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## High performance thin-layer chromatography and image processing analysis of some essential oils from *Perovskia atriplicifolia* leaves extracted by microwave assisted heating

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### ABSTRACT

A high performance thin layer chromatography (HPTLC) with support of an image processing method has been developed and validated for qualitative and quantitative analysis of  $\beta$ -caryophyllene and *d*-camphor from *Perovskia atriplicifolia* essential oil extracted by microwave assisted heating and hydrodistillation methods. The HPTLC analysis was performed in a twin trough chamber on silica gel 60 sheet using toluene-ethyl acetate (95:5, *v*:*v*) as mobile phase and anisaldehyde solution as derivatizing reagent. The scanned digital images of sheets were analyzed by means of an ImageJ software. The  $R_f$  values examined by ImageJ for  $\beta$ -caryophyllene and *d*-camphor were  $0.20\pm 0.05$  and  $0.85\pm 0.30$ , respectively. A linear relationship was found in the range of 0.905-5.43  $\mu\text{g}/\text{spot}$  for  $\beta$ -caryophyllene and 0.025-0.15  $\text{mg}/\text{spot}$  for *d*-camphor. The quantification for both analytes were found to be 1.79  $\mu\text{g}/\text{spot}$  and 0.13  $\text{mg}/\text{spot}$ , and detection limit 0.53  $\mu\text{g}/\text{spot}$  and 0.04  $\text{mg}/\text{spot}$ , respectively. The results obtained in validation represent good accuracy and precision of the developed HPTLC methods.

**Keywords:** *Perovskia atriplicifolia*, high performance thin layer chromatography, ImageJ,  $\beta$ -caryophyllene, *d*-camphor

### INTRODUCTION

Genus *Perovskia* belongs to Lamiaceae family. The plant is silver-gray with violet-blue flowers. The flowers appear in July and August [1]. There are thought to be nine species in this genus [2]. *P. atriplicifolia* widely grows in rocky places of Afghanistan, Iran and Pakistan. This plant is used as cooling medicine for treatment of fevers. The

flowers can be eaten in salads or as adorn and are very attractive for bees [3]. Natural products are rich source of bioactive compounds and most of their chemical properties associated with its constituent. Essential oil is one of the principle components of plants widely used as medicines, cosmetics and spices for thousands of years [4].

Traditionally, essential oils are extracted by conventional extraction methods, that is, hydro-distillation (HD), steam distillation (SD) and solvent extraction [5,6]. These techniques are endured some shortcomings, including long extraction time, loss of volatile compounds and low extraction efficiency [7]. Modern extraction techniques have been widely utilized for extraction of essential oils from natural plant products. These techniques include supercritical CO<sub>2</sub> extraction (SCE), subcritical water extraction (SWE) and microwave extractions [8-10]. Different microwave methods, for instance microwave assisted extraction (MAE), microwave assisted hydrodistillation (MAHD), microwave accelerated steam distillation (MASD), solvent free microwave extraction (SFME) and microwave hydrodiffusion and gravity (MHG), have been developed for extraction of essential oils [11-15]. The main advantages of above mentioned techniques are as follow: shortening extraction time, improving extraction yield, reducing organic solvent consumption and enhancing extract quality. Microwave heating has already been applied for extraction of essential oils of several species of Lamiaceae family [16-20]. Extraction of essential oil from areal part of *P. abrotanoides* was done by SFME compared to HD which resulted a high amount of oxygenated mono and sesquiterpene than that of obtained from HD [21]. Pourmortazavi and his co-workers have been used supercritical carbon dioxide method for isolation of essential oil from *P. atriplicifolia* [3]. So far, any microwave extraction has not been reported for extraction of essential oil of *P. atriplicifolia*.

The extraction methods are highly affected on chemical compositions and quality of essential oils. Therefore, the chemical composition analysis of essential oils is performed [22]. Several gas-chromatographic (GC) and gas-chromatographic-mass-spectroscopic (GC-MS) methods have been reported for analysis of essential oil of *P. atriplicifolia* [23, 24]. Study on essential oils from *P. atriplicifolia* which has grown in Afghanistan represents a total of 1.9% yield with the main chemical composition of  $\alpha$ -pinene (27%),  $\beta$ -pinene (1%), camphene (10%),  $\alpha$ -terpineol (8%), 1,8-cineole (31%), camphor (3%), borneol (6%), bornyl acetate (5%) and  $\beta$ -caryophyllene (3%) which confirm Rao's result which was done in 1962. Studies on essential oil extracted from *P. atriplicifolia* cultivated in Iran and Pakistan have been done separately and have shown different chemical composition [25,26]. Sefidkon has found sabinene, myrcene, limonene, humulene and guaiol in addition but the amount of humulene was in trace (0.4%) compared with the result 5.7% from *P. atriplicifolia* grown in Pakistan. In addition, Iranian samples contained 40% limonene which was not found in Afghanistan and Pakistan *P. atriplicifolia*.

Thin layer chromatography (TLC) is a rapid, economic and facilitate technique for identification, characterization and determination of chemical compounds. Recently, TLC has been applied for quantitative analysis of main volatile components of essential oils by developing some advanced methods. High performance thin layer chromatography (HPTLC) can be successfully applied for analysis of water soluble and low vapour pressure compounds [27]. A slit-scanning densitometry system is a widely known method for quantification of TLC, but the

equipment is cost-high. By development of digital electronics in particular imaging devices like charge coupled device (CCD) and complementary metal oxide semiconductor (CMOS), the quantitative and qualitative analysis of gel electrophoresis and TLC were provided by digital imaging and analyzing [28]. Various image analysis softwares have been developed. MATLAB program with image processing functions, ImageDecipher-TLC (BioDit Technology, Co.), JustTLC, Sorbfil TLC Videodensitometer (Sorbpolymer, Russia) are frequently used softwares for image analysis. On the other hand, development of open source concept has changed laboratories situation [29]. ImageJ (U.S. National Institute of Health) is a most often used and free software available for both PC and Mac [30]. Several TLC-Image analysis method using ImageJ software have been reported [33, 34]. Implementation of ImageJ software for determination of ochratoxin A in wine and aflatoxin B1 in peanuts by HPTLC method using a CCD camera has been reported by Welke and her co-workers [31, 32]. More recently, Sakunpak was used a TLC-densitometer and an image-analysis method for determination of barakol in *Senna siamea* leaves extract and  $\gamma$ -oryzanol in rice bran oil. In these researches they compared the results obtained from image-analysis using and ImageJ software with those obtained by a densitometry method [33, 34].

In this study, a convenient HPTLC by support of an image analyzing method for quantification of  $\beta$ -caryophyllene and *d*-camphor in essential oils extracted from *P. atriplicifolia* using hydrodistillation and microwave extraction methods has been developed and validated.

## MATERIALS & METHODS

### Materials

*P. atriplicifolia* leaves were collected, before flowering stage from rocky places of Guldara district of Kabul province, Afghanistan, in early Jun 2015. The leaves were dried on shadow and were kept in cold and dry place. The  $\beta$ -caryophyllene and 1,8-cineole standards were obtained from Wako (Japan), *d*-camphor from Kanto (Japan), while *L*-bornyl acetate was purchased from ACROS (Japan). The anisaldehyde reagent used for visualization was from Kanto (Japan). The solvents toluene, ethyl acetate, methanol, and sulfuric acid were purchased from Wako (Japan), while acetic acid was purchased from Kanto (Japan). All reagents were analytical grade.

Stock standard solutions were prepared in toluene at 2  $\mu$ L/mL for  $\beta$ -caryophyllene, 0.5 g/mL for *d*-camphor, 5 $\mu$ L/mL for 1,8-cineole and 5 $\mu$ L/mL for *L*-bornyl acetate, respectively. The anisaldehyde visualizing solution was prepared from 0.5 mL anisaldehyde in 10 mL acetic acid, 85 mL methanol and 10 mL conc. sulfuric acid in chronological order.

## Extraction procedure

### Hydro-distillation

50 g of dried leaves of plant was subjected to hydro-distillation by using a Clevenger type apparatus for 3 h in 500 mL water. The extracted oil was recovered by a micro syringe and stored in a vial in a refrigerator before analysis. A digital multimeter (Wens precision) with support of Ts DMM Viewer driving program for windows was used for temperature monitoring during extraction process.

### Microwave assisted hydro-distillation

5 g of dried powdered leaves of plant was placed in a microwave extractor (Green Motif, J-Sci. Lab.) and distilled with 25 mL water for 15 min. Usually, the multi-mode microwave reactor has been applied for extraction of essential oils but a single-mode reactor is useful when less amount of sample is available. Therefore, a single-mode microwave reactor with the maximum power of 300 W was utilized in this study. A thermocouple-used temperature meter (M-6000, METEX) with support of IDX-GM V2.00 driving program for Windows was used for temperature monitoring during extraction process.

### Chromatographic condition

Analysis was performed on 10 x 10 cm HPTLC 0.2 mm silica gel 60 F<sub>254</sub> sheet (Merck, Darmstadt, Germany). The extracted essential oils from both methods (HD & MAHD) and standard solutions of  $\beta$ -caryophyllene, *L*-bornyl acetate, 1,8-cineole and *d*-camphor were applied as spots at 1.5 cm from the bottom edge of a sheet using 0.5  $\mu$ L capillaries. The sheets were developed on 7.5 cm distance in a twin trough glass chamber (CAMAG) presaturated with mobile phase vapor for 15 min prior the experiment.

Separation was performed using toluene-ethyl acetate (95:5, v: v) as mobile phase. After developing the sheet was dried and visualized with anisaldehyde derivatizing reagent. A dipping method was used for visualizing. The sheet was heated for 5 min at 100-105°C on a hotplate [35].

### Image analysis method

The qualitative and quantitative analyses of spots on HPTLC sheets have been done with support of an image software. The qualitative analysis of spots has been done by comparing  $R_f$  values and color of spots of standards with those of separated samples on HPTLC sheets. The sheets were scanned with a scanner (Canon. LiDE 220) and the photos were saved in a format of the joint photographic experts group (JPEG). The images were analyzed using ImageJ. ImageJ is one of the many image processing tools issued by the U.S. National Institute of Health (NIH) and freely available on their website (ImageJ, <http://rsbweb.nih.gov/ij/>) [36]. The procedures for image processing and analyzing used in this study were based on Olech et al. paper [37].

## Validation of the HPTLC method

International Conference of Harmonization (ICH) guidelines [38] were followed for validation of the analytical method developed (linearity, precision, accuracy, LOD (limit of detection), LOQ (limit of quantification), ruggedness and robustness). Six concentrations of standard stock solution of  $\beta$ -caryophyllene (0.905-5.43  $\mu\text{g}/\text{spot}$ ) and *d*-camphor (0.025-0.15  $\text{mg}/\text{spot}$ ) were spotted on HPTLC sheets in triplicate and analyzed by ImageJ. The calibration curves were made by plotting peak areas against concentrations. The inter-day and intra-day precision (%RSD) were determined by analyzing standard solution of  $\beta$ -caryophyllene and *d*-camphor over the calibration range for three different days and three times on the same day. The accuracy of the analyses was compensated by calibration curve prepared experimentally. The LOD and LOQ were calculated for both  $\beta$ -caryophyllene and *d*-camphor. The LOD was determined as 3.3 (S.D)/S, while the LOQ was determined as 10 (S.D)/S, where S.D represents the standard deviation of response and S represents the average of slope of the calibration curves. The robustness of the method was assayed by introducing small changes in the mobile phase composition (95:5 and 94:6) and development distance (7.0 cm and 7.5 cm). The robustness of the method was examined three times at a concentration level of 0.9  $\mu\text{g}/\text{spot}$  of  $\beta$ -caryophyllene. The %RSD of the peak areas were calculated. The ruggedness of the method was examined by spotting of  $\beta$ -caryophyllene three times by different analysts using same equipment and HPTLC sheets. The % RSD of the peak areas were calculated.

## RESULTS & DISCUSSION

### Comparison of extraction kinetics and extraction yield

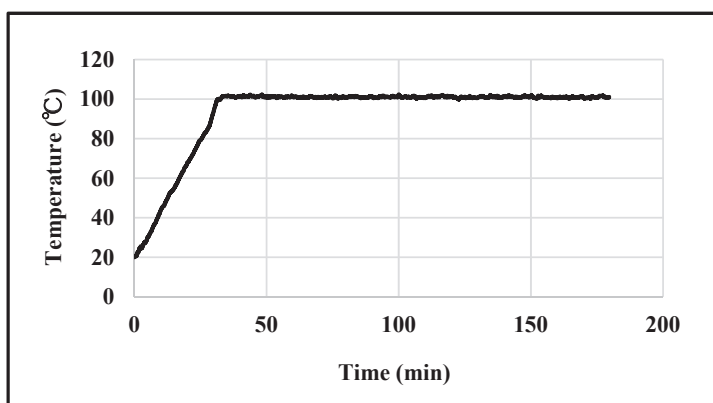
Kinetics of essential oil extraction from *P. atriplicifolia* using MAHD has been compared with that of HD. Extraction with MAHD started at much earlier time than that with HD (4 min vs. 30 min), respectively. For HD, the extraction started at the boiling point of water (100 °C) while for MAHD the extraction started at a target temperature of 110 °C. With MAHD, the boiling point was reached in 4 min, while in the case of HD it was at 30 min. The recovered essential oils for MAHD and HD was 1.4% (v/w) vs. 1.1% (v/w), respectively. The final yield of essential oils extracted by MAHD after 15 min of extraction was greater than that obtained by HD after 3 h of extraction 1.4 % (v/w) vs. 1.1 % (v/w), which resulted a significant time saving.

### Qualitative and quantitative analysis of *P. atriplicifolia* essential oil

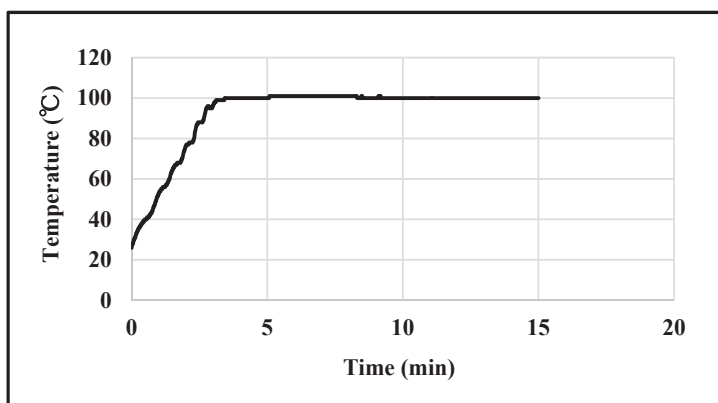
The constituents of the essential oils extracted by both MAHD and HD were similar. In total, four constituents ( $\beta$ -caryophyllene, *d*-camphor, 1,8-cineole and *L*-bornyl acetate) were identified in MAHD and HD extraction, respectively. The HPTLC-image analysis method was used to determine the  $\beta$ -caryophyllene and *d*-camphor content in *P. atriplicifolia* essential oil extracted by both MAHD and HD methods. Figure 2a shows a cut-area image of HPTLC sheet obtained by the photo scanner and Figure 2b shows a HPTLC-chromatogram obtained by ImageJ software. Table 1 shows

the  $R_f$  values of four chemicals of  $\beta$ -caryophyllene,  $d$ -camphor, 1,8-cineole and  $L$ -bornyl acetate contained in *P. atriplicifolia* essential oil extracted by both HD and MAHD methods with its total yield percentage.

**a**



**b**

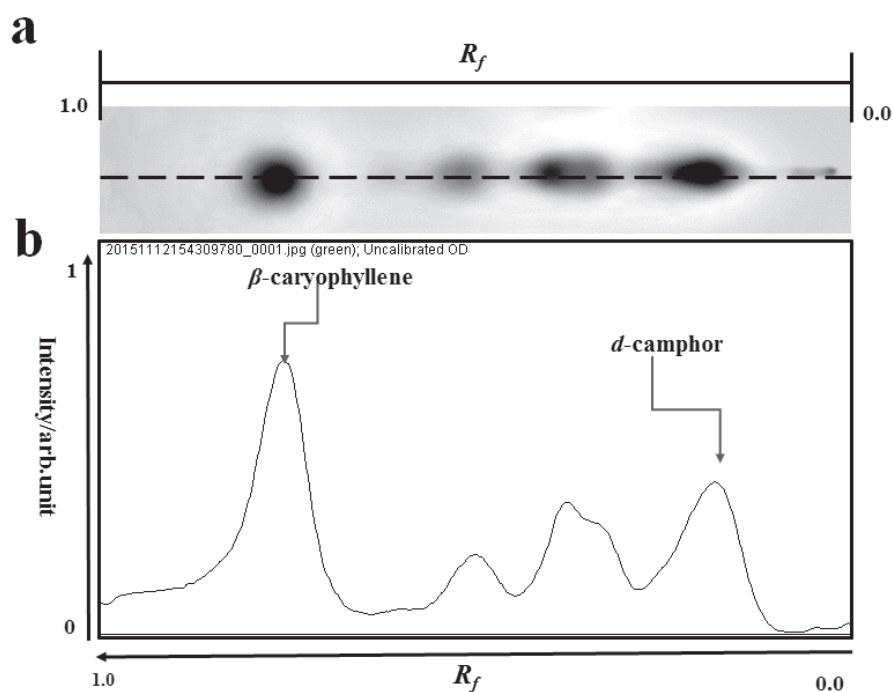


**Figure 1.** **a.** Temperature monitoring of extracted essential oil by HD using a digital multimeter with support of driving program (Ts DMM Viewer). **b.** Temperature monitoring of extracted essential oil by MAHD using a thermocouple with support of driving program (IDX-GM V2.00).

**Table 1.**  $\beta$ -caryophyllene and *d*-camphor content in *P. atriplicifolia* essential oil and  $R_f$  values determined by HPTLC-image analysis method

Composition	$R_f$	HD (%)	MAHD (%)
$\beta$ -caryophyllene	0.20±0.05	2.64	0.88
<i>d</i> -camphor	0.85±0.30	74.8	15
1,8-cineole	0.71±0.03	+	+
<i>L</i> -bornyl acetate	0.46±0.09	+	+
Total yield		1.1%	1.4%

Symbol ‘+’ shows detected compounds on HPTLC sheet.



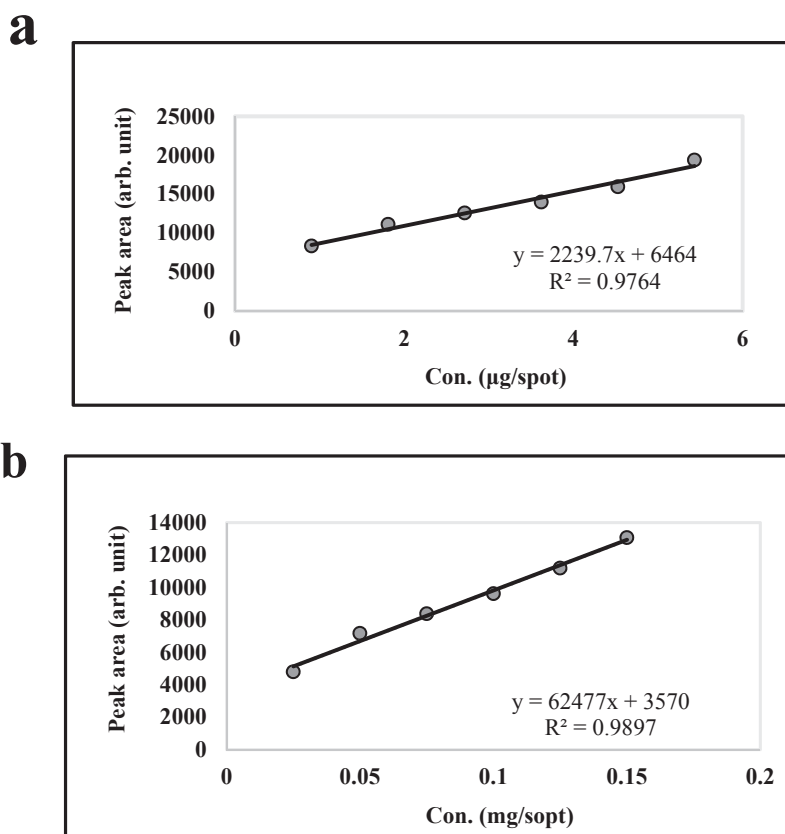
**Figure 2.** a: A cut-area image of HPTLC sheet obtained by the photo scanner. b: A HPTLC-chromatogram obtained by ImageJ software with the relative intensity of 0-1. The higher intensity is given by darker area in Figure 2a.

### Method validation

The calibration curve on HPTLC-image analysis method was found to be a straight line for  $\beta$ -caryophyllene and *d*-camphor, over the concentration range of 0.905-5.43  $\mu$ g/spot and 0.025-0.15 mg/spot, respectively. The



regression equations were  $Y=2239.7X+6464$  for  $\beta$ -caryophyllene and  $Y=62477X+3570$  for  $d$ -camphor with the correlation coefficient  $R^2=0.9764$  and  $R^2=0.9897$ , respectively as shown in Figure 3. The relative standard deviation values for inter-day and intra-day assay of both standards were less than 1%, which indicate precision of the method. The LOQ values obtained were 1.79  $\mu\text{g/spot}$  and 0.31  $\text{mg/spot}$  and the LOD values were 0.53  $\mu\text{g/spot}$  and 0.04  $\text{mg/spot}$  of  $\beta$ -caryophyllene and  $d$ -camphor, respectively. Small changes in the mobile phase composition resulted in % RSD of 1.07 and 1.35 (for mobile compositions of 95:5 and 94:6). And small changes in developed HPTLC distance resulted in %RSD of 1.39 and 1.16 (for developed HPTLC distance of 7.0 cm and 7.5 cm), which represent the robustness of the purposed method. The ruggedness of the method was observed by comparison of %RSD values of peak areas tested by two analysts obtaining in low %RSD values of 1.16 and 1.15, which shows reproducibility of the method. Table 2 shows the validation data of HPTLC-image analysis method.



**Figure 3.** Calibration curve of  $\beta$ -caryophyllene (a) and  $d$ -camphor (b) produced by HPTLC-image analysis method.

**Table 2.** Validation data of HPTLC-image analysis method.

Parameters	$\beta$ -caryophyllene	<i>d</i> -Camphor
Linear range	0.905-5.43 $\mu$ g/spot	0.025-0.15 mg/spot
Linear equations	Y=2239.7X+6464	Y=62477X+3570
R <sup>2</sup>	0.9764	0.9897
Precision (%RSD)		
Interday	0.32	0.31
Intraday	0.29	0.30
LOQ	1.79	0.13
LOD	0.53	0.04
Robustness (%RSD)		
Mobile phase proportions		
toluene: ethyl acetate (95:5)	1.07	
toluene: ethyl acetate (96:4)	1.35	
Developing distance		
7.5 cm	1.39	
7.0 cm	1.16	
Ruggedness (%RSD)		
Analyst 1 <sup>st</sup>	1.16	
Analyst 2 <sup>nd</sup>	1.15	

## CONCLUSION

The extraction of essential oil of *P. atriplicifolia* grown wild in Afghanistan has been first performed under optimal condition by means of microwave assisting heating. The microwave extraction method has shown higher yield in a short time compared to hydrodistillation method. Microwave energy seems to cause rapid rupture of plant cells containing essential oils. Therefore, it accelerates extraction process and increases extraction yield.

We first reported the validated HPTLC method with support of an image-processing software developed on open source concept for qualitative and quantitative analysis of  $\beta$ -caryophyllene and *d*-camphor in essential oil *P. atriplicifolia*.

This work demonstrated that open source concept [29] may change laboratory situation and computer based analytical methods can be used in quality control laboratories with limited resources in many parts of the world, especially in developing countries.

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