„BABEŞ-BOLYAI” UNIVERSITY, CLUJ-NAPOCA
Faculty of Chemistry and Chemical Engineering

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IDENTIFICATION OF SPECIFIC MARKERS FOR WATER SAMPLES AND VEGETABLES BY USING MASS SPECTROMETRY AND CHEMOMETRIC METHODS

PhD Thesis Summary

Scientific advisor
Prof. Emerit Dr. Ioan Oprean

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Introduction

In summary of PhD thesis are presented briefly a aprt of original results of experimental research, general conclusions and selected references. As

În rezumatul tezei de doctorat se prezintă pe scurt o parte din rezultatele originale a cercetărilor experimentale, concluziile generale şi bibliografia selectivă. As novelty, the thesis brings relevant contributions to both the surface water field, through characterization from the organic compounds and elemental content point of view, as well as for food domain, by identifying specific markers that can be used to select the growth regime (organic vs. conventional) or growth conditions (greenhouse vs green). For large obtained data sets, statistical methods have been used, namely: variance analysis (ANOVA), cluster analysis (CA), principal component analysis (PCA) and linear discriminant analysis (LDA).

The PhD thesis titled ***"Identification of Specific Markers for Water and Plant Tests Using Mass Spectrometry and Chemometric Methods"*** is structured in two parts: **Part I** - *Theoretical Considerations* and **Part II** - *Original Contributions*.

Part I, *Theoretical Consideration*, comprises 4 chapters:

Chapter 1, *Surface water pollution*, presents information about surface water pollution, the main sources of pollution, and mentions the main classes of organic and metallic pollutants. The sampling methods and main preparation techniques for surface water are also described.

Chapter 2, *The importance of authentication of vegetables by determining isotopic and elemental content,* presents issues related to the need for isotopic and elemental authentication, for both consumers and producers. The preparation methods of vegetables, for the isotopic and elemental analyzes, are briefly discussed.

Chapter 3, *Applied analytical techniques for the determination of organic compounds, isotopic and elemental content*, describes the main analytical methods used in the experimental part of this paper, for the identification of organic pollutants in surface waters and for determination of the isotopic and elemental content in the vegetables.

Chapter 4, *Chemometric methods applied in analytical data processing obtained from the analysis of various matrices*, sums up some of the chemometric methods used for a more efficient interpretation of the data. There are also some applications where chemometric methods have been proven their effectiveness.

Part II, *Original Contributions*, is structured in 5 chapters:

Chapter 5, *Researches on the spatial and temporal assessment of organic pollutants in surface waters in Transylvania using mass spectrometry and multivariate statistical techniques*, presents a screening study of the main rivers in Transylvania in terms of the organic pollutants content. The statistical methods used have been able to group the sampling points according to the organic load, and principal components analysis has highlighted the sources of pollution associated with each compound.

Chapter 6, *Development of extraction method and analysis using gas chromatography coupled with mass spectrometry (GC/MS) for isolate flame retardands from aqueous samples,* presents an optimized and validated method for the concentration of flame retardants from aqueous samples, followed by analysis using the GC/MS technique.

Chapter 7, *Researches regarding the spatial and temporal evaluation of metals in surface water from Transylvania using inductive coupled plasma mass spectrometry and multivariate statistical techniques,* presents a screening study of the main rivers in Transylvania from the elemental content point of view. As with organic pollutants, cluster analysis was used to group sampling points and principal component analysis was used in order to identify pollution sources.

Chapter 8, *Researches regarding isotopic and elemental markers identification for characterisation of organic and conventional vegetables,* presents the classifications of vegetables according to the growing regime (organic vs conventional), the growth conditions (greenhouse vs field) or the place of edible parts (underground vs. above ground).

Chapter 9, *Researches regarding isotopic and elemental markers identification for geographical origin and agricultural practices discrimination of carrots*, is a study that has been conducted on a larger set of carrot samples, in order to remove the influence of species variation. The main isotopic and elemental markers have been identified, and can differentiate organic vegetables from conventional ones, or those who can geographically differentiate carrots. A differentiation of samples from the Transylvanian region was also achieved, with the highlighting of the main markers.

Chapter 10, *General Conclusions*, summarizes the results presented in the paper, the general conclusions and a series of recommendations.

The main objective of this paper was to identify specific markers for surface and vegetal water samples using mass spectrometry (GC/MS, ICP-MS and IRMS) and chemometric methods (ANOVA, CA, PCA, LDA) thus providing both clear and less observable information by simply analyzing experimental data, but which may be essential. To demonstrate the versatile character of this combination of techniques, surface waters and food (vegetables) were studied as complex matrices, both beeing of particular importance to the health of the population. A special focus on this work was given to the samples collected from the Transylvanian area, for both matrices. To achieve the main objective, the following specific objectives have been reached:

- Analysis of water samples using GC/MS and ICP-MS techniques.
- The chemometric processing of the experimental results by identifying the specific pollutants for each basin and the sources of pollution.
- Development, optimization and validation of the SPE extraction method for determination of flame retardants in water samples.
- Analysis of vegetable samples in terms of isotopic and elemental content
- Chemometric processing of experimental results by identifying specific markers for organic (conventional vs. conventional) and growing conditions (greenhouse vs. field).

Chapter 1.

Researches regarding the spatial and temporal evaluation of organic pollutants from surface waters from Transylvania using mass spectrometry and multivariate statistical methods

Monitoring programs include frequent sampling from various points, followed by determination of parameters that usually have the highest variability. As a consequence, monitoring studies generate very large and complex databases that are difficult to interpret. In order to explore the information included in this data, different chemometric methods can be applied. These techniques allow the interpretation of the obtained analytical data, the reduction of the data set, but without losing information.

This chapter aims to characterize the rivers in Transylvania (Olt, Mureş and Cibin) from organic pollutants content point of view. GC/MS coupled technique was used to accomplish this goal and the results were efficiently processed using chemometric techniques in order to assess the effect of seasonal variations on the concentrations of organic compounds and to extract the parameters that are representative of each basin.

1.1. Surface water sampling

Two sampling sessions were conducted, corresponding to the rainy season (October) and to dry season (July). There were nine sampling points along the three rivers as follows: two points on the Cibin River (Cristian and downstream of Sibiu), three points on the Olt River (Bradu, Fagaras and Voila-aval Fagaras) and four points on the Mures River (Târgu-Mureş, Cipău-aval Târgu-Mureş, Cuci, Luduş).

1.2. The concentration of organic pollutants using solid phase extraction (SPE) and chromatographic analysis coupled with mass spectrometry (GC/MS)

A volume of 500 mL of water sample were used to prepare the samples, which were filtered using the vacuum system and Teflon filters with a diameter of 47 mm. The samples were then extracted using solid phase extraction with OASIS HLB cartridges (hydrophilic lipophilic balance, 3 ml, 60 mg). The eluents were transferred to a conical flask and evaporated with a rotary evaporator. The samples were reconstituted by adding one mL of isooctane, and then 2 μL were injected into the GC/MS system. All samples were prepared in duplicate. In parallel, a blank sample was extracted to eliminate potential interference from sample manipulation or from the SPE procedure. No significant interferences were observed.

The samples were analyzed using a GC/MS method presented in another paper (Moldovan, 2006). For the quantitative assessment (Figure 1), the internal standard method was used. The internal standard used was PCB-30, 1000 ng·L^{-1} concentration in heptane. GC/MS analysis was performed by electron impact ionization (EI) at 70 eV. The temperatures of the ion source and injector were 250 ° C. A capillary column DB-5MS, 30 m × 0.25 mm i.d. and the transfer line between the chromatograph and the spectrometer had a temperature of 300 °C. The temperature program of the chromatograph was as follows: the initial temperature of 90 °C for 1 minute increased by 10 °/min to 120 °C, then increased again by 4 °/min to 300 °C. These temperature gradients are necessary for a good separation of the compounds and to eliminate coelutions. The carrier gas was helium at a flow rate of 1.5 mL·min^{-1}. The quantitative analysis was made by comparing the mass spectra obtained with NIST library literature or those reported in the literature. The quantitative assessment was made by correlating each area with that of the internal standard.

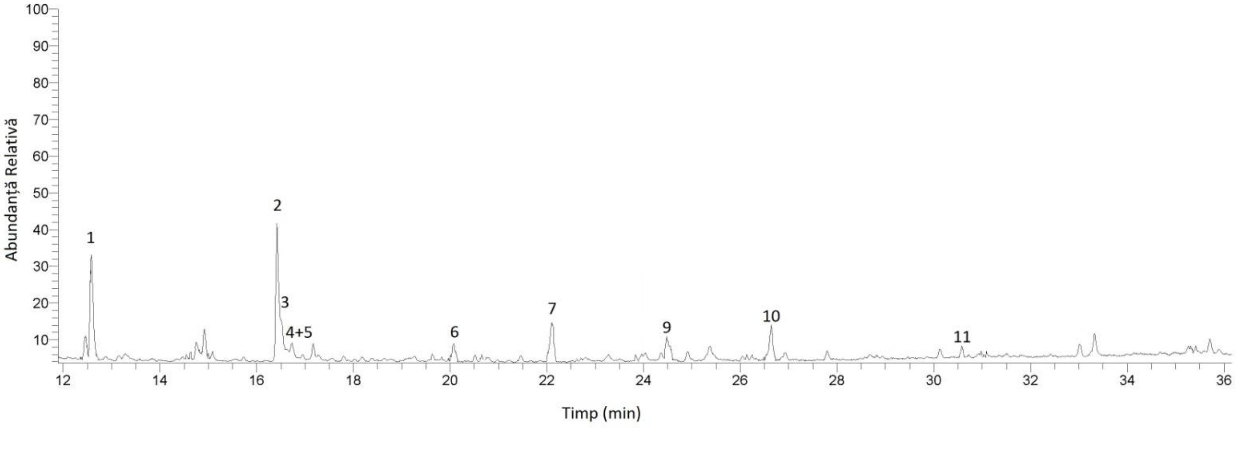


Figure 1. *The obtained chromatogram for water sample collected from Cibin River in rainy season.Compounds are: (1)DBP, (2)BZP, (3)AMI, (4)DNTP, (5)MHJ, (6)DBNP, (7)GALA, (8)TONA, (9)BHA, (10)TPP, (11)EHMC*

1.3. Chemometric interpretation

ANOVA analysis (5% confidence interval) was applied to highlight compounds whose concentrations were influenced by the increased flow rate from the rainy season. Only two compounds were influenced by the increase flow from the rainy season ($p < 0.05$), namely: MHJ ($p = 0.043$) and BZP ($p = 0.001$). Another goal for which ANOVA has been applied was to identify pollutants that can differentiate the three rivers. DBNP ($p = 0.016$) and BHA ($p = 0.004$) showed higher values in the Olt river than in Mureş. Cibin, could not be distinguished from the other two rivers by any organic compound. These comparisons between the three rivers were made using Tuckey post hoc tests. The dendrogram shown in Figure 2 was obtained using the Ward's method of clustering with the Euclidian distance as a measure of similarity between clusters.

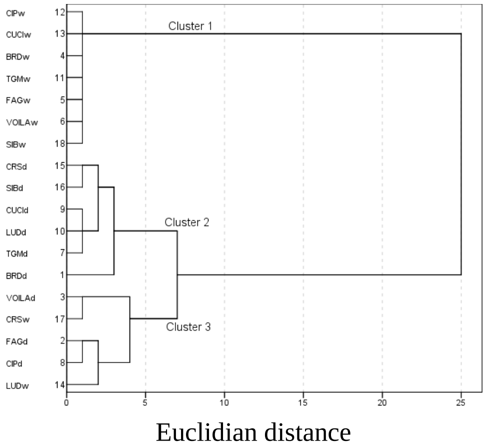


Figure 2. *Sampling points dendrogram according to organic pollutants from water samples*

Sampling points belonging to the same cluster have similar characteristics. This means that for a quick screening it is not necessary to analyze all cluster samples, because a single sample can be representative for entire cluster. This sampling point is a reference to the spatial assessment of water quality. Cluster 1 grouped the CIPw, CUCIw, TGMw, BRDw, FAGw, VOILAw, SIBw sampling sites and these are the least polluted places due to the increased flow of the river in the rainy season. Cluster 2 comprises the most polluted sampling sites of all and all of these samples were collected during the dry season. These sites are: CRSd, SIBd, CUCid, LUDd, TGMd, BRDd. Cluster 3 grouped VOILAd, CRSw, CIPd, FAGd and LUDd, and these points correspond to sites with moderate pollution. The most significant contribution to this high degree of pollution is represented by direct discharges into natural waters, domestic sewage, or agricultural land washing. Another significant cause for the high pollution level is the under-dimensioning and the age of waste water treatment plants from small towns such as Voila (VOILAd) and Fagaras (FAGd). The results obtained from the CA analysis revealed the most polluted sampling sites for organic compounds. Pollutants with the highest degree of discrimination were highlighted by applying the ANOVA analysis using the variable obtained as a result of CA analysis as a independent variable. Only two components were statistically significant, namely BZP ($p = 0.01$) and AMI ($p = 0.058$), even if the value was greater than 0.05 was considered for interpretation. A future monitoring program can be optimized based on these results because samples can only be collected from significant points, thus reducing sampling, time analysis and costs. Similar results have been obtained in previous studies reported in the literature by other researchers when CA was used to group outpoints on a Argentine river (Potrero de los Funes). The procedure used generated three clusters that grouped the points sampling with the same characteristics and with the same sources of pollution. PCA was applied on a matrix containing the concentrations of each analyzed organic compound, from both sampling sessions, at each sampling point. The significant parameters that were used for this method were: the extraction method used was principal components, and only components with their eigenvalue greater than 1 were retained. The rotation matrix was Varimax method with Kaiser normalization. After PCA analysis, five main components were obtained, sumarising 87.3% of the total variation of the data set. The graphical representation of each principal component relative to its eigenvalue is shown in **Figure 3**.

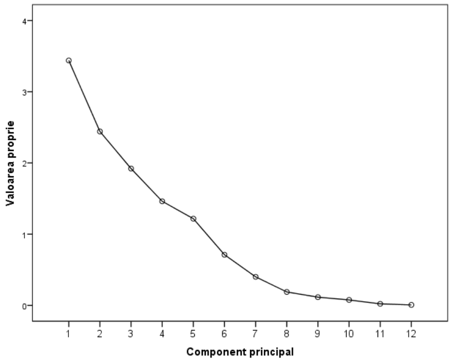


Figure 3. *Representation of eigenvalues for every principal component (scree plot)*

According to **Table 1**, the highest loadings from principal component 1 are given by DBP, TPP, AMI, GALA and TONA. This PC contains the largest number of representative parameters and can indicate the effect of direct discharges of wastewater effluents, mainly from industry or without any preliminary treatment.

Table 1. *Loadings of organic contaminants after Varimax Rotation (five components extracted) for water samples collected from 18 sampling points (values >0.5 are bold)*

Organic parameter	Principal Component 1	principal component 2	Principal component 3	Principal component 4	Principal component 5
DBP	**0.788**	0.317	0.008	0.351	-0.022
DNTP	-0.046	**0.919**	-0.235	0.037	-0.099
DBNP	-0.028	**0.859**	0.187	-0.092	0.373
BHA	-0.036	0.105	0.062	0.110	**0.978**
TPP	**0.916**	0.192	0.050	-0.171	-0.211
MHJ	-0.023	-0.099	0.118	**0.935**	0.157
AMI	**0.551**	-0.286	-0.143	0.145	0.005
GALA	**0.945**	-0.145	-0.103	-0.041	0.092
TONA	**0.721**	-0.153	-0.175	-0.444	0.022
BZP	0.043	**0.559**	0.244	**0.583**	-0.451
PRM	-0.102	-0.080	**0.909**	0.262	-0.123
EHMC	-0.105	0.050	**0.958**	-0.018	0.172

Eigenvalue	3.44	2.44	1.92	1.46	1.22
Explained variance (%)	28.7	20.3	16.0	12.2	10.1
Cumulative variance (%)	28.7	49.0	65.0	77.2	87.3

Principal component 2 and 3 has loadings of DNTP, BAYER, BZP and PRM and EHMC. Nitroaromatic compounds are antioxidants derivates known to be carcinogenic for aquatic organism and their major uses are oil industry for improving resistance to oxidation process. The main uses of PRM and EHMC are UV filter in sunscreen products and UV stabilizers for plastic products. This component can be ascribed to point pollution sources represented here by direct input from recreational activities. Principal component 4 has loading in MHJ and BZP and the last principal component 5 has loadings of BHA. MHJ is a fragrance ingredient used in many fragrance mixtures. It may be found in fragrances used in decorative cosmetics, fine fragrances, shampoos, toilet soaps or household cleaners and detergents. The fact that BZP had high loadings in two PCs can suggest that this compound had two different sources of pollution. BHA is a preservative found very often in many food products, in order to prevent the oxidation of fats and oils. Also it can be used in rubber, petroleum products and wax food packaging.

Chapter 2.

Development of extraction and analysis method, using gas chromatography coupled with mass spectrometry (GC/MS), for flame retardants izolation from aqueous samples

Organophosphorus compounds (OPFRs) are chemicals that are frequently utilized as plasticizers and flame retardants in products such equipments, building materials, textiles, plastics, paints, glues, lacquers and varnishes (Binici *et al.*, 2013). Another important usage of OPFRs is as antifoaming agents and additives, in products such lubricants and hydraulic fluids (Marklund, 2005). *The novelty of this study is the development, optimization and validation of extraction method of flame retardants from aqueous samples.*

2.1. Eficiency study of organic solvents function of stationary phase of SPE cartridges

At this stage of the study, four types of cartridges were tested, namely: *STRATA X, OASIS HLB, Lichrolut EN/RP, Chromabond C18.* For the conditioning and elution step of the cartridges, 3 solvents were tested, namely *methanol, dichloromethane and ethyl acetate* **(Table 2).** All extractions were done in duplicate. For this step, 5 samples (4 samples for the 4 cartridges and one black for each set of extractions) were prepared using 500 mL of river water, to which a mixture containing all spiked samples was added. The mixture containing the compounds of interest was made in acetone and the final concentration in the water sample was 500 ng·L^{-1} for each compounds.

Table 2. *SPE method description for isolation of compounds*

SPE steps	Metod I	Metod II	Metod III
Conditioning	2×3 mL methanol 2×3 mL HPLC water	2×3 mL metilen chloride 2×3 mL acetonitrile 2×3 mL HPLC water	2×3 mL ethyl acetate 2×3 mL HPLC water
Sample flow rate	500 mL sample	500 mL sample	500 mL sample
Drying	3 mL HPLC water	3 mL HPLC water	-
Elution	2×3 mL methanol	3 mL metilen chloride +acetonitrile 3 mL metilen chloride	2×3 mL ethyl acetate

2.2. Separation and identification of obtained extracts using GC/MS

The parameters used were: injector temperature 250 °C, capillary column, DB - 5MS of 30 m × 0.25 mm id and the temperature of the transfer line and the ion source was 300 ºC, respectively 250 ºC . The temperature program used began at 90 °C, where it was maintained for 1 minute, increased by 10 °C min $^{-1}$ to 180 °C, then increased by 15 °C min^{-1} to 250 °C, where it was maintained for 8 minutes. The carrier gas used was helium at a flow rate of 1.5 mL·min^{-1}. Using the above conditions, a chromatogram **(Figure 4)** was obtained in which the identification of the compounds was based on the selected characteristic ion, and the quantification was done by measuring the chromatographic areas, there being a good separation of the three compounds and no other interferences.

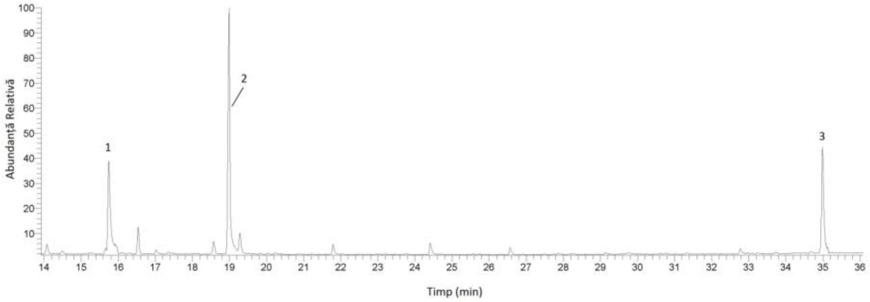
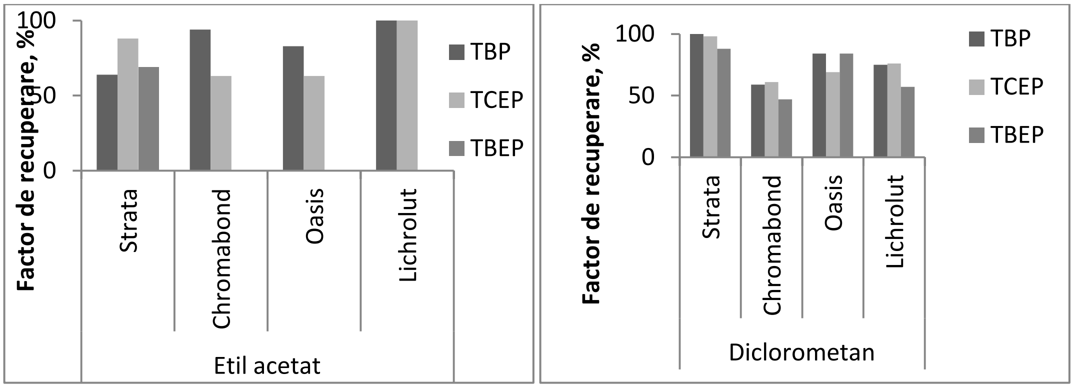
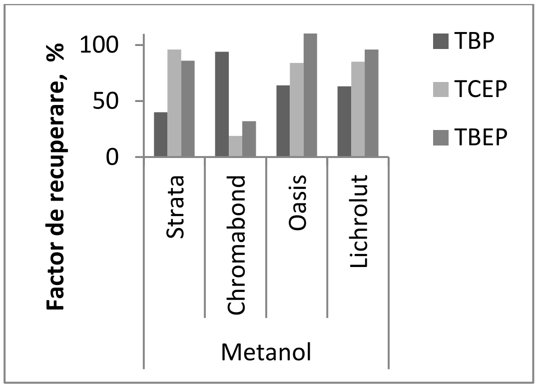


Figure 4. *Obtained chromatogram for extraction using dichloromethane and Strata cartridges. Compounds are: (1)TBP(2)TCEP(3)TBEP*



a)

b)



c)

Figure 5. *Recovery of investigated compounds eluted with a) ehtyl acetate, b) dichloromethane and c)methanol*

As can be observed, the best combination of cartridge and solvent capable of providing the best recovery rates for simultaneous extraction of OPFRs in surface waters is given by STRATA X with dichloromethane.

2.3. Method validation. Identification of performance parameters of the method and uncertainity estimation

Calibration curves were performed in the range of 20-1000 ng·mL^{-1} for TBP and 50-1000 ng·mL^{-1} for TCEP and TBEP. Correlation coefficients obtained from the calibration curves showed values greater than 0.998. The repeatability and reproducibility obtained were good, with relative standard deviations being less than 10%, except for TBEP (16% for repeatability and 13% for reproducibility). The detection and quantification limits are shown in **Table 3.**

Table 3. *LOD and LOQ for investigated compounds*

Parametru	TBP (ng·mL^{-1})	TCEP (ng·mL^{-1})	TBEP (ng·mL^{-1})
LOQ	2.9	20.2	3.3
LOD	0.9	6.1	1.0

The uncertainty associated with the measurement is given by:

$$\frac{u(C)}{C} = \sqrt{\left(\frac{u(V_{proba})}{V_{proba}}\right)^2 + \left(\frac{u(V_{solvent\ SPE})}{V_{solventSPE}}\right)^2 + \left(\frac{u(iC_8)}{iC_8}\right)^2 + \left(\frac{u(V_{material\ referinta})}{V_{material\ referinta}}\right)^2 + \left(RSD_{aparat}\right)^2}$$

$$\frac{u(C)}{C} = \sqrt{0.0026424^2 + 0.005847^2 + 0.005487^2 + 0.005779^2 + 0.053^2}$$

$$\frac{u(C)}{C} = 0.054$$

$$u(C) = C \times 0.054$$

The standard uncertainty for TBP, for example, is: u (c) = 317.4 ng/L x 0.054 = 17.1. Considering a confidence level of 95% with an extension factor k = 2, the following uncertainty value follows: Uextins = 2 × 17.1 = 34.2

Concentrations of inhibitors can be declared as follows: C TBP = 317.4 ng/L ± 34.2 ng / L or 317.4 ng/L ± 10.7%

2.4. Utilization of proposed method for real surface water samples

To test the developed method, 10 surface water samples were taken from the Somes River. The results were centralized in Table 4.

Table 4. *Concentrations of Somes River Combustion Inhibitors, expressed in ng · L-1 ± confidence interval*

Sample number	**TBP ($ng \cdot L^{-1}$)**	**TCEP ($ng \cdot L^{-1}$)**	**TBEP ($ng \cdot L^{-1}$)**	**TCPP ($ng \cdot L^{-1}$)**
1	4418±250	<LOD	<LOD	<LOD
2	2187±92	<LOD	<LOD	661±58
3	604±8	<LOD	<LOD	102±12
4	39659±1080	2347±80	<LOD	<LOD
5	653±62	<LOD	<LOD	<LOD
6	420±70	<LOD	<LOD	288±20
7	465±64	<LOD	<LOD	267±18
8	582±38	<LOD	<LOD	176±22
9	1216±102	<LOD	<LOD	262±32
10	380±46	<LOD	<LOD	<LOD

Chapter 3.

Researches regarding the spatial and temporal evaluation of metals in surface water from Transylvania using inductive coupled plasma mass spectrometry and multivariate statistical techniques

Among water pollutants, heavy metals are the most frequent and they cannot be biologically or chemically degraded, as organic compounds. It also tends to accumulate or might be transported long distance from source points (Simeonov *et al.*, 2000).

In this study, the basins of Cibin, Mures and Olt have been selected for monitoring the concentrations of metals. *The novelty of this study is the combination of sensitive analytical techniques used for water analysis and chemometric techniques for a better interpretation of the results.*

3.1. Statistical analysis of experimental data obtained for metals

One way ANOVA (at 5 % level of significance) was used to assess the influence of precipitations upon metal concentration levels and to find out significant differences between the three investigated rivers. The obtained results showed that the only elements which were influenced by increased level of precipitation in wet seasons were Cr (p=0.028), As (p=0.000), Mg (p=0.014), K (p=0.039) and Na (p=0.05). Unexpectedly, the three rivers could not be distinguished by any characteristic element.

The HCA was performed in order to group the sampling points (cases) according to their metal content. Generally the objects (sampling points) have similar properties within a cluster and different proprieties between clusters (Yang *et al.,* 2014). The result of cluster analysis is usually illustrated by a dendrogram, presented in Figure 2. Cluster analysis was run using Ward method of clustering, with Squared Euclidean distance as a measure of similarity between clusters.

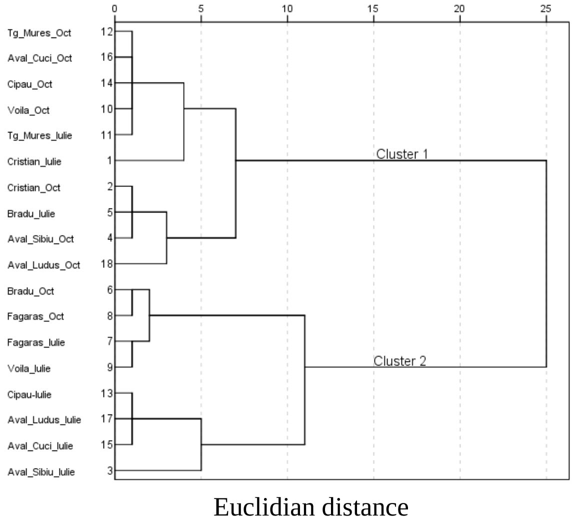


Figure 6. *Dendrogram resulted from cluster analysis, where sampling points are grouped according to metal content*

The results showed that the main elements that distinguish between clusters are: Ti (p=0.044), Mn (p=0.046), Ca (0.000), Mg (0.049) and Na (p=0.001). By running PCA on our data set 5 principal components were retained (eigenvalues higher than 1) encountering a total variance of 86.4 %. The graphical representation of every principal component against its eigenvalues is presented in **Figure 7.**

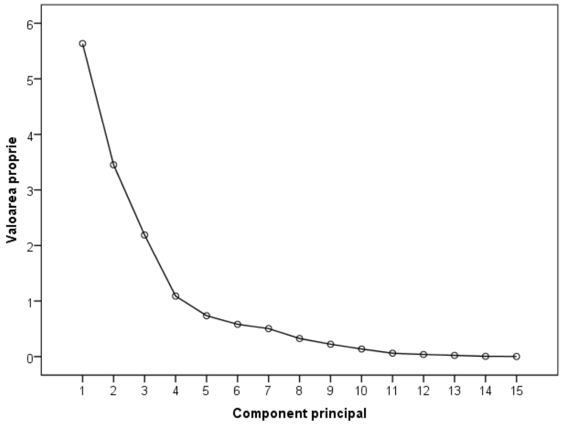


Figure 7. *Representation of eigenvalues for every principal component (scree plot)*

The loadings of each principal component extracted are presented in **Table 5.** First principal component explained 34.4 % of total variance and had high loading of Cu, Pb, Al, Mn and Fe, PC 2 (18.1 % of total variance) included Na and Mg, while PC 3 (17.3 %) was loaded by K. The last two PC had 9.7 % and 6.8 % from total variance and were loaded by Ti and Ca, Cr and Cd, respectively.

Table 5. *Components matrix. Bold values represent elements with signifiant loadings in principal components*

Parameter	**Principal component 1**	**Principal component 2**	**Principal component 3**	**Principal component 4**	**Principal component 5**
Cr	0.280	0.205	-0.273	-0.025	**0.750**
Ni	0.660	0.478	0.207	0.069	0.395
Cu	**0.896**	-0.135	0.038	-0.108	0.265
As	-0.078	-0.546	0.667	0.274	-0.263
Cd	0.101	-0.158	-0.093	-0.186	**0.921**
Pb	**0.861**	0.181	0.301	0.091	0.110
Al	**0.843**	-0.009	-0.410	-0.020	-0.047
Ti	0.006	-0.044	-0.075	**0.841**	-0.138
Mn	**0.757**	0.121	-0.178	0.470	0.013
Fe	**0.913**	0.087	-0.125	0.010	0.123
Zn	**0.685**	-0.495	0.425	0.065	0.129
Ca	0.104	0.200	0.336	**0.836**	-0.043
Mg	0.076	**0.860**	-0.103	0.229	-0.030
K	-0.019	0.171	**0.896**	0.021	-0.208
Na	0.018	**0.944**	0.187	-0.035	0.000
Eigenvalue	5.155	2.715	2.601	1.460	1.026
% from variance	34.4	18.1	17.3	9.7	6.9
Cumulative variance %	34.4	52.5	69.8	79.5	86.4

The sources of pollution from the first PC could be of geogenic origin (Levei *et al.*, 2014), natural, due to the influence of river beds and sediments, by the desorption of metals at trace level of the Al, Fe, Mn hydroxides, which have a particular affinity for Pb and Cu (Mihailović *et al.*, 2014). PC2 reflected the processes of natural washing due to the dissolution of Mg and Na silicates. For example, a limestone rock releases sodium, while dolomite releases magnesium (Avdullahi *et al.*, 2013). The pollution sources suggested by the third PC are with anthropogenic influence. Potassium is predominantly released (87%) from the washing of silicate minerals. Another important source of potassium is associated with the use of fertilizers applied on agricultural land because it is a vital nutrient required in large quantities by all plants (Lenntech 2015). The fourth component also reflected a natural influence on Ca and Ti concentrations. Titanium is a component of minerals such as rutile and anatase. So only a small amount of titanium gets into the water by washing the rocks. It can be dissolved from limestone rocks, marble, calcite and dolomite (Lenntech 2015).

The last major component suggests a contribution of the existing industry to the studied area. Therefore, a major source of chromium is represented by the metallurgical industry, but also by dyeing plants where chromium compounds are used as catalysts. Similarly, cadmium can also come from industrial activities (Boyacioglu, Boyacioglu, 2011).

Chapter 4.

Researches regarding isotopic and elemental markers identification for characterisation of organic and conventional vegetables

Organic products are considered by some consumers to be healthier, more natural and environmentally friendlier than that from conventional production (Schmidt *et al.*, 2005, Mondelaers, Verbeke, Huylenbroeck, 2009, Camin *et al.*, 2011). Increasing the consumer demand for organic food, introduction of certification and premium prices have created a financial enticement to mislabel and try to pass off cheaper conventionally grown products as organic. In order to differentiate these two categories, the association of robust analytical methods able to distinguish organically and conventionally produced foods is needed for authentication purposes.

The aim of this work was to establish the most suitable markers, that are able to differentiate organic from conventional vegetables, or which might differentiate greenhouse grown vegetables from those grown in the field. These classifications were established using chemometric tools applied upon isotopic and multielemental experimental data.

4.1. Chemometric processing

The first purpose of this study was *the identification of specific particularities of each vegetable from isotopic and elemental point of view*. For this aim, in order to find the best markers that could differentiate among different vegetables types, LDA statistic treatment was applied to all investigated samples. . Since six groups (formed by salads, tomatoes, potatoes, cabbages, garlic and leek samples) were compared, each representing one type of vegetable, five discriminant functions were obtained. All five had statistical significance ($p < 0.05$), the first two explaining 92.2 % from total variance of dataset (Wilks lambda was 0.009 and 0.077). The sample distribution is presented in **Figure 8**. The initial classification was 93.3 %, while the cross-validation procedure managed to reclassify a percent of 88.9 % from cases. This classification was made based on the strongest predictors, P, Sr, $\delta^{18}O$, $\delta^{15}N$ and $\delta^{13}C$. It was observed that these differences among vegetables are mainly related to the plant physiological factors and given by the isotopic ratios of oxygen ($^{18}O/^{16}O$), nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$). This could be explained through the fact that, the isotopic enrichment degree depends on the plant species, even for the species grown in the same geographic (Magdas, Puscas, 2011).

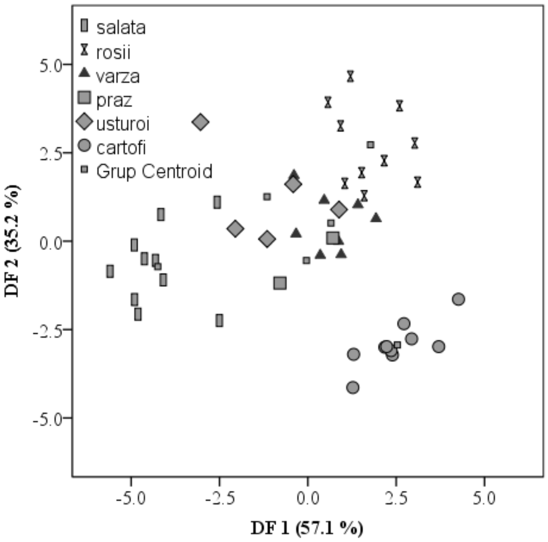


Figure 8. *Samples distribution according to vegetable type, using two discriminant obtained function*

For the identification of suitable markers that could *differentiate greenhouse grown vegetables from those produced in field*, LDA test was applied and the classification criterion was the growing condition of vegetables. The parameters obtained as being the best predictors for this criterion were: Ni, Mo, Cd, Pb, and δ^{13}C. Also, the obtained discriminate function was statistically significant ($p = 0.001$) for agricultural practices discrimination (Wilks lambda 0.231) and explained 100 % of data variance. The graphical representation is in **Figure 9.** 100 % of the original samples were correctly classified, while a percent of 93.3 % was obtained in cross-validation. Very negative δ^{13}C - value are indicative for greenhouse cultivation and could be also explained by the influence of depleted δ^{13}C values of the natural gas heating source on the available CO_2 for plant growing (Schmidt *et al.,* 2005). The application of some fertilizers or animal manures to agricultural soil could increase some potentially toxic elements (i.e. Cd, Pb, Ni) in soil, which may be absorbed by plants and, thus, increase the risk of contamination in agricultural products (Nookabkaew *et al.,* 2016). The differences in these metal concentrations could appear due to the different pesticide types that are used for greenhouse vegetable cultivation as compared with those used for field.

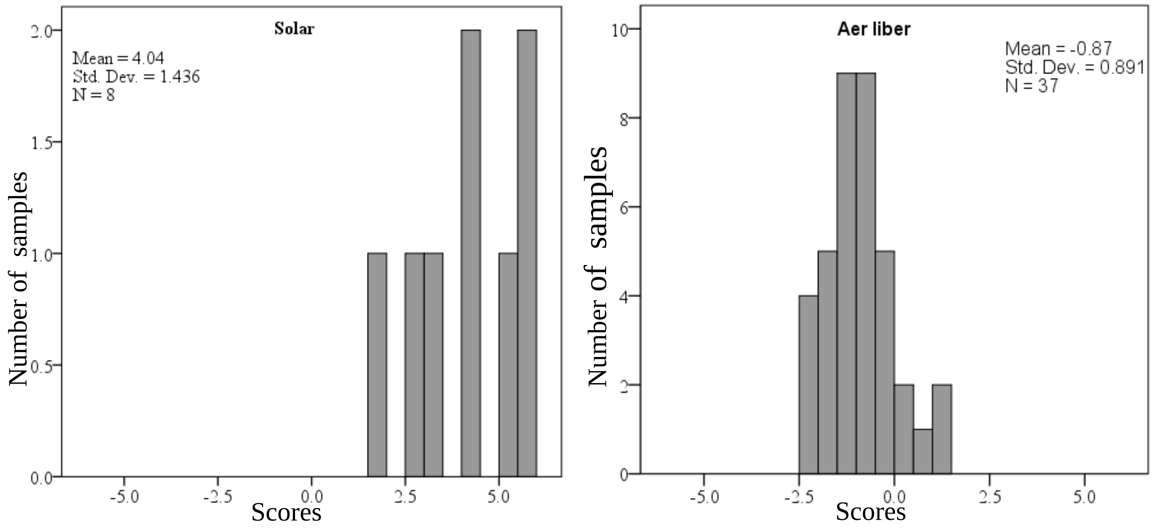


Figure 9. *Vegetables discrimination according to growing conditions*

The last differentiation, which was made by using LDA, consists in the *discrimination of organic grown vegetables from those cultivated under conventional practices.* When all analyzed samples were taken into consideration, the two compared vegetables groups (organic and conventional) were initially classified in percent of 84.4 %. The obtained significant function ($p = 0.01$, Wilks lambda 0.553) was tested and the same percent as initial was obtained in cross-validation. Five samples from organic group and two samples from conventional group were misclassified, both in initial and cross validation tests. The main predictor for this purpose was $\delta^{15}N$. The explanation for this consists in the fact that synthetic nitrogen fertilizers have $\delta^{15}N$ values significantly lower (from – 6 ‰ to 6 ‰) than the manures and fertilizers (from 1 ‰ to 37 ‰) permitted in organic agriculture (Bateman, Kelly, 2007) and thus, the differences between these two inputs could contribute to the differentiation of products obtained through these two agriculture practices types. The graphical separation is presented in **Figure 10**. The weak separation between organic and conventional grown vegetables, that was obtained, is due to the large amount of vegetable type that was compared. In this case the differences that appear, in terms of elemental and isotopic content, among vegetables types, are overlapping with the particularities of organic and conventional grown plants.

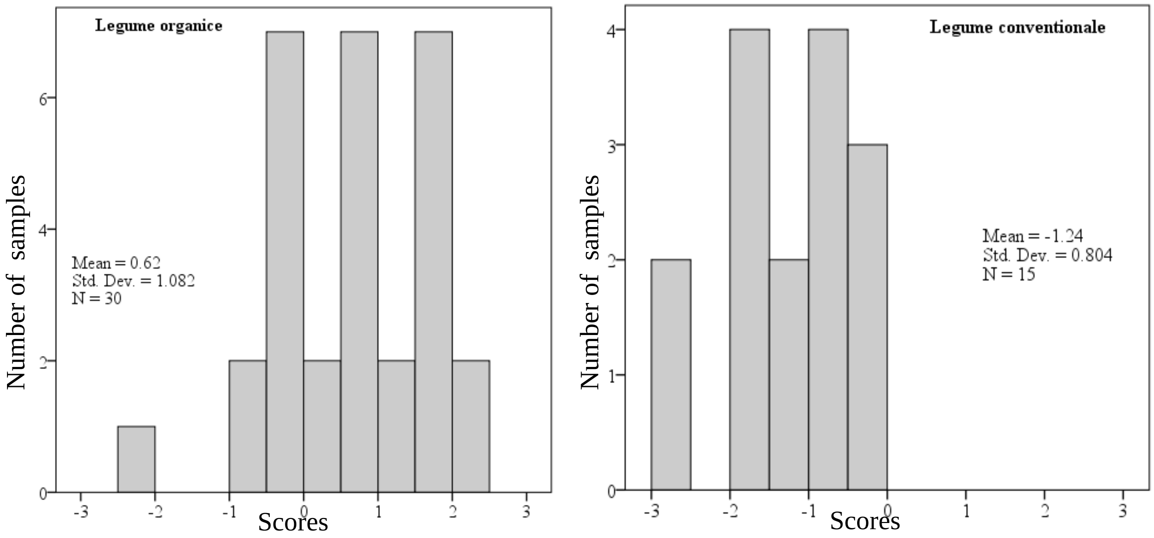


Figure 10. *Vegetables differentiation according to agricultural practices*

It has been found that the results obtained reflect the mark of plant type particularities and the predictors obtained are perhaps not the most representative for the proposed classification. As a result of this study, it has been found that, for the establishment of specific predictors of a certain category (regime or growth, or type of plant), the distribution of the data set is of particular importance. This should include a relatively uniform and balanced number of samples for each category to be compared. Moreover, within the same category, there should be representative samples, meaning that all the properties and characteristics of the samples should be highlighted in that dataset. It was possible to observe the increase of the classification percentage when working on small groups of samples.

Chapter 5.

Researches regarding isotopic and elemental markers identification for geographical origin and agricultural practices discrimination of carrots

Carrots are among the most popular and widely used vegetable, being one of the first vegetable introduced in infant's alimentation. For this reason, an even enhanced control of this product is required, with respect to their growing conditions, agricultural practices, as well as geographical origin, especially now when new emerging key technologies (i.e. renewable energy, electronics) involve new pollutants and also while new generation of fertilizers and pesticides are used. Despite the considerable progress that was made for understanding the eco-toxicological behaviour of more traditionally used elements like As, Cd, Cr, Cu, Hg or Pb, the environmental and eco-toxicological effects of the rapidly increasing use of other trace elements (Ga, Ge, In, Te, Nb, Ta, Tl), the Platinium Group Elements (PGEs: Pt, Pd, Rh, Os, Ir, Ru) and most of the Rare-Earth Elements (REEs: Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Yb, Lu) are yet not well understood (Cobelo-Garcia *et al.*, 2015). Previous studies reported that, discriminations either between organically from conventionally grown vegetables or from different geographical origin involved REEs as powerful markers (Gundersen *et al.*, 2000, Bertoldi *et al.*, 2016).

The aim of this study was the differentiation of carrots according to growing regiome and geographical area, based on isotopic and elemental content (inlcuding rare earth elements).

5.1. Discrimination of organic and conventional grown carrots

The first performed test was ANOVA and had shown that the markers which have statistical significance in the differentiation of organic from conventional vegetables, proved to be: $^{15}N/^{14}N$ (0.001), Ti (0.014), Cr (0.002), Mn (0.003), Fe (0.039), Ga (0.025), Sb (0.003), Ba (0.013), Sc (0.031), La (0.001), Ce (0.004), Pr (0.002), Nd (0.005), Yb (0.043), Lu (0.012), Tm (0.005), Th (0.018). Some of these elements: Ti, Sb, and Lu were found to have statistical significance in the discrimination of organically from conventionally onions samples (Gundersen *et al.*, 2000).

It was emphasised, by previously reported studies, the direct relationship between the adsorption capacity of Rare-Earth Elements (REEs) and the manganese oxides content of soil (Pang, Li, & Peng, 2001). In this study, the mean Mn concentration found in organically grown samples (4.6 mg/kg) was higher as compared with manganese content of carrots produced under conventional conditions (1.7 mg/kg). This result had an opposite trend as the results reported by Kelly & Bateman (2010) for the differentiation of organically grown tomatoes and lettuce samples from those conventional, which found the higher manganese content in conventional samples. A possible explanation for our obtained results could be the composition of organic fertilizers which are commercialized on Romanian market and which contain a significant content of manganese between 1 % and 32 %. Also, other authors Kelly & Bateman (2010) had assumed that the use of manganese as an additive to livestock feed supplements could also be a source of Mn in organic agriculture, where manure is used

In order to obtain a better picture concerning the relationship between the Mn content and REEs concentration and also to confirm that such a correlation and interdependence exists, Pearson correlation matrix was made. Concerning the high number of employed descriptors, the obtained matrix was very large. Thus, according to Liu, Lin and Kuo (2003), values between 0.2-0.5 means the correlation is weak, values between 0.5-0.75 means moderate correlation, while values higher than 0.75 means the correlation is very strong. In the present study, just very strong correlation were taken into discussion, and only for descriptors that were obtained as being significant after applying ANOVA test.

It was found that the content of both Cr and Mn were correlated with a high number of elements belonging to the REEs group, namely Sc, La, Ce, Pr, Nd, Lu, Th, while other elements, Co, Ni, Sr, are all positively correlated with Nd. Among above mentioned REEs, a strong correlation was observed, most probably due to the fact that the REEs have close related electronic configuration leading to very similar physical and chemical behaviour (Shtangeeva, 2014). In the present case, no negative correlations among analyzed elements were observed.

By applying LDA (stepwise method) on the markers that were found to have statistical significance after the application of ANOVA test, for the differentiation of organically from conventionally grown carrots, the best discrimination markers proved to be: the isotopic ratios of nitrogen $^{15}N/^{14}N$ along with La and Ga content. The initial classification of organically versus conventionally grown carrots was 83.3 % whereas; the obtained percent at cross-validation was 81 %. Previously studies performed by (Bateman *et al.*, 2007) on a carrot sample set (30 samples) had suggested that there is no apparent difference in the nitrogen isotope composition between organic and conventional samples. They assume that a possible explanation of the limitation in using the $^{15}N/^{14}N$ for the differentiation of organically grown vegetables, from those grown in conventional regime, are related to the lower nitrogen requirement of carrots as compared to other vegetables types (i.e. tomatoes or lettuces) and consequently, generally lower levels of fertilizer applications. Similar results regarding the apparent unsuitability of nitrogen isotope ratios for the differentiation between organically and conventionally grown field-crops, having a low nitrogen demand was also reported by Schmidt *et al.* (2005).

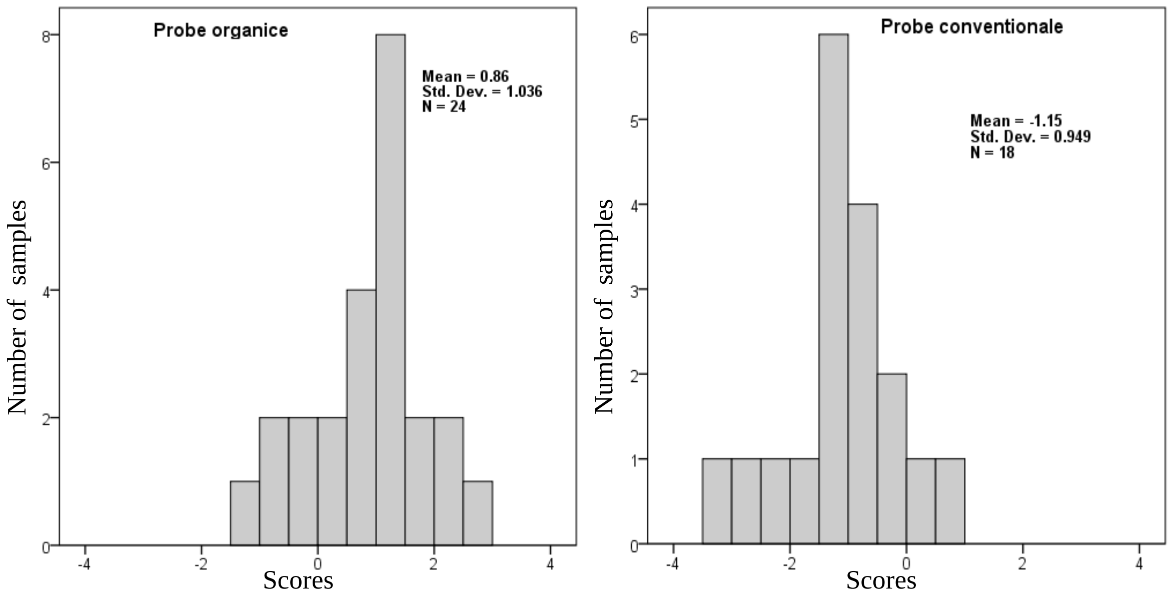


Figure 11. *Graphical representation of carrots samples, according to agriculture regime, obtained after aplying LDA*

5.2. Discrimination of Transylvanian and other areas grown carrots

For identification of parameters that are characteristic for samples grown in Transylvania area, a 103 samples set, having as descriptors the same elemental content, mentioned above, along with isotopic values was used. After applying ANOVA test on this matrix, having as factor the geographical origin, the following variables were obtained: Ti ($p=0.001$), Cr ($p=0.001$), Mn ($p=0.001$), Ga ($p=0.001$), Sb ($p=0.014$), Cs ($p=0.010$), Ba ($p=0.035$), Tb ($p=0.006$) and Pb ($p=0.049$). Similar discrimination variables, Ba, Cr, Cs, Ga and Mn, were obtained by other authors (Bertoldi *et al.*, 2016), which successfully managed to reclassify the samples according to area of provenance in proportion of 100 %. Among these elements, Ga and Mn presented the largest standardized coefficients in the obtained canonical functions.

For a more comprehensive picture of elements that are able to differentiate, the carrots samples grown in Transylvania or in different areas, LDA was applied and solely variables obtained as being statistical significant, after ANOVA, were used. Since two variables were compared, only one function was obtained (Wilk's lambda 0.767 and $p < 0.001$), which was able to explain 100 % of data variance. The most powerful variables for discrimination between the carrot samples grown in Transylvania from the rest, were Mn and Tb (initial and cross validation percent of 75.7 %).

5.3. Discrimination of carrots grown in three Transylvanian areas

Another goal of this study was to *identify elemental or isotopic markers, which might differentiate the three Transylvanian regions*, from where most of the samples were collected: Cluj, Salaj and Bihor. As in previous case, ANOVA test was applied using 62 carrots samples and the statistically significant predictors (Ti $p=0.044$, $\delta^{18}O$ $p= 0.041$ and $\delta^{2}H$ $p=0.003$) were used further for LDA. The most powerful discrimination markers were, in this case, $\delta^{2}H$ followed by $\delta^{18}O$. It is known that, isotope ratios of oxygen and hydrogen represent the best markers for geographical origin discrimination (Longobardi et al., 2015; Bontempo, Camin, Larcher, Nicolini, Perini, & Rossmann, 2008). On the other hand, no specific marker for agricultural practices was found; this fact indicated that the agriculture regime among producers from Transylvania is similar.

After applying LDA, only one marker was obtained, namely δ^2H. The percents of initial and cross validation were not very high (66.1 % and 64.5 %, respectively), due to the fact that those areas are very close one another. It can be seen from the Fig. 2 that all samples from Salaj area are overlapping Cluj area.

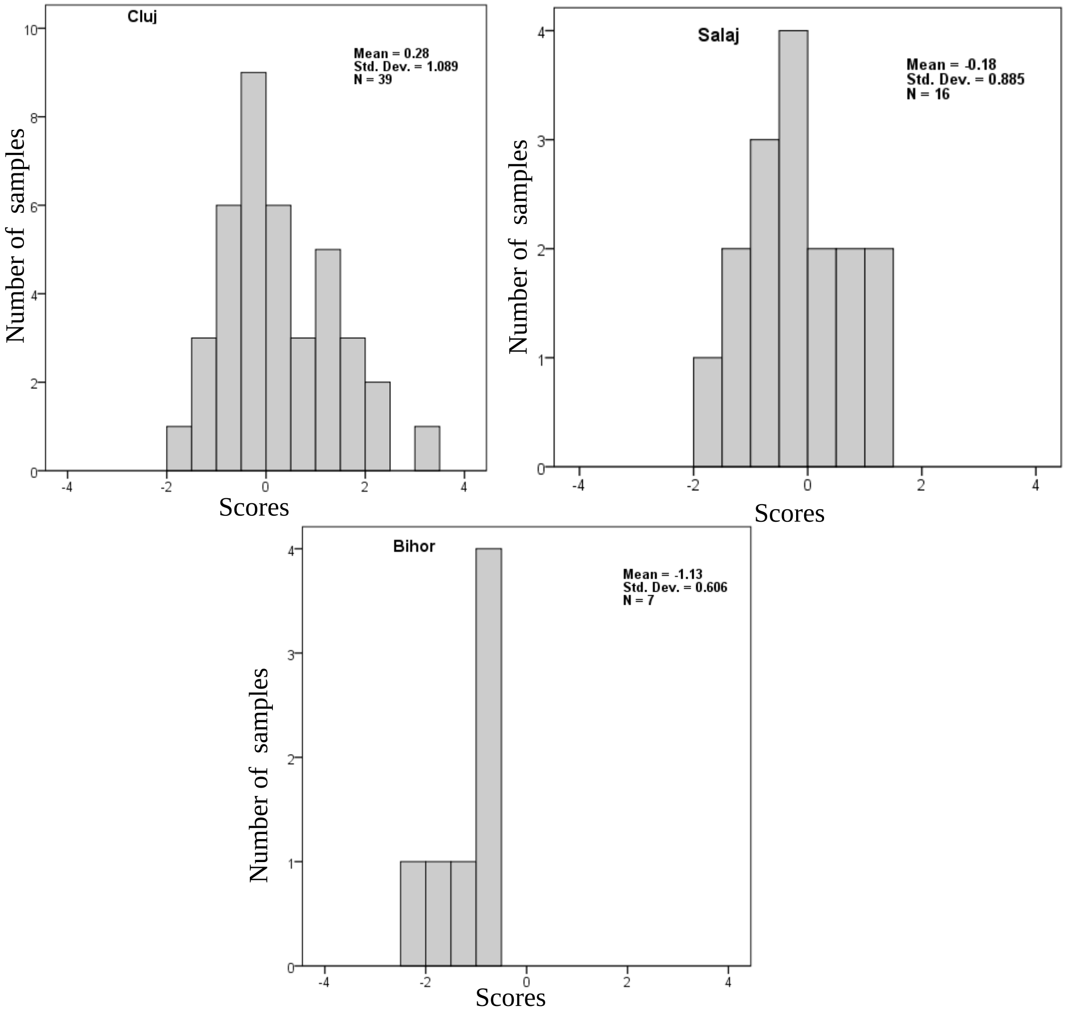


Figure 12. *Carrots samples distribution according to three areas from Transylvania, using LDA*

This overlapping appeared because the two regions are quite similar, from the point of view of climatic conditions, and both are supplied with water from the same source (i.e. Somes catchment). The better discriminate potential of hydrogen δ^2H as compared with δ^{18}O is given by the fact that the isotopic composition of oxygen in plants keeps the isotopic fingerprint of groundwater and precipitation from plant's location and evapotranspiration effect, which is affected by humidity and temperature (Rossmann *et al.*, 1999). On the other hand, the hydrogen content of plants, originated from water, is taken up by the and in this case, the evapotranspiration process is not present, the carrot being a root vegetable

Chapter 6

General conclusions

The main purpose of this paper was to exploit the potential of the chemometric and spectrometric methods, in order to highlight specific markers for surface waters and for vegetables, according to certain criteria. In the first and second chapter of the experimental results, the surface waters from three basins (Olt, Mureş and Cibin) from Transylvania were analyzed in terms of organic and elemental content. For assessing the impact of precipitation on the concentrations of the investigated compounds, 36 samples of surface water were taken in two different seasons. It also sought to identify representative parameters for each basin.

For organic pollutants identifying, water samples were extracted using solid phase extraction (SPE), followed by gas chromatography coupled with mass spectrometry (GC/MS) analysis. All analytical results were subjected to chemometric techniques (ANOVA, CA, PCA and LDA). Among all organic compounds, the highest concentration was BZP, at the sampling point on the Olt River (BRDd). Precipitation influenced only two compounds of those analyzed, namely: MHJ and BZP. The compounds that could differentiate the Olt River from Mureş are DBNP and BHA, which showed higher values in the Olt River than in Mureş. The Cibin River could not be distinguished from the other pools by any compound. Applying the cluster analysis, grouping the sampling sites according to the organic load in three clusters with small, medium and large concentrations respectively was performed. In this way the sampling plan can be reduced to one representative point in each cluster, which reduces the number of analyzes and implicitly the time and cost associated with the measurements. By applying the PCA to the whole set of experimental data, it was found from the examination of the correlation matrix that there are pollutants from the same sources. The first main component obtained was attributed to direct pollution of wastewater without proper treatment.

The second main component was attributed to industrial pollution, while the third corresponds to pollution from recreational activities.

Also, in this study of organic pollutants in surface waters a solid extraction method was developed for the extraction of flame reatrdants from water samples. The SPE method was optimized by testing four different types of cartridges and three elution solvents. Also, the developed method has been validated by calculating the main validation parameters and uncertainty associated with the measurements. The method was tested using 10 surface water samples from the Somes River, where some of the investigated compounds were identified.

For elemental content determinations, samples were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) without a complex processing of the samples before analysis. The experimental results were subjected to the same statistical treatments (ANOVA, CA, PCA, LDA). The highest concentrations were found for Na, Ca, Mg and K. These elements showed maximum values in all three rivers. The high level of precipitation influences only the concentrations of Cr, As, Mg, K and Na. By studying the correlation matrix, more correlations could be observed, than in the case of organic compounds. The cluster analysis revealed two clusters, where the sampling points were grouped according to the sampling season. Following the PCA analysis, it was found that the first component represents a source of pollution of geogenic origin (Al, Mn and Fe). The second component can be associated with a natural source of pollution due to Na and Mg elements. The third component suggests sources of pollution from agricultural activities due to fertilizers used for plant growth. The fourth component also reflects a natural influence on Ca and Ti concentrations. Titanium is a component of minerals such as rutile and anatase. So only a small amount of titanium gets into the water by washing the rocks. It can be dissolved from limestone rocks, marble, calcite and dolomite. The last major component suggests a contribution of the existing industry to the studied area.

In the last two chapters, vegetables were studied in terms of isotopic and elemental content. All experimental results were subjected to chemometric processing (ANOVA and LDA). Thus at the beginning of the study we studied 45 samples of vegetables from different species. The aim of the study was to differentiate *(i)* vegetables grown in the open air from those grown in the sun *(ii)* vegetables from organic farming versus those from conventional agriculture *(iii)* several species among them.

The main purpose of this work was to exploit the potential of chemometric and spectrometric methods, in order to highlight specific markers for surface waters and for vegetables, according to certain criteria. In the first and second chapter of the experimental results, the surface waters from three basins (Olt, Mureş and Cibin) from Transylvania, were analyzed in terms of organic and elemental content. For assessing the impact of precipitation on the concentrations of investigated compounds, 36 samples of surface water were sampled in two different seasons. It also sought to identify representative parameters for each basin. For identification of organic pollutants, water samples were extracted using solid phase extraction (SPE), followed by gas chromatography coupled with mass spectrometry (GC/MS) analysis. All analytical results were subjected to chemometric analysis (ANOVA, CA, PCA and LDA). Among all organic compounds analyzed, the highest concentration was BZP, at the sampling point from Olt River (BRDd). Precipitation influenced only two compounds from those analyzed, namely: MHJ and BZP. The compounds that could differentiate the Olt River from Mureş are DBNP and BHA, which showed higher values in the Olt River than in Mureş. The Cibin River could not be distinguished from the other rivers by any compound. By applying the cluster analysis, the sampling sites were grouped according to the organic load in three clusters with small, medium and large concentrations. In this way, the sampling plan can be reduced to representative point in each cluster, which reduces the number of analysis and implicitly the time and cost associated with the measurements. By applying the PCA to the whole set of experimental data, it was found from the examination of correlation matrix that there are pollutants from the same sources. The first main component obtained was attributed to direct pollution of wastewater without proper treatment. The second main component was attributed to industrial pollution, while the third corresponds to pollution from recreational activities.

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For the determination of the elemental content, the samples were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) without a complex processing of the samples before analysis. The experimental results were subjected to the same statistical treatments (ANOVA, CA, PCA, LDA). The highest concentrations were found for Na, Ca, Mg and K. These elements showed maximum values in all three rivers. The high level of precipitation influences only the concentrations of Cr, As, Mg, K and Na. By studying the correlation matrix, more correlations could be observed than in the case of organic compounds. The cluster analysis revealed two clusters, where the sampling points were grouped according to the sampling season. Following the PCA analysis, it was found that the first component represented a source of pollution of geogenic origin (Al, Mn and Fe). The second component could be associated with a natural source of pollution due to Na and Mg elements. The third component suggested sources of pollution from agricultural activities due to fertilizers used for plant growth. The fourth component also reflected a natural influence on Ca and Ti concentrations. Titanium is a component of minerals such as rutile and anatase. So only a small amount of titanium gets into the water by washing the rocks. It can be dissolved from limestone rocks, marble, calcite and dolomite. The last major component suggests a contribution of the existing industry in the studied area.

In the last two chapters, vegetables were studied in terms of isotopic and elemental content. All experimental results were subjected to chemometric processing (ANOVA and LDA). Thus at the beginning of the study, 45 samples of vegetables from different species were investigated. The purpose of the study was to differentiate *(i)* vegetables grown in the field from those grown in the greenhouse *(ii)* vegetables from organic farming versus those from conventional agriculture *(iii)* several species among them. This aspect of data variability has been eliminated in the final chapter of experimental results by studying a single food matrix (carrots with a better representation for all the criteria discussed. Thus, 103 carrot samples were analyzed from the isotopic and elemental point of view. The results were processed using ANOVA, and significant markers were further used in the LDA model. The main purposes were to establish markers for *(i)* the identification of the growth regime and *(ii)* to establish the geographical origin of the carrots. The main markers that can be used to establish the growth regime were $\delta^{15}N$, along with La and Ga. Geographical differentiation was carried out in two stages, the first was the differentiation of Romanian samples, and the second was done only for the samples taken from Transylvania, where it was attempted to differentiate three areas, which is quite difficult to achieve because of the proximity and the precipitation or thermal conditions may be the same. Mn, Tb and $\delta^{2}H$ were the strongest markers for this criterion.

Thus, the main elements of originality and novelty made in this paper are: - Characterization of Transylvanian rivers in terms of organic and elemental content, using high resolution mass spectrometric methods, with high sensitivity and specificity, and multivariate statistical methods ; - Development, optimization and validation of the extraction method for combustion inhibitors in water samples;- Determination of isotopic and elemental markers, using multivariate statistical methods, for different species of vegetables, depending on the growth regime and the growing conditions;- Analysis of rare earth elements in vegetables and selection of representative elements for the above-mentioned criteria, using the statistical methods. This paper demonstrated the increase of the mass spectrometry potential by association with the chemometric methods, which are able to extract from the large number of analyzed variables the ones representative for each criterion. Also, the novelty of these studies on vegetables is the analysis of rare earths elements and the attempt to establish some sources of contamination with them. Future research directions can be focused on the study of other classes of organic pollutants with negative environmental impact. Rare earths can also be measured in other vegetable species. A future approach would be the identification of pollution sources with rare earths elements and toxicological and risk assessment that these elements pose.

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