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FINGERPRINT HPTLC ANALYSIS OF MARKETED BOTANICALS**

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Abstract

Traceability of botanical food supplements (botanicals) is a prerequisite for their validation, utility and security. Botanicals are usually based on a single herb or on the combination of several herbal drugs, giving rise to a complicated analytical problem, due the great number of different constituents. Against the usual approach based on standards or markers, a metabolomic approach is reported based on HPTLC fingerprint. The results obtained in case of mono- or multi-ingredient botanicals are reported to evidence and testify the utility of the method.

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Introduction

Revival of natural substances opened to new utilizations, novel products and additional scientific challenges. Actually, consumers' attention and preferences are focused on food supplements [1]. In principle, food supplements are foods intended to complement the normal diet by ingestion of substances with physiological effects. These products radically influenced the utilization of natural products, owing to the great acceptance by consumers [2]. However, in the last years consumers' prevalence changed types and targets of food supplements. Initially, food supplements started as vitamins, minerals, proteins, carbohydrates, but nowadays "other substances" are dominant. Other substances are essentially extracts of medicinal plants, often the same ones used as foods or spices. Therefore currently, these new products are properly named botanical food supplements, or simply botanicals.

The impact of these products, based on their nutritional and physiological effects, can be evidenced by the market data. In 2009 the world market for food supplements was reported as about 45-50 billion euros and in the EU about 10-15 billions [3], with "other substances" accounting for half of this figure. In "other substances" products Italy is the leader with about 1454 millions euros and one third of the population regularly consuming food supplements [4]. Every year in Italy botanicals mark an increment of about 11%, despites the economic difficulties, accounting in 2011 for 1861 millions of euro, with pharmacy dominating sales.

As a consequence, complexity in composition of food supplements increased exponentially with predominance of botanical "other substances".

Botanicals are hybrids born from the traditional medicine and the modern technology. They start from herbal drugs whose processing changes deeply form and composition. The importance of composition cannot be overestimated. The distance between the starting raw material and the marketed product, that means identification and traceability, can be overcome only by the chemical quality control. As a matter of fact, any step of the procedure production can deserve an important impact on the composition, that means effects on efficacy and security. The definition of the composition is a prerequisite for the utilization of any food, but very important in botanicals, that are concentrated extracts. Prescription drugs are subjected to severe controls and medical supervision and decision, whereas herbal products are self-administered and generally regarded as harmless, despite any contrary evidence.

Despites the possible changes during production steps, the analysis first must detect the identities of the species used for the production. If the composition of the botanical is different from that reported in the label, all the process and any utilization loose any meaning, including alarm for the security. Thus we first focused on this target, starting from the monoherbal analysis to face the challenge of the multiherbal botanicals in order to obtain an adequate low cost, reliable, rapid, useful quality control.

Quality control in botanicals

Usually traditional herbal remedies are water decoction of a mixture of 2-12 different herbs. Botanicals mimic the traditional remedy, but usually they are based on technological extraction by maceration in aqueous ethanol at different temperatures of plant drugs and subsequent processing to obtain the adequate marketed form. In accordance with traditional medicine, in several botanicals extracts are mixed together, giving rise to a multiherbal product. A difficult analytical challenge, since any herbal drug contains a myriad of compounds pertaining to many different classes in chemical interaction, forming a complex matrix. Constituents can interact each other, influencing solubility, stability and resorption, including actions of residual enzymes.

So far, chromatographic quality controls of multiherbal botanicals followed the usual targeted approach, based on the

use/comparison/determination of marker constituents, as reported in all Pharmacopoeias. The limits of this method appeared consistent on the light of new pharmacological and medical approaches based on all the extract, the phytocomplex, acting as the active principle with different or complementary action [5]. Also in case of the most simple determination, like that of the identity of a powdered botanical drug, the target method can fail, since, as well known, similar plants contain similar main constituents, and in any case differences in the secondary ones can be important. Although the target approach is still useful in many cases and has its historical relevance times are ready to test other methods.

We used an untargeted approach, based on metabolome and fingerprint. In metabolome analysis of botanicals the target is to detect as many small molecules as possible, that means consider the entire extract as the analytical target [6,7]. Therefore, the analyst device must be very sensible and exerts a great separation capacity.

Here we propose for quality control of botanicals an untargeted metabolome analytic approach obtained using the HPTLC devices, specifically developed by CAMAG, Switzerland, for analysis of natural products [8]. HPTLC, High Performance Thin Layer Chromatography, has great separation capacity due to the utilization of silica gel with smaller particles (5 mm vs. 15 mm) and a set of automatic devices insuring repeatability, robustness and efficiency [9]. Furthermore, the results can be stored as digital data and easily interpreted and compared.

HPTLC fingerprint analysis of botanicals

The utility of HPTLC in quality control of marketed products [10-14], as well as in the presence of adulterants has been already reported [15-16]. However, the challenge raising from the analysis of a combination of herbal drugs constitutes a further current problem of overall difficulty, derived from the number of constituents. A metabolome approach is here reported, based on HPTLC fingerprint, for identification and quality assessment of marketed multi-ingredient botanicals, and the results obtained in case of multi-ingredient botanicals. A chromatographic fingerprint is the individual track representing, as near as possible, the mixture of produced organic substances [17].

HPTLC is able to generate a chromatographic fingerprint in the form of an unique sequence of peaks nearly corresponding to the analyzed sample in its chemical fullness. By the fingerprint approach, it is possible to obtain a proper identification of the plant material, but also determine and assert biological changes, without necessary identifying nor quantifying a specific compound(s). In HPTLC tracks of the same species, variations are mainly quantitative, not qualitative.

The analytic method here proposed is based on the comparison of the fingerprints between that of the marketed multiherbal product and that independently obtained as a mixture of the extracts of the herbal components. In a monoherbal product, the identity of the used plant can be obtained by the comparison of the fingerprint of the marketed product with that of the extract of the reported species. Now the usefulness of a similar approach was tested in case of a multiherbal product.

Analysis of a monoherbal botanical

In Fig. 1 the HPTLC fingerprint was applied to the analysis of a monoherbal botanical based on *Hypericum perforatum* (aerial flowering plants) in comparison with raw materials of the same species collected in different parts of Italy (tracks 1-5). The fingerprints differ only for quantitative presence of the nìmain constituents and for the presence of red spots at higher Rf values. However, these spots are usually due to fatty acids and similar substances, whose occurrence can change according to the freshness of the used drug. The analysis can be visualised in different ways, i.e. at different wave light and with different derivatization, each affording useful information. Further conversion by

densitometry allows a better comparison of the fingerprints.

see Fig. 1.

see Fig. 2.

Analysis of a multiherbal botanical

In Fig. 3 the HPTLC fingerprint analysis of a multiherbal marketed product containing as constituents species *Salvia officinalis* (leaves) and *Juniperus communis* is reported, in comparison with the fingerprint of the constituents drugs. In this case the number of the spots are clearly numberous. Track 4, is the fingerprint of the solution obtained by the mixture of the extracts of tracks 2 and 3. Track 4 can be compared with track 1, showing the fingerprint of the marketed analysed product, evidencing the correspondence of the great majority of the spots. Again differences in spots at high Rf must considered on the light of the presence of lipophilic substances.

see Fig. 3

Conclusion

Recently the problem related to the complexity of botanicals composition was increased by the massive introduction in the market of products based on many plant raw materials, including first for importance the TCHM (Traditional Chinese Herbal Medicine). These medicines, being derived by traditional medicines, are based on multi-ingredient preparations representing *in toto* the activity of the herbal preparation.

Modernization of traditional medicine afforded by botanicals starts from quality control. This is in particular true for TCHM preparations, that are registering an increasing interest in the Western hemisphere, as well as a general alarm for low quality of raw materials, presence of contaminants, substitutions causing intoxication and adulterations by synthetic products in order to increase activity. A lot of scientific work is in progress for the validation of TCHM, including metabolome fingerprint with different tools [18]. Therefore, it is important to develop new strategies for the analyses of such products, including the HPTLC fingerprint multiherbal method here reported, verifying its efficacy in case of the presence of many herbs.

Experimental

Chemicals, Reagents, Materials

Methanol and other solvents for analysis and HPLC grade solvents were purchased from Sigma-Aldrich (Milan, Italy) and Carlo Erba (Milan, Italy). Information concerning marketed products or collection of plant raw materials can be obtained by request to the corresponding author. Plant raw materials were identified by Dr. Filippo Maggi, University of Camerino (Italy). Extracts utilised as mono- or multiherbal standards were hydroalcoholic 95° extracts obtained from the market. Also hydroalcoholic extracts of the constituent plants (5 mg / 10 ml) were obtained in the lab to be used for comparison as standards to confirm the identities of the marketed ones. Reference drugs or marketed products (400 mg in any case) were extracted with aqueous EtOH (5ml) for 48 h at room temperature.

HPTLC analysis

The samples were deposited in the form of bands of width 6 mm with a Camag 100 microlitre sample syringe (Hamilton – Bonaduz, Switzerland) on precoated HPTLC plate silica gel 60 F 254 (20x10cm) (E. Merck - Darmstadt, Germany) using a Camag Linomat V (Camag – Muttenz, Switzerland). The plates were prewashed by methanol and activated at 110°C for 5 min prior to chromatography. A constant application rate of 150 nL/s was employed and the space between two bands was 12.3 mm. Linear ascending development was carried out with Camag Automatic Developing Chamber – ADC2 (Camag – Muttens, Switzerland). The optimize chamber saturation time for mobile phase was 20 min at room temperature (26.3°C) at relative humi-

dity of 43.6%rH. The length of chromatogram run was 70 mm in 12 min. The developed layers were allowed to dry in air for 5 min and then derivatized with 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). 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 upper and lower (WRT), respectively, at a Camag TLC visualizer.

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Finocchio (Foeniculum vulgare) frutto estratto secco titolato 0.9-1,1 %
in olio essenziale,
Carvi (Carum carvi) frutto estratto secco E:D/1:4 (carvi, maltodestrina),
Anice stellato (Illicium verum) frutto estratto secco E:D/1:4 (anice)
Finocchio (Foeniculum vulgare) frutto olio essenziale,
(Carum carvi) semi olio essenziale,
Cannella (Cinnamomum zeylanicum) corteccia olio essenziale.

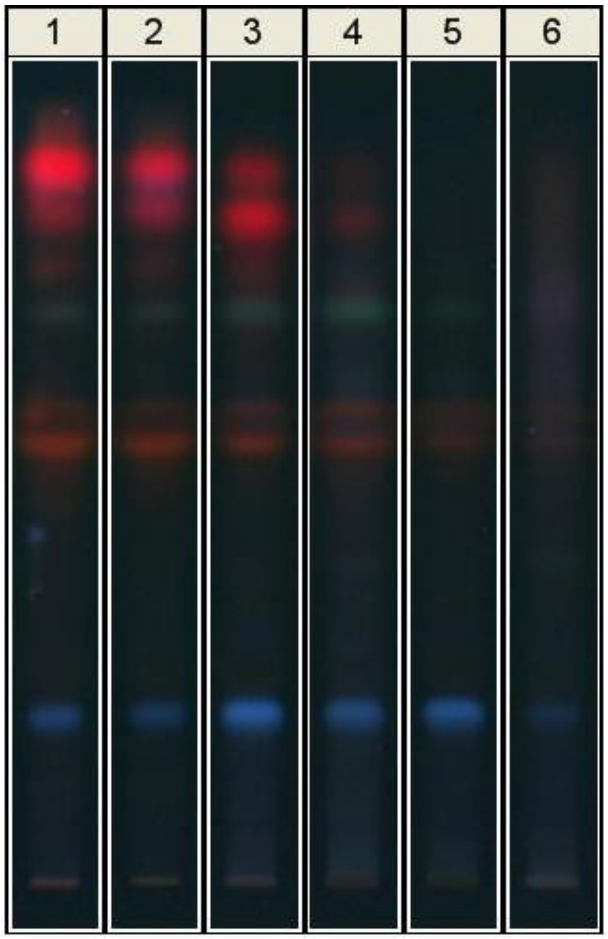

Fig. 1. Comparison between HPTLC fingerprints of St. John's wort, *Hypericum perforatum* L. (aerial flowering plants), collected in different parts of Italy (tracks 1-5) and a marketed product labeled as hydroalcoholic extract of *H. perforatum*. Mobile phase: ethylacetate/dichloromethane/acetic acid/formic acid/water 100:25:10:10:11 (v/v/v/v/v). Visualization: UV 366 nm. Derivatization: NP Reagent.

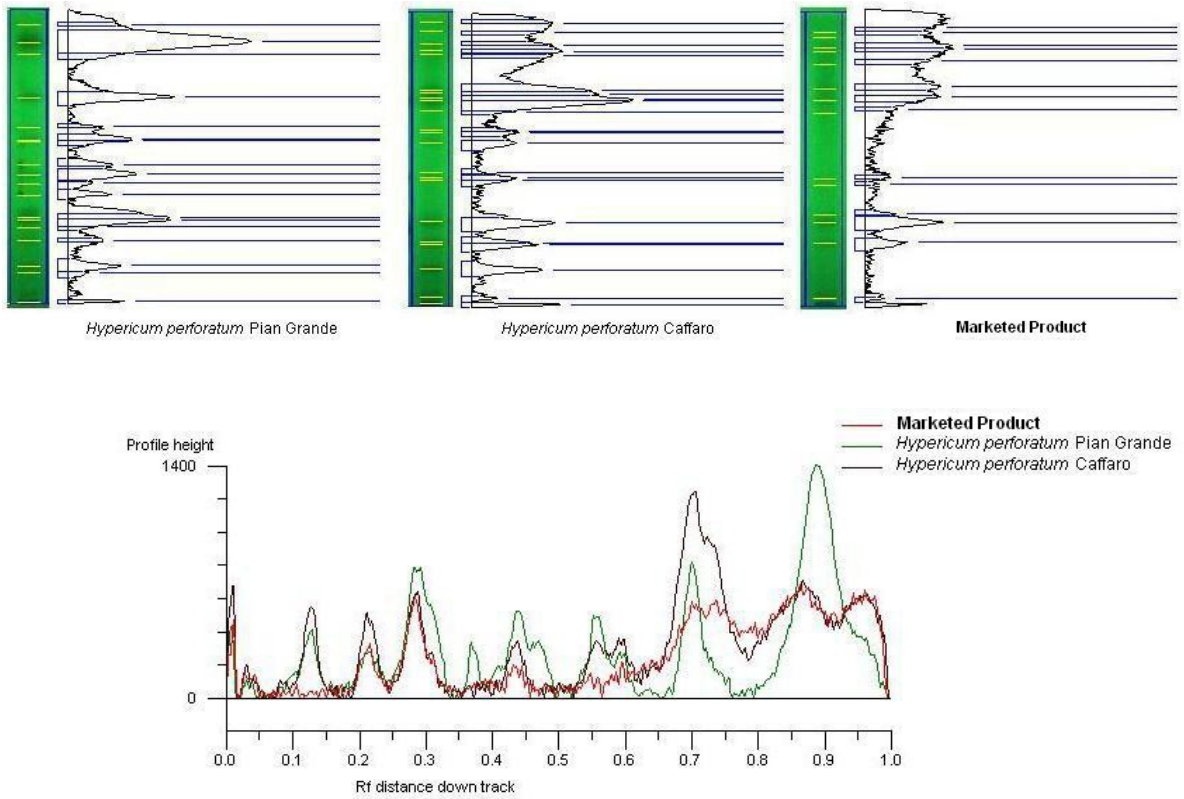

Fig. 2. Densitometric conversion of selected samples of *Hypericum perforatum* and the analysed marketed product.

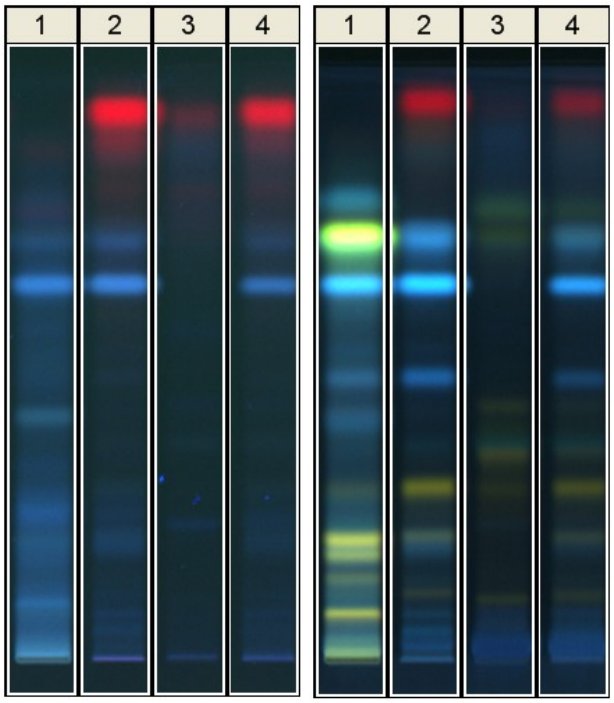

Fig. 3. HPTLC analysis of a multiherbal marketed product labeled with the occurrence of common sage, *Salvia officinalis* L. (leaves), and common juniper, *Juniperus communis* L. (berries). Mobile phase: ethyl acetate/dichloromethane/acetic acid/formic acid/water 100:25:10:10:11 (v/v/v/v/v) Visualization: UV 366 nm. Derivatization: before (left plate) and after NP Reagent treatment (right plate). Tracks: 1, multiherbal marketed product; 2, *Salvia officinalis* hydrolcoholic extract; 3, *Juniperus communis* hydrolcoholic extract; 4, mixture of extracts of 2 and 3 fingerprints.