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CHARACTERIZATION AND CLASSIFICATION OF MEDICINAL PLANT EXTRACTS ACCORDING TO THEIR ANTIOXIDANT ACTIVITY USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND MULTIVARIATE ANALYSIS

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ABSTRACT. Fuzzy divisive hierarchical clustering (FDHC) alongside with principal component analysis, hierarchical cluster analysis and linear discriminant analysis are efficiently employed for the characterization and clustering of some medicinal plants according to their antioxidant capacity. These methods are applied to the numerical data obtained from the chromatographic profiles monitored at 242, 260, 280, 320, 340 and 380 nm by high-performance liquid chromatography with a multistep isocratic and gradient elution system and diode array detection (HPLC-DAD). The samples were successfully classified according to the antioxidant activity determined using the DPPH assay. A correct classification rate of 100% was obtained when the samples were divided into two groups corresponding to high antioxidant activity and low antioxidant activity. Moreover, it is suggested to use the scores obtained applying principal component analysis and unprocessed data (the processed data by scaling and normalization did not improve the results), the analysis being faster with the same results. The proposed methodology could be considered as a promising tool with future applications in plant material investigations and other analytical fields.

Keywords: *Fuzzy clustering, chemometrics, high-performance liquid chromatography, antioxidant capacity, medicinal plants*

INTRODUCTION

Nature has always involved in human development providing the necessary means in order to live a healthy and careless live through natural resource such as fruits, vegetables or medicinal plants. Herbal medicine, as

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the name suggests, uses plants as a replacement for chemically synthesized drugs, because they are cheaper, less toxic and have no side effects. The medicinal plant's therapeutic properties are sustained by the bioactive compounds that are produced through different processes that take place in the plant's cells called secondary metabolites. Thanks to the so-called secondary metabolites represented by alkaloids, sterols, terpenes, flavonoids, tannins, glycosides, resins, volatile oils, etc., the alternative medicine started to play an important role in the treatment of diseases all over the world, mostly because the medicinal system in many underdeveloped countries is still inexistent. Due to the above-mentioned considerations, the World Health Organization (WHO) has developed a strategic plan to promote alternative medicine by publishing four volumes containing 118 monographs regarding medicinal plants. The main purpose of WHO is to train people to develop their monographs due to the diversity of the flora that is characteristic from one territory to another [1-12].

It is also well known that the medicinal plants act as radical scavengers of free radicals that appear in the human body through metabolism, pollution, contaminants and different medical treatments, factors that lead to the appearance of some serious diseases such as diabetes, cancer or neurological disorders. The composition and antioxidant activity of plant extracts has been determined using various spectrophotometric or chromatographic methods [3, 8, 13, 14]. However, high-performance liquid chromatography (HPLC) is recommended by WHO and European Medicine Agency (EMA) for analysis of plant-based samples (additives, toxins, residues or food adulteration) [15-21]. Using these methodologies, a large volume of data may result leading to a difficult or even impossible interpretation of the obtained results, therefore chemometric methods like principal component analysis (PCA), hierarchical cluster analysis (HCA) or linear discriminant analysis (LDA) are successfully employed [22-26].

Considering all the above, the aim of this study is to characterize and classify 42 hydroalcoholic extracts prepared from medicinal plants using the chromatographic profile obtained at 242, 260, 280, 320, 340 and 380 nm according to the antioxidant capacity obtained using the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) procedure.

RESULTS AND DISCUSSION

The 42 samples of the commercially available plant extracts from the Romanian flora subjected to a comprehensive holistic characterization and classification according to their antioxidant activity estimated by radical scavenging assay using the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) procedure and chromatographic profiles are presented in Table 1.

Table 1. Name and total radical scavenging capacity (RSC %) determined by DPPH$^{\bullet}$ assay of the investigated hydroalcoholic extracts

No.	Name	Scientific name	RSC* %	Antioxidant activity
1	Blueberry	*Vaccinium myrtillus*	46.71	High
2	Lingon berry	*Vaccinium vitis-idaea*	36.58	High
3	Rosemary	*Rosmarinus officinalis*	31.01	High
4	Hoary willowherb	*Epilobium parviflorum*	29.82	High
5	Lady's mantel	*Alchemilla vulgaris*	29.61	High
6	Quaking aspen	*Plopus nigra*	27.97	High
7	Lemon balm	*Melissa officinalis*	27.36	High
8	Sage	*Salvia officinalis*	27.36	High
9	Silver brich	*Betula pendula*	26.27	High
10	Saint John's wort	*Hypericum perforatum*	21.12	Moderate
11	Hawthorn	*Crataegus monogyma*	18.74	Moderate
12	Breckland thyme	*Thymus serpyllum*	15.48	Moderate
13	Burdock	*Arctium lappa*	13.98	Moderate
14	Great celandine	*Chelidonium majus*	12.86	Moderate
15	Lady's bedstraw	*Galium verum*	11.16	Moderate
16	Common juniper	*Juniperus communis*	10.13	Moderate
17	Yarrow	*Achillea millefolium*	9.45	Moderate
18	Spinycockle-bur	*Xanthium spinosum*	9.44	Moderate
19	Lavender	*Lavandula augustifolia*	8.93	Moderate
20	Artichoke	*Cynara scolymus*	7.42	Moderate
21	Liquorice	*Glycyrrhiza glabra*	4.93	Low
22	Gentian	*Gentiana asclepiadea*	4.46	Low
23	Echinacea	*Echinacea purpurea*	4.38	Low
24	Comfrey	*Symphytum officinale*	4.32	Low
25	Milk thistle	*Silybum marianum*	3.75	Low
26	Nettle	*Urtica dioica*	3.69	Low
27	Heart's ease	*Viola tricolor*	3.06	Low
28	Motherwort	*Leonurus cardiaca*	2.78	Low
29	Ginger	*Zingiber officinale*	2.26	Low
30	Valerian	*Valeriana officinalis*	2.09	Low
31	Shepherd's purse	*Capsella bursa-pastoris*	1.78	Low
32	Horsetail	*Equisetum arvense*	1.70	Low
33	Dill	*Anethum graveolens*	1.62	Low
34	Garlic	*Allium sativum*	1.45	Low
35	Mistletoe	*Viscum album*	1.20	Low
36	Elder	*Sambuctus nigra*	1.19	Low
37	Chili pepper	*Capsicum annuum*	1.05	Low
38	Sweet flag	*Acorus calamus*	1.00	Low
39	Hogweed	*Heracleum sphondylium*	0.68	Low
40	Wolf's-foot clubmoss	*Lycopodium clavatum*	0.37	Low
41	Celery	*Apium graveolens*	0.25	Low
42	Ramson	*Allium ursinum*	0.22	Low

The visual examination of the profile of antioxidant activities highlights two or three groups: high antioxidant activity (26.27-46.71%), group of samples 1-9, moderate antioxidant activity (7.42-21.12%), group of samples 10-20, and low antioxidant activity (0.22-4.93%), last group of samples 21-42.

Fuzzy divisive hierarchical clustering

The partitions obtained applying fuzzy divisive hierarchical clustering (FDHC), using the chromatographic data (without any preprocessing), are presented in Table 2. This fuzzy algorithm provides only two classes in the majority of cases: the first one includes the plant extracts with high and moderate antioxidant activity and the second one the plant samples with low antioxidant activity. In addition, the 2D-representation of the degrees of membership (DOMs) corresponding to the two fuzzy partitions (242 nm) presented in Fig. 1 shows that there are some "anomalies": samples 21 and 22 which belong to the class with low antioxidant activity are classified in the group with high antioxidant activity and samples 14, 16, 18 and 20 are classified wrong as well.

Table 2. The fuzzy clustering results obtained applying fuzzy divisive hierarchical clustering

Fuzzy partition history	242 nm		260 nm		280 nm		320 nm		340 nm		380 nm	
	Sample	DOM (range)	Sample	DOM (range)	Sample	DOM (range)	Sample	DOM (range)	Sample	DOM (range)	Sample	DOM (range)
A1	13, 19, 21, 6, 17, 4, 10, 5, 15, 11, 8, 2, 3, 1, 22, 9, 12, 7	0.503 - 0.714	11, 27, 21,6, 19, 2, 15, 4, 9, 22, 5, 1, 8, 3, 12, 7	0.506 – 0.707	19, 6, 17, 13, 9, 4, 5, 1, 3, 8, 12, 7	0.524 – 0.740	15, 6, 1, 13, 17, 3, 8, 12, 7	0.509 – 0.745	11, 9, 27, 15, 6, 17, 1, 13, 3, 12, 8, 7	0.506 – 0.732	21, 3, 15, 2, 27, 1, 9, 5	0.514 – 0.663
A2	33, 39, 14, 27, 16, 24, 23, 35, 32, 38, 26, 25, 29, 18, 41, 42, 40, 34, 37, 36, 30, 20, 31, 28	0.510 – 0.943	14, 39, 17, 13, 24, 33, 38, 35, 23, 26, 16, 10, 25, 29, 41, 32, 42, 34, 36, 37, 18, 40, 30, 20, 31, 28	0.505 – 0.945	14, 11, 21, 15, 2, 27, 22, 39, 23, 24, 18, 25, 26, 16, 29, 41, 10, 42, 35, 34, 37, 38, 40, 36, 30, 20, 33, 32, 31, 28	0.519 – 0.970	11, 39, 27, 9, 19, 21, 2, 23, 24, 5, 18, 26, 22, 14, 20, 4, 40, 16, 29, 30, 41, 42, 38, 34, 25, 10, 37, 36, 35, 33, 28, 32, 31	0.526 – 0.981	39, 2, 21, 22, 19, 23, 24, 5, 26, 18, 16, 14, 20, 29, 33, 30, 40, 41, 42, 34, 25, 36, 10, 35, 38, 4, 37, 28, 32, 31	0.574 – 0.971	6, 11, 8, 16, 25, 22, 17, 19, 12, 7, 14, 35, 4, 33, 39, 30, 10, 36, 13, 29, 41, 42, 34, 24, 38, 23, 37, 40, 20, 32, 18, 31, 26, 28	0.503 – 0.986

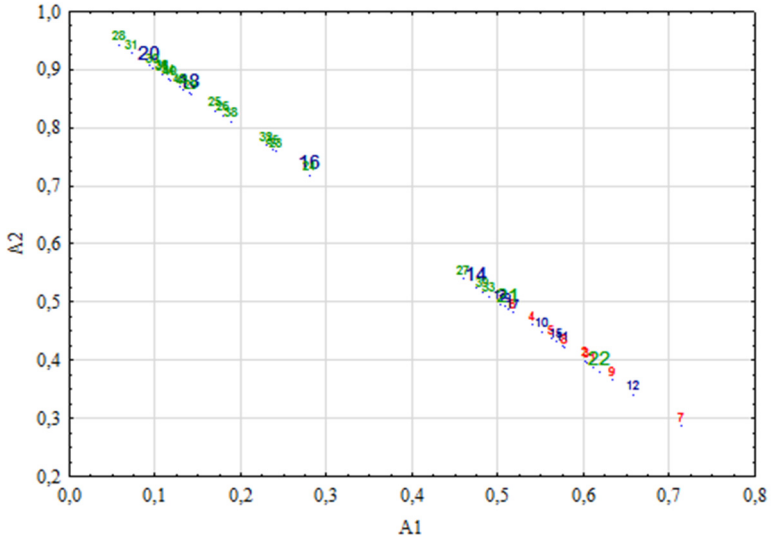

Figure 1. 2-D scatterplot of DOMs corresponding to the two hierarchical fuzzy partitions (A1 and A2)

Principal component analysis

The results obtained from PCA analysis using again the raw data matrix indicate a significant reduction in the number of variables (Fig. 2).

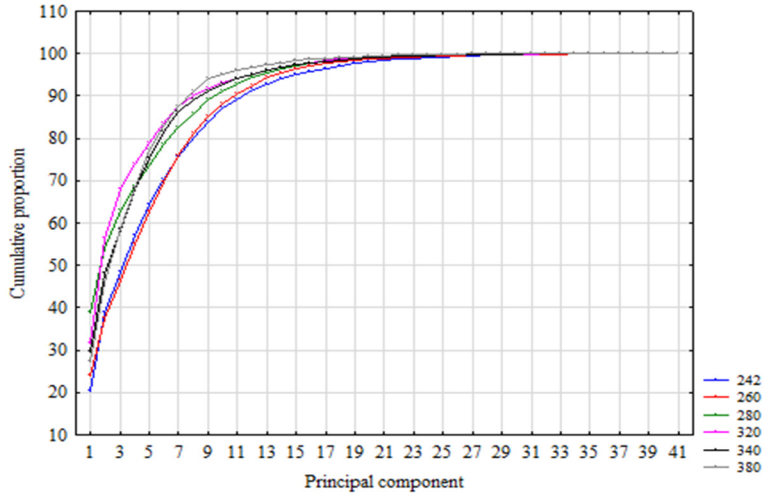

Figure 2. Cumulative proportion profile

In all cases, the first 41 PCs explain the total variance (100%) of the data. The variance corresponding to each PC1 is not so different, accounting, for example, more than 38% (the highest proportion) in the case of data corresponding to 280 nm detection wavelength and only 20% (the smallest proportion) for 242 nm (Table 3). However, surprisingly, the graphical representation of the samples using the first two components corresponding to data obtained at 242 nm, for example, indicates a satisfactory separation of samples according to the antioxidant activity (Fig. 3) in good agreement with the fuzzy clustering results discussed above.

Table 3. Proportion of the first five PC's obtained after PCA method was applied on the data obtained at 242, 260, 280, 320, 340, and 380 nm.

Proportion %						
PC's	242 nm	260 nm	280 nm	320 nm	340 nm	380 nm
PC1	20.33	23.81	38.72	31.45	29.67	27.33
PC2	18.50	13.59	15.40	24.87	18.28	18.66
PC3	9.58	9.07	8.96	11.54	10.50	12.62
PC4	8.73	8.55	5.63	5.94	9.52	9.58
PC5	7.16	7.76	4.93	5.11	7.25	8.89

The PC1 profile shows a similar contribution to the separation of the samples according to the antioxidant activity for 242, 260, 280 and 320 nm. It also can be seen a high contribution of PC1 to the separation of sample number 6 (quaking aspen) sample that in a PC1 vs PC2 representation appears as an outlier (Fig. 3). Besides, all the results obtained by applying PCA supports the idea of using the orthogonal and clean scores corresponding to the first 41 PCs in HCA and LDA classification of medicinal plant extracts according to their antioxidant activity.

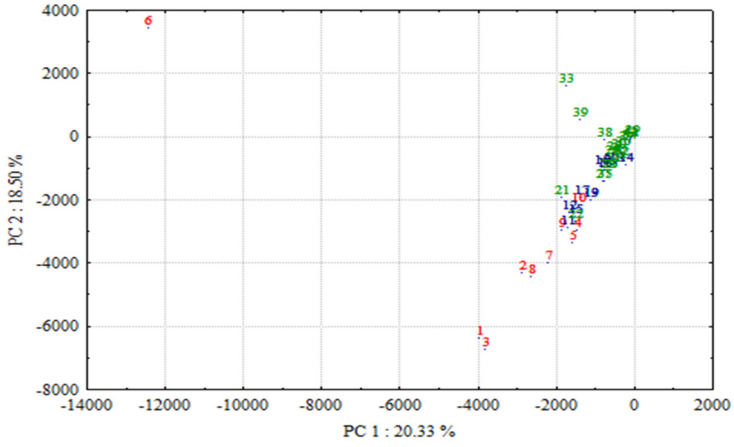

Figure 3. PC1-PC2 scatterplot for the score obtained at 242 nm

Hierarchical cluster analysis

The dendrogram (Fig. 4) was obtained applying the hierarchical cluster analysis (the Ward method as linkage method and Manhattan distance as a measure of similarity) to the data corresponding to the 41 PCs (242 nm), highlighting well defined groups of plant extracts in good agreement to all the results obtained by using fuzzy clustering method and also PCA. As it can be seen in Fig. 4 there are 2 major clusters: one contains the samples with high and moderate antioxidant capacity with few exceptions (samples 21 and 22), and the group of samples with low antioxidant capacity including the following exceptions, namely 6, 10, 16, 18, 19 and 20 samples. HCA shows samples that are the most similar, that is are the closest in the sense of having the lowest dissimilarity, the group with low antioxidant capacity containing more similar plants regarding the characteristic taken into consideration (antioxidant capacity).

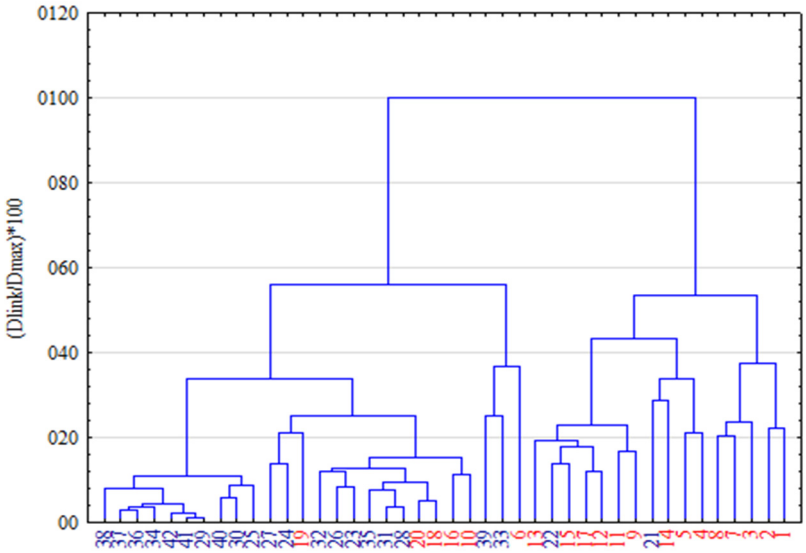

Figure 4. Dendrogram corresponding to the medicinal plant extracts obtained using the Ward method as linkage method and Manhattan distance as a measure of similarity

Linear discriminant analysis

The combination of PCA with LDA led to the most efficient discrimination of the investigated medicinal plants in two classes. The results obtained applying forward stepwise LDA to the first 41 PCs indicate a very good separation of the samples in almost all cases according to the correct classification rate of original

PC score data (Table 4): the highest value (100%) was obtained for raw data in the majority of cases (242, 260, 280, 320 and 340 nm) and the lowest value (78.6%) for the score data corresponding to the 380 nm. All of the above statements concerning the efficiency of this methodology are well supported by the values of quality performance features obtained by applying the leave-one-out (LOO) cross-validation approach. The results of the cross-validation presented also in Table 4 pointed out a correct classification rate in good agreement to the results obtained for the classification of the original PC score data: the highest value (100%) was obtained again for the PC score data corresponding to 254 nm and the lowest value (76.2%) for the score data obtained at 380 nm.

Table 4. Values of quality performance features from PCA-LDA approach applied to each wavelength (242, 260, 280, 320, 340, 380 nm) for medicinal plants classification according to their antioxidant capacity

Data	DAD wavelength detection											
	242 nm		260 nm		280 nm		320 nm		340 nm		380 nm	
	Original %	*LOO %	Original %	LOO %	Original %	LOO %	Original %	LOO %	Original %	LOO %	Original %	LOO %
[2]Raw data	100.0	100.0	100.0	95.2	100.0	95.2	100.0	97.6	100.0	92.9	78.6	76.2
[2]Normalized	88.1	85.7	100.0	97.6	100.0	97.6	95.2	90.5	85.7	78.6	92.9	85.7
[2]Autoscaled	100.0	97.6	100.0	90.5	100.0	95.2	95.2	90.5	100.0	100.0	85.7	85.7
[3]Raw data	92.9	90.5	85.7	81.0	83.3	76.2	85.7	78.6	85.7	76.2	69.0	64.3
[3]Normalized	71.4	57.1	88.1	81.0	78.6	66.7	85.7	73.8	72.9	60.3	73.8	73.8
[3]Autoscaled	88.1	81.0	92.9	81.0	83.3	76.2	76.2	64.3	85.7	76.2	90.5	78.6

*Leave-one-out validation; [2]two predefined classes; [3]three predefined classes

CONCLUSION

This study used the chromatographic data collected at different wavelengths (242, 260, 280, 320, 340, 380 nm) for characterization and classification of medicinal plants according to their antioxidant capacity. The best results, according to the multivariate methods employed are those obtained at 242 nm detection wavelength (wavelength where the majority of the antioxidant compounds present in the samples are detected). The FDHC including classical methods PCA, HCA and LDA separated the samples into well defined groups with few exceptions: *Glycyrrhiza glabra, Gentiana Asclepiades, Chelidonium majus*, *Juniperus communis*, *Xanthium spinosum*, and *Cynara scolymus.* The presence of these samples in the wrong class is made according to the concentration

and not by the type of compounds (compounds that are more or less similar) found in the medicinal plant extracts that have antioxidant activity. The leave-one-out cross-validation also suggested that the samples are divided into two main classes with a correct classification rate of 100% compared with 90.5% for three classes. Moreover, this study highlights that the data can be utilized without any preprocessing: the normalization and autoscaling didn't bring any improvements of the clustering results. Another important conclusion of this study is that the scores corresponding to the principal components that explained 100% of the variance (41 PCs) can be used instead of the initial variables (4501 variables), the same results being obtained, minimizing the speed and time of the analysis.

EXPERIMENTAL SECTION

Chemicals and plant samples

The reagents used in this study were of analytical grade, the HPLC grade ammonium acetate and acetonitrile were from Merck (Darmstadt, Germany) and the plant samples belong to Dacia Plant manufacturer (Brașov, România) (Table 1). A number of 42 plant samples commercially available were obtained, according to the manufacturer using different parts of plants and different water: ethanol ratios comprised within the range 35-80% ethanol. The label that accompanies the alcoholic extracts offers the necessary information regarding the quality of the vegetal material, used to obtain the final products, namely, the plants meet the highest standards of quality, and are procured from their own cultures or partner producers.

The HPLC protocol

The commercialized extracts were analyzed in order to separate the phytoconstituents using an HPLC-DAD approach. The device used to perform the separation is an Agilent 1200 HPLC system (Waldbronn, Germany) which is equipped with an on-line vacuum degasser, quaternary pump, temperature-controlled sample tray, automatic injector, a column thermostat compartment, and a DAD detector. The chromatographic column used to perform the separations was a Zorbax SB-C18 column (250 mm × 4.6 mm, 5 µm particle size) also from Agilent. The parameters used were: an injection volume of 30 µL (0.22 µm filtered extract), a column temperature of 30 °C and the flow rate of 1 mL/min. In order to optimize the method, several preliminary tests were employed using different experimental conditions. The final results were

obtained (maximum number of compounds separated and maximum resolution) using a multistep isocratic and gradient elution system: solvent A, 10 mM ammonium acetate pH 5 and as solvent B acetonitrile. The steps were as follows: 0-2 min isocratic at 5% B, 2-10 min from 5 to 35% B, 10-20 min from 35% to 45% B, 20-25 min from 45% to 95% B, 25-28 min from 95% to 100% B, 28-32 min isocratic at 100% B and 32-32.1 min back to 5% B where was kept until 35 min. The detection of the compounds in the UV-Vis range was performed using the DAD detector that measured the entire spectrum in 240-750 nm region (2 nm resolution), every 2 seconds and the chromatograms were monitored at 242, 260, 280, 320, 340 and 380 nm. The chromatograms and the mean spectra of the main chromatographic peaks were exported and analyzed using advanced chemometrics.

Antioxidant activity assay

The antioxidant capacity of the investigated samples was determined using the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) method. In order to perform the analysis, the extracts needed to be diluted at a dilution rate of 1:50 with a mixture of ethanol-water (60:40). The total radical scavenging capacity (RSC parameter expressed as a percent of consumed $DPPH^{\bullet}$ radical) of the hydroalcoholic extracts was measured at 518 nm (absorbance of $DPPH^{\bullet}$ solution) after 30 minutes, the required amount of time for the reaction between DPPH and samples to take place. The ratio between the investigated samples and the $DPPH^{\bullet}$ solution (150 μmol L^{-1} prepared in ethanol) was 0.1 mL: 3 mL. The $DPPH^{\bullet}$ solution was daily prepared and protected from light throughout the analysis time in order to minimize the free radical decomposition.

Chemometrics

Cluster analysis is a large field, both within fuzzy sets theory and beyond it. Clustering and classification are useful since they allow meaningful generalizations to be made about large quantities of data by recognizing among them a few basic patterns. In classical cluster analysis, each object must be assigned to exactly one cluster. This is a source of ambiguity and error in cases of outliers or overlapping clusters and allows a loss of information. This kind of vagueness and uncertainty can, however, be taken into account by using the *theory of fuzzy sets* introduced by Zadeh in 1965 [27, 28]. A *fuzzy set* or a fuzzy subset is a collection of ill-defined and not-distinct objects with un-sharp boundaries in which the transition from membership to non-membership in a subset of a reference set is gradual rather than abrupt. The theory of fuzzy set is basically a theory of graded concepts. A central concept in the fuzzy set theory is that it is permissible for an element to belong partly to a fuzzy set (partition). It provides

an adequate conceptual framework as well as a mathematical tool to model the real-world problems which are often obscure and indistinct, namely fuzzy [29-32].

HCA was extensively used to group experimental variables or samples into clusters, based on similarity within a class and dissimilarity between different classes, according to a predefined criterion. The most common clustering procedure is known to be Ward's method with Euclidean distance as a similarity measure.

LDA is a classification procedure (supervised technique), which needs an initial sample classification into predefined classes. The LDA model contains linear discriminant functions that can classify data, after a predefined criterion. The model is usually validated through leave-one-out classification, which means that each sample is tested, using the model, as an unknown sample. The higher the percentage of correct classification rate by cross-validation, the better the model is. Unlike the other two classical methods, which are clustering/ classification methods, PCA aims to transform the original variables to a new set of variables, uncorrelated and cleaned of noise, called principal components. The high variance of the data set is explained in many cases by the first principal components.

All the graphs and some chemometric methods were performed using Statistica 8.0 (StatSoft, Inc. 1984–2007, Tulsa, USA) software and leave-one-out cross-validation with IBM SPSS Statistics (International Business Machines Corp., New York, USA).

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