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Solid phase extraction of ayurvedic lipid based formulations for the analysis and the determination of active botanical ingredients by HPTLC

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Abstract: Ayurvedic lipid based formulations falling under "Sneha Kalpana" in ayurvedic formulary were made by boiling the polyherbal decoction with oil or ghee with paste of other botanicals until the evaporation of water. The lipid nature and the contents of oil or ghee were tending to interfere with the determination of active botanical ingredients (ABIs) from Ayurvedic lipid based formulations in preparing the samples for the chromatographic analysis. Most of the earlier methods used for the sample preparation for the analysis of ayurvedic lipid based formulations were utilized either dissolving directly in a nonpolar solvent or liquid-liquid extraction with aqueous methanol (mostly 90% methanol) in determination of ABIs by HPLC, HPTLC and GC. Solid phase extraction on silica gel columns using hexane and 90% methanol was performed for the sample preparation. Both fractions were analyzed by HPTLC analysis on pre-coated silica gel 60 GF254 aluminium plates using Toluene: Ethyl acetate: Methanol (7:2:1) as mobile phase. The results have shown that the ABIs were maximally extracted in the methanol, the polar fraction whereas the non-polar fraction by hexane did not show the presence of ABIs. The visualization under UV light followed by densitometric analysis have shown an increased number of spots as well as highly concentrated spots with respect to the sample prepared from the formulation dissolved in hexane. The samples prepared by solid phase extraction produced the clear spots with good resolution in HPTLC when compared to the samples prepared by conventional methods and can be used for the determination and quantification of ABIs in these formulations.

Keywords: HPTLC; Solid phase Extraction; Ghrita, Taila; Ayurveda

1. Introduction

Analyzing herbal formulations was always a herculean task because of the complex array of active ingredients and again complicated by polyherbal nature. Extraction of active botanical ingredients (ABIs) and further analysis by chromatographic analysis offers a good choice for fingerprinting the ABIs in the given solvent system using planer chromatography such as TLC and HPTLC. But the traditional formulations prepared by using oils and ghee pose a problem in detection of ABIs because of interferences from the lipid base. Ayurvedic lipid based formulations falling under "Sneha Kalpana" in ayurvedic formulary were made by boiling the polyherbal decoction with oil or ghee with paste of other botanicals in appropriate proportions until the water was got evaporated. The lipid nature and the contents of oil or ghee were tending to interfere with the determination of active botanical ingredients from these formulations by conventional instrumental analysis. Chromatographic techniques like HPTLC, HPLC and GC were the major techniques used regularly for the analysis and determination of ABIs in the polyherbal formulations with known biomarkers or chemical markers. The analysis further complicated by the limited choice of sample preparation techniques and usually prepared by dissolving the sample in either nonpolar solvent or liquid-liquid extraction with methanol.[1-5] The common problems associated with the analysis of these formulations by planer chromatography like TLC, HPTLC are poor resolution of spots, disappearance of spots because of lower concentration, and the number of spots detected by various visualization techniques. The resolution of spots or peaks in those chromatograms would affect the analysis in the detection and quantifications of ABIs since the effects of fronting, tailing, spreading and broadening or the overlapping spots.

In order to find a solution to above said problems, it is necessary to separate the ABIs from the interferences of the aforesaid formulations. Extraction of ABIs and further analysis could offer a solution but hindered by the unavailability of the nature/property of the major active ingredients, which should be required to decide the solvent for extraction. It was observed from these ayurvedic lipid based formulations that they were prepared from polyherbal decoction and the major ABIs would have been transferred from the decoction alone.

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Since the decoction was expected to have predominantly polar ingredients which led us to develop a conclusion that the polarity differences exist between the carrier (oil or ghee) and the ABIs (transferred from decoction). If such a vast polarity difference existing then it was easier to decide the solvents for extraction to separate the ABIs fraction for HPTLC analysis. Most of the earlier methods used for the sample preparation for the analysis of ayurvedic lipid based formulations were utilized either dissolving directly in a nonpolar solvent or liquid-liquid extraction with aqueous methanol (mostly 90% methanol) in determination of ABIs by HPLC, HPTLC and GC but none of the method was optimized for the maximum extraction of ABIs in the fractions for the analysis.[6-15] Solid phase extraction may offer maximum extraction of ABIs in the fractionation and so far, no methods have been reported using solid phase extraction for sample preparation of Ayurvedic lipid based formulations for the chromatographic analysis.

Solid phase extraction (SPE) is very fast and used for selective sample preparation and is a powerful tool for isolation of even trace amounts in different sample matrices.[16-19] The degree of improvement relies on the selection of adsorbent, strength of interaction of the sample with adsorbent and the eluting solvent. The compounds of interest either retained in the column or eluted along with the solvents depended on the polarity. So it was decided to use solid phase extraction on the sample preparation of these ayurvedic lipid based formulations for the maximum extraction and determination of ABIs by HPTLC. For this study, we have chosen to work with some of the model ghritas [Mahakalyanaka ghrita (MKG), Brahmi ghrita (BG) and Aragwadhadi ghrita (AG)] to observe the effect of solid phase extraction on the analysis of ayurvedic lipid based formulations.

2. Result and Discussion

2.1. Solid Phase Extraction

We have chosen to work with three ghrita preparations to show the effect of SPE on the sample preparation, detectability and the resolution of spots in the HPTLC plates. From the results, it was observed that in these formulations the polar active ingredients from polyherbal decoction were incorporated into the nonpolar lipid base. For sample preparation using either liquid-liquid extraction or solid phase extraction, polarity differences must exist between the matrix and the components and this would guide oneself in deciding the solvents for the extraction. The Methanol (90%) in water was selected as polar solvent for the SPE from the fact that most of the ABIs were from polyherbal decoction that predominantly contained water-soluble active ingredients and methanol was serving as an excellent extraction solvent to dissolve polar ingredients dispersed in a lipid phase. Hexane was selected as nonpolar solvent since its ability to dissolve lipids and oils. Methanol extractive values have been provided in **Tables 1**, **3** and **5** for MKG, BG and AG respectively which showed that a small portion of the lipid base which contained all the ABIs and methanol soluble was extracted into the methanol fraction.

Table 1. Mahakalyanaka Ghrita (MKG) Separation of ABIs

Ghrita		Separated material	
Volume (mL)	Weight (g)	Weight (g)	Percentage (%)
15	13.58	1.86	13.7

1 mL of ghritam is equivalent to 0.9061 g

Table 2. Showing the Rf values and corresponding AU (Area under the peak) of ABIs visualized under UV light at 254 and 366 nm of Mahakalyanaka Ghrita (MKG)

S. No	at 254 nm				at 366 nm			
	Rf		Area (AU)		Rf		Area (AU)	
	Ghrita	B	Ghrita	B	Ghrita	B	Ghrita	B
1		0.09		263.1	0.15	0.18	178.2	366.2
2		0.22		575.9	0.26	0.30	701.3	1119.4
3		0.25		780.1	0.39	0.43	2098.1	3692.4
4		0.33		828.4	0.52	0.54	1097.5	424.5
5		0.37		785.1	0.57		3314.3	
6		0.53		70.4	0.69	0.64	1889.0	5863.1
7	0.56	0.66	546.6	559.4	0.88	0.93	5643.0	1579.5
8	0.79	0.73	1077.4	1464.1		0.99		368.4
9	0.92	0.99	22259.7	1467.5				

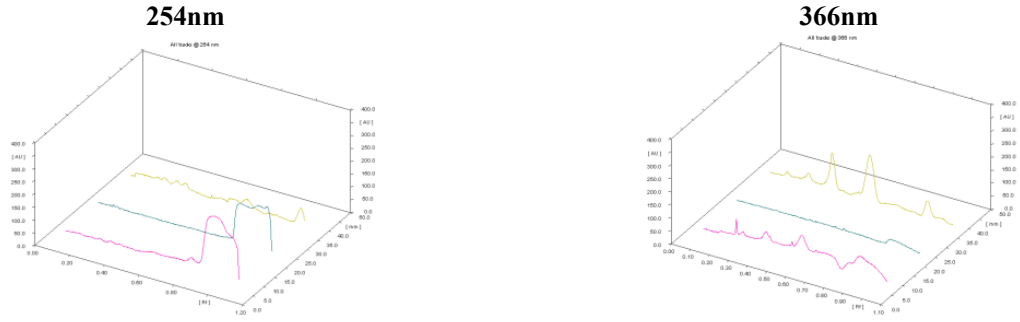


Figure 1. HPTLC profile of Mahakalyanaka Ghrita (MKG): 3D overlay chromatograms showing the behavior of Ghrita (Track1), Fraction A (Track 2) and Fraction B (Track 3) viewed under UV light at 254 and 366 nm respectively of MKG

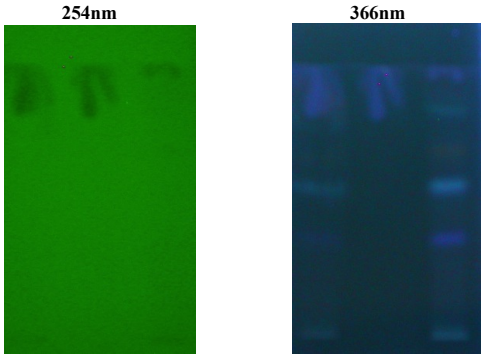


Figure 2. Visualization of HPTLC plates under UV light at 254 and at 366 nm of Mahakalyanaka Ghrita (MKG)

2.2. High Performance Thin Layer Chromatography

From the visualization of the HPTLC plates under UV light and 3D chromatograms of all ghritas it was observed that the hexane fraction did not show the presence of any active ingredients at 254 and 366 nm whereas the methanolic fraction showed the presence of full spectrum of ABIs in comparison to the chromatogram obtained from ghrita itself. Moreover methanolic fractions have shown improved and additional spots that have been not detected when the ghrita was run alone in the HPTLC plates. 3D overlay chromatograms showing the behavior of Ghrita, Fraction A (hexane) and Fraction B (methanol) viewed under UV light at 254 and 366 nm respectively of MKG, BG and AG, were shown in **Figure 1**, **3** and **5** respectively. Visualization of HPTLC plates under UV light at 254 and 366 nm of MKG, BG and AG were shown in **Figure 2**, **4** and **6** respectively.

2.2.1. HPTLC of Mahakalyanaka Ghrita (MKG)

The HPTLC analysis of MKG was shown in **Figure 1, 2** and **Table 2** which clearly showed that the ABIs were completely extracted into the methanolic fraction whereas the hexane fraction showing no evidence of ABIs. The AU of methanolic fraction in **Table 2** showed that clear, concentrated peaks were obtained with good resolution with respect to the peaks obtained in MKG sample prepared in hexane.

2.2.2. HPTLC of Brahmi Ghrita (BG)

For Brahmi Ghrita (BG) as shown in **Figure 3** and **4** the individual chromatograms were obtained for the first and second fractions of hexane and methanol respectively to study the variation in the solid phase extraction. As anticipated both hexane fractions did not extract the ABIs as shown in their chromatograms. **Table 4** showed the difference in the methanolic extraction obtained after first and second fractions in terms of number and intensity of the peaks obtained with respect to the BG itself. The AU (Area Under the Peak), indicator of the concentration has showed that concentrated peaks (spots) were obtained with respect to the peaks obtained in Brahmi ghrita prepared in hexane comparatively in the same sample size.

Table 3. Brahmi Ghrita (BG) separation of ABIs

Ghrita		Separated material	
Volume (mL)	Weight (g)	Weight (g)	Percentage (%)
15	13.2	3.17	24
2nd Fraction		0.73	5.5

1 mL of ghritam is equivalent to 0.8798 g

Table 4. Showing the Rf values and corresponding AU (Area under the peak) of ABIs visualized under UV light at 254 and 366 nm of Brahmi Ghrita (BG)

S. No	at 254 nm						at 366 nm					
	Rf Value			Area (AU)			Rf Value			Area (AU)		
	Ghrita	B_1	B_2	Ghrita	B_1	B_2	Ghrita	B_1	B_2	Ghrita	B_1	B_2
1		0.13	0.14		594.1	1310.0			0.10			371.4
2			0.17			740.3	0.16	0.13	0.14	993.8	2264.9	4028.2
3		0.24	0.25		521.9	1296.8			0.23			393.8
4			0.28			1164.7		0.29	0.30		1198.7	2118.1
5		0.33	0.33		396.6	781.3			0.38			2844.6
6			0.36			1505.8		0.41	0.45		1292.8	2632.6
7		0.48	0.46		4704.4	685.3		0.56	0.61		539.8	371.2
8	0.53	0.54	0.53	1994.3	2921.5	13743.4		0.62	0.67		1362.3	3931.2
9		0.59	0.60		6597.9	4571.2			0.77			568.8
10	0.63		0.65	2080.2		15670.5		0.90	0.87		941.3	931.6
11	0.73	0.71	0.71	503.4	1883.9	3505.8		0.96	0.93		518.8	1694.9
12		0.81	0.76		382.4	5163.2						
13			0.88			1213.4						

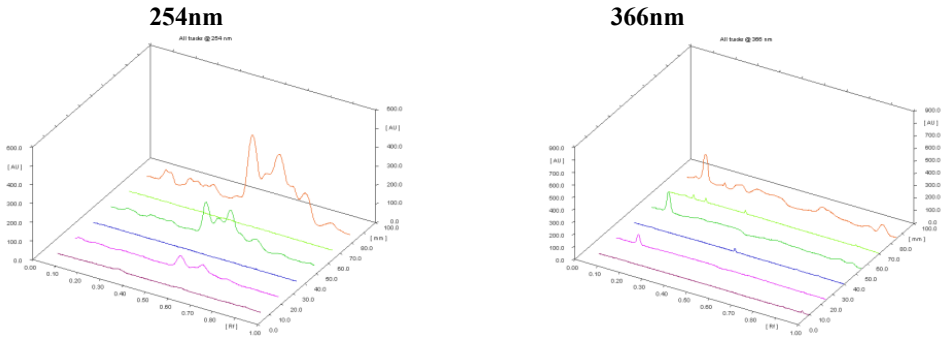


Figure 3. HPTLC profile of Brahmi Ghrita (BG): 3D overlay chromatograms showing the behavior of Ghee (Track 1), Ghrita (Track 2), Fraction A_1 (Track 3), Fraction B_1 (Track 4), Fraction A_2 (Track 5), Fraction B_2 (Track 6) viewed under UV light at 254 and 366 nm respectively of BG

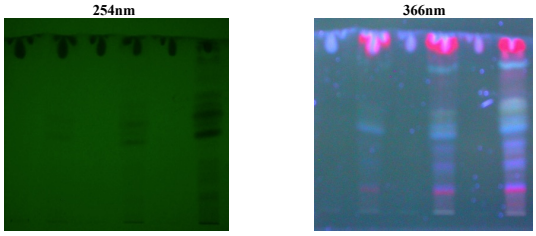


Figure 4. Visualization of HPTLC plates under UV light at 254 and 366 nm of Brahmi Ghrita (BG)

2.2.3. HPTLC of Aragwadhadi Ghrita (AG) and Tailas

In Aragwadhadi Ghrita (AG) the individual methanolic fractions were run alongside after first and second methanolic extract. The similar results were obtained as discussed in the HPTLC chromatograms and AU of Brahmi Ghrita. The results were shown in **Figure 5**, **6** and **Table 6**.

Table 5. Aragwadhadi Ghrita (AG) separation of ABIs

Ghrita		Separated material	
Volume (mL)	Weight (g)	Weight (g)	Percentage (%)
15	13.65	1.59	11.6

1 mL of ghritam is equivalent to 0.9147 g

Even though the comparative chromatography with original preparations was not performed for tailas (medicated oils), **Figure 7** showed the clear chromatographic finger printing of methanolic extract derived from solid phase extraction.

Table 6. Showing the Rf values and corresponding AU (Area under the peak) of ABIs visualized under UV light at 254 and 366 nm of Aragwadhadi Ghrita (AG)

S. No	at 254 nm						at 366 nm					
	Rf Value			Area (AU)			Rf Value			Area (AU)		
	Ghrita	B1	B2	Ghrita	B1	B2	Ghrita	B1	B2	Ghrita	B1	B2
1			0.11			358.2			0.19			287.4
2		0.16	0.18		122.9	1952.1			0.27			676.6
3			0.22			2229.9			0.37			1727.7
4		0.27	0.28		257.8	552.8			0.39			1502.7
5			0.33			178.0		0.45	0.45		734.6	3541.6
6			0.39			623.9		0.51	0.51		2045.8	5093.9
7		0.44	0.45		179.0	201.3		0.55	0.55		1164.4	2384.8
8	0.54	0.53	0.53	230.5	259.5	359.9		0.64	0.64		3144.7	7532.8
9	0.57		0.62	297.3		1411.8	0.68	0.71	0.71	895.4	1369.4	2384.1
10	0.78	0.80	0.80	217.9	506.1	769.4	0.82	0.80	0.80	4337.3	16331.5	24996.8
11	0.80	0.86	0.88	182.5	449.4	702.6	0.88	0.87	0.87	375.9	2420.4	3083.8
12	0.97			769.5								

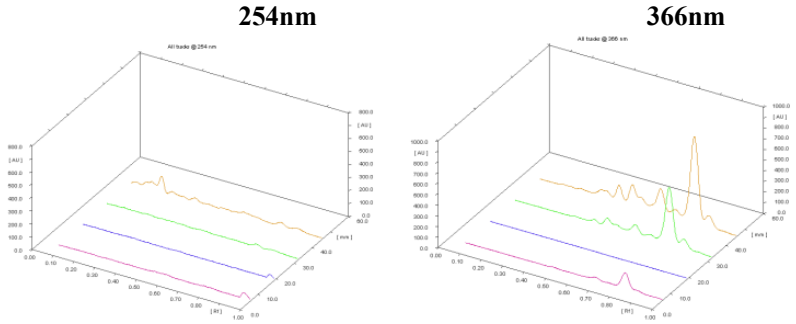


Figure 5. HPTLC profile of Aragwadhadi Ghrita (AG): 3D overlay chromatograms showing the behavior of Ghrita (Track1), Fraction A (Track 2) and Fraction B1 (Track 3), Fraction B2 (Track 4) viewed under UV light at 254 and 366 nm respectively of AG

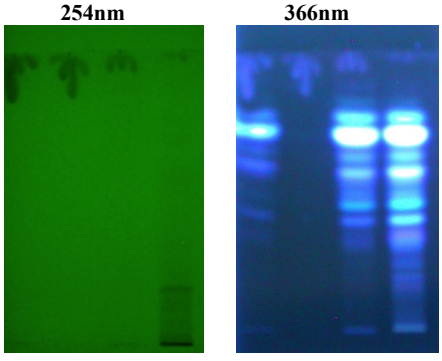
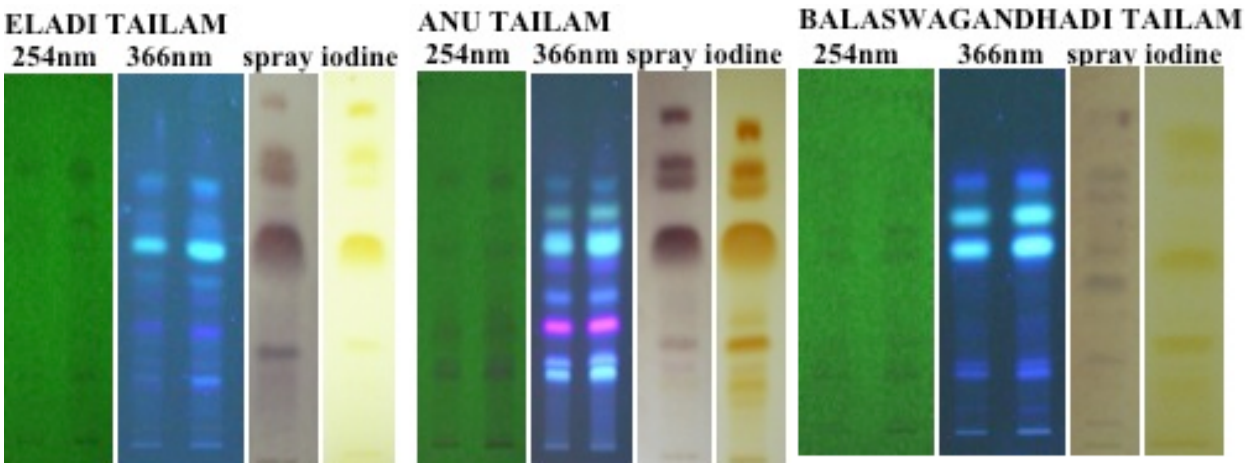


Figure 6. Visualization of HPTLC plates under UV light at 254 and 366 nm of Aragwadhadi Ghrita (AG)

3. Conclusion

From the above analysis, it was observed that the spots appeared in HPTLC plates of methanolic extract obtained through the solid phase extraction has shown concentrated, clear spots than corresponding spots in the ghrita itself. The chromatogram also produced some additional peaks, which were not appearing when the sample was run without SPE, which showed the effectiveness of this method. In conclusion it was found that SPE produced effective samples for the chromatographic analysis by HPTLC and this will aid the determination and quantification of ABIs in these kinds of formulations using marker compounds.



4. Experimental

Materials and Methods:

All the reagents and solvents used were of analytical grade, which were purchased from Sigma-Aldrich, and they were used without further purification. The Mahakalyanaka ghrita, Brahmi ghrita and Aragwadhadi ghrita were purchased from AVN Arogya Ayurvedic Pharmacy, India. The silica gel (60-120 mesh) for chromatography was purchased from Merck, India. Borosil, India supplied the fabricated glass column with a sintered glass filter. Pre-coated silica gel GF_{254} aluminium plates for normal phase were purchased from Merck, India to carry out HPTLC analysis on CAMAG HPTLC system, Switzerland.

4.1. Solid Phase Extraction (SPE) [16, 17]

Ghrita sample (15 mL) was weighed as dissolved in hexane (15 mL) and transferred into a silica gel column (15 cm x 1.0 cm) packed with 75 g of silica in hexane. The column was first eluted with hexane (2x50 mL) followed by methanol (90%) in water (2x50 mL). The fractions were collected separately and the solvents were recovered by boiling under reduced pressure and evaporated to obtain the residue of constant weight and dried weight was taken. The samples (~2 µg) were taken in a sample bottle and dissolved in 2 mL of the respective solvents used for the elution and used for the chromatographic analysis. The solid phase extraction was performed with deliberate minor modifications in the ghritas taken for the analysis and explained in the respective sample preparation and HPTLC analysis

4.2. Sample preparation

The ghrita samples were prepared by dissolving 15 mL of the ghrita in equal amount of hexane. Hexane and methanolic fractions obtained were evaporated to obtain a constant weight and dissolved in 10 mL of hexane and 5 mL of methanol respectively to prepare samples for the HPTLC analysis

4.3. High Performance Thin Layer Chromatography (HPTLC)

Samples of 2 µL each were applied as 8 mm bands in four tracks on pre-coated silica gel 60 GF_{254} aluminium plates with the help of Linomat 5 applicator attached to CAMAG HPTLC system, which was programmed through WINCATS software. The detection was performed using Densitometry TLC Scanner 3 at 254 and 366 nm in UV cabinet. The plates were developed in the TLC chambers pre-saturated with mobile phase, Toluene: Ethyl acetate: Methanol (7:2:1)[18, 19]

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