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Low-parachor solvents extraction and thermostated micro-thin-layer chromatography separation for fast screening and classification of spirulina from pharmaceutical formulations and food samples

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ABSTRACT

The goal of this paper is to demonstrate the separation and detection capability of eco-friendly micro-TLC technique for the classification of spirulina and selected herbs from pharmaceutical and food products. Target compounds were extracted using relatively low-parachor liquids. A number of the spirulina samples which originated from pharmaceutical formulations and food products, were isolated using a simple one step extraction with small volume of methanol, acetone or tetrahydrofuran. Herb samples rich in chlorophyll dyes were analyzed as reference materials. Quantitative data derived from micro-plates under visible light conditions and after iodine staining were explored using chemometrics tools including cluster analysis and principal components analysis. Using this method we could easily distinguish genuine spirulina and non-spirulina samples as well as fresh from expired commercial products and furthermore, we could identify some biodegradation peaks appearing on micro-TLC profiles. This methodology can be applied as a fast screening or fingerprinting tool for the classification of genuine spirulina and herb samples and in particular may be used commercially for the rapid quality control screening of products. Furthermore, this approach allows low-cost fractionation of target substances including cyanobacteria pigments in raw biological or environmental samples for preliminary chemotaxonomic investigations. Due to the low consumption of the mobile phase (usually less than 1 mL per run), this method can be considered as environmentally friendly analytical tool, which may be an alternative for fingerprinting protocols based on HPLC machines and simple separation systems involving planar micro-fluidic or micro-chip devices.

1. Introduction

High-resolution metabolomic approach, involving gas (GC), liquid (HPLC) or electrophoretic (CE) separation followed by mass spectrometry (MS) detection, is the most commonly applied methodology for the characterization of biological and environmental samples [1–3]. However, a number of complex problems can be effectively resolved using relatively low-resolution analytical methods. For that reason, simple separation protocols involving *e.g.* micro-chip or micro-fluidic paper-based devices are extensively developed [4–6]. Such methods allow fast pre-separation, detection and quantification of complex samples, without sample processing *via* classical gas or liquid chromatographic techniques. In comparison to micro-chip or micro-fluidic devices, a planar chromatography (TLC) technique exhibits a number of advantages. This is mainly due to simple and inexpensive equipment needed for the technique and parallel sample processing. In many cases a single use of a TLC plate allows fast screening of raw materials without time-consuming sample pre-purification [7–10]. One of the advantages of planar chromatography over its column counterpart is that each TLC run can be performed using a non-previously used stationary phase. Therefore, it is possible to fractionate or separate complex samples characterized by heavy biological or environmental matrix loading. In the case of column chromatography including GC and HPLC such samples must be accurately pre-purified and usually diluted. Particularly, all substances that are strongly adsorbed by the stationary phase must be carefully removed to increase the life of the column.

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To extend the separation power of the classical TLC plate, a high-performance thin-layer chromatography (HPTLC) and/or two-dimensional developing mode can be easily selected. In practice the typical separation capacity of non-forced flow rate HPTLC system lies between 10 and 20 spots per plate measured in one direction. However, working under 2D-TLC mode the number of spots separated can be significantly increased even by one order of magnitude [9,11,12]. Our experimental data have revealed that the 2D developing protocol involving micro-HPTLC plate is capable of separating more than 240 spots consisting of low molecular mass compounds like steroids or herb extract components [12,13]. Noteworthy, under proper experimental conditions, in which the heat evolved during the solvent adsorption and the mobile phase "distillation process" is minimized, micro-planar chromatographic systems can be very suitable for separation at elevated and sub-ambient temperatures [12,13]. This is mainly because of the low flow rate and small amount of the mobile phase that is necessary to perform the separation process. Moreover, there is no Joule's heat evolved due to the electric current flow as in classical planar electrophoresis systems. Depending on the plate size, solvent viscosity and temperature, the results of HPTLC separation can be obtained within a short period of time of less than 5 min [13].

It is noteworthy to say that modern high-performance planar chromatography is an excellent illustration of how an old technique may evolve due to the development of new sensitive detectors and acquisition data methods in the last decade [14–16]. Most recently, a number of new detection methods involving mass spectrometry techniques were introduced and extensively utilized [17–20]. Such MS-based sophisticated analytical tools including matrix-assisted laser desorption/ionization mass spectrometry (TLC–MALDI-MS), electron impact ionization mass spectrometry (TLC–EI-MS) or desorption electrospray ionization mass spectrometry (DESI-MS) were successfully applied for the analysis of complex biological samples allowing the use of planar chromatography in metabolomic studies [21–25].

Growing demand for the development of environmentally friendly analytical protocols usually results with miniaturization of the existing and well established methods. In the case of liquid chromatography, modern micro-separation systems may work with a low volume mobile phase, of less than 1 mL. Particularly, in comparison to high-performance liquid chromatography (HPLC) or extreme high-pressure liquid chromatography (X-LC) involving typical analytical columns and eluent flow rate ranging from 1 to 10 mL/min, the miniaturized systems such as microbore columns and/or nano-LC technique, may deal with an eluent flow at μL or even nL/min volumes, respectively [25,26]. Moreover, to reduce increasing consumption of the organic liquids characterized by high elution power, like tetrahydrofuran or acetonitrile, a number of different protocols based on the isocratic type separations with water only or water/organic binary eluents have been developed [27–31]. In analytical practice, a modern high-performance thin-layer chromatography (HPTLC) involving reversed phase (RP) plate is particularly suitable for the method of miniaturization. Using modern HPTLC plates, the mobile phase developing distance can be reduced to less than 50 mm, which is well documented in the literature [32–35]. Generally, this conclusion is based on the observation that the minimum values of the plate height (H) can be achieved if the solvent migration distance along the HPTLC plate ranges from 30 to 40 mm [11,33]. Under such conditions total analysis time can be dramatically reduced in comparison to chromatographic separations performed on typical 10 or 20 cm long TLC plates. Moreover, it allows the miniaturized planar chromatographic devices to work with μL volumes of the mobile phase to complete the whole analytical run [12,13,34–36].

Spirulina belongs to a group of cyanobacteria organisms that has attracted worldwide attention due to their utilization as human and animal nutritional protein sources [37,38]. Spirulina is easy to harvest and is the only cyanobacteria to be grown on an industrial scale [39,40]. Such organisms contain more than 55% of crude protein on a dry weight basis. This species is also rich in microelements and antioxidants like carotenoids or vitamins, and, therefore, many medical uses of spirulina have been described. Particularly, this can be used as a complementary diet for patients suffering from malnutrition, allergy, glycemia, and hyperlipidemia [41–44]. A number of spirulina low-molecular mass metabolites, especially a wide range of colored substances, including carotenoids, chlorophyll, and phycobiliproteins, can be used as safe and ecological friendly food and cosmetic colorants [45,46]. Despite the liquid-phase separation protocols involving column chromatography or capillary electrophoresis techniques [46,47] thin-layer chromatography seems to be still the most commonly applied method for fast qualitative and quantitative analysis of lipids, antioxidants, and colored substances from raw and extracted spirulina samples [7,48–54]. This is mainly due to fast and parallel sample processing without pre-purification of the raw material and simple detection of spirulina dyes under visible light as well as using sensitive visualization reagents for a variety of UV–vis transparent bioactive analytes [49,51,54].

This work is continuation of our research focusing on development of micro-TLC platform for the fast analysis of low-molecular mass compounds from spirulina samples [55–60]. The main goal of this paper is to demonstrate the separation and detection capability of micro-TLC technique for the classification and assessment of spirulina and other selected herbal pharmaceutical and food products. Based on our previous research concerning spirulina dye fractionation, in this study the target components were extracted using relatively low-parachor liquids. A number of the spirulina samples originated from pharmaceutical formulations and food products, which were isolated using a simple one step extraction method involving methanol, acetone or tetrahydrofuran. Rich in chlorophyll dyes, herb samples were analyzed as reference materials. Quantitative data were explored *via* simple chemometrics tools including cluster analysis and principal components analysis. The described methodology can be applied as a fast screening or fingerprinting tool for classification of spirulina and herb samples, particularly in a commercial setting for quality control. Moreover, the proposed approach allows a non-expensive fractionation of target substances including cyanobacteria pigments in raw biological or environmental samples.

2. Experimental

2.1. Chemicals

Methanol (LiChrosolv 99.8% for liquid chromatography) was obtained from Merck, Darmstadt, Germany. Acetone (99.9% HPLC grade) was received from Sigma–Aldrich, Steinheim, Germany. *n*-Hexane 95% was a product of Fluka Chemie AG, Buchs, Switzerland and tetrahydrofuran (HPLC 99.9%) was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, USA. Iodine (cryst., ACS, pure P.A.) was a product of POCH SA, Gliwice, Poland.

2.2. Micro-TLC chromatography

Separation experiments were performed on glass-based HPTLC RP18W plates that were products of Merck (Darmstadt, Germany). Before sample application, the factory-prepared plates (100 mm × 100 mm) were cut to a working size of 50 mm × 50 mm. In each case, a sample starting line was placed 5 mm from the plate bottom edge, allowing a maximum eluent front migration distance of 45 mm. Micro-planar separations were performed using a

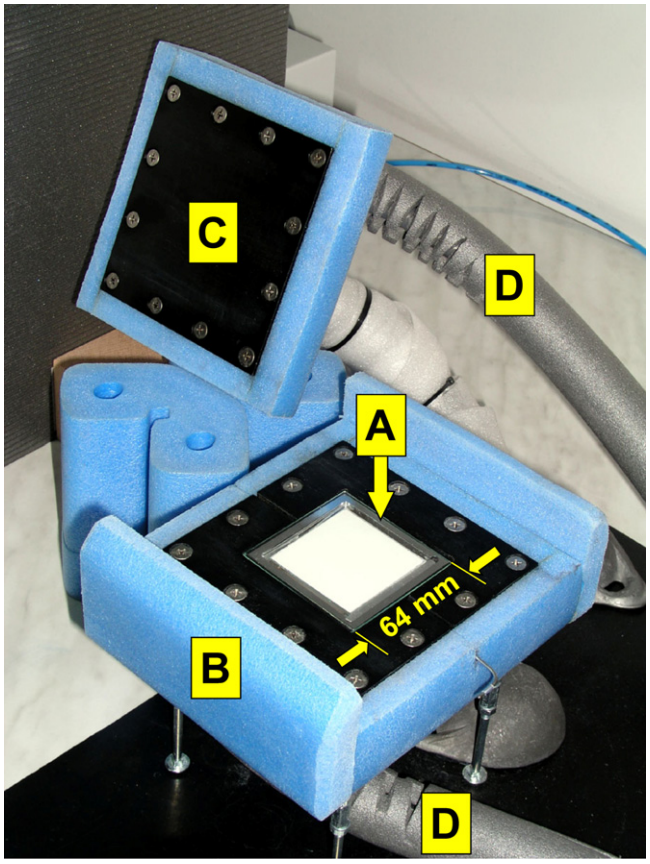

Fig. 1. Analytical setup of temperature controlled micro-planar chromatographic device: horizontal chamber unit (A) working inside temperature controlled metal oven (B) equipped with movable cover (C) and connected to external liquid circulating thermostat through foam insulated pipes (D).

home-made temperature-controlled removable horizontal micro-TLC chamber unit (Fig. 1), described previously [13]. Particularly, a chromium-coated brass unit was working inside a foam insulated metal oven connected to an external liquid circulating thermostat (Ultra-Low Refrigerated Circulator FP51-SL, Julabo, Seelbach, Germany) filled with ethanol as a circulating liquid. The system provided a constant TLC plate temperature, which was set at 30 °C temperature with an accuracy of ±0.02 °C. Under such conditions, a plate peak capacity of at least 20 spots/lane and a plate development time of 5 min were obtained.

To obtain chromatograms, the following chamber working protocol was applied: a micro-TLC plate with samples spotted on the starting line was positioned horizontally inside a chamber module with the stationary phase layer placed up side down. Afterwards, the chamber module was transferred into a thermostating oven cavity and sealed using a 1 mm thin glass cover. Then, the movable cover of the oven was slid so as to reach the position above the TLC chamber module and the temperature equilibration step was performed for 15 min. The chromatographic process was started after injecting a given eluent in a volume from 250 μL to 1 mL through an injection pipe into a mobile-phase application bar. Finally, the TLC plate was removed from the chamber module immediately after the mobile-phase front reached the plate edge located opposite to the application bar.

Chromatographic separations were performed under unsaturated chamber conditions using 3:7 (v/v) acetone:*n*-hexane mobile phase. Spot patterns developed on the plates were acquired by direct scanning under visible light conditions. Additional spots, corresponding to visible light transparent substances were detected by iodine vapor exposure (room temperature; 30 min).

2.3. Samples extraction

Spirulina, herbs and food material (specified in Table 1) were powdered manually using a small ceramic mortar, and 150 mg samples were transferred into 5 mL glass tubes. The samples were mixed with 1 mL of the extraction liquid, which were methanol, acetone or tetrahydrofuran. Afterwards, the tubes were sealed and sonicated for 1 h at room temperature using an ultrasonic bath Sonic 1 (80W, Polsonic, Warszawa, Poland). Next, the tubes were centrifuged (5 min, 5800 rpm; MPW-53; MPW Med Instruments Spółdzielnia Pracy, Warszawa, Poland) and clear extract was transferred onto the TLC plate start line.

2.4. Micro-TLC plates application protocol

Given liquid samples were spotted on TLC plates using Linomat 5 semi-automatic application instrument (Camag, Switzerland), controlled through the Planar Chromatography Manager (winCATS software, 1999–2008, version 1.4.4.6337). Using the spray-on technique narrow 4 mm long bands were formed along start line, which was located 5 mm from the bottom edge of TLC plate.

2.5. Chromatograms digitalization and quantitative data proceeding

Picture acquisition was performed using a Plustek OpticPro S12 USB scanner (Plustek, Taipei, Taiwan) with an 8-bit per RGB channel color deep mode, 600 DPI resolution, and saved as TIFF files without compression with the help of image-acquisition software: Image Folio v.4.2.0 (1991–2000, NewSoft Technology Corporation). After data acquisition an appropriate TLC plate area was cropped from the original frame size, and subsequently auto-balance or gray scale conversion filters were applied. Selected cross-sections of the chromatographic lanes were extracted from the digital pictures with the help of ImageJ software (ver. 1.43q Wayne Rasband, National Institutes of Health, USA; http://rsb.info.nih.gov/ij). Chromatographic spot quantification was performed manually using the peak height/area above baseline method (Table 2; available at the online supplementary data appendix). Quantitative data concerning separated band intensity were inspected with agglomerative hierarchical clustering and PCA statistical procedures using XLSTAT-Pro/3DPlot (version 2008.2.01) provided by Addinsoft, Paris, France.

3. Results and discussion

Extraction techniques that have recently been developed for the isolation of a wide range of bioactive substances from spirulina samples involve direct liquid, supercritical fluid, pressurized liquid, accelerated solvent, and subcritical water extraction protocols [48,49,45,61,62]. Proper optimization of an extraction protocol is critical for a metabolomics study and this step may produce substantial loss of various classes of metabolites, particularly from cyanobacteria material [63,64]. Moreover, extraction efficiency is the key parameter to establish of eco-friendly protocols used for the isolation of low-molecular mass compounds from natural products [65]. Our previous study revealed that parachor can be a convenient and simple physicochemical factor to evaluate the extraction efficiency of spirulina dyes by organic liquids with polarity ranging from water to *n*-hexane [55]. The parachor concept was invented by Sugden in 1924 [66]. This physicochemical factor still has a broad application for solving various structural problems and has also been used as a measure of intermolecular interactions or to

Table 1
Product data and composition of investigated spirulina, herbs pharmaceutical formulations and food samples.

No.	Product name (A) Production date (B) Expiry date (C) Series number	(A) Product manufacturer (B) Product dealer	Spirulina/herb species	Specified formulation ingredients	Formulation type (A), total mass (B) spirulina or herb mass (C)
1	Spirulina (A) Non-specified (B) 01.2009 (C) 620022	(A) A-Z Medica Sp. Z o.o. ul. Władysława IV 13C, 80-547 Gdańsk, Poland	*Spirulina maxima*	Spirulina	(A) Capsule
		(B) As above		Capsule contents: gelatin, water, dyes: E171, E104, E122, E131, E151	(B) Non-specified
					(C) 450 mg
2	Spirulina (A) Non-specified (B) 03.2011 (C) 831036	(A) A-Z Medica Sp. Z o.o. ul. Władysława IV 13C, 80-547 Gdańsk, Poland	*Spirulina maxima*	Spirulina, magnesium stearate	(A) Capsule
		(B) As above		Capsule contents: gelatin, water, dyes: E171, E131	(B) Non-specified
					(C) 450 mg
3	Spirulina 500 mg (A) MFG070208 (B) EXP060211 A (C) L C8B0358	(A) Walmark, a.s., Oldrichovice 44, 739 61 Trinec, Czech Republic	Non-specified	Spirulina 500 mg	(A) Tablet
		(B) Walmark Sp. Z o.o., ul. Teatralna 9, 41-200 Sosnowiec, Poland		Tablet contents: tiamin 0.2 mg, riboflavin 0.3 mg, vitamin B6 0.1 mg, vitamin E 0.6 mg, microcrystalline cellulose, carboxymethyl cellulose, magnesium stearate, amorfic silicon dioxide	(B) 640 mg
					(C) 500 mg
4	Spirulina (A) Non-specified (B) 30.09.2009 (C) 30.09.2009	(A) MedPharma, spol. S r.o. Botanicka 53a, 602 00 Brno, Czech Republic	Non-specified	Spirulina 400 mg	(A) Tablet
		(B) MedPharma Polska Sp. z o.o., ul. Komorowicka 43, Bielsko-Biała, Poland			(B) 400 mg
					(C) 400 mg
5	Spirulina (A) Non-specified (B) MHD: 01.12.2012 (C) L: 8731107	(A) Natura Vitalis BV, Groeneweg 12, 6041 AX Roermond, Holland	*Spirulina platensis*	Spirulina 400 mg	(A) Tablet
		(B) Natura Vitalis Sp. z o.o., ul. Zaporoska 35, 53-519 Wrocław, Poland		Tablet contents (in 15 tablets): pantothenic acid 2.4 mg, vitamin PP 0.9 mg, vitamin B1 0.13 mg, vitamin B2 0.12 mg, vitamin B12 4.7 μg, iron 4.2 mg, carotenoids 23.8 mg	(B) 400 mg
					(C) 400 mg
6	Spirulina (A) Non-specified (B) 10.2009 (C) 011007	(A) Krakowskie Zakłady Zielarskie "Herbapol" w Krakowie S.A., ul. Chałupnicka 14, 31-464 Kraków, Poland	Non-specified	Spirulina 280 mg	(A) Capsule
		(B) As above		Capsule contents: gelatin, titanium dioxide, iron oxides	(B) 356 mg
					(C) 280 mg
7	Spirulina (A) Non-specified (B) 30112009 (C) 1 11 07	(A) A.H.A. International Co., Ltd. China	Non-specified	Spirulina, silica dioxide, magnesium stearate	(A) Tablet
		(B) S.P.R.P. GAL L.P.M.Ł. Marek s.j. 61-012 Poznań, ul. Krótka 4, Poland		Tablet contents (in 4 tablets): phosphorus 18.0 mg, calcium 14.0 mg, chlorophyll 14.0 mg, magnesium 8.0 mg, beta-carotene 3.0 mg, iron 2.6 mg, riboflavin 70.0 μg, zinc 64.0 μg, thiamin 62.0 μg, iodine 28.0 μg, vitamin B6 16.0 μg, vitamin B12 6.4 μg	(B) 500 mg
					(C) <500 mg

Table 1 (*Continued*)

No.	Product name (A) Production date (B) Expiry date (C) Series number	(A) Product manufacturer (B) Product dealer	Spirulina/herb species	Specified formulation ingredients	Formulation type (A), total mass (B) spirulina or herb mass (C)
8	Spirulina Pacifica (A) 31.12.2007 (B) 31.12.2010 (C) 1002031113	(A) Cyanotech Corp., Kailua-Kona Hawaje 96740, USA	*Spirulina platensis*	Spirulina, silica	(A) Tablet
		(B) KENAY AG – 62-800 Kalisz, ul. Częstochowska 25, Poland		Tablet contents: beta-carotene 2.33 mg (including: 9-cis 0.26 mg, 15-cis 0.02 mg, 13 cis 0.085 mg), zeaxanthin 0.16 mg, gamma-linolenic acid 5 mg, vitamin B12 1.18 μg, niacin 0.1 mg, inazitol 0.34 mg, chlorophyll 4 mg, phytocyanin 60 mg, super oxide dismutase (S.O.D.) 1200 units, potassium 7.5 mg, phosphorus 5.2 mg, sodium 3.33 mg, magnesium 2.4 mg, iron 0.53 mg	(B) 500 mg (C) <500 mg
9	Spirulina (A) Non-specified (B) 07/2009 (C) LOT# 906891 0003	(A) NOW Foods, Bloomingdale, IL 60108, USA	*Spirulina platensis* (Spirulina Pacifica)	Spirulina, amorphous silicon dioxide	(A) Tablet
		(B) PRO NATURA, ul. Gen. Okulickiego 133D, Szczecin, Poland			(B) 525 mg (C) 500 mg
10	Spirulina (Algi morskie; Marine Algae) (A) Non-specified (B) 05.2010 (C) Non-specified	(A) TECH-FOOD TRADING Sp. z o.o., 97-220 Rzeczyca, m. Glina 103, Poland	Non-specified	Spirulina	(A) Dry spirulina cells
		(B) TECH-FOOD TRADING Sp. z o.o., ul Młynarska 35, 01-175 Warszawa, Poland		Sample contents (in 5 g): proteins 3 g, carbohydrates 0.72 g, fats 0.35 g, beta-carotene 9.75 mg, vitamin C 5.15 mg, B1 0.19 mg, B2 0.19 mg, E 0.48 mg, iron 3.52 mg	(B) 25 g (C) 25 g
11	Spirulino – krąg życia (A) Non-specified (B) 04.2010 (C) 002	(A) Zakład Produkcyjny 97-220, Rzecznica, Glina 103, Poland	Non-specified	Spirulina	(A) Dry spirulina cells
		(B) Bio-Active Dystrybucja Sp. z o.o., ul. Bukowiecka 92, 03-893 Warszawa, Poland		Sample contents (in 5 g): proteins 3 g, carbohydrates 0.72 g, fats 0.35 g, beta-carotene 9.75 mg, vitamin C 5.15 mg, B1 0.19 mg, B2 0.19 mg, E 0.48 mg, iron 3.52 mg	(B) 25 g (C) 25 g
12	Ananas + Spirulina (A) 03.2006 (B) 02.2009 (C) 361482	(A) Sarl Produit Nature, 397 Chemin de Deze, 40990 Herm, France	Non-specified	Spirulina 125 mg, pineapple 125 mg	(A) Capsule
		(B) HomeoMed Sp. z o.o., ul. Nadarzyńska 38, 05-805 Kanie, Poland		Capsule contents: carbohydrates 220.75 mg; proteins 11.4 mg; fats 8.325 mg	(B) 250 mg (C) 125 mg

Table 1 (*Continued*)

No.	Product name (A) Production date (B) Expiry date (C) Series number	(A) Product manufacturer (B) Product dealer	Spirulina/herb species	Specified formulation ingredients	Formulation type (A), total mass (B) spirulina or herb mass (C)
13	Belissa hydro (A) Non-specified (B) 02.2010 (C) 04AF0208	(A) AFLOFARM Fabryka Leków Sp. z o.o., ul Szkolna 31, 95-054 Ksawerów, Poland	Non-specified	Tablet contents: spirulina 100 mg, horsetail extract 150 mg, hydrolyzed collagen proteins 50 mg, vitamin C 15 mg, vitamin E 5 mg, beta-karoten 0.9 mg; microcrystalline cellulose, hydroxypropyl methylcellulose, titanium dioxide, magnesium stearate, D-alpha-tocopheryl succinate, silicone dioxide, hydroxypropyl cellulose, beta carotene, indigotine, quinoline yellow	(A) Tablet
		(B) As above			(B) Non-specified (C) 100 mg
14	Spirulina Super Forte (Aquarium fish feed) (A) Non-specified (B) 11.2009 (C) CH117/FO	(A) TROPICAL Tadeusz Ogrodnik, ul. Opolska 25, PL 41-507 Chorzów, Poland	*Spirulina platensis*	Sample contents: plant plankton inclusing spirulina 36%; dried fish meat, grain seeds, proteins extracts, dried mollusk and crustaceans, yeasts, fats, vitamin A 38,200 units/kg, vitamin D_3 2100 units./kg, vitamin E 130 mg/kg, vitamin C 540 mg/kg, lecithin, dyes, antioxidants	(A) Flakes
		(B) As above			(B) 12 g (C) <36%
15	Liść pokrzywy Urticae folium (A) 2008 (B) 06.2009 (C) 724.2008	(A) Zakład Zielarski "KAWON-HURT" Nowak Sp.J.; 63-800 Gostyń, Krajewice 119, Poland	N/A	Stinging nettle leafs; Urticae folium	(A) Dried leafs
		(B) As above			(B) 50 g (C) 100%
16	Liść mięty pieprzowej; FP V Menthae piperitae folium (A) 2008 (B) 03.2009 (C) 01 03 2008 B	(A) Herbapol Białystok S.A., ul. Składowa 3, 15-399 Białystok, Poland	N/A	Mentha piperita; FP V Menthae piperitae folium	(A) Dried leafs
		(B) As above			(B) 50 g (C) 100%
17	Liść melisy; FP V Melissae folium (A) 2007 (B) 12.2008 (C) 02.12.2007B	(A) Herbapol Białystok S.A., ul. Składowa 3, 15-399 Białystok, Poland	N/A	Lemon balm; FP V Melissae folium	(A) Dried leafs
		(B) As above			(B) 50 g (C) 100%
18	Japanese Green Tea (A) 2008 (B) 4.5.2009 (C) –	(A) Japan	N/A	Japanese Green Tea	(A) Dried leafs
		(B) Japan			(B) 100 g (C) 100%

investigate the mass transfer between organic fluids [67–69]. Based on this criterion and the results of principal components analysis, three relatively low-parachor extraction solvents; methanol, acetone and tetrahydrofuran were selected for the classification study of dry spirulina samples from pharmaceutical formulations and food samples. As the reference material selected herb samples (green tea, stinging nettle, peppermint and lemon balm) that are rich with chlorophyll dyes were also investigated.

For purpose of this study the samples were derived from commercially available pharmaceutical formulations and food products, which are listed in Table 1. Tissue extracts were separated *via* thermostated micro-TLC technique using plates coated with low-carbon load stationary phase (RP18W) and binary acetone/*n*-hexane mobile phase at elevated temperature. This approach allows for a fast separation time (around 5 min) and simultaneous analysis of 9 independent samples using one micro-plate. Resulting chromatograms with arrays of the separated target substances observed under visible light conditions and detected after exposure to iodine vapor are presented in Fig. 2. As can be seen micro-TLC technique working under isocratic and unsaturated chamber conditions is capable of separating a whole range of colored substances from dry samples. Close inspection of

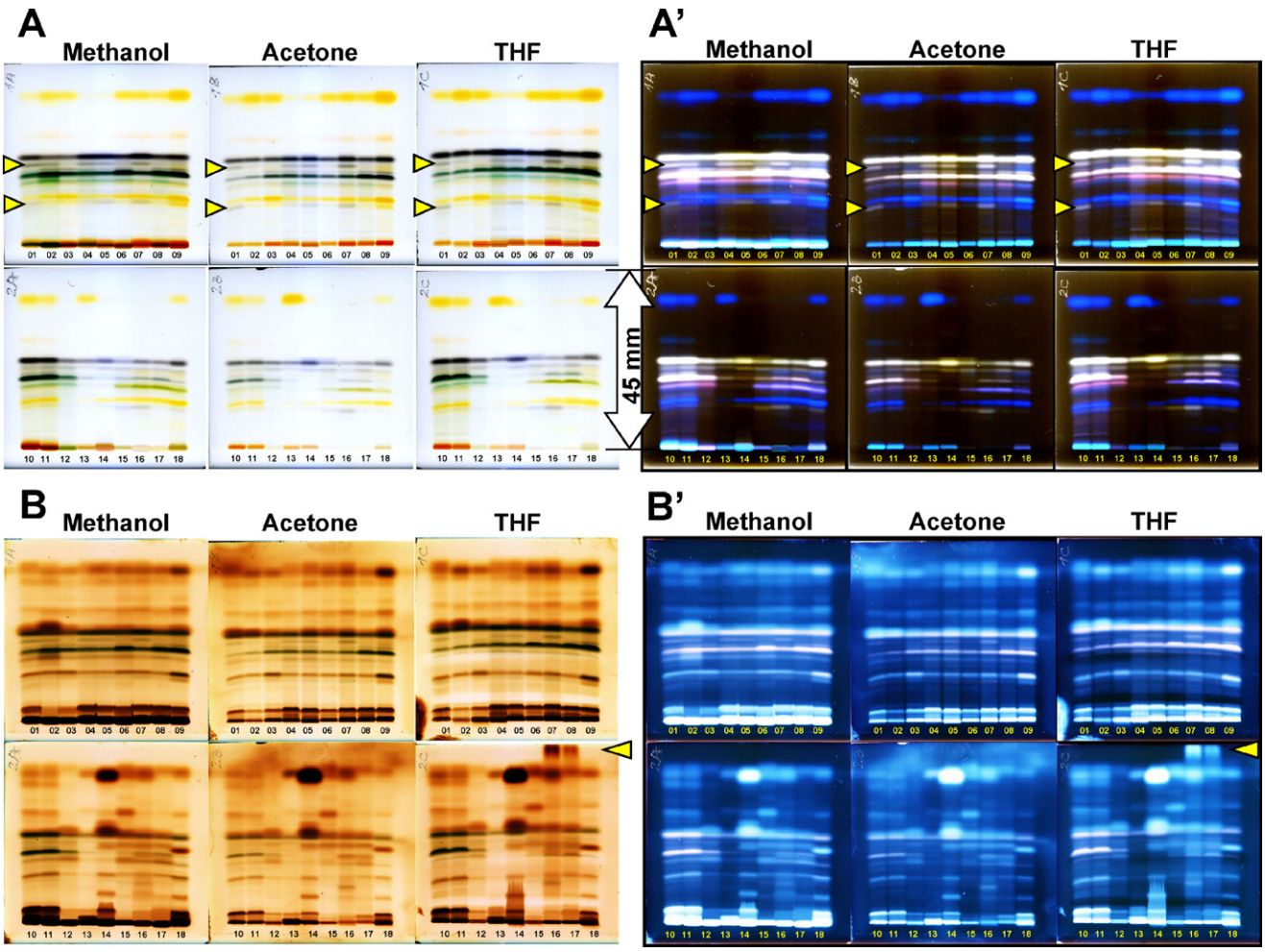

Fig. 2. Micro-TLC arrays of dyes and low-molecular mass compounds of cyanobacteria cells and herbs leafs extracted from pharmaceutical formulations and commercial food products listed in Table 1. Extraction was performed using organic solvents characterized by relatively low parachor values including methanol, acetone and tetrahydrofuran. Analytical conditions: sample volume – 3 μL; sample application – 4 mm bands using spray-on technique (Linomat 5); separation temperature +30 °C; stationary phase – HPTLC RP18W; mobile phase composition – 30% (v/v) acetone:*n*-hexane; unsaturated chamber; separation time 5 min; detection – visible light scan (A; A′ inverted colors) and after iodine vapor exposure at room temperature (B; B′ inverted colors); chromatogram acquisition method – digital scan using Plustek OpticPro S12 USB office scanner.

densitometric profiles acquired from 18 samples that were extracted with 3 solvents revealed the presence of 12 spots under visible light conditions. The main advantage of this approach is that it can produce confident quantitative data that can be acquired using manual peak matching on the analyzed chromatograms. Importantly, manual peak matching is currently considered as the best approach for the processing of relatively small data sets (approximately 20–50 peaks per chromatogram), particularly, in the case of irregular shaped base line and overlapping peaks [70]. Typical densitometric profiles derived from acetone extracted spirulina sample are present in Fig. 3. Exposure of developed microplates to iodine vapors at room temperature significantly improved the detection of a wide range of visible light transparent substances. Under such conditions around 20 individual bands can be detected on the analyzed micro-TLC plates. These chromatographic profiles as well as individual spots (like indicated by triangle markers in Fig. 2A and B) may contain important information concerning chemotaxonomy or sample quality in relation to product storage time.

In practice, the composition of dyes and low-molecular mass substances can be affected by a number of independent factors including spirulina/herbs species, raw product quality and preparation conditions, pharmaceutical formulation type, formulation additives, ingredients and stabilizers, production expiry date as well as final product storage conditions including temperature, humidity and light exposure. From that point of view, sample classification can be considered as a typical multivariate problem. The results of cluster analysis (CA) based on the initial matrix composed of 1728 data points (18 objects – spirulina/herb samples versus 96 variables – chromatographic peaks intensities acquired from all solvents and detected under visible light as well as after iodine exposure; Fig. 2) are presented in the dendrogram form (Fig. 4). As can be seen the genuine spirulina products (object nos. 1–11) form one cluster, which is away from the remaining materials comprised of complex spirulina pharmaceutical formulations and food samples mixed with other tissues as well as non-spirulina herb extracts. These results may indicate that investigated samples can be effectively fingerprinted and characterized by a simple extraction using low-parachor solvents and a quantification protocol involving micro-TLC platform.

To obtain additional quantitative data concerning spirulina and herb clustering, as well as to determine latent information from the raw data set, a principal components analysis (PCA) was performed. The main advantage of PCA is to reduce the dimensions of the large data matrix to fewer uncorrelated variables. For the initial data matrix (18 objects versus 96 variables) the first three factors explained over 60% of the total variability. The number of principal

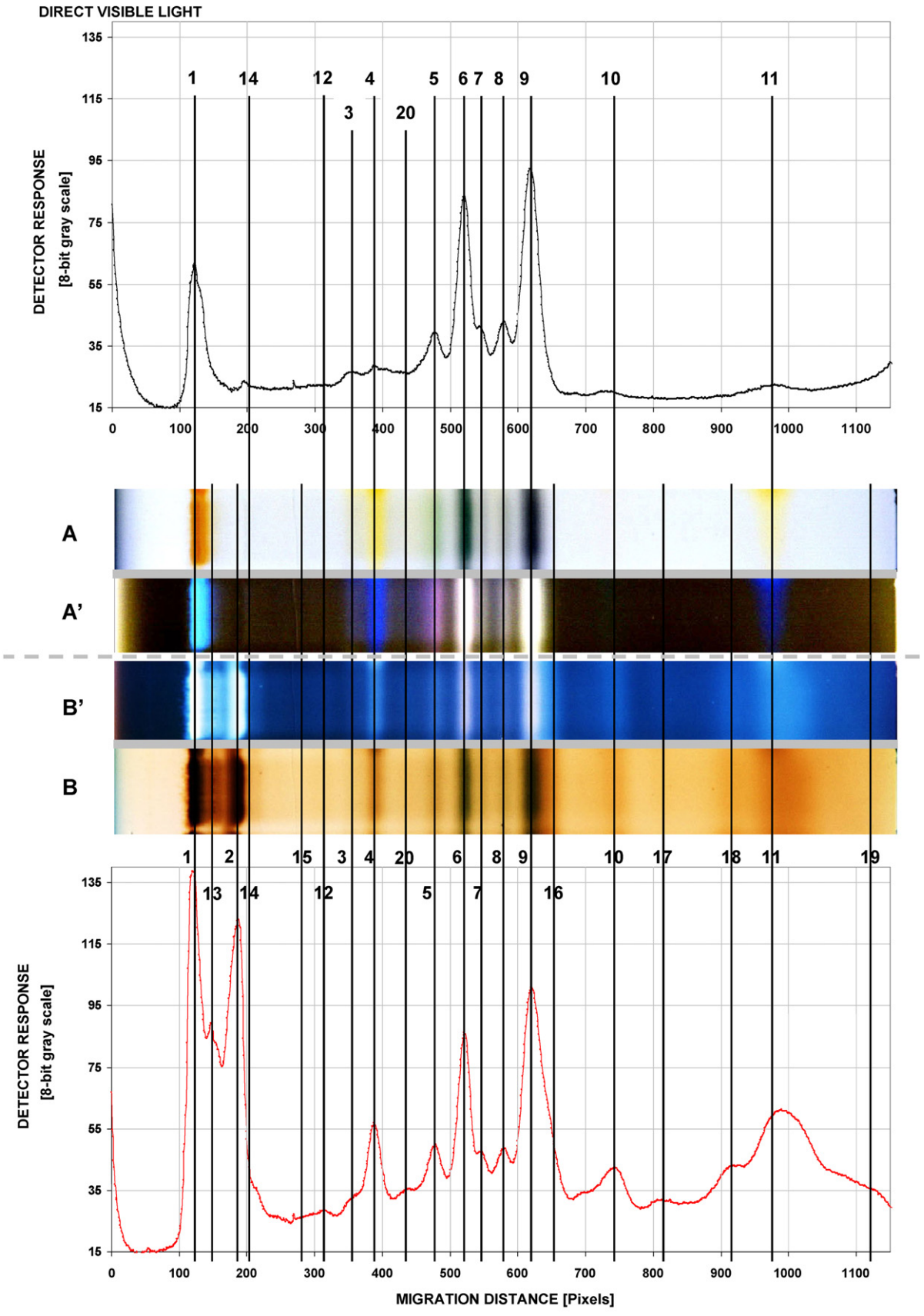

Fig. 3. Typical densitometric profiles and peaks ID of raw dyes (top) and after iodine exposure (bottom) corresponding to lane 4 located within acetone plate presented in Fig. 2. Raw scans (A, B), inverted colors (A′, B′).

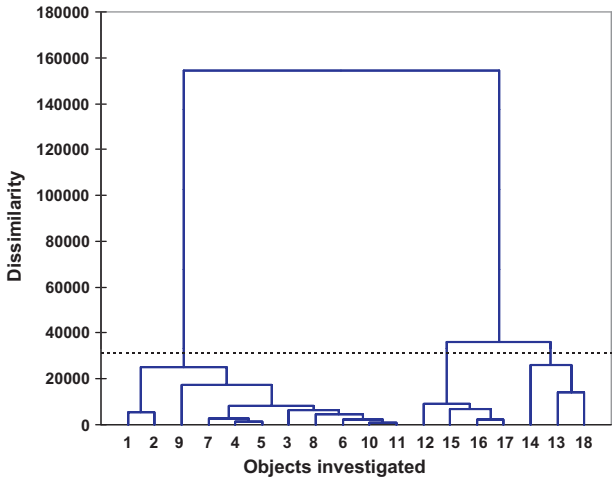

Fig. 4. Clustering of spirulina and herbs samples according to the chromatographic band intensities derived from micro-TLC plates presented in Fig. 2. Dendrogram of agglomerative hierarchical cluster analysis involving Ward's method as the aggregation criterion was based on data matrix consisted of 1728 experimental points made up of 18 spirulina and herbs samples (objects investigated) characterized by 96 variables (chromatographic peaks intensities). Objects numbers correspond to samples numbers listed in Table 1.

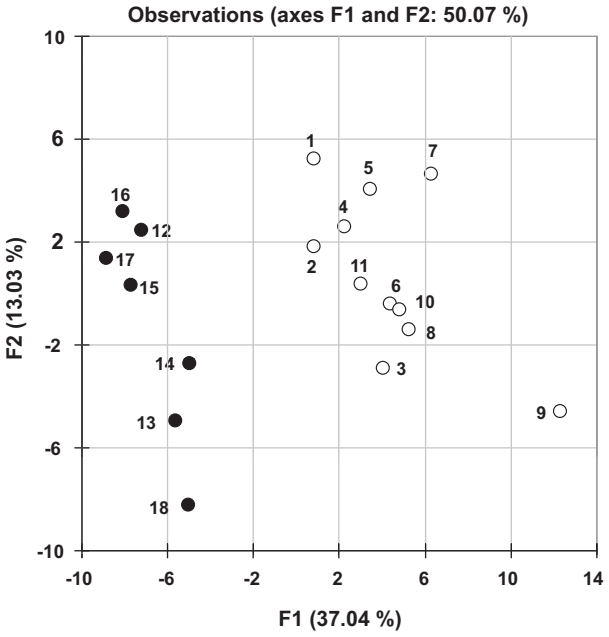

Fig. 5. Results of principal components analysis involving data set used for cluster analysis (matrix composed of 1728 points) presented in Fig. 4. Objects marked as white circles correspond to genuine spirulina products. Objects numbers are the same as listed in Table 1.

components (PCs) characterizing our data set was determined by considering the percentage of variance criterion and the Factorial Scree Test (Cattell Test). The result of this graphical test revealed that the first two factors (accounting for 37 and 13% of variance) should be investigated and were supposed to contain key information concerning the object grouping. Corresponding PC score plot for the objects investigated is presented in Fig. 5. From this plot it is clearly seen that genuine spirulina samples are well separated from remaining materials along the *X*-axis, as it was previously visualized by the CA dendrogram. PCA analysis confirms that proposed analytical protocol is capable of distinguishing significant differences between the samples investigated.

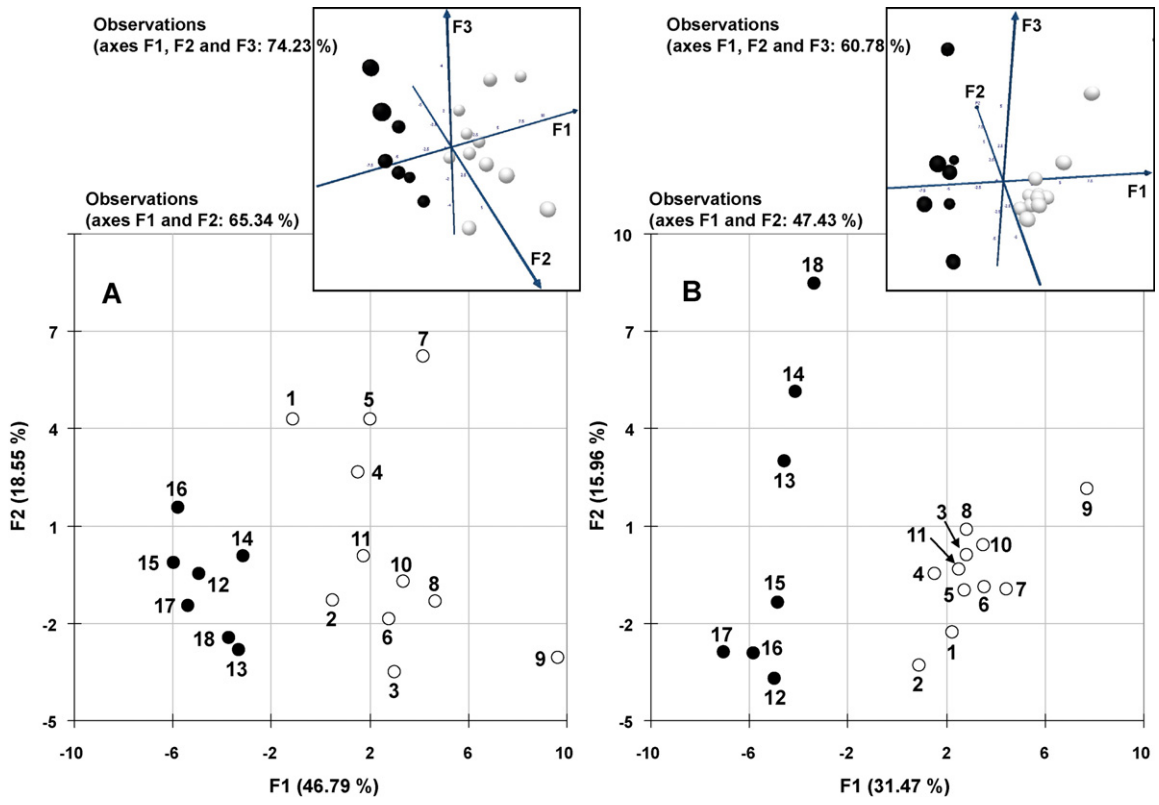

Fig. 6. Principal component plots showing relationships between objects investigated with respect to 1 and 2 factor scores. Computations involved two data sets derived from micro-TLC plates with raw dyes (A) and after iodine exposure (B) were based on 702 and 1026 data matrices, respectively. Object numbers and labeling is the same as it specified within capture of Fig. 5.

In order to find a rational meaning for the virtual PC scores the initial matrix was divided into two data sets related to raw dyes observed under visible light conditions (Fig. 2A) and TLC bands acquired after iodine exposure (Fig. 2B; 702 and 1026 data points, respectively). In the case of dyes observed under visible light the first three factors explain more than 74% of the total variability, while the data set derived from iodine derived micro-plates account for more than 60%. Fig. 6 shows the PC score plots observed in two and three-dimensional space for the objects investigated. Under both detection conditions the genuine-spirulina samples are clustering away from the remaining samples. Noteworthy, clustering of spirulina samples within F1 and F2 space is much more compact considering the iodine derived data set (Fig. 6B). This suggests that the first two factors may reflect the basic information directly related to chemotaxonomy of the samples investigated. Particularly, spirulina and non-spirulina samples can be identified considering F1 factor (Fig. 6A and B). Along the F2 axis (Fig. 6B) the non-spirulina tissues can be clearly distinguished especially, green tea (18), stinging nettle (15), peppermint (16) and lemon balm (17). Interestingly, contrary to methanol and acetone the THF can efficiently extract a volatile oil fraction from peppermint and lemon balm. Those compounds can be detected under iodine exposure as the dark spots migrating close to the solvent front (marked in Fig. 2B and B′ within 16 and 17 THF lanes). Close inspection of the raw visible light chromatograms shows the presence of a few biodegradation spots (triangle marked in Fig. 2A and A′). Those samples were characterized by a long shelf storage time, close or beyond the product expiry date at the time of chromatographic analysis. Genuine spirulina clusters within Fig. 6A have revealed that factor F2 may be related to pharmaceutical formulation quality in terms of *e.g.* shelf storage time (sample nos. 1, 4 and 7) or potential problems with improper storage conditions of commercial product (sample no. 5). This observation may be important from practical point of view because spirulina dyes mixture is recognized as a strong antioxidant and free radicals scavenger. The proposed analytical approach involving simple chemometric tools can be used as an efficient method for the rapid assessment of spirulina pharmaceutical formulations or to evaluate food product quality.

4. Conclusions

It has been demonstrated that a simple liquid extraction involving selected low-parachor liquids followed by micro-TLC separation is a fast screening tool of spirulina and herb samples. The main advantages of isocratic non-forced flow rate micro-planar chromatography are its simplicity, easy operation and the use of inexpensive equipment. Contrary to column chromatography and due to planar chromatography method principles, all sample components, including substances that are strongly retarded or chemisorbed by the stationary phase on the start line, can be visualized after plate development. From that point of view the described micro-TLC technique can be a promising and non-trivial technique for preliminary screening and classification of high level metabolites in complex biological samples. Our investigations have revealed that multivariate data processing of micro-TLC data involving direct visible light dye detection and non-selective detection of visible light transparent metabolites *via* iodine derivatization can be fast, convenient and an effective tool for sample classification in chemotaxonomic or pharmaceutical formulations and food product quality control studies. Using this method it is possible to distinguish fresh from old samples and to identify biodegradation peaks within the micro-TLC profiles. Due to low volume of the mobile phase (usually less than 1 mL per run), this method can be considered an environmentally friendly analytical tool, which can be an alternative for fingerprinting protocols based on HPLC machines or simple separation systems involving planar micro-fluidic or micro-chip devices.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.065.

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