TWO DIMENSIONAL CHROMATOGRAPHIC ANALYSIS AS A QUALITY MEASURE OF HERBAL EXTRACTS – *SALVIA OFFICINALIS*.

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Abstract
Background
Homoeopathic mother tinctures and herbal extracts are used worldwide for medicinal purposes on the basis that the plant extract contains the active components essential for medicinal use. Quality analysis of samples ensures that the correct active components are present for medicinal use. Thin layer chromatography has been used extensively to determine the quality of plant extracts and could just as readily be applied to the assessment of quality of homoeopathic mother tinctures. The development of a multidimensional technique allows for greater quality analyses of these extracts.

Materials and Methods
The Salvia officinalis sample was extracted and the one dimensional thin layer chromatographic plate development of extracted sample was performed according to the German Homoeopathic Pharmacopoeia (GHP). Before the two dimensional thin layer chromatographic plates where run, different solvent systems were tested through a comparison of the resultant one dimensional thin layer chromatographic plates with that of the plate developed according to the GHP. The two dimensional thin layer chromatographic plate was developed in one direction using the eluent given in the GHP, the plate was then rotated 90 degrees and further developed using neat dichloromethane.

Results and Conclusion
The two dimensional thin layer chromatogram for the selected sample *Salvia officinalis* was successfully established. This results in a more extensive profile regarding the identification of the individual components present in the homoeopathic mother tincture. The further production of two dimensional chromatograms is essential to the expanded detailed analysis of individual products and potential raw material production of even higher standards.

Keywords
Salvia officinalis, two dimensional thin layer chromatography, homoeopathic mother tincture

Introduction

The analysis of complex samples is a frequent problem in the development of chromatographic methods. The number of components in many samples (plant samples in particular) are unknown and may be as high as several hundred. It is accepted that when analysing plant extracts for quality control purposes, the first step is one dimensional thin layer chromatography. The properties of the absorbents used in TLC are well known and when combined with a broad range of eluents, provide good prospects for the analysis of complex mixtures (Markowski and Matysik, 1993).

Plant extracts are usually rich in groups of substances with various physiochemical properties. Substances with similar physiochemical properties cause difficulties in separations. Separation of these compounds, having similar compositions, is often impossible in one chromatographic run (Chitlange, 2008). Two dimensional separations can be relatively easily performed on one chromatographic plate with two eluents of different selectivity. It makes possible the separation of multi component natural mixtures on one plate by the use of non-aqueous and perpendicularly aqueous eluents (various properties and selectivity's) (Hawryl *et al.*, 2011) and the continued pursuit of this method may provide avenues for the construction of fingerprints of various herbal extracts and these may be effectively used in quality control of herbal materials and their associated antioxidative activity (Hawryl and Waksmundzka-Hajnos, 2013; Author, 2013)

Homoeopathy is one of the most frequently used derivative forms of herbal medicines and there is a great market for homoeopathic products around the world (World Health Organization, 2013). Despite its increasing use, very few countries regulate these medicines.

The safety and the quality of homoeopathic medicines has become a key concern for health practitioners, pharmaceutical industries and consumers due to the worldwide increase in the use of homoeopathic medicines and market globalisation (Calixto, 2000). Due to the associated technical methods of preparation, the requirements and methods for the quality control of finished proprietary homoeopathic medicines are complex, particularly for the combined or mixed homoeopathic medicines. The analysis of the quality of a homoeopathic medicine (other than the assessment for impurities) in high dilution is also difficult to assess. Products which meet high quality standards are necessary, to allow the patient to make safe use of the homoeopathic medicines (World Health Organization, 2009). Quality assurance is becoming crucially important where many of the raw materials and medicines used in the homoeopathic profession may originate from different sources or countries. It would be advantageous to ensure that the starting substance is at least of high quality value (Author, 2011).

The advantages of TLC to assist as a tool for early quality assurance include short analysis time, low solvent usage, a high degree of accuracy, and the ability to detect the separated zones by physical and chemical methods (Glensk *et al.*, 2001). One dimensional TLC does not, however, always provide adequate separation of the highly complex plant extracts and thus additional methods for the determination of the quality of plant extracts should be investigated (Giddings, 1990).

When using two dimensional thin layer chromatography (2D-TLC), only a single use of the plate is possible, yet there are no limits as far as mobile phase components are concerned. Multiple detection techniques of the plate enable the analysis of a wide spectrum of compounds (Ceiśla and Waksmundzka-Hajnos, 2009). The advantageous features of 2D-TLC in the analysis of medicinal plants enables solving many analytical problems encountered in the separation of multi-component mixtures (Nyiredy, 2001).

Salvia officinalis (Sage) is a member of the family Lamiaceae (Blumenthal, 1998). It is native to the Mediterranean regions but can be cultivated worldwide in sheltered shady area of light soil (Greive, n.d.). *Salvia officinalis* is collected at the start of flowering in May and June, in the Northern hemisphere (Hoffman, 2001) Sage is a woody plant, which grows to 60cm tall, with large, long greyish leaves with wrinkled veins. The flowers are purple or blue (Garland, 2004). For medicinal purposes, the leaves are used to produce the mother tincture (Hoffman, 2001).

Constituently it contains up to 3.6% essential oils, where alpha and beta thujone (20-60%) are the major compounds, seen in Figure 2.12 (Van Wyk and Wink, 2004). It also contains smaller amounts of camphor (14-37%), 1,8-cineole (6-16%), beta caryophyllene and limonene (Dewick, 2009). Also present are caffeic acid derivatives, namely rosmarinic and chlorogenic acid (Kraft and Hobbs, 2004). Diterpenes, namely carnosolic acid, flavonoids, and triterpenes are also present (Van Wyk and Wink, 2004).

For the purposes of potential improved quality analysis, this paper aimed to demonstrate the two dimensional chromatographic technique as a more accurate instrument for comparison of the composition of plant extracts (fingerprints) in addition to standard one dimensional thin layer chromatographic using of *Salvia officinalis*.

Material and Methods

Plant Material: The *Salvia officinalis* sample used was sourced from a study conducted on the quality of homoeopathic mother tinctures available in South Africa. The sample was found to be of superior quality during this study and therefore was selected to be utilized in the two dimensional TLC plate development. This *Salvia officinalis* sample was grown and manufactured in England.

Extraction Process: 10ml of mother tincture was extracted with three 5ml portions of pentane in a separation funnel. The combined organic phases were filtered under vacuum, through Watman no.1 filter paper, and dried over anhydrous sodium sulphate. The remaining solution was reduced to dryness in a Buchi water vaporiser at 30°C. The residual was dissolved in 1ml of methanol and placed in a labelled polytop.

2D-TLC

1D-TLC Plate development: TLC was performed on 10cm x 20cm aluminium plates coated with silica gel 60F containing UV 254 fluorescent indicator (Merck, South Africa). In order to conserve the use of the plates and the time taken to run the TLC plate, they were cut to 10cm x 10cm. The *Salvia officinalis* sample was applied to the plate, 10mm from the bottom using a capillary tube. The plates were developed to a distance of 80mm with a mixture of 20ml of diisopropyl ether and 80ml of toluene, in a TLC chamber previously saturated with the mobile phase vapour for 10 minutes. After removal from the chamber, the plates were completely dried in air at room temperature (approximately 25°C).

The plate was visualised under UV 254nm and any fluorescent spots were circled in pencil. The plate was then sprayed with anisaldehyde solution, made up by adding 80ml of ethanol, 4ml of sulphuric acid, 1.2ml of glacial acetic acid and 1.6ml of anisaldehyde, in a fume hood. The plate was heated to 105°C in an oven for 5-10 minutes and examined and photographed using a Sony Cyber-shot DSC W300, set at 13 megapixels on manual exposure shooting, with no flash, in daylight within 10 minutes thereafter (German Homoeopathic Pharmacopoeia, 2003).

2D-TLC Plate Development: 2D–TLC was performed on 10 x 20cm aluminium plates coated with silica gel 60F containing UV 254 fluorescent indicator (Merck, South Africa). The plates were cut into 10cm x 10cm plates.

Before the 2D-TLC plates where run, different solvent systems were tested through a comparison of the resultant one dimensional thin layer chromatographic plates with that of the plate developed according to the GHP. This was conducted in order to identify the most viable mobile phases which would provide for the clearest separation when utilized in the two dimensional thin layer chromatography (Table 2.1).

Table 2.1: Test mobile phases for *Salvia officinalis* Sample E

Mobile Phase Used	**v/v Ratio**
Dichloromethane	Neat
Hexane: Ethyl acetate	2: 1
Hexane: Dichloromethane: Methanol	18: 2: 1
Toluene: Hexane: Acetone	5: 5: 1

Each plate that was developed using a different mobile phase (Table 2.1) was examined and compared to the original TLC plate developed according to the GHP, highlighted in 2.3.1 above. The mobile phase for the first run of the 2D-TLC plate development is the original mobile phase stipulated in the GHP for standardized TLC procedure for *Salvia officinalis* mother tinctures.

The mobile phase that was chosen for the second run of the 2D-TLC plate development was done according to which plate showed a different spot separation when compared to the original TLC plate development, as well as the clear separation of the spots in the sample (Table 2.2).

Table 2.2: Mobile phases used for 2D-TLC for *Salvia officinalis* Sample E

Run	**Solvent System**	**v/v Ratio**
First Run	Diisopropyl ether: Toluene	20: 80
Second Run	Dichloromethane	Neat
	Toluene: Hexane: Acteone	5: 5: 1

The *Salvia officinalis* sample was spotted onto the 2D-TLC plate, 10mm from the bottom and 10mm from the left hand side of the plate, using a capillary tube. The plate was developed to a distance of 90mm with the mobile phase for the first run (Table 2.2) in a TLC chamber previously saturated with the mobile phase vapour for 10 minutes. After removal from the chamber, the plate was completely dried in air at room temperature (approximately 25°C). The plate was then turned 90 degrees and developed to a distance of 90mm with the mobile phase for the second run (Table 2.2) in a TLC chamber saturated with the mobile phase vapour for 10 minutes. After removal from the chamber, the plate was completely dried in air at room temperature (approximately 25°C).

The plate was visualised under UV 254nm and any fluorescent spots were circled in pencil. The plates were then sprayed with anisaldehyde solution, made up by adding 80ml of ethanol, 4ml of sulphuric acid, 1.2ml of glacial acetic acid and 1.6ml of anisaldehyde, in a fume hood. The plates were heated to 105°C in an oven for 5-10 minutes and examined and photographed in daylight within 10 minutes.

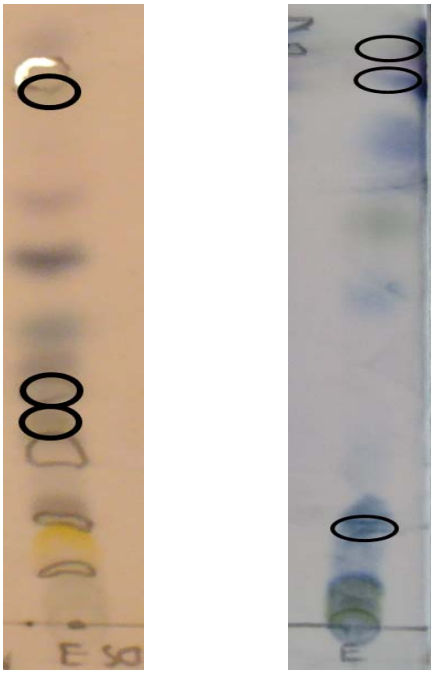
Results and Discussion

Eluent Selection: The R_f values and spot colours for the sample run in the eluent diisopropyl ether: toluene (80:20, v/v) and neat dichloromethane are shown in Table 3.1. The active compounds can be visualized in Plate B with R_f values of 0.16 0.88 and 0.90. The R_f values of the active compounds from Plate A are 0.36, 0.40 and 0.87. In comparison of both plates, Plate B shows a greater degree of separation of the components between the first and second active compounds. There is a more clear separation of the sample, run in diisopropyl ether: toluene (80:20, v/v) (Plate A), giving rise to an additional spot throughout the separation as well as clearer spot colours.

Table 3.1: Chemical marker components obtained for the *Salvia officinalis* Sample E, by TLC on aluminum backed TLC plates. (a) Eluent: diisopropyl ether: toluene (20:80, v/v). (b)Eluent: neat dichloromethane. Derivatization with anisaldehyde solution

Plate A		**Plate B**	
Sample E		**Sample E**	
R_f Value	**Colour**	**R_f Value**	**Colour**
0.04	Green	0.06	Blue green
0.13	Yellow	0.16	Dark blue
0.17	Blue purple	0.25	Light blue
0.31	Dark green	0.58	Light blue
0.36	Blue	0.68	Green
0.40	Purple	0.80	Dark blue
0.45	Blue	0.86	Dark blue
0.57	Dark purple	0.88	Pink
0.65	Purple	0.90	Light green
0.87	Green	0.94	Blue
0.92	Purple		

Figure 3.1 illustrates the difference between the two types of eluents and the positions of the active compounds of the sample which have been circled. From Plate A, the Rf values of the active components are as follows; borneol Rf 0.36, 1,8- cineole Rf 0.40 and bornyl acetate Rf 0.87. From plate B, the change of eluent allows for different separation distances resulting in the Rf of borneol to be 0.16, the Rf of 1,8-cineole to be 0.88 and the Rf of bornyl acetate to be 0.90. Although there is a less clear separation and an appearance of fewer spots of Sample E when run in neat dichloromethane (Plate B), the separation is adequate showing a different separation profile when compared to Plate A. Plate B shows the presence of more spots between the first and second active compounds.



A B

Fig 3.1 Thin layer chromatography of *Salvia officinalis* sample on aluminium backed TLC plate. Sprayed with anisaldehyde solution
(A) Eluent: diisopropyl ether: toluene (20:80, v/v)
(B) Eluent: neat dichloromethane

Two Dimensional Analysis: Relevant to the 2D-TLC, Figure 3.2 shows the chromatogram using the eluent stipulated by the GHP for the first development and neat dichloromethane (the best performing eluent in the one dimensional experiments) for the second development. The figure clearly indicates the more distinct separation of the spots in an orthogonal direction, showing a greater resolution of the components of the sample. All active compounds were identified visually and circled on the plate. The blue and purple spots in the middle of the plate, separated out into additional spots, showing that in the one dimensional analysis more than one component was present in each of those spots.

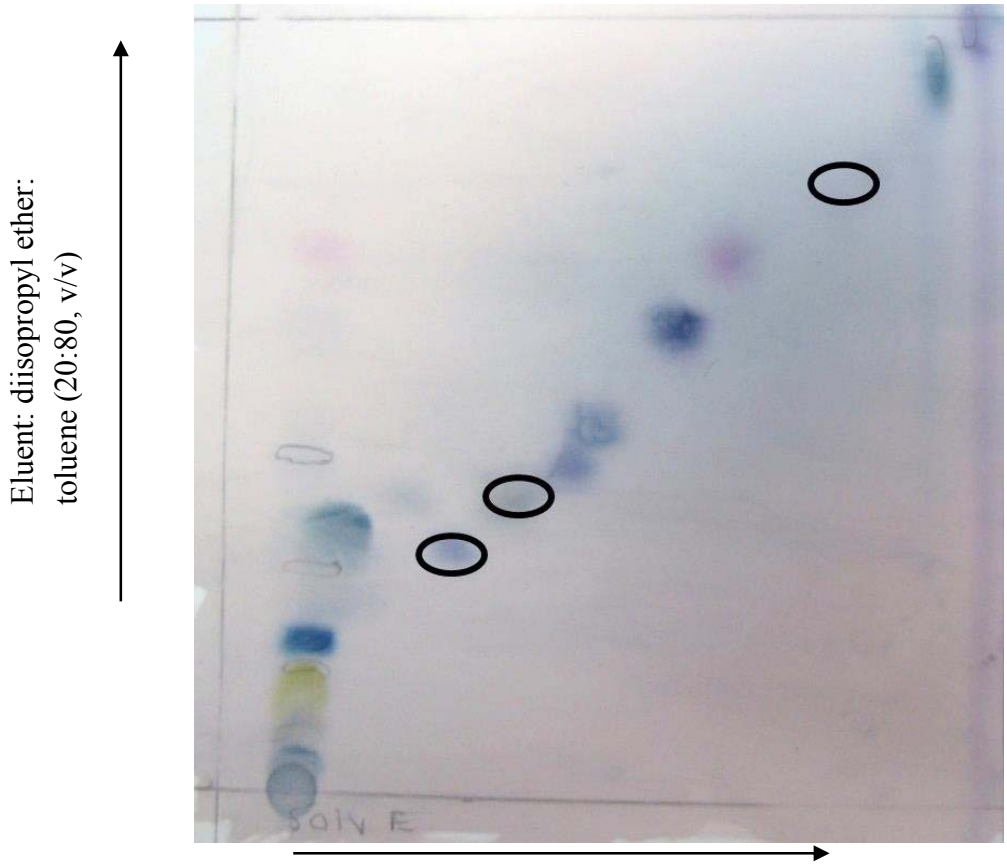


Fig 3.2 2D TLC separation of *Salvia officinalis* sample on aluminium backed TLC plate with diisopropyl ether: toluene (20:80, v/v) for the first development and neat dichloromethane for the second development. Sprayed with anisaldehyde solution

Discussion: Two dimensional thin layer chromatographic analyses demonstrate the separation of the sample in two directions, allowing for a more detailed fingerprint and the identification of additional components in the sample.

From Table 3.1 and Figure 3.1, a comparison was made between the sample separations using the eluent stated in the GHP, diisopropyl ether; toluene (20:80, v/v) (Plate A) and the experimental eluent neat dichloromethane (Plate B). On Plate B, the separation was clear, showing all the active components of the sample, and good colouring and concentrations of the spots plus the presence of additional spots. As seen in Figure 3.1, Plate B shows a different separation to that of Plate A, with a shift in the R_f values of the components of the sample.

Relevant to the 2D-TLC, Figure 3.2 shows the chromatogram using the eluent stipulated by the GHP for the first development and neat dichloromethane for the second development. The separation occurred in an orthogonal direction giving rise to additional spots appearing throughout the plate. The presence of these additional spots clearly shows that running a plate only in one dimension does not provide the complete picture of all the components of a sample. The presence of dark, concentrated spots in the one direction may indicate that more than one component is present in that area.

The main application for the TLC field is to prompt fingerprint analysis of herbal mixtures (Medic-Saric *et al.*, 2008). Natural components have to be separated from each other in order to be identified and determined as individual entities forming a composite phytochemical fingerprint characteristic of a single plant species (Waszkuc, 2011).

Future trends allow the use of multidimensional chromatography by the herbal analyst to construct these fingerprints in order to ensure quality control of the products being sold into the public market (Medic-Saric *et al.*, 2008). Botanical identification of plant raw materials is the critical step in the quality control of any herbal preparation (Waszkuc, 2011). The construction of standardised two dimensional thin layer chromatograms for plant species identification and quality control is not yet a standard practice. Given the advantages of the technique with its potential to produce a greater spot capacity, and with the versatility of coupling of additional techniques, more two dimensional chromatograms should be produced for individual plant species (Lian *et al.*, 2004). The increased use of multidimensional chromatography within laboratories for the assessment of quality control, will encourage manufacturing companies to use these chromatograms as standardised fingerprints. Two dimensional chromatography is a popular method for solving problems encountered in the analysis of complex samples of natural origin, and always lends itself towards improvement (Glowniak *et al.*, 2005).

Conclusions: New multidimensional planar chromatography methods may provide a viable and more specific solution in everyday phytochemical analysis due to its simplicity and far superior detailing of chemical composition. Both TLC and 2D TLC are inexpensive methods and also very suitable for rapid separation and identification of various compounds present in the extracts examined.

The two dimensional thin layer chromatogram for the selected sample for *Salvia officinalis* was successfully established. This results in a more extensive profile regarding the identification of the individual components present in the homoeopathic mother tincture. Two dimensional thin layer chromatograms may be used further to more accurately compare the quality of homoeopathic mother tincture or herbal extract products as guidelines for their successful reproduction are now established. The further production of two dimensional chromatograms is essential to the expanded detailed analysis of individual products and their manufacture of even higher standards.

Further studies and experimentation is recommended for the construction of two dimensional chromatograms as the standard profiles for homoeopathic / herbal mother tinctures. The separation of components in a second direction allows for a more complete picture of the components of the samples tested. The use of two dimensional chromatograms will ensure the potential for greater compliance by manufacturers to the quality standards stipulated by recognised homoeopathic pharmacopoeias and those of Good Manufacturing Practice (GMP).

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