

Article

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HPTLC fingerprint: a modern approach for the analytical determination of Botanicals

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Abstract: Availability of rapid and reliable methods for detection of quality in plant raw materials and botanicals is urgently needed. The recent HPTLC instrumentation allows to obtain fingerprints useful to ascertain identity and composition. The results of direct application of HPTLC devices in selected cases, using the fingerprint approach, are here reported, considered and compared with other methods. HPTLC is proposed as an useful tool for analytical validation of the novel forms of natural products.

Introduction

Recently, the market of natural substances dramatically changed after the appearance of several new products characterized by elements of novelty as well as confusion, including a plethora of imaginative denominations, *i.e.* nutraceuticals, food supplements, botanicals, dietary supplements, medical devices, multifunctional foods, herbal drug preparations and others. As a matter of fact, these are modern forms of natural products utilizations, inheriting natural origin from the tradition like herbal medicines and herbalist products and offering solutions for the treatment of increasing minor pathologies or health maintenance. However, they urgently need adequate analytical controls, since their nowadays success could continue and be insured only by quality assessments, first by the exact determination of the composition. So far limited but significant cases of adulteration, substitution or changes, with the aim of improving efficacy, could invert the positive phenomenon. Dangers clearly also come from the utilization method: registered products are prescription drugs, that must be used under medical supervision, whereas herbal products are self administered and generally regarded as being harmless because of their natural origin. Therefore, the availability of analytical tools specially tailored to face the complexity of natural products mixtures is

crucial. Controls should be based on simple, viable, comprehensible and low cost methods.

From the beginning, started with the first application by Michael Tsweet (Pelick et al., 1966), the future of the chromatography was marked: suited for separation and identification of plant constituents. Separation allowed the identification of thousands and thousands of natural substances, generating the construction of an amazing catalogue of more or less complicated structures, a flippant evidence of the endless chemical fantasy of plant metabolism and the necessary basis for any further phytochemical advancement, also in the analytical frontiers. The central position must be conceived to the planar chromatography, in particular TLC, the daily tool of any phytochemical student. Nowadays, TLC remains the immediate, simplest, reliable analytical friend in the laboratory of organic chemistry to check the presence and the identity of known marker compounds, to follow the improvement of a synthesis, to test column fractions separation trend, and others. However, the current challenge of complex mixtures, as those present in the botanicals, needs more effective proper analytical tools (Kaiser, 1988).

Materials and Methods

Preparation of the samples before HPTLC was simply performed by filtration on cotton. All chemical

standards were of analytical grade. Arbutin (AR, purity $\geq 96\%$) and 3,4-dihydroxybenzoic acid (IS, purity $\geq 97\%$), hydroquinone (purity 99.0%), were purchased from Sigma-Aldrich (Milan, Italy). Rutin ($\geq 98.5\%$) and gallic acid were procured from Prodotti Gianni (Milan, Italy); other flavonoid standards were obtained in other researches and their purity checked by NMR analysis.

HPLC grade solvents were purchased from Sigma-Aldrich (Milan, Italy), Merck (Darmstadt, Germany) and Carlo Erba (Milan, Italy). HPLC-grade water was prepared with a Milli-Q gradient (Millipore, Vimodrone, Italy) water purification system. Formic acid was purchased by BDH prolabo (Poole, England). Analysed products were obtained from the market. Market materials were procured from different Italian companies of raw plant material or were obtained from the market. The origins of *Arctostaphylos* spp. raw materials were from Macedonia, Canada, Mexico, Albania and Siberia. Neem products from India and Italian different importer companies.

Filtered solutions were applied to HPTLC 60 silica gel glass-backed layers (Merck, Darmstadt, Germany). For this purpose, a Camag Linomat IV sample applicator (Muttentz, Switzerland) with nitrogen flow was used. The operating conditions were as follows: syringe delivery speed, $10 \text{ s } \mu\text{L}^{-1}$; injection volume, $10 \mu\text{L}$; band width, 6 mm; space between bands, 9 mm; start position, 9 mm; distance from bottom, 8 mm. The HPTLC plates were developed in a horizontal chamber (Camag 20x10) after saturation with the same mobile phase. The optimised chamber saturation time for the mobile phase was 20 min. at room temperature. The length of the chromatogram run was 80 mm. The developed layers were dried in an oven at $100\text{-}105 \text{ }^\circ\text{C}$ for 15 min and then detected. Initially, the separated components were visually detected. The layers were allowed to dry in air for 30 min and then analysed under the proper detection way.

For the densitometric analysis a Camag TLC scanner 3 linked to winCATS software was set at 350 nm, after multi-wavelength scanning between 250 and 400 nm in the absorption mode had first been tried. The sources of radiation were deuterium and tungsten lamps. The slit dimension was kept at $6.00 \times 0.45 \text{ mm}$ and the scanning speed used was 20 mm s^{-1} .

Results and Discussion

HPTLC vs. TLC

HPTLC arises from the need for major separation capacity, has it is obtained by the use of precoated plates with smaller particles (5 vs. $15 \mu\text{m}$), in order to obtain the efficacy needed for plant mixtures, but problems for TLC have also other origins. Planar

chromatography is mainly based on critical manual steps: it is an open system, dependent on environmental factors (temperature, light, fumes, humidity), that can influence the resulting data; also using the best rigor, despite all the extracautions of the same operator, results can not be fully controlled and analyses are not totally reproducible. In other words, data are scientifically not totally reliable and confident. TLC still remains a craftsman's performance. HPTLC, High Performance Thin Layer Chromatography - the TLC of the 21st Century - is the last evolution of planar chromatography, whose mission is change the weakness into strengths. In modern HPTLC, the plate is the central tool of a complex automatic instrumentation system developed to control analysis conditions, to optimize reproducible results and to allow a complete comparison between different laboratories. The operator is the director of a tentative to reproduce Nature's symphony tricking with sophisticated machines. In this regard, HPTLC is much more complicated than TLC, but complex problems need complex solutions. Being a multistep process, HPTLC performance requires separated devices for each step of the sequence: sample application, chromatogram development, derivatisation, visualization and documentation. The full power of HPTLC comes from the proper use, compatible and complementary, of each device in an integrated system (Reich & Schibli, 2007).

Frequent goal of HPTLC is the fingerprint, as authentic maker of the biological complexity. A fingerprint is the individual chromatographic track representing, as near as possible, a mixture of organic substances. By the fingerprint approach, it is possible to obtain a proper identification of the plant material, but also determine and accept the limits of the biological changes. Variations in HPTLC tracks of the same species are mainly quantitative, not qualitative.

HPTLC vs. HPLC

Although HPLC remains the current best analytical tool, other instrumental possibilities have been proposed. Nowadays, HPTLC is ready to become one of the best methods for control of quality, purity, stability and identity, in one word validation of the composition of complex botanical products. The final coherent step of an evolution of analytical applications in natural products determination. Besides the achieved great increasing in efficacy, due to the use of minor size silica gel that means larger surface, it is possible to perform high quality HPTLC analytical determinations, due to a novel dedicated machinery, in order to finally achieve the necessary reliability, repeatability and flexibility. Substantially, the advantages of HPTLC were the rapidity and the

possibility of analysing many samples at the same time under the same chromatographic conditions. Therefore, the consumption of the mobile phase is reduced and the results are rapid because HPTLC only needs chamber saturation and running time is only a few minutes. The mobile phase is completely removed from the plate during detection so that interferences may be eliminated, costs are limited, and all of the sample components may be seen together as part of the chromatogram. The same HPTLC plate can be visualised with and without derivatisation using different light sources. Reference materials can be chromatographed side by side on the same plate, that means exactly in the same conditions. Among the advantage of the HPTLC is important to report the sensitivity: the limit of detection are very low in accordance with the tradition of TLC, and, using the proper standard, quotient of each area can be defined and used for quantitative analysis.

The main feature is in flexibility and 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 (R_f) and colour of the separated zones, also in reference to a data bank, using a computerized digitalic data bank. Furthermore, densitometry allows a quantitative analysis. Analogous and complementary data can be obtained using the NMR fingerprint of the total extract or parts due to simple treatment. The fingerprint approach is very useful in the analysis of complex mixtures. Usually, operator set HPLC analysis on a single standard or a class of substances, therefore other compounds are invisible or not detected.

Therefore, botanicals can be easily and simply compared using their fingerprint and presence of adulterants as well detected. Main default concerns the still high cost of instrumentation and main advantage consists into the clear and simple evidence of the understandable result typical of TLC, avoiding the limits derived by the use of too specialized analyses.

The successful use of HPTLC performed in our laboratory is here reported in cases concerning:

- a) adulteration, the use of other constituents besides those reported in label composition
- b) substitution, the utilization of botanical species not reported in pharmacopoeias and other regulatory references instead of the official ones
- c) comparison, between extracts of the same species differently obtained.

HPTLC vs. NMR

NMR has been proposed as a possible tool in natural products fingerprint (Krishnan et al., 2005; Piccin et al., 2009). However, also using spectrometer

of high resolution, the possibility of overlapping remains very high. The difficulty is enhanced in the case of comparison of species taxonomically near and in the absence of typical markers. Therefore, sensitivity is in contrast with the appearance of signals of the same constituent in several parts of the spectrum, whose unity must be reconstructed. On the contrary, the NMR study remains crucial in the identification of single substances.

Adulterations

Herbal products spiked with synthetic drugs are dangerous to consumers and noxious for future correct developing of the use of natural products. In 2010 HPTLC was used in our laboratories to evidence several adulterations in food supplements commercialized in Italy: a) presence of synthetic substances: nimesulide in a herbal remedy marketed for relieving pain (Mobile phase: toluene: ethylacetate 2:8 v/v; Detection: UV 254 nm); b) presence of hemisynthetic products: bromexine in a product commercialized for antiallergic properties, containing *Adathoda vasica* among other plant extracts; bromexine is obtained by chemical transformation of vasicine, the main alkaloid present in *A. vasica* (Mobile phase: toluene: ethylacetate: diethylamine 5:4:1 v/v/v; Detection: Dragendorff Reagent); c) presence of natural substances: several terpenoid alkaloids of reserpine type probably from a *Rauwolfia* sp. were detected (Mobile phase: toluene: ethylacetate: diethylamine 5:4:1 v/v/v; Detection: Dragendorff Reagent) in a herbal product sold for hypotensive effects, whose composition reported plants without any alkaloid relevant presence.

As well known, most problems come from the products that can be obtained by web site selling, including herbal dietary supplements marketed as natural products for the enhancement of sexual function. In particular, HPTLC plates clearly showed an anomalous relevant constituent in a herbal product marketed from China as a potent aphrodisiac, named Sensual Tea or Jinshenkang, heavily present in internet sites as able to rapidly solve any sexual problem of females and males without any collateral effect (www.thesensualtea.com.mx; www.okokchina.com/p/Herbal-Medicine/Herbal-Jinshenkang-better-than-Viagra). The product examined, also marketed in Italy, came from Spain, where later the product was removed from the market.

HPTLC showed a great spot very strong at UV lamp at 366 nm, as evident clue of the adulteration (Figure 1). Direct HPTLC comparison with sildenafil and vardenafil - the PDE-5 inhibitors approved as anti-impotence drugs for the treatment of erectile dysfunction, excluded the identity with these substances. Also, densitometry analysis confirmed the differences,

showing different UV absorption. A simple extraction with ethylacetate completely removed the substance from the product and the NMR spectrum of the extract resulted in an almost pure compound identified as thiosildenafil, by analysis of spectral data (Balayssac et al., 2009). The analysed Jinsenkahn product came from 2009 market, but the analysis of a 2008 product revealed the presence of sildenafil (1): therefore, thiosildenafil (2) has the same structure of sildenafil with a S instead of O, and it was artfully inserted to bypass the controls, too specific and specialised, as really happened. Both HPLC and HPTLC spectrometric analyses allowed to ascertain a quantity of the adulterant compound slightly superior to that contained in a tablet of Viagra. Thiosildenafil has been detected in another Chinese similar supplement, but using complicated 2D and 3D DOSY ^1H NMR spectroscopy experiments (Balayssac, 2009).

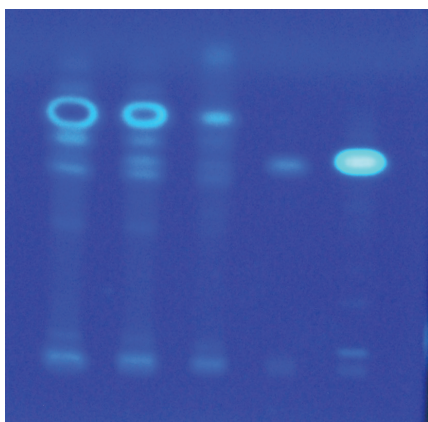
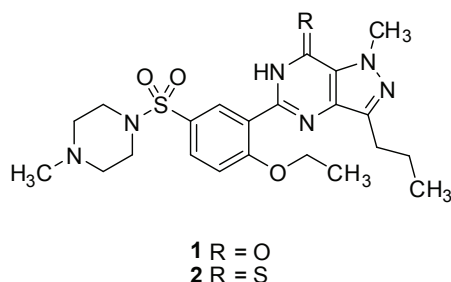


Figure 1. The HPTLC plate of Jinshenkang. From left to right, the three first spots are the product in different concentrations (1, total extract; 2, 1:2 total extract/ethyl acetate; 3, 1:5 total extract/ethyl acetate) and the last two vardenafil and sildenafil, respectively. Mobile phase: dichloromethane: methanol (9:1 v/v). Detection: UV 366 nm.



Substitution

Regulatory documents on medicinal plants, including Pharmacopoeias, are resistant to modifications and not able to follow the changes of the market. In the global market, several new species are already

used from several years and are still waiting a legal recognition. Meanwhile, these species are used and confused with old ones. The use of HPTLC fingerprint allows to obtain an easy identification of the different species, certifying in some cases also the geographic origin, as reported in the HPTLC volume of the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2009).

In particular, we applied the fingerprint method to the study of *Arctostaphylos uva-ursi* and *A. pungens*. The second species is nowadays increasingly used instead of the first one, traditionally employed as medicinal plant to treat urinary infections. *A. pungens* is collected in good quantity in Mexico, whereas *A. uva-ursi* is even more difficult to find and costly. The activity is attributed to arbutine, a glucosidic parachinone metabolically converted into the corresponding aglucone, hydroquinone (WHO, 1996; Frohne, 1970). However, HPTLC analysis clearly showed a different composition for the two species: arbutine is practically absent in *A. pungens*, which is very rich in flavonoids. Also, NMR confirmed this absence, whereas HPLC analysis was able to verify a very low presence of arbutine, below the 0.5% (Nicoletti et al., 2010a). However, recent pharmacological studies showed a potent activity of flavonoids against bacterial infection of the urinary tract, validating the use of *A. pungens*.

Other similar studies were obtained for products containing *Equisetum* sp. (Gallo, 2011) (Mobile phase: ethyl acetate:formic acid:water 82:9:9, v/v/v; Detection: NPR). According to the European Pharmacopoeia only *E. arvense* should be used, but other species, i.e. *E. maximum*, resulted present in the marketed products. Also in this case pharmacological data are lacking about the effects of other *Equisetum* species.

Comparison

HPTLC is a potent tool for easy comparison of similar products. Lots of the same product can be compared in the same plate and quantities assessed by densitometric inspection. In particular, we were able to examine the composition of several products marketed by the name of neem cake. Neem tree, *Azadirachta indica* A. Juss, features a long traditional utilization in agriculture, for the body care (cosmetics) and medicine (Mordue & Blackwell, 1993) but currently, there is a huge amount of products obtained from this multipurpose tree, due to the anti-fungal, anti-bacterial and insecticide properties. The most commercially important product is the neem oil extracted from the seeds, also known as margosa oil. Neem, identified by WHO/UNEP1989 as an environmentally powerful natural pesticide, is considered to be one of the most promising trees of the 21st century for its great potential

in pest management, environment protection and medicine. Neem cake is the raw material derived by cold pressing neem kernels from handpicked and cleaned neem fruits and seeds. Neem cake is commercially important for its use as fertilize material, as well as potential insecticide (Nicoletti et al., 2010b). By the HPTLC neem cakes resulted different depending by the extraction process used, the different origins and the method of commercialization (Neem America Incorporated, 2010). In particular, the content of fatty constituents appeared highly variable, as evident in the plates (Figure 2). Therefore, a different utilization of neem cakes should be inferred according to the compositions.

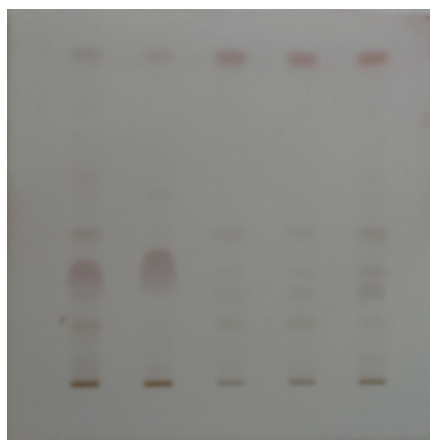


Figure 2. Different compositions of neem cakes from the market. Mobile phase: toluene: ethylacetate (8:2, v/v). Detection: anisaldehyde reagent (sulphuric acid (10 mL) added to methanol (170 mL) and acetic acid (20 mL) and to further anisaldehyde (1 mL). The main spots evident in the

middle of the first two tracks were identified as linear fatty acids after separation and NMR analysis, whereas spots on the top were identified as methyl esters of fatty acids.

The fingerprint approach is extremely effective in the comparison of populations of the same species or lots of the same extract. It was used to verify the different production of flavonoids and hypericine in *Hypericum perforatum* plants collected in different parts of Italy. As evident in Figure 3, the contents resulted very different and this is in contrast with the current regulation requiring a fixed relationship between the constituents.

Conclusion

The utilization of HPTLC, owing to the high automatization recently obtained, can be considered as an useful tool in the analysis of complex mixtures of natural products, such as those introduced nowadays in the market. Still some aspects remain to be improved, like the cost of instruments, and the sensibility, still not comparable with HPLC. Also in this field, like in the other analytic approaches, the use of hyphenated techniques, as already obtained in GC/MS. Hyphenated HPTLC/MS instruments are already available, and HPTLC/NMR are under study, to solve in one step the problem of separation and identification.

Adoption of HPTLC by USP (USP, 2007), Ph. Eur., AHP and PhPRC (Chinese Pharmacopoeia, 2009) constitute a clear recognition of the importance of this technique as the method of choice for handling complex analytical task involving herbal drugs and botanicals.

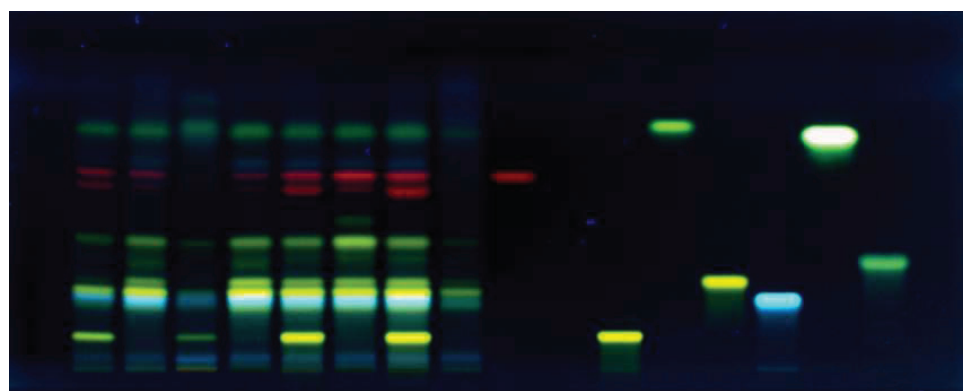


Figure 3. Comparison between different populations of *Hypericum perforatum*. On the left: tracks of *Hypericum perforatum* collected in different Italian regions at the same vegetative stage including a commercial extract (tract 3); on the right: the flavonoids standards, track 10 hyperoside (not visible in these conditions), 11 rutin (yellow spot at low Rf), 12 quercetin (green spot), 13 isoquercetin (yellow spot), 15 luteolin (white spot) and 16 apigenin; hypericine was track 9, evident in red, and chlorogenic acid in track 14 was used as standard. Mobile phase: formic acid: water: ethyl acetate 10:5:85, v/v/v. Detection: the layers were treated with a solution containing the Natural Product Reagent (diphenylborinic acid aminoethylester (1 g) in ethyl acetate (200 mL)), dried in the open air and then dipped into Macrogol reagent (polyethylene glycol 400 (1 g) in dichloromethane (20 mL)).

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