-transfer complexes are perhaps the best example. Also we take into consideration the van der Waals forces [3] and the steric hindrance effect and the mesomeric effect.

## RESULTS AND DISCUSSION

In the binary systems containing (+)-fenchone, methyl chavicol and *trans*-anethole the values of the experimental activity coefficients, $\gamma_i$ range from ca. 0.75 to ca. 1.08. The experimental fugacity coefficients, $\varphi_i$ are very close to unity. This means a quasi-ideal behaviour of the liquid phase and ideal behaviour of the vapor phase.

The binary system methyl chavicol + (+)-fenchone shows positive and negative deviations from ideality (Fig. 2, 3).

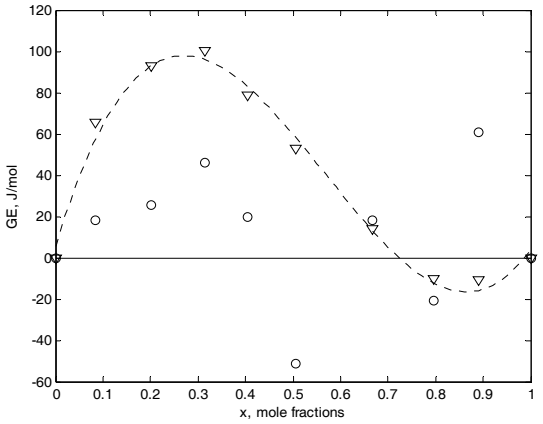



**Figure 2.** Variation of the molar excess Gibbs energy, $G^E$ with mole fraction $x_i$, for the binary system (+)-fenchone (1) + methyl chavicol (2) at a constant pressure $P$=4000 Pa: (o), - experimental; (▽), – calculated using binary parameters of the NRTL model (the object function - the boiling points condition).

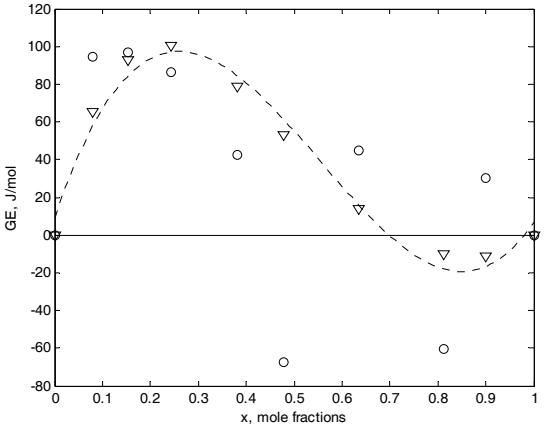



**Figure 3.** Variation of the molar excess Gibbs energy, $G^E$ with mole fraction $x_i$, for the binary system (+)-fenchone (1) + methyl chavicol (2) at a constant pressure $P$=4270 Pa: (o), - experimental: (▽), - calculated using binary parameters of the NRTL model (the object function - the boiling points condition).

The binary system *trans*-anethole + (+)-fenchone shows only negative deviations (Fig. 4).

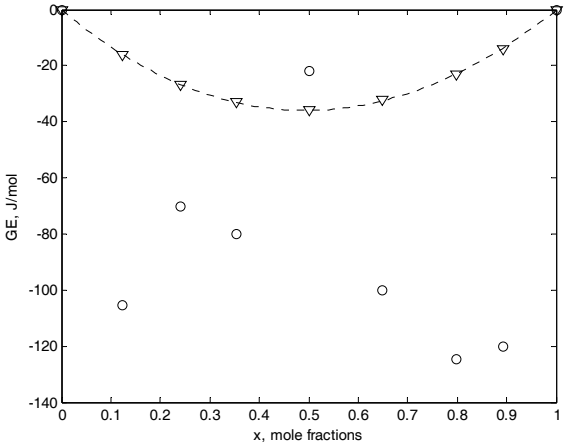



**Figure 4.** Variation of the molar excess Gibbs energy, $G^E$ with mole fraction $x_i$, for the binary system (+)-fenchone (1) + *trans*-anethole (2) at a constant pressure $P$=4000 Pa: (o), - experimental; (▽), – calculated using binary parameters of the NRTL model (the object function - the boiling points condition).

The binary system methyl chavicol + *trans*-anethole shows only positive deviations from ideality (Fig. 5).

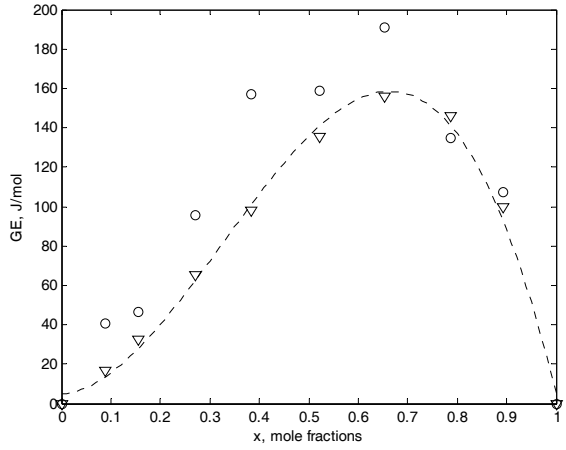
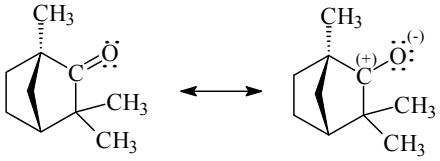



**Figure 5.** Variation of the molar excess Gibbs energy, $G^E$ with mole fraction $x_i$, for the binary system {methyl chavicol (1) + *trans*-anethole (2)} at a constant pressure $P$=4000 Pa: (o), - experimental; (▽), - calculated using binary parameters of the NRTL model (the object function - the boiling points condition).

The dispersion of the experimental values of $G^E$ (Figs. 2, 3, 4) is owing to the small values of $G^E$ and to the errors of the temperature measurements, ($\sigma_T$ = 0.1 K) and of the vapor and liquid phase compositions measurements ($\sigma_{x,y}$ = 0.003 mol. fr.) as well as due to maintaining the pressure, $P$ within 30 Pa around the desired values ($\sigma_P$ = 60 Pa). The values of $G^E$ were calculated using the binary parameters of the NRTL model published in [5].

The calculated dipole moment of the (+)-fenchone, methyl chavicol and *trans*-anethole are: 2.73 D, 1.17 D and 1.41 D, respectively. The values of the calculated dipole moment were obtained using, for geometry optimization - hamiltonian: B3LYP (density functional theory); basis set: 6-31G(d) (Gaussian98) and for dipole moment -hamiltonian: B3LYP; basis set: 6-31G(d) (Gaussian98).

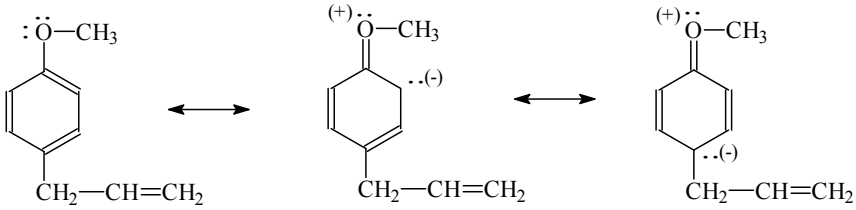
(+)-Fenchone could be assimilated with a simple cyclic ketone, having only an active functional group, >C=O. The ketone group, >CO is sterically hindered by three adiacent methyls, -$CH_3$ (Scheme 1).



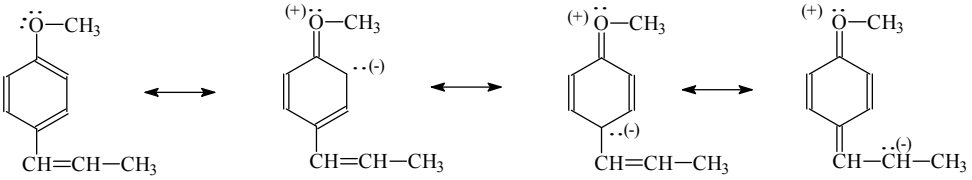
***Scheme 1***

In mixtures containing ketones the like strong dipole/dipole, >CO/>CO interactions occur due to the high value of the dipole moment.

Methyl chavicole and *trans*-anethole contain three active functional groups, oxygen, –O-; phenylene, –$C_6H_4$- and double bond, -CH=$CH_2$, in methyl chavicol or –CH=CH-, in *trans*-anethole (Scheme 2, 3).



***Scheme 2***



***Scheme 3***

It is possible that double bonds, -CH=$CH_2$, from allyl position, to be disturbed, therefore to behave like a simple alkene, but the oxygen from methoxy is very different from a dialkylic ether. Between phenylene, –$C_6H_4$- and double bond, -CH=$CH_2$ there is a proximity effect.

In methyl chavicol and *trans*-anethole complex intermolecular interactions and mesomeric effect occur. To the like weak dipole/dipole, -O-/-O- interactions the dipole/induced dipole interactions, –O-/$\pi$, from -$C_6H_4$- (phenylene) and -CH=$CH_2$ or –CH=CH- (double bonds) and the induced dipole/induced dipole interactions, $\pi/\pi$, from -$C_6H_4$-/-$C_6H_4$-, -$C_6H_4$-/-CH=$CH_2$ or -$C_6H_4$-/-CH=CH- and -CH=$CH_2$/-CH=$CH_2$ or -CH=CH-/-CH=CH- are added. In methyl chavicol the mesomeric effect is extended only on the ether group, -O- and phenylene, -$C_6H_4$- due to the allyl position of the double bond, -CH=$CH_2$ in the molecule (Scheme 2) while in *trans*-anethole the mesomeric effect is extended on the ether group, -O- and phenylene, -$C_6H_4$- as well as on the double bond, –CH=CH- (Scheme 3). The more extended mesomeric effect in *trans*-anethole explains the big difference between the boiling temperatures of the two isomeric compounds, methyl chavicol (216 $^oC$/760 mmHg) and of its more stable isomer, *trans*-anethole (236 $^oC$/760 mmHg).

In order to understand the thermodynamic properties of the mixtures containing (+)-fenchone, methyl chavicol and *trans*-anethole have to discuss the intermolecular interactions in more simple binary systems containing the same functional groups.

*Linear ketones* exhibit fairly large deviations from ideality, e.g. the binary system (nonan-5-one + hexane) has a $G^E$ of the order of 300 J $mol^{-1}$, at 340 K [6]. *Cyclic ketones*, e. g., cyclohexanone, have larger deviations than linear ketones. $G^E$ ($x_1$=0.5) at 298 K of the binary system (cyclohexanone + heptane) is ca. 830 J/mol [7]. Due to additional alkyl groups and to the steric hindrance by three adjacent methyls groups, the binary system (+)-fenchone + n-decane has smaller $G^E$. At ca 370 K, $G^E$ ($x_1$=0.5) is ca. 450 $Jmol^{-1}$ [4].

*Dialkylic ethers* exhibit fairly large deviations from ideality, e.g. the binary system (methyl, 1,1-dimethyl ethyl ether + hexane) has a $G^E$ ($x_1$=0.547) of the order 172 J $mol^{-1}$ at 329 K [8]. *Semi-aromatic ethers*, e.g., methyl phenyl ether (anisole) have larger deviations than *dialkylic ethers*. $G^E$ ($x_1$=0.5) at 353 K of the binary system (methyl phenyl ether + hexane) is ca. 720 J $mol^{-1}$ [9]. In methyl phenyl ether (anisole) to the like weak dipole/dipole, -O-/-O- interactions the dipole/induced dipole interactions, –O-/$\pi$, from -$C_6H_4$- (phenylene) are added. A mixture of *semi-aromatic ether* with *unsaturated terpenoid hydrocarbon* e.g. the binary system (methyl phenyl ether + beta-pinene) has smaller $G^E$. At 393 K, $G^E$ ($x_1$=0.6) is ca. 533 $Jmol^{-1}$ [10]. In beta-pinene the induced dipole/induced dipole interactions, $\pi/\pi$, from >C=$CH_2$/>C=$CH_2$ compensate the methyl phenyl ether's interactions.

*Aromatic* π/π interactions occur between aromatic rings, often in situations where one is relatively electron rich and one is electron poor. There are two general types of π/π interactions: face-to-face and edge-to-face, although a wide variety of intermediate geometries are known [3]. In *aromatic hydrocarbons* the induced dipole/induced dipole interactions, π/π, from -$C_6H_4$-/-$C_6H_4$- show fairly large deviations from ideality, e.g. the binary system (benzene + hexane) has a $G^E$ of the order of 384 J mol$^{-1}$ at 298 K [11].

In *unsaturated hydrocarbons* the induced dipole/induced dipole interactions, π/π, from $H_2C$=CH-/$H_2C$=CH- show very small deviations from ideality, e.g. the binary system (1-hexene + hexane) has a $G^E$ of the order of 25 J mol$^{-1}$ at 328 K [11].

The unlike dipole/induced dipole interactions, –O-/π, from -$C_6H_4$- (phenylene) compensate the induced dipole/induced dipole interactions, π/π, from -$C_6H_4$-/-$C_6H_4$- e.g. the binary system (methyl butyl ether + benzene) has a $G^E$ ($x_1$=0.5) of the order 40 J mol$^{-1}$ at 343 K [11].

The unlike induced dipole/induced dipole interactions, π/π, from $H_2C$=CH-/-$C_6H_4$- compensate the induced dipole/induced dipole interactions, π/π, from -$C_6H_4$-/-$C_6H_4$- e.g. the binary system (1-hexene + benzene) has a $G^E$ ($x_1$=0.5) of the order 246 J mol$^{-1}$ at 298 K [11].

The major contribution to the non-ideality of the mixtures containing (+)-fenchone, methyl chavicol and trans-anethole comes from the like strong dipole/dipole, >CO/>CO interactions in (+)-fenchone and from the like weak dipole/dipole, -O-$C_6H_4$-/-O-$C_6H_4$- in methyl chavicole as well as the like weak dipole/dipole, -O-$C_6H_4$-HC=CH-/-O-$C_6H_4$-HC=CH- in *trans*-anethole.

In mixtures with (+)-fenchone the complex intermolecular interactions from methyl chavicol and *trans*-anethole are compensated by the like dipole/ dipole interactions, >CO/-O-, as well as the dipole/induced dipole interactions, CO/π, from -$C_6H_4$- and -CH=$CH_2$ or –CH=CH- and the like dipole/dipole interactions, >CO/-O-$C_6H_4$-, respectively >CO/-O-$C_6H_4$-HC=CH-and this results in a small deviations from ideality. The binary system methyl chavicol + (+)-fenchone has a $G^E$ ($x_1$=*0.4*) of the order of 40 J mol$^{-1}$ at 380 K (Fig. 2, 3) while the binary system *trans*-anethole + (+)-fenchone has a $G^E$ ($x_1$=*0.4*) of the order of -80 J mol$^{-1}$ at 384 K (Fig.4). Due to higher dipole moment in *trans*-anethole, the like dipole/dipole interactions, >CO/-O-$C_6H_4$-HC=CH- are stronger than the like dipole/dipole interactions, >CO/-O-$C_6H_4$-, in methyl chavicol and this results in negative deviation from ideality in binary system (+)-fenchone + *trans*-anethole.

Due to the hydrocarbon part of the involved molecules, *hydrophobic interactions* could not be negligible. The hydrophobic effect arises mainly from the attractive forces between hydrophobic parts of the molecules. It creates a higher degree of local order, producing a decrease in entropy that

leads to an unfavorable Gibbs energy. The *hydrophobic interactions*, in addition with van der Waals and $\pi/\pi$ attractions, contribute to positive and negative deviations from ideality in the binary system methyl chavicol + (+)-fenchone (Figs. 2, 3) and only negative deviations from ideality in the binary system *trans*-anethole + (+)-fenchone (Fig. 4) and only positive deviations from ideality in the binary system methyl chavicol + *trans*-anethole (Fig. 5).

## CONCLUSION

We have analyzed in terms of intermolecular forces the thermodynamic properties of the mixtures containing (+)-fenchone, methyl chavicol and *trans*-anethole. The chemical structures of the compounds contain the following active functional groups: carbonyl group, >C=O; ether group, -O-; phenylene, $-C_6H_4-$ and double bonds, $-CH=CH_2$ in methyl chavicol or –CH=CH- in *trans*-anethole. The oxygenated functional groups are in mesomeric relationship or are subjected to other electronic effects and steric hindrance effect. The compounds present complex intermolecular interactions. Due to the compensation of the intermolecular interactions the mixtures show quasi-ideal behaviour of the liquid phase and ideal behaviour of the vapor phase. The binary system methyl chavicol + (+)-fenchone shows positive and negative deviations from ideality and the binary system *trans*-anethole + (+)-fenchone shows only negative deviations while the binary system methyl chavicol + *trans*-anethole shows only positive deviations from ideality. The values of the molar excess Gibbs energies, $G^E$ calculated from the isobaric *T-x-y* measurements range from ca. -135 $Jmol^{-1}$ to ca. +190 $Jmol^{-1}$.

## EXPERIMENTAL SECTION

In previous papers [12, 13, 14] we reported experimental vapor-liquid equilibrium data for the following binary and ternary systems: methyl chavicol + (+)-fenchone; *trans*-anethole + (+)-fenchone; methyl chavicol + *trans*-anethole; methyl chavicol + *trans*-anethole + (+)-fenchone.

A series of isobaric *T-x-y* measurements were performed at (4000±30) Pa [12, 13, 14]. Another series of *T-P-x* measurements were performed at three constant liquid-phase compositions [12, 13]. For the binary system (+)-fenchone (1) + methyl chavicol (2) a series of isobaric *T-x-y* measurements were performed at (4270±30) Pa [12].

VLE data in binary systems were found to be thermodynamically consistent as tested by using the maximum likelihood multimodel fitting method described by Panaitescu [15]. The standard deviations for pressure, temperature and liquid and vapor phase compositions were set to $\sigma_P$ = 60 Pa, $\sigma_T$ = 0.1 K, $\sigma_x$ = 0.003 mol. fr. and $\sigma_y$ = 0.003 mol. fr., respectively. According to this test

the isobaric *T-x-y* measurements are considered consistent if the values of the statistic criterion of selection of the each experimental point (*Ro*) and of the all experimental points (*global Ro*) are less than 2.45. At (4000±30) Pa the values of the (*global Ro*) criterions and of the all values of the (*Ro*) criterions are less than 2.45.

The thermodynamic consistency of the isobaric *T-x-y* measurements in the ternary system was checked using the McDermott-Ellis method [16] modified by Wisniak and Tamir [17]. According to these references two experimental points *a* and *b* are considered thermodynamically consistent if the local deviation, *D* is less than maximum deviation, $D_{max}$. For all the experimental points reported, *D* never exceeded 0.156 while the smallest value of $D_{max}$ was 0.500.

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# PROTEIN CHARACTERIZATION OF ROMANIAN BUFFALO MILK COMPARED TO COW MILK

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**ABSTRACT**. Buffaloes have been studied in relation to the exclusive use of their milk for the manufacture of high-quality dairy products. Despite cow milk proteins having been extensively studied, there is still a substantial lack of characterisation on proteins fractions from Romanian buffalo milk. We report here a detailed protein analysis of buffalo skim milk, whey and whole milk protein fractions in comparison with values gathered from cow milk protein analysis. Major protein components, i.e. β- caseins, k-caseins, α-lactalbumin and β-lactoglobulin, were characterized through HPLC with the subsequent amino acids (GS) providing a scientific basis to coagulation/cheese making processes used in dairy productions. The mean data collected from the samples of buffalo milk were compared using the Origin 8.5 program, ANOVA test. The Romanian buffalo milk proved to be a high value raw material for further processing into dairy products, with more essential amino acids than cow milk. It is further suggested that buffalo milk should be considered as functional food through protein input in human diet.

***Keywords:*** *buffalo, proteins*, *k-caseins, β- caseins, amino acids.*

## INTRODUCTION

Milk is the most important food for young mammals and a common source of proteins and microelements for adults. Its main components, namely caseins, lactoglobulins, have specific biological functions that have been already reviewed [1]. The knowledge of protein chemistry has been used by the dairy manufacturing industry to develop/optimize more modern technologies [2] and also it has been indispensable for detection of adulteration in raw materials used for high-quality products [3]. Despite cow milk proteins having been extensively studied in a number of studies [4,5], there still is a substantial lack of characterization on the proteins from Romanian buffalo. The Romanian

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buffalo breed stems from the Common European water buffalo, Mediterranean type. Because of its economic value in our country it has been studied in regard to protein polymorphism detection [6] but a full proteomic evaluation in regard to cow milk has not yet been established. The main goal of this work was to obtain new data on the quali-quantitative composition of the major proteins and amino acids in Romanian water buffalo milk, useful for animal selection purposes.

The field literature has established that the protein content of buffalo milk is higher than in cow milk, with 80% of total proteins being caseins and whey proteins being higher in colostrum [7]. Almost all casein of buffalo milk is present in the micelles form (90–95% in cow milk) [8]. The size of the micelle in buffalo milk ranges from 80–250 nm with the majority being 110–160 nm compared to 70–110 nm in cow milk [9]. The voluminosity of the buffalo casein micelle is 2.7–3.7 ml/g in the temperature range of 25–37° C [10]. Solvation of the casein micelle as calculated from voluminosity is 2.6–2.9 g water/g casein.

## RESULTS AND DISCUSSION

The results obtained after analyzing the mean concentration of proteins in buffalo milk were higher (Table1) than the one stated in the field literature [11]. These differences can be explained through the fact that in the determination the skimmed milk was used and not the whole one like in the literature mentioned data.

**Table 1.** Total protein concentration (mg/ml milk) in skimmed cow and buffalo milk

| Samples | Absorbtion at λmax = 595nm | Total proteins (mg/ml skimmed milk) |
|---|---|---|
| *Skimmed cow milk* | 0,5932 | 28,97 |
| | 0,6269 | 36,50 |
| | 0,7084 | 54,71 |
| **Average – cow milk** | | **40,06** |
| *Skimmed buffalo milk* | 0,6988 | 52,61 |
| | 0,700 | 52,88 |
| | 0,7409 | 62,03 |
| **Average – buffalo milk** | | **55,84** |

The protein concentration in lactoserum was higher in buffalo milk (14.13 mg/ml) than the one found in cow milk (11.79 mg/ml). Also the casein profile and soluble proteins varies according to the specie. The lowest content of casein was found in cow milk, while the highest in buffalo milk (Figure1). At other buffalo species like the African buffalo [12] the values obtained were lower than in our study.

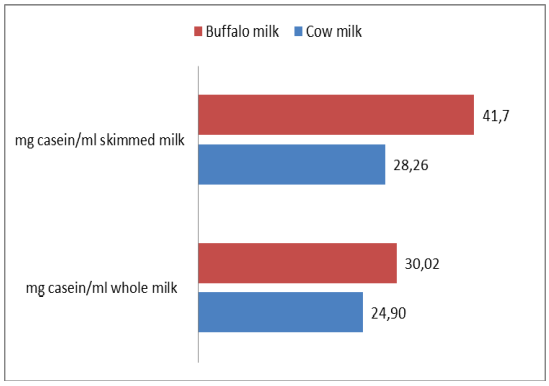


**Figure 1.** Casein concentration (mg/ml) in whole and skimmed cow and buffalo milk

After the total protein content was established the protein fractions were evaluated through HPLC separation both from skimmed and whole milk (Figure 2).

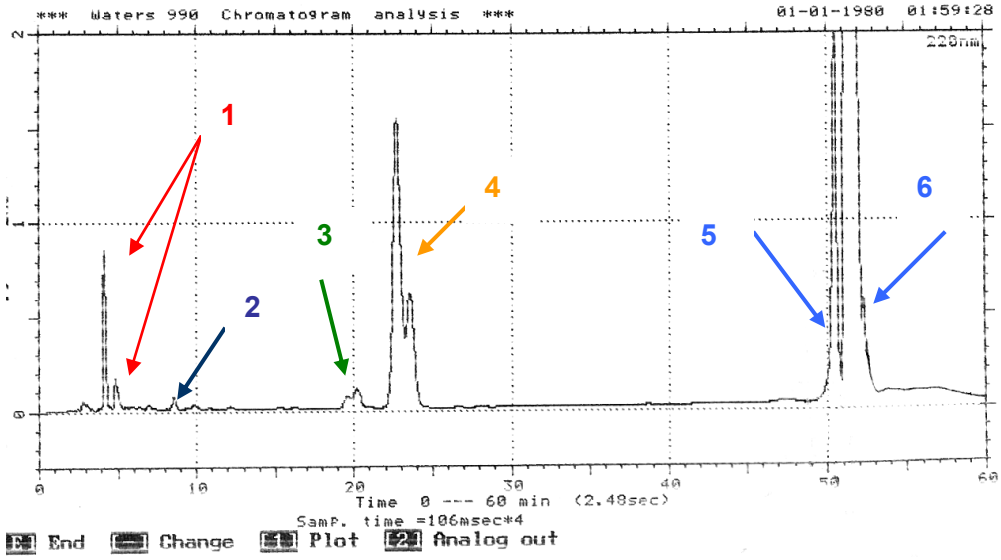


**Figure 2**. Identification of separated fractions from whole milk:1 - k cazeine; 2 - αs2 cazeine; 3 - αs1 cazeine; 4 - β cazeine; 5-αlactalbumin; 6 - β lactoglobuline

Recent proteomic studies are concerned on the polymorphisms of these β or k caseins, lactalbumins given the fact that it influences their quantity and product quality.

The buffalo k casein is similar to the cow's k casein and has 7 main fractions of which k4 and k5 show two more (a and b) and k7 four more (a, b, c and d). Fraction k1 represents respectively 40% of the total k-casein in buffalo and is very similar to kB1-casein in the cow, where it accounts for only 25% of total k-casein [13]. The amino acid composition of the k-casein of the two species differs in the quantity (mole/mole protein) of N-acetylgalactosamine (0–4.3 and 0–6.7 respectively in buffalo and cow) and sialic acid (5.5–8.5 and 3.5–4.3 in buffalo and cow).

As casein is constituted by $\alpha_{s1}$ and $\alpha_{s2}$ fraction, $\alpha_{s1}$ casein we found out that it does not differ much in the two species and it consists of fractions $\alpha_{s0}$ [14], $\alpha_{s1}$-II and $\alpha_{s1}$-I which are differentiated respectively by the presence of eight, seven and six phosphate groups. Buffalo's β casein is similar to that of the cow and has two variants. Of these, A has been found only in Venezuelan buffalo and differs from B in three amino acids [15]. The two variants A and B closely resemble cow's βA2 casein, differing from the latter by four and five amino acids respectively. The β and $\alpha_{s1}$ casein fractions make up 70% of the micelle network of proteins.

In figure 3 there are revealed the chromatograms obtained in the separation of the amino acids (two different extractions), being also revealed the retention time. The quantitative analysis results are shown in table 2, these representing the average of 10 parallel determinations.

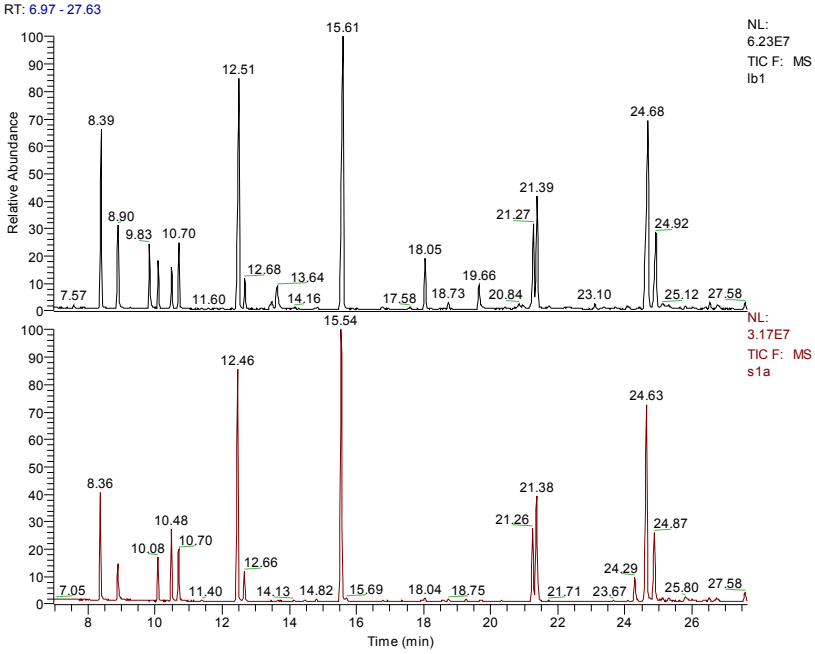


**Figure 3.** The comparative chromatograms of amino acids' separation in two cow milk samples obtained by two different extraction protocols

In the case of buffalo milk there was a very similar amino acids profile to the one revealed in the cow milk samples. The quantitative numbers are shown in table 2. Also, in figure 4 there is a representative chromatogram obtained through gas-chromatographic separation of the amino acids in buffalo milk.

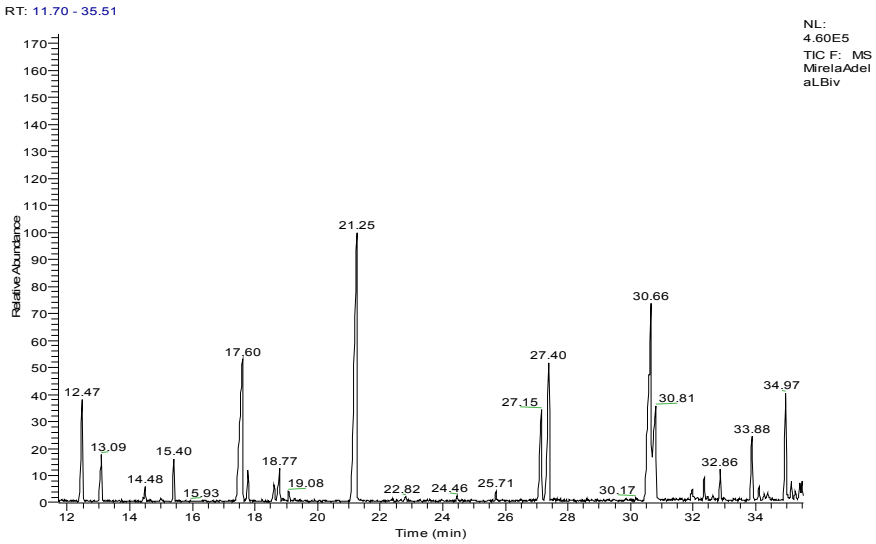


**Figure 4.** Representative chromatogram of gas-chromatographic separation of the amino acids in buffalo milk

There are high variations in the amino acids profile at these species. Some amino acids like cysteine, aspartic acid, arginine, histidine have been high lightened only in cow milk, whereas ornithine has been revealed in significant quantities only in buffalo milk, varying from 20.8 – 27.6 g%. significant differences have been shown in the quantity aspect also at other amino acids: alanine, glycol, glutamic acid and proline are found in two or even three times higher amounts than in cow milk; tyrosine, methionine, isoleucine, serine are found in lower quantities in buffalo milk.

Analyzing the amino acids composition in the protein fractions investigated, αS-casein, β-casein, K-casein, we revealed a high amount of glutamic acid in the β- casein fraction (Table 3) and also in K-casein fraction of buffalo milk. These results were in contrast with Addeo et al. 1977 and Ganguli, 1976 which obtained lower amounts. Given the fact that these studies were made on Murrah buffalo milk we consider that the results obtained serve as data base for Romanian buffalo with these particularities in the amino acids composition. Also the some of the essential amino acids for human nutrition were found in high amounts, like leucine and phenylalanine in β-casein and threonine in K – casein (table 3).

**Table 2.** Amino acids content in cow and buffalo milk

| AMINO ACIDS | Average quantity (g/100g) | | SEM | ANOVA |
|---|---|---|---|---|
| | Cow milk | Buffalo milk | | |
| Alanine | 7,58 | 14, 84 | 6.23 | *** |
| Glycol | 2,05 | 7, 34 | 3.2 | *** |
| Serine | 3,84 | 3, 00 | 0.34 | * |
| Valine | 5,11 | 5, 99 | 0.21 | NS |
| Leucine | 20,29 | 23, 68 | 1.09 | * |
| Isoleucine | 2,20 | 1, 83 | 0.56 | ** |
| Cysteine | 6,81 | - | - | - |
| Proline | 25,55 | 47, 86 | 5.4 | *** |
| Methionine | 1,13 | 0,56 | 0.44 | * |
| Aspartic acid | 3,39 | - | - | - |
| Phenylalanine | 5,65 | 12, 52 | 4.34 | *** |
| Tyrosine | 5,32 | 0,56 | 3.20 | *** |
| Glutamic Acid | 19,88 | 51, 64 | 5.32 | *** |
| Lysine | 9,59 | 23, 79 | 6.3 | ** |
| Arginine | 7,62 | - | - | - |
| Histidine | 4,45 | - | - | - |
| Ornithine | - | 25, 99 | - | - |

Significance, NS, not significant P>0,5; * P< 0,5; ** P<0.01; *** P<0.001; Data is presented as least square mean. SEM, standard error of the mean.

**Table 3.** Average amino acids composition in αS-casein, β-casein, K-casein of buffalo and cow milk

| Amino acid | αS-casein | | β-casein | | k-casein | |
|---|---|---|---|---|---|---|
| | Buffalo | Cow | Buffalo | Cow | Buffalo | Cow |
| Aspartic acid | 7.7 | 7.5 | 4.4 | 4.8 | 7.8 | 7.1 |
| Tyrosine | 5.8 | 6.2 | 6.5 | 7.6 | 7.8 | 7.2 |
| Serine | 6.1 | 6.5 | 6.9 | 7.4 | 5.7 | 6.4 |
| Glutamic acid | 22.7 | 22.5 | 30.6 | 26.8 | 21.2 | 18.2 |
| Proline | 7.9 | 8.4 | 15.9 | 15.8 | 10.1 | 8.6 |
| Glycine | 2.8 | 2.9 | 1.6 | 2.7 | 0.3 | 1.5 |
| Alanine | 3.1 | 3.3 | 1.7 | 1.8 | 5.8 | 5.5 |
| *Valine | 5.2 | 4.9 | 8.7 | 10 | 7.5 | 5.2 |
| *Methionine | 2.3 | 3.1 | 3.6 | 3.2 | 1.5 | 0.8 |
| *Isoleucine | 5.8 | 6.4 | 5 | 5.5 | 7.4 | 6.8 |
| *Leucine | 8.9 | 8.7 | 12.6 | 10.7 | 5.9 | 6 |
| *Threonine | 2.3 | 3.2 | 3.6 | 5.6 | 10.2 | 5.9 |
| *Phenylalanine | 5.4 | 5 | 6 | 5.3 | 3.3 | 4.1 |
| *Lysine | 8.6 | 9 | 6.7 | 6.5 | 5.7 | 5.8 |
| *Tryptophan | 2.2 | 2 | 0.5 | 0.6 | 1.4 | 1 |

* Essential amino acids in human nutrition

## CONCLUSIONS

Buffalo milk is an important source of proteins with higher levels of amino acids like alanine, glycol, glutamic acid and proline which are found in two or even three times higher amounts than in cow milk. The Romanian buffalo should be exploited better, even on an international level, given the fact that this particular breed produces a high quality product from the protein content point of view. In regard to human nutrition buffalo milk should be considered as functional food through protein input in human diet.

## EXPERIMENTAL SECTION

### Samples

The samples were gathered from a semi–subsistence buffalo dairy farm situated in north Transylvania, and a dairy farm situated in the same region. Each month 10 samples were collected from buffaloes and cows fed on the same dietary regiment, including green forages (constitutes 70% of dietary intake during spring and summer) and a mixture of crop residues.

### The analysis of milk proteins

Casein concentrations were revealed the volumetric method was made in accordance to the protocol initiated by Ciurdaru et al. (2001) [18], the etalon curve and the protein dosage according the Bradford method [19], and their separation using the HPLC method. The separated proteins were identified using the protocol described by Miranda et al. (2004) [20]. The only alteration made in our protocol was the type of the column used: the INRA research group from Paris used the Vydac column type C4, while we used the Vydac column type C8, which explains the slightly delay (2-3 minutes) of the retention time.

Another protocol used for casein identification was the one presented by Morra-Gutierrez et al. (1991) [21] and applied by us in accordance. The protein identification from the whey was done according to Trujillo et al. (1999) [22] and Moatsou et al. (2005) [23] who described it on goat and ewe milk, both using the same technique, RP-HPLC, their final isolation using a high tech method – mass spectrometry.

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# DFT VIBRATIONAL ANALYSIS OF METAL-HYDROPEROXO BLEOMYCIN COMPLEXES

**RADU SILAGHI-DUMITRESCU***

**ABSTRACT.** As part of our current efforts to understand the mechanisms of oxygen-oxygen bond cleavage in biological metal complexes, density functional theory (DFT) calculations are employed to calculate the vibrational spectra of the Fe(III)-hydroperoxo adduct of bleomycin and of its more stable cognate Co(III)-hydroperoxo, as models for the ABLM form of bleomycin thought to be key in its biological activity. In addition to bleomycin, we examine another Fe(III)-hydroperoxo complex, $[Fe(III)(N4Py)(OOH)]^{2+}$. The theoretical predictions show errors of 20-100 $cm^{-1}$ with respect to experiment, and reproduce some of the experimental trends, with the important observation that the vibrational modes are far more complex than usually assumed.

***Key words**: bleomycin, cobalt, iron, DFT, vibrational spectrum*

## INTRODUCTION

Bleomycin is a drug whose action involves chelating a metal center and then damaging DNA within living cells. A key intermediate in bleomycin's anti-DNA action is a species known as activated bleomycin, ABLM, which, on the basis of spectroscopic and theoretical studies, appears well described as a bleomycin-ferric-hydroperoxo adduct (cf. Figure 1). ABLM's instability has to some extent precluded detailed structural characterization.[1-3] A somewhat more stable cognate, the Co(III)-hydroperoxo bleomycin adduct, has been characterized spectroscopically, and its inferred structural features appear very similar to those of ABLM.[4-12]

The reactivity of the Fe-O-OH moiety is central to bleomycin's therapeutic activity. Arguably, the most direct spectroscopic probe available to date for bonding in metal-hydroperoxo bleomycin adducts is vibrational spectroscopy. Although vibrational data is not available for the Fe-O-OH modes in ABLM, a detailed vibrational analysis of Co(III)-hydroperoxo bleomycin has recently been reported.[5,6] Here, we attempt to employ density functional (DFT) calculations to calculate the vibrational spectra of Fe(III)-hydroperoxo and Co(III)-hydroperoxo bleomycin. In addition to bleomycin, we examine an

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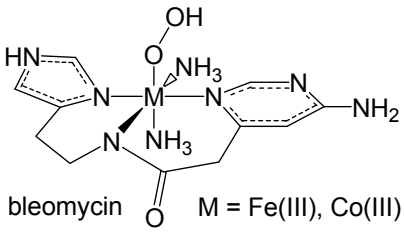
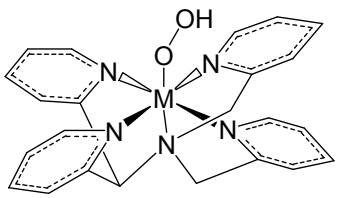
Fe(III)-hydroperoxo complex for which vibrational data *is* available, namely, $[Fe(III)(N4Py)(OOH)]^{2+}$.[13,14] The theoretical predictions show errors of 20-100 $cm^{-1}$ with respect to experiment, and reproduce some of the experimental trends, with the observation that the vibrational modes are far more complex than usually assumed.

## RESULTS AND DISCUSSION

Figure 1 shows the models employed in the present work. Table 1 lists key calculated geometrical parameters for the three models of Figure 1. These parameters are in good agreement with previously reported data on related iron-hydroperoxo species.[3,13-22]




$[Fe(III)(N4Py)(OOH)]^{2+}$

**Figure 1.** Models employed in the present work. The two ammonia ligands in the bleomycin structure are modelling terminal amine groups otherwise connected to the rest of the macro-chelate unit.

Table 2 lists calculated vibrational modes for the Co-OOH moiety. At the outset, we note that the $\nu_{Co\text{-}OOH}$ and $\nu_{O\text{-}OH}$ modes are far from pure: they are mixed with each other as well as with several other metal-nitrogen and bleomycin-based modes, such that several calculated vibrational modes have consistent $\nu_{Co\text{-}OOH}$ and $\nu_{O\text{-}OH}$ character. Resonance Raman experiments on Co(III)-OOH bleomycin as well as on various Fe(III)-OOH complexes have consistently identified *one* single experimentally detectable vibrational mode for $\nu_{Co\text{-}OOH}$ and *one* for $\nu_{O\text{-}OH}$.[3,6,10,13,14,23] The calculated frequencies are within 20-90 wavenumbers from the experimental data.

**Table 1.** Key calculated bond lengths (Å) and angles for the three models of Figure 1.

| | | | |
|---|---|---|---|
| **Co(III)-OOH bleomycin** | | | |
| Co-O | 1.90 | Co-N(amide)[b] | 1.96 |
| O-OH | 1.50 | Co-N(imid)[c] | 2.00 |
| Co-O-OH | 112º | Co-N(py)[d] | 1.99 |
| Co-$NH_3$(cis)[a] | 2.04 | Co-N(trans)[a] | 2.04 |
| **Fe(III)-OOH bleomycin** | | | |
| Fe-O | 1.80 | Fe-N(amide)[b] | 1.96 |
| O-OH | 1.53 | Fe-N(imid)[c] | 2.03 |
| Fe-O-OH | 113º | Fe-N(py)[d] | 2.03 |
| Fe-$NH_3$(cis)[a] | 2.09 | Fe-N(trans)[a] | 2.09 |
| **[Fe(III)(N4Py)(OOH)]$^{2+}$** | | | |
| Fe-O | 1.79 | Fe-N(axial) | 2.03 |
| O-OH | 1.48 | Fe-N(py)[d] | 2.00 |
| Fe-O-OH | 120º | | |

[a] positions cis and trans relative to the OOH ligand. [b] amide nitrogen atom.
[c] imidazole nitrogen atom. [d] pyridine/pyrimidine nitrogen atoms (for [Fe(III)(N4Py)(OOH)]$^{2+}$, the average of four distances is shown).

**Table 2.** Calculated vibrational modes for the bleomycin Co(III)-OOH model. Shown in bold is previously reported experimental data.[6,10]

| vibration | Co(II)-OOH |
|---|---|
| $\nu_{Co-OOH}$ | 517, 520 / **548** |
| $\nu_{O-OH}$ | 744, 748, 750, 787 / **828** |
| $\nu_{OO-H}$ | 3579 |

Table 3 lists calculated vibrational modes for the Fe-OOH moiety in the bleomycin model. Similar to the cobalt case discussed above, the $\nu_{O-OH}$ mode is far from pure, contrary to previous B3LYP results.[3] Table 4 lists vibrations calculated for the related complex, [Fe(III)(N4Py)(OOH)]$^{2+}$, for which experimental data exists.[14]

**Table 3.** Calculated vibrational modes for the bleomycin Fe(III)-OOH model. Shown in bold is previously reported theoretical data.[3]

| vibration | Fe(III)-OOH |
|---|---|
| $\nu_{Fe-OOH}$ | 465 / **575** |
| $\nu_{O-OH}$ | 647, 664, 672, 737 / **879** |
| $\nu_{OO-H}$ | 3568 |

A comparison of the O-OH stretching frequencies in the three models reveals that they do not correlate with the calculated bond length: the order is Co-OOH > Fe-OOH(bleomycin) > Fe-OOH(N4Py) for the calculated frequencies and Fe-OOH > Co-OOH(bleomycin) > Fe-OOH(N4Py) for the calculated O-O bond lengths. On the other hand, the experimentally-determined frequencies are in the order Co-OOH > Fe-OOH(bleomycin) > Fe-OOH(N4Py), in good agreement with experiment. A similarly good agreement with experiment is found when comparing the metal-oxygen(OOH) stretching frequency, with Fe-OOH(N4Py) > Co-OOH. This order correlates well with the distinctly shorter calculated metal-oxygen bond in the iron model compared to the cobalt.

**Table 4.** Calculated vibrational modes for the $[Fe(III)(N4Py)(OOH)]^{2+}$ model. Shown in bold is previously reported experimental data.[14]

| vibration | Fe(III)-OOH |
|---|---|
| $\nu_{Fe-OOH}$ | 576 / **632** |
| $\nu_{O-OH}$ | 638, 688, 700, 707 / **790** |
| $\nu_{OO-H}$ | 3546 |

## CONCLUSIONS

In models of activated bleomycin and in related structures, the theoretical predictions on vibrational spectra show errors of 20-100 $cm^{-1}$ with respect to experiment in terms of peroxide-related vibrations, and reproduce some of the experimental trends; however, the vibrational modes are far more complex than usually assumed.

## EXPERIMENTAL SECTION

Geometries were optimized and vibrational spectra were computed using the BP86 functional. This functional employs the gradient-corrected exchange functional proposed by Becke,[24] and the correlation functional by Perdew.[25] The 6-31G** basis set was used, as implemented in Spartan. [26] For the SCF calculations, a fine grid was used, and the convergence criteria were set to $10^{-6}$ (for the root mean square of electron density) and $10^{-8}$ (energy), respectively. For geometry optimization, convergence criteria were set to 0.001 a.u. (maximum gradient criterion) and 0.0003 (maximum displacement criterion).

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