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Ecosystem discrimination and fingerprinting of Romanian propolis by hierarchical fuzzy clustering and image analysis of TLC patterns

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ABSTRACT

The fingerprinting capacity of thin layer chromatography (TLC) and image analysis in the case of propolis samples collected in different area in Romania has been investigated. Fuzzy divisive hierarchical clustering approach was used as a powerful tool of samples discrimination and fingerprinting according to the geographical origin and local flora. The fuzzy partition and patterns obtained by membership degrees plot were in a very good agreement with floral origin and geographic location of Romanian propolis samples, and clearly illustrate the fuzziness concerning their similarities and difference. The results obtained strongly support that TLC via image analysis can be successfully employed in the fingerprinting methodologies if they are combined with appropriate fuzzy clustering method. The method developed in this paper might be also extended in the authenticity and origin control of fruits, herbs or derived products.

1. Introduction

Propolis (sometimes called "bee glue") is the super-sticky, gooey material gathered by bees from the buds, leafy stalks and young twigs of certain trees. Propolis comes from the sap or juice secreted by trees which fights their own infection and disease and heals cuts. Propolis is usually chestnut or greenish-brown in color. It gives off a pleasant aroma of poplar buds, honey and vanilla. The bees use propolis to seal cracks and varnish the inside walls of the hive stabilizing the inner temperature around 35 °C, strengthening the combs but its major role is in defending their community as a "chemical weapon" against intruders mummifying them after they were killed. The hive is kept aseptic and clean mainly due to antibacterial, antifungal, antitrypanosomal, antimicrobial activities of propolis [1–3]. Several studies proved other important biological activities such as antioxidant [4,5], anti-inflammatory [6,7], antihepatotoxic [8] or beneficial effects in dental care [9], gastric ulcer [10], cartilage protection [11] and immune system [12]. Some of these biological activities were known from centuries all over the world, and is still used in folk medicine [13] but also in modern medicine [14] as ingredient in commercial products such as vitamin C, toothpaste, medicinal syrups, creams and so on.

The aspect, texture and chemical composition of propolis is very variable and depends upon the climate, season and mainly upon the local flora which is exploited by the bees, thus on the geographic characteristics of the region [15–19]. Propolis contains approximately 50% resins and balms, 30% wax, 10% etheric oils and 5% pollen, 5% other organic substances, including amino acids, trace elements (iron, copper, manganese, zinc). It has high vitamin content, especially the valuable bioflavonoids [20,21]. The results showed, for example, that propolis from Europe and Brazil had similar activity despite the drastic differences in chemical composition.

The development of a suitable analytical procedure to separate and evaluate all the constituents of propolis is practically impossible to realize and time waste. The global assessment of the propolis samples is recommended, instead of focusing on individual compounds. Such a possibility is offered by the fingerprinting methods, which are in fact emphasizing and comprehensive characterizing the analyzed sample [22]. The fingerprinting method was first introduced for the characterization of the herbal medicines and extended to other types of vegetal materials. Even more, it is widely involved in the authenticity and origin control of fruits, herbs or derived products [23]. The Food and Drug Administration [24] and the European Medicines Agency [25] recommend that the appropriate fingerprinting procedure involves chromatographic techniques. The accepted chromatographic techniques are including thin layer chromatography (TLC) [26], high performance liquid chromatography (HPLC) [27], gas chromatography (GC) [28], highly speed counter current chromatography (HSCCC) [29] and so on. The spectroscopic techniques may also offer important infor-

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Table 1
Propolis samples code, location and description.

Types of propolis	Sample group codes	Number of samples	Origin place of samples (place, county)	Origin coordinates	Flora type	Color	Texture
T1	T11	4	Nadaselu, Cluj	46°50′N/23°27′E	Meadow, low content mixture of deciduous forest	Brown	Rigid powder
	T12	3	Sarmasu, Mures	46°45N/24°12′E		Brown	Rigid powder
	T13	4	Gledin, Bistrita-Nasaud	46°57′N/24°33′E		Yellowish brown	Waxy powder
T2	T21	1	Pestera, Constanta	44°11′N/28°07′E	Grassy meadow	Brownish yellow	Waxy
	T22	2	Livada, Arad	46°13′N/21°23′E		Yellowish brown	Waxy
	T23	1	Sanleani, Arad	46°12′N/21°23′E		Dark greenish brown	Rigid waxy
	T24	2	Carei, Satu-Mare	47°40′N/22°25′E		Brownish yellow	Rigid waxy
T3	T31	2	Cristian, Sibiu	45°48′N/24°02′E	Meadow, high content of mixture of deciduous forests	Brown	Waxy
	T32	4	Orlat, Sibiu	45°45′N/23°57′E		Brown	Waxy
T4	T41	2	Brebi, Salaj	47°13′N/23°10′E	Mixture of deciduous and resinous forests	Reddish brown	Rigid
	T42	2	Romuli, Bistrita-Nasaud	47°32′N/24°24′E		Reddish brown	Rigid powder
	T43	2	Fagaras, Brasov	45°47′N/24°58′E		Brown	Rigid waxy
T5	T51	7	Romuli, Bistrita-Nasaud	47°32′N/24°25′E	Mixture of deciduous forests	Light reddish brown	Rigid waxy
	T52	2	Gura Raului, Sibiu	45°43′N/23°58′E		Light brown	Rigid waxy
T6	T6	1	Pastoral[a]	Pastoral	Complex	Dark brown	Rigid waxy

[a] Pastoral-refers to the fact that the beehive was moved to various places during a season.

mation that may be used to discrimination and fingerprinting of samples [30–35].

The main goal of this study has been focused on the development of an effective, simple and fast method for the discrimination and authentication of various samples of propolis based on fuzzy clustering of TLC data via image analysis. The results obtained are in very good agreement with the geographical origin and local flora of the Romanian propolis and illustrate the fuzziness concerning the similarities and difference of the propolis samples. It has been clearly demonstrated that the fuzzy clustering may be also considered a more illuminating and competitive strategy in the fingerprinting approaches.

2. Experimental

2.1. Sampling and sample preparation

Thirty-nine propolis samples originating from several geographical and floral places of Romania were collected by professional beekeepers with assistance from a specialized chemist and then deposited at −20 °C. Details on the origin, color and texture of the studied propolis samples are listed in Table 1. The raw propolis was homogenized using a grinding mortar and a pestle as soon as the samples were removed from the freezer, while the propolis was still brittle.

2.2. Extraction and thin-layer chromatography

An accurately weighted sample of grinded powder was introduced into the flask and a precise volume of ethanol 95% (v/v) was added so that the final concentration of each sample to be 200 mg solid sample/mL solvent. The suspension was ultrasonicated for 1.5 h and then incubated for 48 h at 37 °C in a shaker at 200 rpm. Finally, the samples were centrifuged at 1600 rpm for 1 h. The clear supernatant was collected and used for further analysis. The sample solutions were applied bandwise by means of a Camag Linomat 5 semiautomatic applicator (CAMAG, Muttenz, Switzerland) on HP-TLC silica gel 60 pre-coated plates 10 cm × 10 cm (Merck, Germany). The application conditions were: carrier gas, air; syringe delivery speed 50 nL/s; application volume 0.5 μL; bandwidth 5 mm; distance from bottom 10 mm. The mobile phase consisted of toluene–ethyl acetate–formic acid (30:12:5, v/v/v), each of them of analytical grade purity, was added into a chromatographic chamber to saturated it for 15 min. The plate in the chamber was developed upward over a path of 8 cm and sprayed after drying with methanolic solution of 0.2% diphenylboryloxyethylamine (NTS) and 4% polyetheleneglycol. The fluorescent images were examined under UV 366 nm by using a UV viewer cabinet. They were captured with a camera and processed using TLC Analyzer software [36,37].

3. Hierarchical fuzzy clustering

In general, a fuzzy divisive hierarchical clustering algorithm with objective function can be formulated as follows [38–42]: let $X = \{x^1, \ldots, x^n\} \subset \mathbf{R}^p$ be a finite set of feature vectors, where n is the number of objects (samples) and p is the number of the original variables, $x^j = [x_1{}^j, x_2{}^j, \ldots, x_p{}^j]^T$ and $L = (L^1, L^2, \ldots, L^c)$ be a c-tuple of prototypes (supports) each of which characterizes one of the c clusters composing the cluster substructure of the data set; a partition of X into c fuzzy clusters will be performed by minimizing the objective function:

$$J(P, L) = \sum_{i=1}^{c}\sum_{j=1}^{n}(A_i(x^j))^2 d^2(x^j, L^i) \quad (1)$$

where $P = \{A_1, \ldots, A_c\}$ is the fuzzy partition, $A_i(x^j) \in [0,1]$ represents the membership degree of feature point x^j to cluster A_i, $d(x^j, L^i)$ is the distance from a feature point x^j to the prototype of cluster A_i, defined by the Euclidean distance norm:

$$d(x^j, L^i) = \left\| x^j - L^i \right\| = \left[\sum_{k=1}^{p} (x_k{}^j - L^i)^2 \right]^{1/2} \quad (2)$$

The optimal fuzzy set will be determined by using an iterative method where J is successively minimized with respect to A and L.

Supposing that L is given, the minimum of the function $J(\cdot,L)$ is obtained for:

$$A_i(x^j) = \frac{C(x^j)}{\sum_{k=1}^{c} (d^2(x^j, L^i))/(d^2(x^j, L^k))}, \; i = 1, \ldots, c \quad (3)$$

where C is a fuzzy set from X and

$$C(x^j) = \sum_{i=1}^{c} A_i(x^j) \quad (4)$$

It is easy to observe that $C(x^j) \leq 1$, $j = 1, 2, \ldots, n$.

For a given P, the minimum of the function $J(P,\cdot)$ is obtained for:

$$L^i = \frac{\sum_{j=1}^{n} \left[A_i(x^j)\right]^2 x^j}{\sum_{j=1}^{n} \left[A_i(x^j)\right]^2}, \; i = 1, \ldots, c \quad (5)$$

The above formula allows one to compute each of the p components of L^i (the center of the cluster i). Elements with a high degree of membership in cluster i (i.e. close to cluster i's center) will contribute significantly to this weighted average, while elements with a low degree of membership (far from the center) will contribute almost nothing.

4. Results and discussion

According with their floral origin and location, the propolis samples might be assigned to five different types according to the vegetation zone (Table 1). By carefully visual examination of the chromatograms depicted in Fig. 1 it appears clearly that there is a significant difference in chemical composition of the samples according to their geographical origin and local flora. Most of the samples originated from forest regions (T4, T5), for example, do not present the orange bands while all the samples from area with meadows (T1, T2, T3), present the three orange bands. So, it might have concluded that the samples from forests and the samples from meadows forms two distinct groups. However, the (dis-)similarity level of the analyzed propolis samples is impossible to be comprehended by a simple visual screening. In addition, since propolis extracts are very complex mixtures, the chromatographic resolution will be more or less altered. This inconvenient can be overcome by exploit the RGB channels because some compounds have different fluorescent colors (Fig. 2). This is an efficient facility available in the image analysis software packages [36,37].

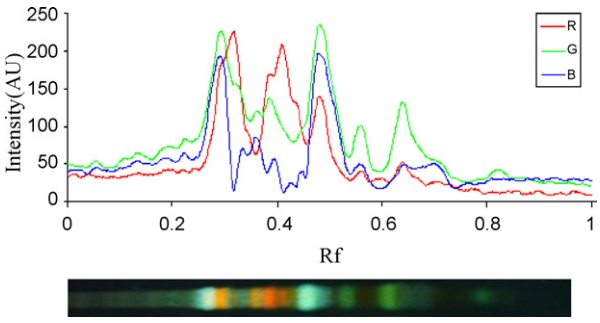

Fig. 2. A chromatogram of a propolis extract in all three channels. Samples which are not separated enough but have different color can been evaluated using a different color channel as is the case of compounds 1 and 2.

The results obtained applying hierarchical fuzzy cluster analysis to the digitized data of TLC fluorescent chromatograms support the reliability of this technique. Hierarchical fuzzy clustering analysis was applied on the matrix resulted from the digitized chromatograms (39 samples × 1000 variables) corresponding to each channel (Fig. 2) after standardization and offsetting of R_F values, i.e. the intensity of each chromatogram was adjusted between 0 and 1, and the R_F of the common compound was fixed to the same value in all chromatograms. The membership degrees to the final fuzzy partition corresponding to each channel are presented in Table 2. The hard partition corresponding to the fuzzy successive partition of the 39 propolis samples produced by using fuzzy divisive hierarchical clustering are presented in Figs. 3–5. The hard partition is obtained by defuzzification of fuzzy partition, namely by assigning the sample to the fuzzy cluster with the higher membership degree. Comparing the partitions in Figs. 3–5 and the membership degrees to the final fuzzy partition obtained in all three cases, one can observe that the best results are obtained by using R and B channels, respectively, i.e. the partitions obtained are in a very good agreement with the nature of samples and their assignment in Table 1.

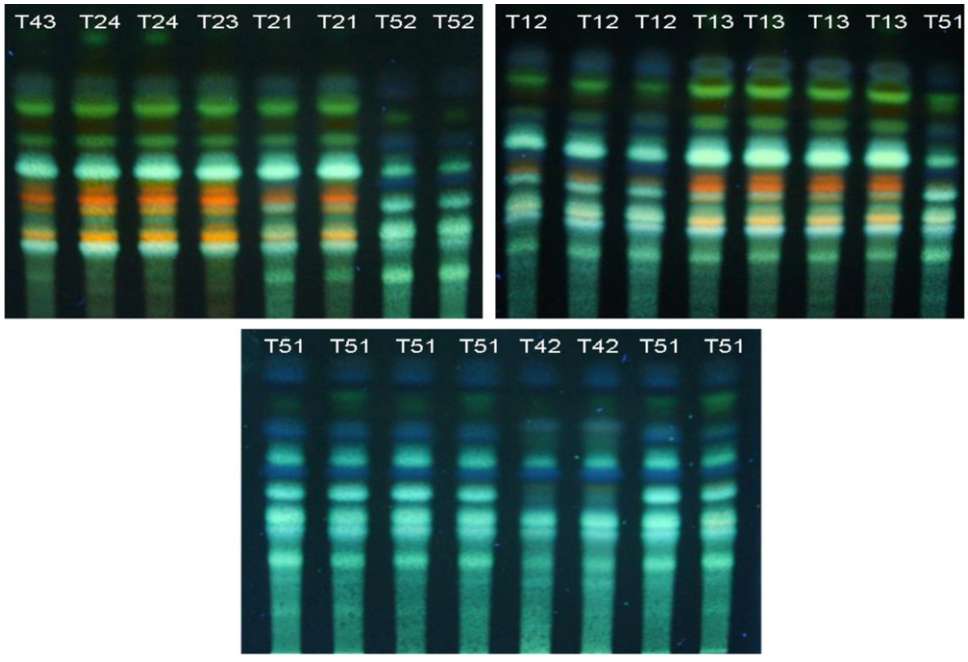

Fig. 1. Chromatographic plates exposed at 366 nm after they were sprayed with NTS. Each lane has been marked with a code depending on which type of propolis they belong to (see Table 1).

Table 2
Membership degrees of the propolis samples to the final fuzzy partition corresponding to the three channels.

No	Code	Location	R-channel				G-channel				B-channel				
			A_{11}	A_{12}	A_{21}	A_{22}	A_{11}	A_{12}	A_{21}	A_{22}	A_{111}	A_{112}	A_{12}	A_{21}	A_{22}
1	T24-1	Carei 1	**0.675**	0.175	0.073	0.078	**0.732**	0.169	0.048	0.051	0.055	0.061	**0.700**	0.099	0.086
2	T24-2	Carei 2	**0.744**	0.125	0.064	0.066	**0.756**	0.134	0.054	0.056	0.057	0.064	**0.687**	0.101	0.092
3	T23-1	Sanleani	**0.673**	0.159	0.083	0.085	**0.718**	0.153	0.063	0.066	0.053	0.065	**0.687**	0.102	0.093
4	T22-1	Livada 1	**0.580**	0.257	0.080	0.083	**0.446**	0.348	0.101	0.105	0.198	0.132	**0.506**	0.084	0.080
5	T22-2	Livada 2	**0.723**	0.147	0.064	0.066	**0.730**	0.168	0.050	0.053	0.073	0.077	**0.707**	0.074	0.069
6	T21-1	Pestera	**0.468**	0.414	0.059	0.058	**0.544**	0.335	0.061	0.060	0.084	0.121	**0.646**	0.081	0.067
7	T13-1	Monor Gledin 1	0.366	**0.464**	0.088	0.083	0.257	**0.584**	0.077	0.082	0.110	**0.553**	0.145	0.099	0.093
8	T13-2	Monor Gledin 2	**0.423**	0.415	0.082	0.079	0.283	**0.572**	0.070	0.075	0.094	**0.557**	0.172	0.091	0.086
9	T13-3	Monor Gledin 3	**0.504**	0.382	0.058	0.056	0.411	**0.508**	0.039	0.042	0.062	**0.622**	0.210	0.056	0.051
10	T13-4	Monor Gledin 4	**0.589**	0.298	0.057	0.056	**0.630**	0.272	0.047	0.051	0.134	**0.423**	0.338	0.054	0.052
11	T12-1	Sarmas 1	0.190	**0.679**	0.067	0.063	0.251	**0.588**	0.081	0.080	**0.527**	0.119	0.152	0.104	0.098
12	T12-2	Sarmas 2	0.131	**0.655**	0.110	0.104	0.148	**0.580**	0.133	0.139	**0.353**	0.160	0.091	0.204	0.192
13	T12-3	Sarmas 3	0.130	**0.598**	0.138	0.134	0.177	**0.636**	0.086	0.101	**0.309**	0.197	0.126	0.173	0.195
14	T11-1	Nadaseni 1	0.217	**0.486**	0.137	0.160	0.161	**0.339**	0.252	0.249	0.204	0.124	0.103	**0.289**	0.280
15	T11-2	Nadaseni 2	0.314	**0.384**	0.151	0.152	**0.480**	0.372	0.071	0.077	**0.281**	0.238	0.292	0.096	0.093
16	T11-3	Nadaseni 3	0.359	**0.393**	0.116	0.132	0.212	**0.556**	0.108	0.124	**0.500**	0.138	0.140	0.114	0.108
17	T11-4	Nadaseni 4	0.252	**0.356**	0.195	0.197	0.284	**0.489**	0.113	0.114	**0.458**	0.116	0.167	0.133	0.125
18	T31-1	Cristian1	0.213	**0.315**	0.264	0.209	0.277	**0.424**	0.149	0.150	0.165	**0.190**	0.156	0.261	0.228
19	T31-2	Cristian2	0.216	**0.309**	0.262	0.213	0.317	**0.395**	0.142	0.147	0.173	**0.199**	0.172	0.240	0.216
20	T32-1	Orlat1	0.074	0.107	**0.542**	0.277	0.059	0.084	**0.499**	0.358	0.086	0.067	0.096	**0.410**	0.341
21	T32-2	Orlat2	0.066	0.107	**0.618**	0.210	0.066	0.098	**0.457**	0.380	0.094	0.070	0.089	0.367	**0.380**
22	T32-3	Orlat3	0.075	0.129	**0.580**	0.216	0.160	0.254	**0.311**	0.275	0.144	0.105	0.116	**0.329**	0.305
23	T32-4	Orlat4	0.038	0.063	**0.575**	0.324	0.043	0.070	0.370	**0.517**	0.070	0.048	0.056	0.370	**0.456**
24	T42-1	Magura 1	0.076	0.102	0.360	**0.462**	0.074	0.093	**0.466**	0.367	0.072	0.047	0.081	**0.442**	0.358
25	T42-2	Magura 2	0.063	0.092	0.411	**0.434**	0.054	0.076	**0.523**	0.347	0.062	0.043	0.063	**0.459**	0.374
26	T43-1	Fagaras1	0.232	**0.300**	0.255	0.213	**0.437**	0.261	0.154	0.148	0.107	0.119	**0.476**	0.160	0.138
27	T43-2	Fagaras2	0.171	0.255	**0.330**	0.243	0.261	**0.276**	0.252	0.211	0.134	0.137	**0.366**	0.207	0.155
28	T41-1	Brebi 1	0.067	0.106	**0.616**	0.211	0.062	0.095	**0.648**	0.194	0.072	0.044	0.061	**0.676**	0.147
29	T41-2	Brebi 2	0.070	0.106	**0.569**	0.255	0.050	0.074	**0.653**	0.223	0.054	0.034	0.048	**0.692**	0.172
30	T51-1	Sat Romuli 25.06.08	0.075	0.102	0.209	**0.613**	0.039	0.056	0.299	**0.606**	0.052	0.034	0.050	0.246	**0.618**
31	T51-2	Sat Romuli 20.07.08	0.087	0.124	0.191	**0.598**	0.055	0.076	0.162	**0.707**	0.063	0.037	0.056	0.172	**0.672**
32	T51-3	Sat Romuli 01.08.08	0.102	0.146	0.183	**0.569**	0.060	0.087	0.170	**0.683**	0.058	0.035	0.048	0.143	**0.716**
33	T51-4	Sat Romuli 07.09.08	0.088	0.122	0.173	**0.617**	0.062	0.088	0.154	**0.696**	0.064	0.039	0.056	0.168	**0.674**
34	T51-5	Romuli 1	0.072	0.113	0.171	**0.644**	0.071	0.109	0.191	**0.630**	0.068	0.046	0.059	0.209	**0.616**
35	T51-6	Romuli 2	0.140	0.218	**0.328**	0.314	**0.286**	0.276	0.199	0.238	0.149	0.123	0.204	0.254	**0.270**
36	T51-7	Romuli 3	0.081	0.116	**0.421**	0.382	0.054	0.074	**0.518**	0.354	0.066	0.045	0.072	**0.463**	0.353
37	T52-1	Gura Raului 2	0.046	0.073	**0.473**	0.408	0.035	0.050	**0.561**	0.355	0.045	0.027	0.042	**0.509**	0.377
38	T52-1	Gura Raului 1	0.067	0.099	0.392	**0.441**	0.052	0.073	**0.575**	0.300	0.067	0.043	0.074	**0.493**	0.322
39		Pastoral	0.077	0.110	**0.561**	0.252	0.077	0.099	**0.602**	0.222	0.084	0.059	0.112	**0.513**	0.231

The maximum values are bolded.

For instance, samples collected within four months (30–33) from the same sampling point have very close membership degrees. Generally, the samples have been assigned to two large groups in a good agreement with their vegetal sampling location: samples originating from meadows area and samples originating from predominant forest area. Within the first group, in the case of B channel, three subgroups were found according to the spreading and variety of the meadow, while within the second group two subgroups were

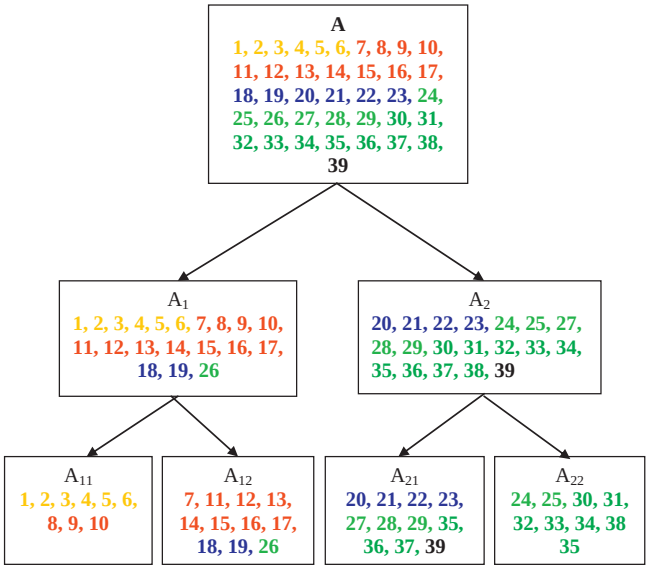

Fig. 3. The hard partition corresponding to the fuzzy hierarchical clustering of the 39 propolis samples (R channel).

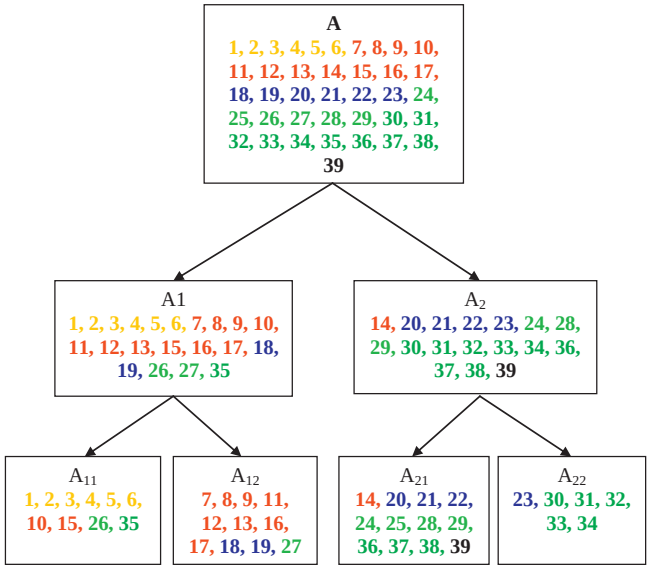

Fig. 4. The hard partition corresponding to the fuzzy hierarchical clustering of the 39 propolis samples (G channel).

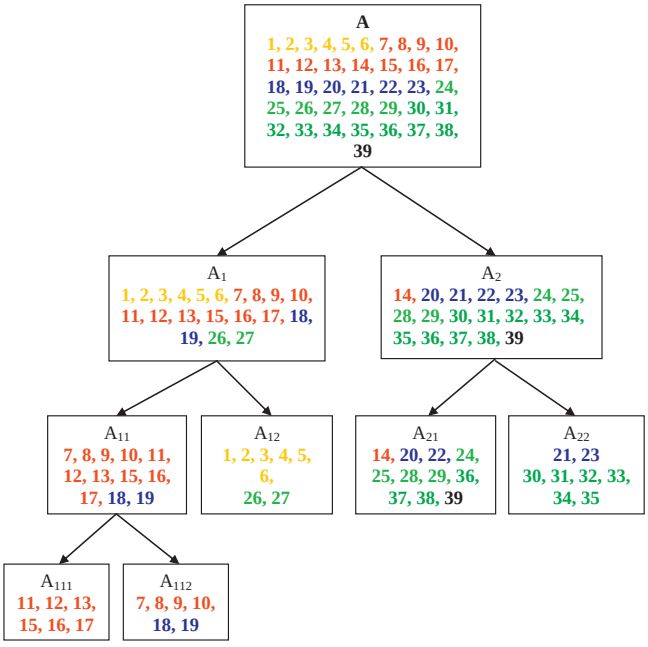

Fig. 5. The hard partition corresponding to the fuzzy hierarchical clustering of the 39 propolis samples (B channel).

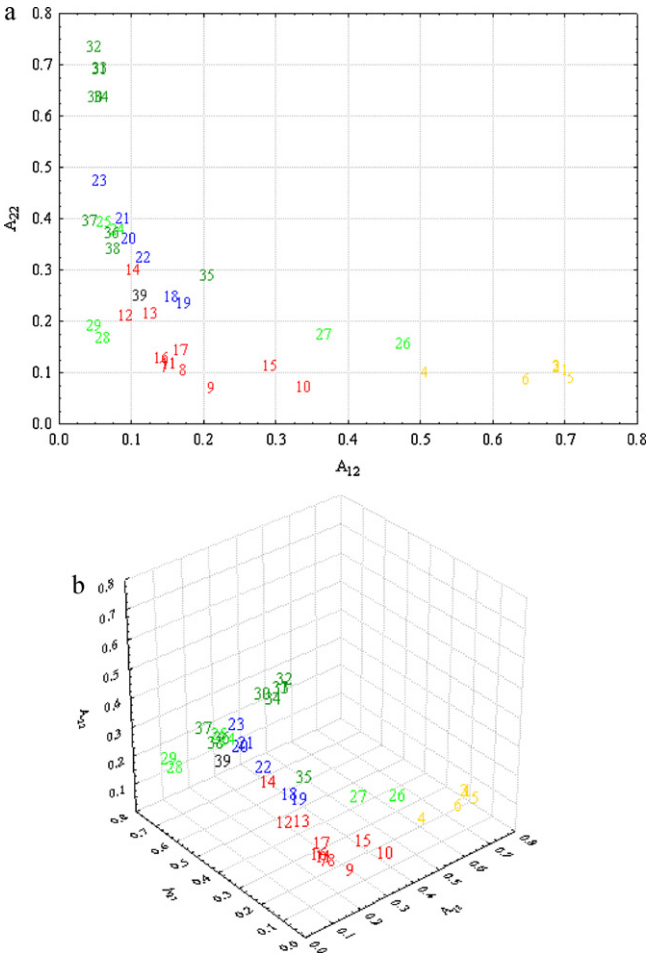

Fig. 6. Projection of the 39 propolis samples (B channel): (a) on the plane defined by the membership degrees to the fuzzy class A_{12} and A_{22}; (b) in the space defined by the membership degrees to the fuzzy class A_{12}, A_{21} and A_{22}.

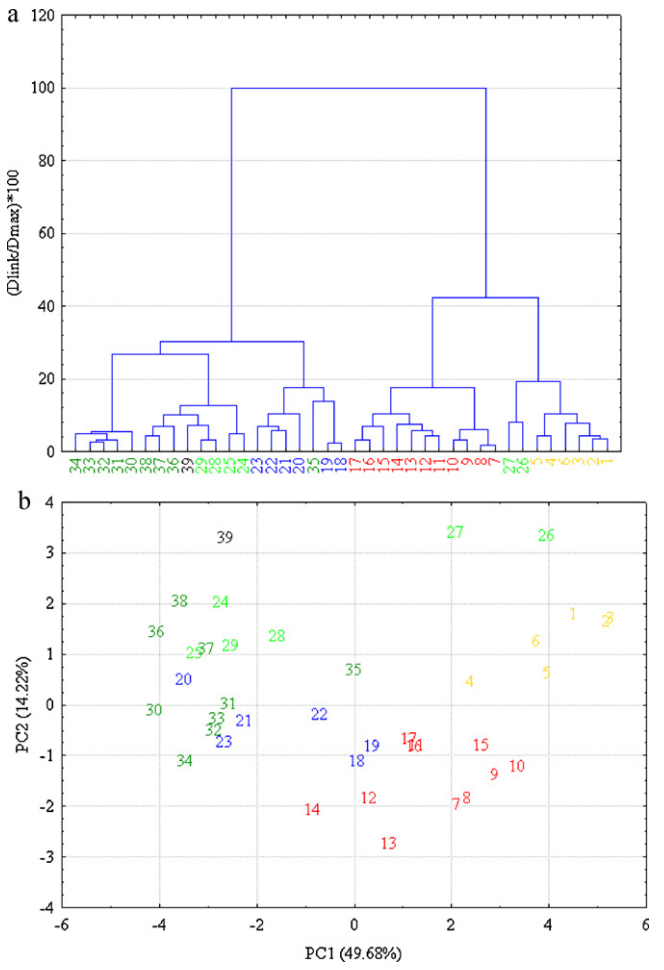

Fig. 7. Dendrogram obtained using Ward's linkage (a) and PC1–PC2 scatterplot (b) corresponding to the 39 propolis samples (B channel).

identified according to the dominant type of the forest, deciduous or resinous. The statements above and the fuzziness of samples are also well illustrated by two and three dimensional representations of the membership degrees in Fig. 6a and b. In addition, all the statements above concerning the informative powerful of TLC image analysis and the efficiency of hierarchical fuzzy clustering are also supported in a good way by the results obtained by applying classical multivariate methods (cluster analysis and principal component analysis) but the fuzziness character of samples is completely lost (Fig. 7a and b).

5. Conclusions

Fuzzy divisive hierarchical clustering and fingerprinting of the propolis samples based on the image analysis of TLC chromatograms allowed an objective interpretation of their similarities and difference considering the membership degree of each sample to the all fuzzy partitions. The results obtained are in a very a good agreement with the floral origin and vegetation zone. Two large types of propolis have clearly been identified, those samples originating from meadows area and samples originating from predominant forest area. Within the first group, three subgroups were found according to the spreading and variety of the meadow, while within the second group two subgroups were identified according to the dominant type of the forest, deciduous or resinous. The method developed in this paper might be also extended in the authenticity and origin control of fruits, herbs or derived products.

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