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HPTLC FINGERPRINTING OF WITHANOLIDES IN COMMERCIAL ASHWAGANDHA FORMULATIONS

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Abstract

Ashwagandha (Withania somnifera) root products are sold as capsules, tablets, powders and liquid extracts, and their labels rarely reveal how much of the active steroidal lactone fraction they actually contain. This narrative review summarises published evidence on high-performance thin-layer chromatography (HPTLC) as a fingerprinting tool for withanolides in commercial formulations. It covers withanolide chemistry, plate development and detection, validation against ICH criteria, and the quality problems reported for marketed herbal products. HPTLC offers a cheap, parallel and visual comparison of many samples on one plate, but quantitative work depends on reference standards and careful validation. This review reports no new experimental data, and published fingerprint studies remain hard to compare.

1. INTRODUCTION

Ashwagandha, the dried root of *Withania somnifera* (L.) Dunal, has been used in Ayurvedic practice for centuries as a rejuvenating tonic, and it now sits on the shelves of pharmacies and supplement shops around the world [1, 2]. Modern interest has grown with clinical reports on stress, sleep and general well-being [3, 4, 5]. Demand has pushed the product into every convenient format: root powder in capsules, compressed tablets, polyherbal churna, syrups, and standardised extracts sold under brand names. The claim is not that every product is poor, only that a consumer or a purchasing pharmacist has very little way of telling. Few retail labels give a marker compound, a plant part or an extraction ratio, and where they do, the figure may rest on a certificate the buyer

never sees [6, 7].

The trouble starts at the label. A bottle that says 'ashwagandha 500 mg' may contain milled root, a concentrated extract, a leaf-derived material or a blend of these, and the amount of the biologically relevant steroidal lactones can differ several-fold between brands [6, 8]. Pharmacopoeial monographs set identity tests and, in some cases, limits for total withanolides, yet these tests were written for raw drug, not for a finished tablet with excipients, binders and other herbs [9, 10]. Variation of this kind is not unique to ashwagandha, but the plant makes a good case study because its chemistry is well described and its commercial use is so broad. Quality failures here would also be easy to detect with methods that already exist, which makes the question of why they are used so unevenly worth asking. Pharmacists asked about a particular brand can only fall back on the manufacturer's word.

Chromatographic fingerprinting is one answer. Instead of chasing a single marker, the analyst records the whole pattern of bands produced by a sample and compares it with an authentic reference [7, 11]. High-performance thin-layer chromatography is well suited to the job because it separates many samples side by side on one plate, tolerates crude extracts, and leaves a permanent visual record that an inspector can read without specialist software [12, 13]. That matters for anyone trying to compare one preparation with another. Two products can carry identical labels and still differ in plant part, solvent and marker content, and a chromatogram is one of the few quick ways to show it on a bench rather than in a brochure [12, 13]. Where a laboratory already owns an applicator and scanner, the running cost per sample is small, and training needs are modest compared with mass spectrometry.

This review draws together published work on HPTLC fingerprinting of withanolides, with a focus on marketed formulations. It covers the chemistry that makes the analysis difficult, how plates are developed and read, how methods have been validated, and what the literature says about adulteration and batch variation. Principal findings are collected in table 1. The article is a narrative synthesis of published reviews and experimental papers and presents no new analytical data. Reading the literature with that in mind, the sections below move from chemistry to method to validation and then to product quality, and each stage is tied back to what has been reported by others rather than to any measurement made for this article. Gaps are noted where they appear.

A short note on scope. Clinical efficacy of ashwagandha is touched on only where it explains why quality matters, since the trials cited here used particular extracts and cannot be assumed to transfer to every product on sale [4, 5, 14]. Readers wanting the clinical picture are better served by the dedicated trials and systematic reviews, which are cited here only for context [3, 4, 5]. Dosing in those studies varied widely.

2. WITHANOLIDE CHEMISTRY AND SOURCES

Withanolides are C28 steroidal lactones built on an ergostane skeleton, usually with a six-membered lactone in the side chain and oxygenated functions on the A and B rings. More than forty have been isolated from *W. somnifera*, and withaferin A, withanolide A, withanolide D and withanone are the ones most often mentioned in quality work [15, 16]. Glycosylated forms, the sitoindosides, were described in earlier pharmacological studies and add further polarity classes to the extract [17]. They are not all equally easy to analyse. Some are aglycones that run well on silica, whereas glycosides sit near the origin in the common solvent systems and need more polar mobile phases or a hydrolysis step before they appear as a clear zone [18, 19]. The lactone ring and the 5,6-epoxide or enone systems in ring A are what chiefly distinguish members of the group from one another in chromatographic behaviour [15, 20].

Their distribution within the plant is uneven. Roots and leaves carry different profiles, with withaferin A generally more prominent in leaf material and withanolide A relatively more associated with root, which matters because leaf is sometimes substituted for or blended with root in commercial products [20, 21]. Metabolomic comparisons with NMR and

mass spectrometry have also shown that fruit and other organs have distinct fingerprints [21, 22]. Practical consequences follow. A laboratory that has only a leaf reference and tests a root capsule may call a genuine sample suspect, while one with only a root reference could pass a leaf blend. Authenticated references of the right plant part are the first requirement of any fingerprint [11, 23]. Such differences are one reason authors recommend that both organs be fingerprinted separately before any blend is judged [21, 22].

Then there are chemotypes. Plants grown from seed of different geographical origin can differ markedly in the withanolides they accumulate, and Israeli, Indian and other collections have been grouped into chemotypes on this basis [22, 24]. A single species name on a label therefore does not guarantee a single chemical profile. Growing conditions, harvest age, drying and storage all add further variation, so the same cultivar may give a different fingerprint from one season to the next [7, 25]. Taken at face value, such variation means that a pharmacopoeial limit written around one chemotype may be too lax or too harsh for another. Published authors have pointed to the need for specifications that name the plant part, the chemotype and the marker, rather than quoting a single percentage for the whole species [24, 25]. For formulators, this is a reminder to specify the source of the raw material in the purchase contract and not just the species.

Table 1: Published evidence relevant to HPTLC fingerprinting of withanolides

Topic	Reported finding or feature	Ref.
Withanolide skeleton steroidal lactones	Ergostane-based lactones; withaferin A and withanolide A are the usual quality markers.	[15, 16]
Plant part root versus leaf	Root and leaf carry different withanolide profiles, so substitution changes the fingerprint.	[20, 21]
Chemotypes origin of seed	Collections differ in withanolide content, so one species name does not fix one profile.	[22, 24]
Plate and layer silica gel 60	Fine-particle silica with automated band application improves resolution over classical plates.	[11, 18, 26]
Detection derivatisation	Anisaldehyde–sulphuric acid gives coloured zones; UV scanning is limited by weak absorbance.	[11, 13, 20]
Sample clean-up tablets, polyherbals	Excipients and other herbs cause streaking and overlapping bands unless extracts are cleaned.	[13, 19]
Validation ICH Q2(R1)	Specificity, linearity, precision, accuracy and robustness must be shown for each matrix.	[19, 27]
Reference standards limited supply	Only a few withanolides can be bought, so most bands remain unidentified.	[15, 20]
Herbal supplements DNA barcoding	A substantial share of tested products contained species not on the label.	[28, 29]
Liver injury case series	Ashwagandha products were linked to mild to moderate liver injury; the causative component was unclear.	[30]
Clinical trials proprietary extracts	Reported benefits relate to specific extracts, not to every marketed product.	[3, 4, 5]

The biological activities attributed to these compounds help explain why anyone cares. Withaferin A has been shown in cell studies to interfere with NF-κB signalling through inhibition of IκB kinase, and related effects on stress-response pathways have been reviewed [31, 32]. Sitoindosides were reported to have immunomodulatory and central actions in animal work [17]. None of this proves that a particular capsule will work, but it supports the idea that the withanolide fraction should be measured rather than assumed [16, 33]. There is a further caution. Pharmacological studies of purified withaferin A often use concentrations that are hard to reach after oral dosing of a root extract, and extrapolating from the cell dish to the capsule is risky. The compound is a good marker of identity, but its value as a marker of clinical benefit is still unsettled [31, 32, 33].

Extraction chemistry deserves a mention too. Root material is typically extracted with methanol, ethanol or hydroalcoholic mixtures for analysis, while commercial extracts may be made with water, ethanol or supercritical carbon dioxide. Each solvent pulls a different slice of the withanolide family, so a fingerprint obtained from a laboratory methanol extract of a finished tablet will not necessarily mirror what a patient swallows [7, 23]. Seasonal and storage effects are less well documented than they ought to be, and most fingerprint papers analyse a single lot, which hides this source of change. Exposure to heat and moisture is a plausible cause of drift in lactone content, though direct stability data on finished ashwagandha products are scarce [25, 27]. Reports of ultrasound-assisted extraction suggest shorter times, though yields vary with solvent.

3. HPTLC FINGERPRINTING METHODS

In HPTLC the sample is applied as narrow bands, usually by an automated spray-on applicator, on a silica gel 60 plate with a fine particle size and a fluorescent indicator. The plate is developed in a saturated chamber, dried and examined. Compared with classical thin-layer chromatography, the finer layer, automated application and densitometric scanning give better resolution and reproducibility, which is the reason the technique is accepted for pharmacopoeial identity tests of herbal drugs [11, 18, 26]. Pharmacopoeial thin-layer tests for herbal drugs now routinely specify plate type, layer thickness, saturation and detection reagent, and a monograph that gives these details can be followed in most well-equipped laboratories without further development work [9, 11]. Training in the technique is also modest, and a new analyst can usually produce a usable plate within a few weeks of supervised practice.

Mobile phases reported for withanolides are generally mixtures of a non-polar solvent with a moderately polar one and a small amount of acid or alcohol, for example toluene with ethyl acetate and formic acid, or chloroform with methanol. The choice is empirical. Withaferin A and withanolide A differ little in polarity, so tuning the ratio to pull the key bands apart on a real extract takes patience and several trial plates [12, 18, 19]. Reported systems also differ in how much attention they pay to chamber saturation and ambient humidity. Unsaturated chambers give edge effects and curved fronts, and plates stored at different humidity before use may give different retardation factors for the same band, so conditioning of the plate is as important as the solvent ratio [18, 26]. Plate-to-plate differences in silica activity can also shift band positions, and many laboratories therefore run a reference mixture on every plate to anchor the pattern [11, 18].

Detection is the second decision. Withanolides contain an enone chromophore and absorb in the short ultraviolet range, so plates can be scanned at around 220 to 230 nm, but many bands in a crude extract absorb there too. Derivatisation with anisaldehyde–sulphuric acid and heating gives coloured zones that are easier to tell apart by eye, and the same plate can then be photographed under white light for the record [11, 13, 26]. Chaurasiya and colleagues showed with liquid chromatography that withanolides are poorly served by UV alone, which is one reason evaporative light scattering detection has been explored [20]. Scanning at several wavelengths adds a second dimension to the fingerprint. A band that is dark at one wavelength and invisible at another tells the analyst something about its chromophore, and spectral overlays between a sample zone and the standard serve as a cheap identity check before any mass spectrometer is involved [12, 20]. For routine release testing, a simpler single-wavelength densitogram may be enough once the identity of the main bands is settled.

What does a fingerprint actually tell you? A test sample is run beside an authenticated root reference and one or more marker compounds. The analyst checks that the characteristic bands are present at the expected retardation factor, that their colours match, and that no unexpected strong bands appear [7, 11]. Image comparison software can overlay tracks, but even a trained eye can pick up a missing zone. The balance of evidence across themes in table 1 is plotted in fig. 1, and it shows how much of the record

concerns method development rather than product testing. Reference tracks matter as much as sample tracks. Running the standard mixture on every plate lets the analyst correct for shifts in the retardation factor, and comparing against a validated botanical reference material rather than a dried sample of unknown history is the better practice in quality control settings [7, 23].

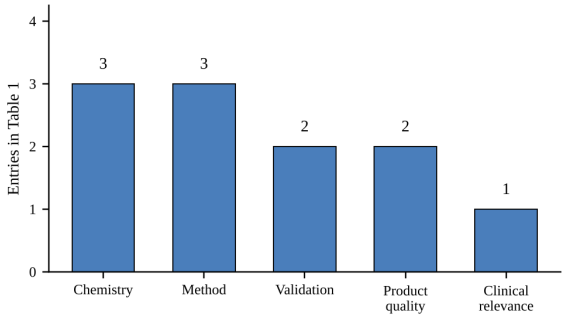


Fig. 1: Number of entries in table 1 by theme

Sample preparation is often where methods succeed or fail. Tablets must be powdered, extracted by sonication or reflux, filtered and sometimes cleaned up by solid-phase extraction to remove sugars and excipients that streak on the plate. Polyherbal formulations are harder, since other herbs contribute their own bands in the same region of the plate and overlapping zones are hard to attribute [13, 19, 23]. Digital documentation has become standard. Plates are photographed under ultraviolet light at two wavelengths and in white light after derivatisation, and the images, with the densitogram, form the batch record that an auditor can review years later [11, 18]. Selective sample clean-up is therefore often the step that decides whether a plate can be read.

4. VALIDATION AND QUANTIFICATION

A visual fingerprint is qualitative. When a laboratory wants to state how much withaferin A or withanolide A a product contains, the method must be validated, and in practice the framework is the ICH Q2(R1) approach, whose familiar parameters (specificity, linearity, range, accuracy, precision, detection and quantification limits, and robustness) are discussed in detail in practical guides to method validation [27]. Reviews of HPTLC assays for pharmaceutical formulations describe how each parameter is handled on a plate and where the technique needs special care [13, 19]. Many published methods follow this order, starting with the optimisation of the mobile phase, then calibration, then recovery experiments in which a known amount of standard is added to a pre-analysed sample. Recovery near the nominal value is taken to show that the extraction and the plate do not lose or create analyte, although the spiked standard may behave differently from the compound bound inside plant tissue [13, 19]. The guideline was written with synthetic drug substances in mind, so analysts of herbal products usually adapt it, accepting wider acceptance limits where the matrix is complex [23, 27].

Specificity is the awkward one. Peak purity can be checked by comparing spectra taken at the start, middle and end of a band, and by showing that the band in the sample co-chromatographs with the standard, but a plate cannot separate every co-eluting compound in a complex extract [12, 19]. Linearity of densitometric response is usually good over a limited range and then curves, so calibration with several levels of reference standard on each plate is the norm rather than a single-point check. Reports often add statements on limits of detection and quantification derived from the calibration line. These depend heavily on the scanning mode and the slit dimensions, so figures from one instrument cannot be copied into another laboratory without checking. Anyone reading such tables should note whether the limit was estimated or measured on spiked plates [18, 27].

Precision on a plate has two parts: the repeatability of application and scanning, and the variation between plates and days. Published method papers generally report acceptable relative standard deviations, but such figures come from the authors' own laboratory and are seldom reproduced independently [12, 18]. Robustness testing, for example by changing chamber saturation time, mobile phase composition or development distance, is described less often than it should be, and humidity effects on silica gel are a known source of drift [19, 26]. The main alternative is liquid

chromatography. Chaurasiya and co-workers analysed withanolides in root and leaf with photodiode array and light scattering detection, and the approach gives better quantitative precision at the price of more solvent, a longer run and a single sample at a time [20]. The two techniques are complementary and not rivals. Where such checks are omitted, a reported relative standard deviation tells the reader little about how the method will behave in someone else's hands.

Reference standards pose a practical limit. Withaferin A and withanolide A are available from commercial suppliers, but others in the family are not, so fingerprints often rest on two or three markers while dozens of other zones go unidentified [15, 20]. Liquid chromatography with photodiode array or mass spectrometric detection can quantify a wider set, and many laboratories use HPTLC for screening and rapid comparison, and liquid chromatography for confirmation [20, 21]. A sensible reading of the literature is that HPTLC suits the first question, whether the product resembles authentic root, and liquid chromatography suits the second, how much of each marker it contains. Using one to answer both has produced some of the least reliable claims in the published record [12, 13, 19]. Cost therefore pushes laboratories toward HPTLC, while risk pushes them toward liquid chromatography, and a balance has to be struck for each product.

For routine quality control the cost argument is real. A plate runs a dozen or more samples in parallel with modest solvent use, which is why HPTLC remains popular in small manufacturing units and in testing laboratories with limited budgets [12, 13]. That economy is only worth having if the method has been properly validated for the matrix in question, and a method validated on raw root should not be silently applied to a tablet [23, 27]. Reproducibility between laboratories is the missing piece. Few HPTLC methods for withanolides have been tested in collaborative studies, so statements about robustness still rest on within-laboratory work. That is a common limitation across herbal analysis and not a special flaw of this method [7, 23].

5. FORMULATION QUALITY AND ADULTERATION

Commercial herbal products have a mixed record. DNA barcoding of products bought in North America found that a substantial share contained species not named on the label, and others contained fillers [28]. An overview of systematic reviews similarly identified contamination and adulteration as recurring problems across herbal medicines [29]. These studies did not target ashwagandha specifically, but they show why independent analysis of a marketed product is more than a formality. Where ashwagandha was concerned, the barcoding work could only reveal botanical substitution, not the chemical strength of the material, and a correctly identified root may still be low in withanolides. DNA tests and chromatographic fingerprints therefore answer different questions, and good control uses both [6, 28]. Such barcoding methods, though, cannot say anything about strength, and chemical fingerprints cannot always separate closely related species.

Value chains matter. Herbal raw material may pass through collectors, traders, processors and formulators before it reaches a tablet press, and each hand-off is an opportunity for substitution, dilution or poor storage [7, 8]. Good manufacturing practice guidance for herbal medicines therefore asks manufacturers to authenticate starting material, retain voucher samples and keep chromatographic reference fingerprints for each plant part used [23, 34]. Auditors and buyers can ask for several documents: a certificate naming the plant part and the country of origin, the fingerprint of the supplied lot compared with the reference, results for heavy metals and pesticides, and a statement of the extraction solvent and drug-to-extract ratio. Few suppliers volunteer all of these, which is why independent testing still has a role [7, 34, 35].

Regulation varies from country to country. European guidance on herbal medicinal products expects a defined herbal substance or preparation with specifications and a chromatographic fingerprint where the active constituents are not known, while the WHO texts set out general methods for

macroscopic, microscopic and chromatographic examination [10, 23, 35]. The Ayurvedic Pharmacopoeia of India offers monographs for the root with thin-layer chromatographic tests, although the age of some of these tests and the lack of finished-product specifications leave gaps [9]. The European approach differentiates between a herbal substance, a herbal preparation and a finished medicinal product, and specifications are expected at each level. For traditional products the regulator may accept a fingerprint in place of a quantified marker when the active constituents are not known with certainty [10]. For a plant where candidate actives exist, a fingerprint plus one or two markers is a defensible compromise. Manufacturers exporting to several markets must therefore keep more than one dossier for the same product [9, 34].

Safety is not a side issue. Case series from Iceland and the United States have linked ashwagandha products to liver injury, usually mild to moderate and resolving after the product was stopped, though the causal ingredient was not established [30]. Concerns of this kind are exactly why identity, strength and purity should be tested, since an unexplained product variation could be mistaken for an inherent toxicity of the plant, or the reverse [6, 30]. Quality problems may also come from the other end. A well-made extract may be diluted by excipients or by cheaper plant material, and the effect is a lower marker content per tablet without any change in the pattern of bands, which a fingerprint alone could miss. This is why densitometric quantification of at least one marker is recommended alongside the visual comparison [19, 27]. Users of herbal products seldom hear about such problems until a report appears in the medical literature.

Table 1 collects the main findings that bear on HPTLC use in this setting, grouped by chemistry, method, validation, product quality and clinical relevance. Two patterns stand out. Method papers cluster around a few marker compounds, and product-quality evidence is mostly indirect, borrowed from studies of herbal supplements in general rather than ashwagandha alone [28, 29]. That asymmetry should shape how far conclusions can be pushed. Taking these threads together, a layered scheme appears in the literature: botanical authentication first, a fingerprint for identity, a quantified marker for strength, and contaminant testing for safety. HPTLC is the second layer, and its usefulness comes partly from how cheaply it can be repeated on every incoming lot [13, 23, 34].

Stability is another worry that receives less attention than identity. Withanolides carry reactive functional groups, and heat, light and moisture can alter an extract during storage. A fingerprint taken on release does not guarantee the pattern at the end of shelf life, and stability-indicating HPTLC work for herbal products is thin on the ground compared with synthetic drugs [19, 27]. Accelerated studies under ICH conditions have been used for some herbal extracts, but marker loss is not always a good guide to loss of activity.

6. LIMITATIONS AND FUTURE DIRECTIONS

The literature has several weaknesses. Many fingerprint papers use a single laboratory, a handful of brands and one reference lot, so they show that a method can work but not how often products fail it. Brands are seldom followed across batches. Marker choice drifts from paper to paper, which makes cross-study comparison hard, and quantitative claims are sometimes reported with little detail about sample preparation [7, 12, 18]. Selection of products is another issue. Studies tend to buy a convenience sample from local markets or online sellers, and brands that were tested are rarely named or traced to lot level, so conclusions about market quality as a whole cannot safely be drawn from them [28, 29]. Reviews of the field regularly call for more rigorous design for exactly these reasons [7, 29].

Several steps could improve the situation. Shared reference fingerprints for root, leaf and common extract types would help, as would collaborative ring trials to measure between-laboratory variation. Hyphenation of HPTLC with mass spectrometry, image analysis with open software, and chemometric pattern recognition are all moving from research papers into routine use

[11, 21, 36]. Bioautographic detection could link bands to activity, which would suit a plant whose benefit is not tied to a single compound [36]. Standards will need to move too. Method details in pharmacopoeias lag behind current practice, and finished-product monographs for multi-herb Ayurvedic formulations are limited, which leaves laboratories to develop their own tests and encourages the inconsistency described above [9, 10]. Funding for shared reference material would probably return more value than another series of single-laboratory method papers.

Clinical studies add a final complication. Trials of ashwagandha have mostly used specific, proprietary extracts at defined doses, and their results may not carry over to a generic powder with an unknown withanolide content [3, 14, 37, 38]. Publishing the chemical profile of the trial material, ideally with a fingerprint, would let readers judge which marketed products resemble it. Regulators and producers could jointly fund open reference libraries, with images of plates run under defined conditions. Such a library would reduce the burden on small laboratories and would make it simpler to see whether a new product fits within the natural range of authentic material [7, 21, 23]. Until then, trial reports and product analyses sit in separate literatures that rarely meet.

A last point concerns reporting. Papers would be easier to use if they gave plate type, chamber conditions, application volumes and derivatisation steps in enough detail to repeat the work, and if they stated plainly which bands were identified and which were not. Authors could do more with the plate itself: record the track images, share the raw files, and describe unexpected bands so that later workers can identify them [19, 36]. Journal editors could help by asking for this at submission.

7. CONCLUSION

HPTLC has earned its place in the quality control of ashwagandha products. It is fast, inexpensive and visual, it handles crude extracts, and a single plate can set a suspect tablet beside authenticated root and the main marker compounds [11, 12, 13]. For identity testing and for screening many samples, the published evidence is encouraging, and the method fits with pharmacopoeial and WHO expectations for herbal materials [9, 10, 23]. For a small laboratory, that combination of low cost and visible results is hard to beat, provided the plates are run with authentic references alongside the samples [7, 18].

The weak points are equally clear. Quantification requires full validation in the matrix tested, reference standards cover only a small part of the withanolide family, and independent comparisons of marketed brands are scarce [20, 27, 28]. Until laboratories share reference fingerprints and report their methods in more detail, results will be hard to compare. Practical advice follows: treat HPTLC as a screening and identity tool, confirm strength by liquid chromatography where decisions depend on it, and ask manufacturers for the profile of the material used [8, 21, 30]. Whether HPTLC is enough for a given product ultimately depends on the question being asked and the consequences of a wrong answer [23, 27].

8. ABBREVIATIONS

API-Ayurvedic Pharmacopoeia of India, EMA-European Medicines Agency, GMP-Good manufacturing practice, HPTLC-High-performance thin-layer chromatography, ICH-International Council for Harmonisation, NF-κB-Nuclear factor kappa B, NMR-Nuclear magnetic resonance, Rf-Retardation factor, UV-Ultraviolet, WHO-World Health Organization

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10. AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

11. CONFLICT OF INTERESTS

Declared none

12. REFERENCES

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