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Phytochemical Techniques in Complex Botanicals. The XXI Century Analytical Challenge

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Abstract

We experimented in natural products a long period of reductionism and simplification to avoid any complexity as a boring and tedious disturb, coupled with countless attempts to adapt plants to the inappropriate role of biofactories of selected substances. However nowadays new approaches are radically changing the natural substances utilizations. Among several others, two factors were determinant: the appearance of a plethora of successful new products and the revival request for Mother Nature's gifts. To be validated the new products are urgently asking for science and technology support. In this paper we will examine some items concerning the analytical aspects of botanicals, using as target the determination of compositions of raw materials and as main tool the HPTLC, High Performance Thin Layer Chromatography, fingerprint approach. Practical examples of HPTLC analyses, derived from applications on market products, are here reported and discussed in order to present an information about the benefits of HPTLC and when it is useful to use it, including limits and crisscross with other analytical tools.

Keywords: HPTLC; Botanicals; Fingerprint; Medicinal plants

Introduction

XXI started as the century of radical changes: cultural changes marked by global communications, climate changes going to remodel planet biosphere, resource changes due to the end of fuel supply, life changes with the main diseases defeated and expectations for a longer time for life. However, main changes are expected in basic needs that mean food and health for new types of living styles.

The new forms of natural substances utilization consists into a composited galaxy of novel products proposals, first qualified as food supplements, now including nutraceuticals, dietary supplements, medical devices, herbal drug preparations and others, collectively named as botanicals for the plant prevalent origin. Next step, as natural evolution of this trend, will be the fusion of nutritional and health aspects in the multifunctional food [1].

In any useful and safe utilization of the new products, the guarantee of composition must be inferred, that means a classic, typical and exclusive analytical matter. In this case, composition must be referred to complex mixtures and related to the claimed activity, avoiding adulterant addition or changes to improve natural properties.

Initially, composition in food supplements was considered as result of the presence of vitamins, minerals, proteins, carbohydrates, but nowadays it is also due to the presence of "other substances". Other substances are essentially medicinal plant extracts, often also used as foods or spices.

The impact of these products, containing ingredients with nutritional and physiological effects, can be evidenced by the market data. In 2009 the EU market of food supplements is estimated about 10-15 billions of euros [2], with "other substances" accounting for half of this figure. Italy is the leader in "other substances" products with about 1454 millions euros involved and one third of the population regularly consuming food supplements [3], in accordance with a long and current tradition in herbal products. Also regional request plays an important role: in Italy the market is mainly covered by probiotics/prebiotics and herbal products, whereas UK, Denmark and Finland are great consumers of fish oil, due to the omega-3 and omega-6 fatty acids products request.

Technological Foods, Gifts from Mother Nature

Natural products used in "other substances" products are essentially of plant origins, obtained from raw materials, wild or cultivated, fresh or dried. Extractions, made in several ways allow the separation and purification of natural products, mainly secondary metabolites, from primary metabolites and basic materials, like components of cell wall. Further steps are focused on obtaining forms similar to pharmaceuticals and able to facilitate acceptance, conservation and utilization. Therefore, post harvesting steps mean a lot of technology to change the natural aspect into a product completely different from the starting plant but appealing in order to be considered useful beside the nutritional aspect. The range of these products is very broad and so far any possibility of contamination between natural products was allowed, including the addition of defined quantity of single substances, like berberine or caffeine, to mixtures of plant extracts. The need of validation of botanicals has generated a scientific tendency to develop efficient analytical tools. This paper is focused on the last advances in analytical quality controls of botanicals, in particular on HPTLC applications.

TLC vs. HPTLC

HPTLC is the new analytical tool of XXI century, as the last evolution of planar chromatography [4]. TLC appeared about half a century ago

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already tailored to separate and visualize organic substances and still remains the immediate, simplest, common, low cost, generally used and useful analytical tool in the laboratory of organic chemistry [5]. The difficulties of TLC to face and solve the problem of complex mixtures are well experienced by analysts and have limited applications, giving space to more reliable and sensitive methods, like HPLC. Furthermore, TLC resulting data can be influenced by several factors: a) as an open system, environmental conditions (temperature, light, fumes, and humidity) are important; b) critical manual steps and interpretation, based on visualization and personal judgement, depend by operator ability.

Any successful evolutionary step is based on the conservation of former basic characters and on their improvement (that means to obtain better results starting from the best of the last acquired) and meanwhile try to transform deficiencies into points of force. Evolution of planar chromatography was achieved in chemical analysis by the transformation and automation of the old TLC into HPTLC. Let's enumerate the most important novelties: a) optimize separation and visualization of constituents, including the minor ones; b) convert the reductionism model, i.e. the knowledge step-by-step = molecule-by-molecule, evident in the sequence extraction/separation/structure elucidation into the opposite holistic approach, where natural complexity is accepted and used to get more and more information; c) obtain the most near picture of the phytocomplex, as the real active principle of the extract. In one word, if so far the target was the separation and identification of one or two particular substances to be used as specific markers, as clearly reported in many monographs of the Pharmacopoeias, now HPTLC allows to give a glance on the Metaboloma [6,7] (Table 1).

HPTLC methodology

Phytochemical analyses based on planar chromatography consist of a sequence of steps including chose of material and conditions, deposition of the sample, separation, detection, and finally data treatment, acquisition and interpretation that are anticipated by metabolites extraction and sample preparation. HPTLC revised and optimized each step.

Material: HPTLC started with the use of more efficient plates and this remained for long time the only character of difference against TLC. Larger superficies of silica gel were obtained by the use of precoated plates with smaller particles (5 µm vs. 15 µm) and this allowed a much better separation and concentration of the spots that appear evidently separated, netted and clean, including a higher sensitivity [8].

Automation: However, for a long time and despite the evident improves, HTPLC remained a niche product, with major cost

	TLC	HPTLC
Separation	Good	Very good
Visualization	Good	Very good
Rapidity	High	High
Easiness	Very high	High
Robustness	Low	Good
Reproducibility	Low	Very high
Quantization	Absent	Present
Flexibility	Very good	Very good
Validation	Very low	In progress

Table 1: Comparison between TLC and HPTLC as analytical tools for chemical determination of botanicals.

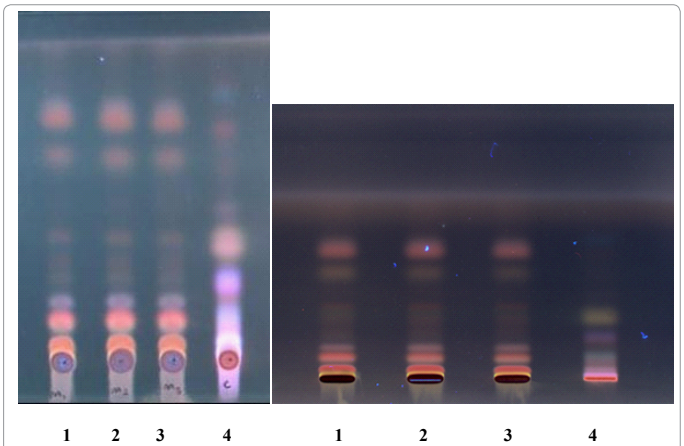


Figure 1: Comparison between TLC (left) and HPTLC (right) analyses of Melissa officinalis extracts. Mobile phase: n-hexane, ethylacetate (9:1). Derivatization: Anisaldehyde. Visualization: UV 366. Tracks: 1-3 marketed hydroalcoholic extracts, 4 mixture of essential oil constituents (citral, limonene, myrcene).

not compensating the positive changes, up to 2010, that showed a remarkable change since the number of dedicated papers jumped to more than one hundred. It was the effect of the automation that solved most of the aforementioned limits of planar TLC. Instrumentation improvements played a major role in turning conventional TLC into HPTLC with high quality results [4,9]. Thus, manual operations are limited to the preparation of the samples, to the movements of the plate in the sequence of devices, as well as to the use of the proper software in the computer. In HPTLC automation step, each step being computer controlled and performed by a special device that defines also environmental conditions. Therefore, the analysis is totally repeatable and reliable as well as quality and type of the results: a chromatogram performed in Rome can be fully compared with that obtained in Nairobi in the same conditions.

Deposition: Among the points of weakness of TLC, the deficiencies due to the manual deposition: despite the extracautions of the operator, sample application to the plate can not be equalized and often chromatography starts in the deposition point, generating disturbs in the separation process (Figure 1). In HPTLC, each sample is deposited by a syringe of an automated device that allows selecting and controlling form, concentration and type of the starting spots, as well as the proper distances, insuring the same best starting conditions. The improvement is not only a matter of separation and visualization; net spots means the possibility of the efficient comparison of the Rfs with the standards.

Development: Silica gel, by far the most used stationary phase used in planar chromatography, is hygroscopic and always in equilibrium with the humidity of the laboratory atmosphere, that can affects the activity of silica. Also temperature is an important factor in the interaction substances/phases. To obtain repetitive and reliable tracks, plates must be run in controlled conditions, that means in special automated development chambers (surpassing the old tank), saturated with mobile-phase vapour, where extent of mobile-phase migration can make a big difference in reproducibility of results. They may also include sensors to detect the advance of the solvent front, so a plate can be automatically withdrawn and dried at a specific level.

Visualization: A 20 x 10 cm HPTLC plate can contain many

samples, up to 72 (usually 20), that can be analyzed side by side in the same conditions, allowing a rapid parallel screening, including also the use of selected standards. Beside the Rf value, the individual character of each spot can observed and recorded, like colour – absent, dark, coloured and fluoreshing - and intensity. After development, the same plate can be visualized in different ways, using lights at different wavelengths, like white light, UV254 and UV366 nm (TLC Visualizer), followed if necessary by particular detection using a derivatization with selected reagents. The final result is that the sample plate can be visualized at least in six different ways. HPTLC analysis of a herbal sample is a set of comprehensive data, based on ordinary visualization at white or visible light, scan in UV at different wavelengths, fluorescence scan, derivatisation with selected reagents, all converted into photo documentation. Albeit it can be considered complicated, often such a wealth of data is the minimum required to compare safely one herbal sample to another. Another consequence of complexity.

The multiple detection can give a lot of information and the visualization can be optimized and stored at the best possibility by the scanning and the acquisition stored into the computer memory. Once in the computer the images of the tracks in different visualizations can be compared with the data-base containing the data and images of plates obtained in other times or in other places. The last point is crucial: the evidence, the most original character of planar chromatography, is exalted and the immediate interpretation of the analysis possible also for a child. Thus, the committent, like a manager or a producer devoid of any chemical knowledge, can discuss directly with the analyst, become satisfied and have the impression to be inside the analysis, trusting much better the results. On the other hand, the total interpretation of the spots in the track is another matter and needs large experience and deep knowledge in phytochemistry and natural products chemistry, and often results must be supported by other analytical techniques.

Sensibility: The best performance of HPTLC consists into the visualization at very low concentrations (up to 2 ppm), that means have conscience of the presence of minor constituents typical of plant extracts, more and more abundant that the main ones and therefore more important in the identification work.

Cost: The cost of HPTLC concerns mainly the initial investment for the devices necessary for the automation, whereas the maintenance expense is very low as well as that for each analysis, and in particular the wasting of solvents is very limited, for instance in comparison with HPLC (acetonitrile dependence is avoided). Furthermore, the mixtures of available and useful solvent systems are enormous and can be easily adapted to the nature of the analysed substances.

Quantization: This is probably the most innovative aspect for planar chromatography. Working so far on the mono or bidimensional feature, TLC suffered the difficulty for getting a quantitative determination, though the intensity of the spot contains evidently this information. HPTLC started from this simple point. Therefore, each track of the planar chromatography can be converted into a sequence of peaks, similarly in HPLC or NMR, whose intensity can be turned into quantity and numbers. Thus the 2D feature is changed into a 3D, using the densitogram method; TLC raised into a Cartesian bidimensional world marked by spots and their Rf values. HPTLC introduced the third dimension with the determination of the quantity that means contents of the substances. For people who used and appreciated TLC all their life, this is a little unexpected miracle, evoked for long time in front of a plate.

Utilization and usefulness: The real force of HPTLC is the correspondence with modernity. HPTLC lives of images. Each image is coupled with a file containing all information concerning the conditions and the devices utilized in the analysis. It is conceivable to expect soon the presence in the web of a reliable data bank of HPTLC fingerprints. Meanwhile, the idea was already realized by the Chinese Pharmacopoeia [10] with the publication of an atlas book collecting the HPTLC fingerprint images of most important Chinese medicinal plants (Figure 2), giving an easy characterization and differentiation of the species. After a short introduction, dedicated to the aim of the book and several technical details, the atlas contains monographies, each reporting images of fingerprints of the plant, certifying also

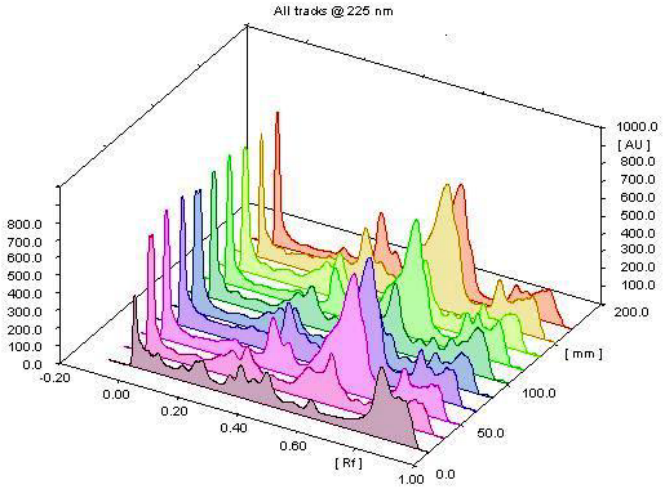


Figure 2: Example of a 3D densitometric chromatograms at 225 nm obtained from HPTLC analysis of commercial extracts of bearberry. Each profile, as sequence of peaks similar to those of HPLC, corresponds to a track where the intensity of the spots can be appreciated and measured in order to obtain a quantification of the separated substances.

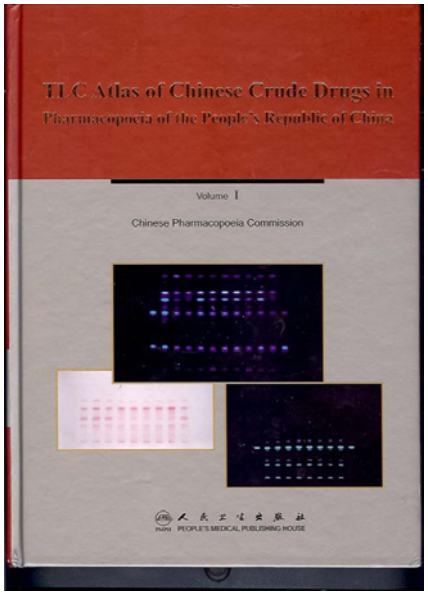


Figure 3: The front page of the TLC Atlas of Chinese Crude Drugs edited by the Pharmacopoeia of the People's Republic of China, totally dedicated to the HPTLC fingerprints of the most important Chinese medicinal plants.

the geographic origin (Figure 3), in order to achieve a validation of marketed plants and strike against the actual dangerous dominating confusion. HPTLC fingerprints have been introduced also in the United States Herbal Pharmacopoeia [11] and times seem to be ready for its introduction in other Pharmacopoeias.

The use of standards: The HPTLC fingerprint based on extract of a species can be confirmed by the comparison with selected standards. In this case, it is possible to use the exact Rf position inside the track of the extract as well the colours typical of the substances. However, in the tracks of the standards, marketed and considered as pure, several additional spots are often evident. This is an ordinary consequence of the high sensitivity of HPTLC: very low quantities of contaminants (for instance not present in the NMR spectrum), are well evidenced by UV light despite the minimum concentrations.

Reliability and robustness: HPTLC main qualities are the simplicity, quickness and comprehensibility. Easy and understandable data in a short time at low cost. However, a validation of the method is also necessary, if results have to be shared. ICH and WHO guidelines afford information about the general recommendation [12-16] that needs to be tailored for HPTLC specificity. So far, generally accepted guidelines for HPTLC validation are absent, although it is obvious that several suggestions and reports are present in the scientific literature. For instance, precision can be achieved by repletion of sample application and measurement of the Rf values or peak area express in term of RSD (relative standard deviation), measuring intraday and interday variation. Several parameters could be considered in robustness, but many are strictly controlled by the devices and probably only concentration levels are necessary. Specificity and peak are probably easier in case of standards since in fingerprints the presence of pure spots is not always achieved. Spiking of tracks with preanalyzed samples and standards is obviously a good method.

HPTLC vs. HPLC

This comparison has been already examined in several papers [8]. Nowadays, HPLC remains the current best analytical tool. However, recently a persistent tendency to enhance sensitivity and specialization has complicated the analytical approach. That means high cost, dedicated operator, wonderful but very complicated devices (from the "simple" multidimensional "comprehensive" GC and LC to high performance tandem quadrupole coupled with sophisticated softwares). The main danger comes from specialization: operator uses to set HPLC analysis on a single standard or a class of substances, therefore other compounds are invisible or not detected.

Substantially, the advantages of HPTLC are the simplicity, rapidity and high number of samples throughup that means the possibility of analysing many samples at the same time under the same chromatographic conditions. The main feature is in flexibility and adherence to complexity: HPTLC is able to generate a chromatographic fingerprint in the form of an unique sequence of peaks corresponding to the analyzed sample in its fullness. The aim is obtain resulting fingerprints that can be compared with respect to the number, sequence, position (Rf) and colour of the separated zones, also in reference to a digitalic data bank.

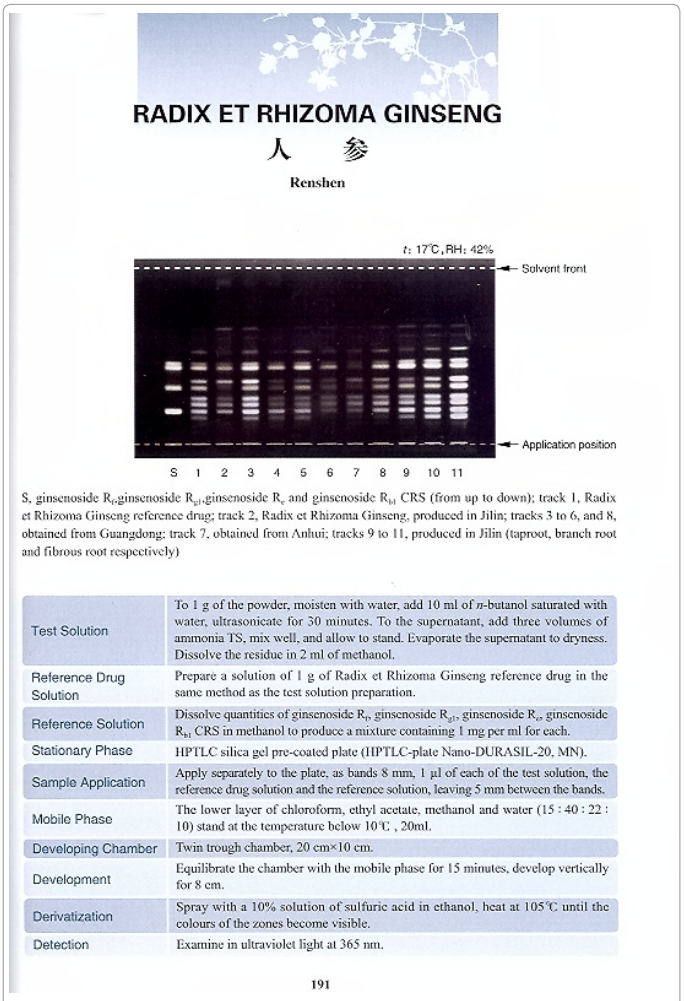
HPTLC vs. NMR

NMR has been proposed as the possible tool in natural products fingerprint and its use reported on foods and medicinal drugs [17-19], including the analysis of adulteration or food supplements spiked with synthetic products, when a single simple HPTLC plate can be sufficient [17-23]. The main problem to face is that also using a spectrometer of high resolution, the possibility of peaks overlapping remains very high. The difficulty is enhanced in the case of comparison of species taxonomically near and in the absence of typical markers. On the contrary, the NMR study remains crucial in the identification of single substances.

The value of fingerprint chromatographic technology has been evidenced by WHO as a reliable strategy for identification and quality evaluation of herbal medicine [15].

The prominence of some analytical tools in the study of specific types of substances is out of discussion. In the case of volatile oils, GC/MS is by far the best method to identify the composition of these very complex mixtures. However, the tendency to obtain automatic identification by simple comparison of MS profile with MS data bank can be origin of misleading. HPTLC fingerprints can be useful in the analysis of aromatic plants, in particular for comparison.

Similar matter for the opposite: the analysis of fixed oils. Several approaches have been recently proposed mainly in food analysis, including the use of high resolution NMR. Sincerely, looking to the


RADIX ET RHIZOMA GINSENG

人 參

Renshen

t: 17℃, RH: 42%

Solvent front

Application position

S 1 2 3 4 5 6 7 8 9 10 11

S, ginsenoside R_g, ginsenoside R_{g1}, ginsenoside R_e and ginsenoside R_{b1} CRS (from up to down); track 1, Radix et Rhizoma Ginseng reference drug; track 2, Radix et Rhizoma Ginseng, produced in Jilin; tracks 3 to 6, and 8, obtained from Guangdong; track 7, obtained from Anhui; tracks 9 to 11, produced in Jilin (taproot, branch root and fibrous root respectively)

Test Solution	To 1 g of the powder, moisten with water, add 10 ml of *n*-butanol saturated with water, ultrasonicate for 30 minutes. To the supernatant, add three volumes of ammonia TS, mix well, and allow to stand. Evaporate the supernatant to dryness. Dissolve the residue in 2 ml of methanol.
Reference Drug Solution	Prepare a solution of 1 g of Radix et Rhizoma Ginseng reference drug in the same method as the test solution preparation.
Reference Solution	Dissolve quantities of ginsenoside R_g, ginsenoside R_{g1}, ginsenoside R_e, ginsenoside R_{b1} CRS in methanol to produce a mixture containing 1 mg per ml for each.
Stationary Phase	HPTLC silica gel pre-coated plate (HPTLC-plate Nano-DURASIL-20, MN).
Sample Application	Apply separately to the plate, as bands 8 mm, 1 μl of each of the test solution, the reference drug solution and the reference solution, leaving 5 mm between the bands.
Mobile Phase	The lower layer of chloroform, ethyl acetate, methanol and water (15 : 40 : 22 : 10) stand at the temperature below 10℃ , 20ml.
Developing Chamber	Twin trough chamber, 20 cm×10 cm.
Development	Equilibrate the chamber with the mobile phase for 15 minutes, develop vertically for 8 cm.
Derivatization	Spray with a 10% solution of sulfuric acid in ethanol, heat at 105℃ until the colours of the zones become visible.
Detection	Examine in ultraviolet light at 365 nm.

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Figure 4: For each species reported in the Atlas, several fingerprints are reported, corresponding to extracts from plants of different regions.

Assignment	-CH=CH-	-CH-OCOR	$-CH_2$-OCOR	$=CH-CH_2-CH=$	$OCO-CH_2-$
Approximated chemical shift value (δ)	5.4	5.3	4.3	2.8	2.3
Assignment	CH_2-CH=CH-	$OCOCH_2CH_3$	-$(CH_2)n$-	CH_2-CH_3	
Approximated chemical shift value (δ)	2.0	1.6	1.3	0.9	

Table 2: Typical set of signals in a ^{1}H-NMR spectrum of fatty acids.

NMR spectra of fixed oils of many plants, most of the peaks profile, generating the fingerprint, appears too similar. In total, also the spectra of animal origin are not so different. In other words, the parts of the molecule generating peaks (-CH=CH-CH_2-, -CHnOCOR, -CH_2-CH_2-, -CH_3) are usually the same and often only changes in intensities of peaks can be observed. A typical spectrum of a mixture of fatty acids is reported in Figure 4 and consequent assignments are reported in Table 2.

In HPTLC, due the great sensibility, fingerprints are highly influenced by relevant quantity of fixed oils and other lipids, appearing all together in big spots evident after derivatization. For these reasons, in the comparison with marketed extracts, a defatting treatment can be necessary to evidence other signals (Figure 5). In the analysis of fatty acids and related products LC-MS remains the good choice.

HPTLC and MS

Although the use of the right standard is often a solution or at least of great help, the identification of the spots is a frequent and unsolved problem in chromatography. Again, Camag suggests the solution. The recent frontier of HPTLC is the coupling with MS spectrometry. HPTLC/MS consists of an interface device able to separate the selected spot, transfer the silica gel containing the separated substance to the apparatus that make possible the direct MS analysis. Next other hyphenated application could be with NMR, once solved some basic problems, like the necessary minimal concentration of the sample.

Studying complexity

A fingerprint is the individual chromatographic track representing, as near as possible, the mixture of produced organic substances [23]. By the fingerprint approach, it is possible to obtain a proper identification

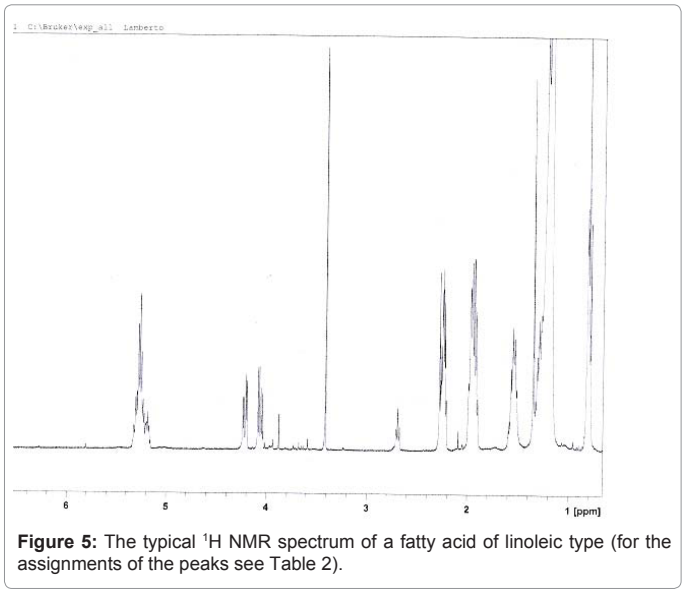

Figure 5: The typical ^{1}H NMR spectrum of a fatty acid of linoleic type (for the assignments of the peaks see Table 2).

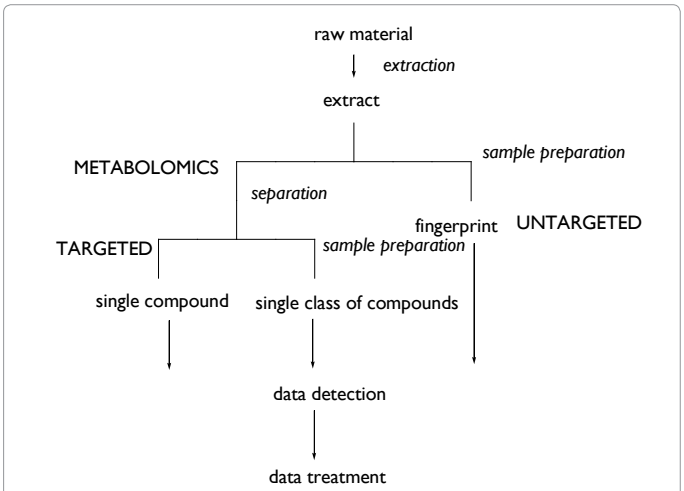

Figure 6: Schematic representation of general classification of metabolomics, with relative steps and targets.

of the plant material, but also determine and assert the limits of the biological changes, without necessary identifying or quantifying a specific compound. In HPTLC tracks of the same species, variations are mainly quantitative, not qualitative. Same plants are more inclined to variability. Variability is an evolution task and a biological resource, but it is difficult to be accepted by pharmacological and clinical trials based on single and defined substances. Limits of variability must be considered and determined for each species and for its marker compounds.

HPTLC fingerprint approach is a product of Metabolomic philosophy, the "study as many small molecules as possible" in an organic system. In our cases the study can be classified as targeted and untargeted (Figure 6). Generally, targeted analyses focus on a specific group of natural products; either a single substance used as marker or considered a reference of special importance, as reported in many monographs of Pharmacopoeias. That often requires extraction metabolites within the group, including if necessary high level of purification and individual identification. In HPTLC the identification can be obtained directly by comparison with standards and quantification by densitometry. In contrast, untargeted metabolomic focuses on the possibility to obtain sufficient data to identify a species, or the effects of biological phenomena like a plant disease or the changes in cultural conditions, or creation of specialized metabolite database useful for comparison, ecc. The target is the metaboloma and each little difference that can be detected. Discrimination, information or predictivity are only faces of the same analytic item.

Materials and Methods

HPTLC analysis

The analysed extracts were obtained from the market. Detailed information, i.e. producers, production conditions, storage method,

etc. can be obtained by directly asking the authors. The dried extracts of marketed products were dissolved in methanol (6 mg/mL). In some cases, also hydroalcoholic extracts of the same plant were obtained in the lab to be used for comparison as standards. Filtered solutions were applied to 60 silica gel glass-backed layers (Merck, Darmstadt, Germany) by a Camag Linomat 5 sample applicator with nitrogen flow. The operating conditions were as follows: syringe delivery speed, 10 µL/s; injection volume, 6 µL; band width, 6 mm; distance from bottom, 15 mm. The HPTLC plates were developed using the best solvent mixture in a horizontal chamber (Camag 20x10) saturated with the same mobile phase for 20 min at room temperature. The length of the chromatogram run was 80 mm from the point of application. The layers were developed in a Camag Chromatogram Immersion Device III and later allowed to dry in air for 5 min. Then they were derivatized with the selected solution, including anhysaldehyde or Natural Product Reagent (NPR), using a Camag Automatic Development Chamber ADC 2 and heated at 115 ˚C by a Camag TLC Plate Heater III. All treated plates were allowed to dry in air for 30 min and then inspected under a UV light at 256 or 366 nm or under white light, respectively, at a Camag TLC visualizer.

Variability and analysis

In any technological application, quality is crucial and therefore analytical tools are requested.

Analytical tools must be chosen in accordance to the target and the most advanced available device. In natural products chemistry, like any biological feature, the variability must be accepted and considered normality, being the response to the changes of the abiotic and biotic factors present in the habitat. Largely considered a phenotype aspect, this variability is also a substantial evolutionary advantage, whose causes and mechanisms are still largely unknown, as a consequence of uncertainty of the effective role of secondary metabolism.

By HPTLC is possible to compare side-by-side the molecular content of populations of the same species present in different habitats and times. Check variation within the fingerprints of species of the same genus can also evidence erroneous collection, like in the HPTLC analysis performed on St John's wort, *Hypericum perforatum*, extracts. The results of the HPTLC analysis can be visualized in Figure 7, following the resulting tracks: 1-4 methanol extracts of aerial parts of flowering plants of *Hypericum hyrcinum* collected in continental parts (1-3) of Italy and in Sardinia (4); 5-9 methanolic extracts of aerial parts of flowering plants of *H. hyrcinum* collected in *H. perforatum*; 10 hypericin coupled with pseudohypericin at lower Rf; 11 iperoside; 12 rutin; 13 quercetin; 14 isoquercetin; 15 chlorogenic acid; 16 luteolin; 17 apigenin. Differences between the tracks of the two *Hypericum* are evident, including the absence of hypericin and pseudohypericin. Clear also the differences in the tracks of the same species, due to the phenotypic metabolomic response to the habitat condition.

The analysis is important considering that in marketed botanicals defined concentrations of hypericine and hyperforine are requested, a condition that appears against the natural situation and opens the door to post-harvesting adjustments.

Other practical applications are reported in Figure 8. The item was the comparison between extracts obtained with a new apparatus working simply by water pressure using different working times; in this case, obviously time means money. In the analysis, the first two tracks were dedicated to the extracts of artichoke, *Cynara scolymus* leaves using the apparatus for 12 hours or 21 days: the fingerprints shows a situation practically identical, as confirmed also by the inspections at other wavelengths lights (UV254 and UV366). The same conclusion concerns the last two tracts, reporting the same extraction on sena, *Cassia senna*, leaves. In the middle, a series of references, and rhein (last track visible at other wavelengths) and extracts of artichoke from the market in tracks 3 and 4: in the extracts obtained by pressure additional spots are visible, testifying the effectiveness and the quality of the new extraction method (Figure 9).

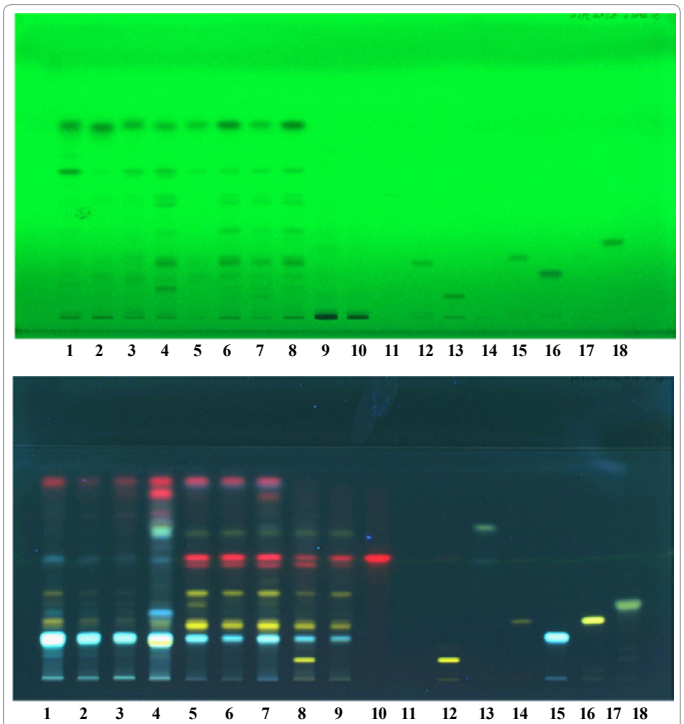

Figure 7: Comparison between Hypercum hyrcinum (tracks 1-4) and H. perforatum (5-9) with selected standards including hypericine (9). Mobile phase: formic acid, water, ethyl acetate (10:5:85). Derivatization: the layers were treated with a solution containing the Natural Product Reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 mL of ethylacetate), dried in the open air and then dipped into Macrogol reagent (1 g polyethylene glycol 400 in 20 mL of dichloromethane). Visualization: UV254 in the upper plate and at UV366 nm.in the lower plate.

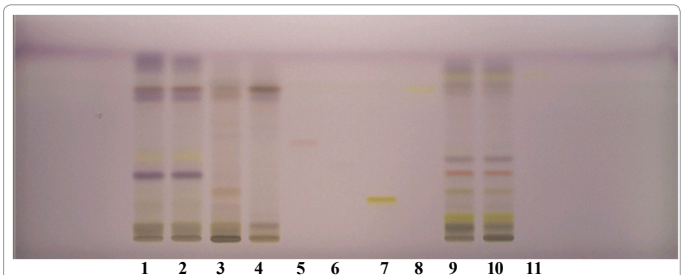

Figure 8: HPTLC analysis of Cynara scolymus and Cassia senna extracts obtained in two different working times (12 hours and 21 days), compared with specific standard. Mobile phase: THF, toluene, formic acid, water (16:8:2:1); Derivatization: Anisaldehyde. Visualization: white light.Tracks: 1,2 extracts of artichoke after 12 hours and 21 days, respectively (differences are minimal), 3,4 extracts of artichoke from the market, 5 cynarine 6 chlorogenic acid, 7 rutin, 8 luteolin, 9,10 extracts of senna after 12 hours and 21 days, respectively (differences are minimal), 11 rhein (not visible in these conditions).

Uses of HPTLC

Market of botanical products need rapid, low cost and understandable replays to simple requests. The cost is important since so far, although probably not in the near future, controls are not requested, and therefore they derive from personal voluntary need of validation. The requests are usually very simple and simple must be the answers, like those evidenced in a HPTLC plate.

Is it the right species, I am using? Am I paying a lot of money for the high quality imported species or is an ordinary domestic one? This raw material looks great, but what about the content in active principles? Does the herbal product comply with the claims on its label? Has the herbal products maintained its composition and stability after one year on the shelf? Has post-harvesting procedure affected the quality? In what my product could be marketed against the other similar products?

All these requests concern validation and quality, but the absolute identification of the utilized botanical species is an essential pre-requisite. In case of a powdered drug or an extract of two species of the same genus or of two entities taxonomically very near, the methods reported in the Pharmacopoeias practically are without any utilities. In these cases differences in morphology are very few, or limited to few crystals of oxalate and residuals of trichomes, whereas the chemical differences can not be evidenced in any way by the essays or a simple TLC based on one or more marker constituents. However, differences must exist and the HPTLC is able to evidence the presence of few additional spots in very similar fingerprints of taxonomically near entities.

Owing to the growing request of natural products by the global market. Actually, several cases are reported of substitution of the classic medicinal plants present in Pharmacopoeias or in books of Pharmacognosy and Phytotherapy by similar species, easier to be found, collected and commercialized al lower prices [24]. The importance of HPTLC sensibility is clear in the comparison between feverfew, *Tanacetum parthenium* L. (= *Chrysanthemum parthenium* (L.) Pers.), and the Mexican feverfew, *Chrysanthellum indicum* Turner (= *Chrysanthellum americanum* L.). The HPTLC respective tracks show a similar fingerprint, both completely different from that of chamomile, a frequent adulterant of feverfew. The tracks show that in some cases, to optimize the comparison, it is useful to clean the extracts from chlorophylls and other lipids (Figure 7). In the plate reported in Figure 10 tracks 1-5 report fingerprints of marketed products with feverfew reported in label, tracks 6 feverfew extract used as reference, track 7 the same extract after elimination of chlorophylls and other lipids, track 8 Mexican feverfew extract used as reference, 9 the same extract of track 8 after elimination of chlorophylls and other lipids, track 10 rutin. Partenolide, considered the constituent marker, is present as blue colored spot, in a very different concentrations in the analysed products, but absent in *Chrysanthellum* were several other additional spots are evident. In other words, the two species very similar on the morphological points of view, but clearly differ in chemical composition. The situation is more complicated when we consider the market products based on extracts obtained with different methods as those reported in Figure 4 and where in some cases the quantity of extract is practically minimal.

Analysis of multi ingredients

In several cases a marketed botanical comes from the mixture of several plant extracts. Contamination is probably going to be a distinctive character of this kind of products, but causes a Mont Everest problem for the analyst. Probably, for this reason so far the argument received not sufficient attention considering the importance. In Figure 11 we report a HPTLC approach, where a marketed multi-ingredient product fingerprint was compared to the fingerprint generated from the mixture of the plants reported in the label, in order to check the composition. Considering the possible variations (extraction method, quality of raw material, storage, ecc.) involved, the possibility to obtain a good result resulted high, although inversely proportional to the number of the involved plants.

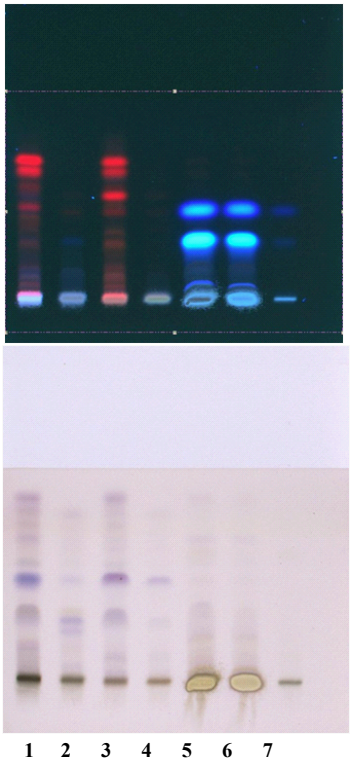

Figure 9: HPTLC of feverfew (tracks 1 methanolic extract and 2 the same extract after defatting treatment) and Mexican feverfew (3-4, with the same treatment) and chamomile (5-6, with the same treatment ,track 7 at lower concentration), another common adulterant of feverfew. Upper plate is visualized at 366 nm and lower plate at white light. The differences with chamomile are evident, as well as the similarities between the first two species, in accordance with their taxonomic relationship.

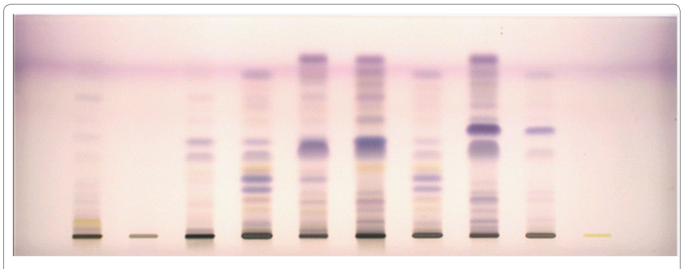

Figure 10: HPTLC fingerprints of extracts of similar marketed species of feverfews. Mobile phase: toluene:ethylacetate (6:4). Derivatization: Anisaldehyde. Visualization: white light. Comparison between feverfew (tracks 6-7) and Mexican feverfew (8-9) against marketed products (1-5) reporting feverfew in the label.

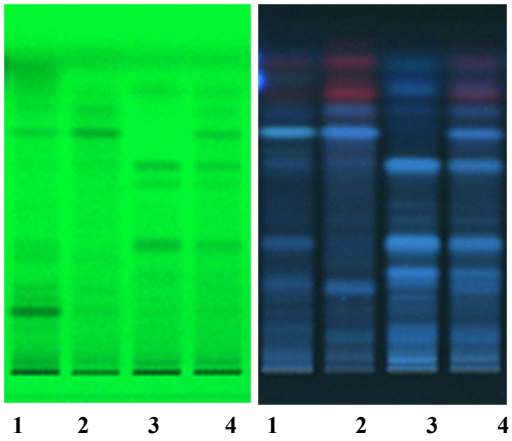

Figure 11: HPTLC analysis of multi-ingredient food supplement by comparison with extracts of constituent raw material. Mobile phase: AcOEt/CH_2Cl_2/CH_3COOH/HCOOH/H_2O 100:25:10:10:11. Left HPTLC: Derivatization: Absent. Visualization: white light. Right HPTLC: Derivatization: Anhisaldehyde. Visualization: UV 366 nm. Comparison between marketed product labelled as extracts of Arctostaphylos uva-ursi and Urtica dioica (track 1), Arctostaphylos uva-ursi marketed extract (track 2), Urtica dioica marketed extract (track 3), mixture of Arctostaphylos uva-ursi and Urtica dioica extracts (track 4).

Limits, shadows and hopes

The future of HPTLC probably be linked to two main aspects: the uses in validation of new incoming products and its introduction into the regulatory systems. Its current heavily presence in the Chinese Pharmacopoeia [10] and into the American Herbal Pharmacopoeia [11] is promising for further recognition of importance.

Once more, first money. Cost of basic HPTLC device is comparable to that of good HPLC apparatus and lower than a high resolution NMR. In any case, the total set of devices is too expensive for a small/medium size industry, and public financial supports in universities and research institutions are practically extinguished. Waiting that the request lowers the prices, the only solution to enlarge the use of HPTLC fingerprint is to create regional or national centres for the control of quality, that can assure consumers and producers.

Second, validation. HPTLC, as well as HPLC-DAD, fingerprint have been recognized by World Health Organization as methods to identify a plant or its preparations by a characteristic chromatographic profile where it is not possible to identify a discriminating active principle [16]. However, still a lot of work must be performed, in setting general and singular conditions, in particular in quantitative analysis. So far, several approaches and methods, including the results comparison between laboratories using the same protocol, have been proposed and discussion about the most adapt is open. Most of the methods suffer for complication, needing too time also to obtain the satisfactory calibration curve, in comparison with easy and rapid qualitative results.

Third, efficacy. The newborn planar chromatography seems to have large possibilities of improve, together with the identification of the role of natural products in maintaining health conditions [25]. Dedication and convincement, as well as invention and technology, will be basically necessary. At the beginning it was sufficient the intelligent and pioneer work of a single research worker, like Michael Twsett, to invent chromatography; later it was necessary the alliance between industry and research, like the couple Merck and Egon Stahl, to obtain the efficient TLC plate; nowadays, the new deal of TLC is possible thanks to the efforts of an industry totally dedicated to the improve of planar chromatography and the typical approach of a modern researcher, that means Camag plus Eike Reich. In any case, the revival of planar chromatography is an important analytical reality.

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