The extraction efficiency of maceration, UAE and MSPD in the extraction of pyrethrins from Dalmatian pyrethrum

Martina GRDIŠA1,2 (✉)
Filip VARGA1,2
Tonka NINČEVIĆ3
Barbara PTIČEK1
Dario DABIĆ4
Martina BIOŠIĆ4

Summary

Dalmatian pyrethrum (Tanacetum cinerariifolium /Trevir./ Sch. Bip.) synthesizes secondary metabolite pyrethrin, known for its potent insecticidal and repellent activity. The present study was aimed at optimizing the maceration extraction parameters that improve the efficiency of pyrethrin extraction from the dried Dalmatian pyrethrum flower heads. Extraction efficiencies under several conditions were investigated: different solvent types, extraction time, the rotational speed of the stirrer, and the solvent volume. The highest recovery values were obtained with 5 mL of acetone, at the rotational speed of 400 rpm, and the extraction time of three hours. In addition, the extraction efficiency of maceration was compared to that of ultrasound-assisted and matrix solid phase dispersion extraction, both previously optimized for pyrethrin extraction. The extractions were carried out on samples of three natural Dalmatian pyrethrum populations (Krk, Mt. Kozjak, and Senj). Both the total pyrethrin content and the HPLC profile varied between different extraction techniques. Across applied methods, the highest efficiency was observed with matrix solid phase dispersion extraction. Evaluation of the differences between data obtained using different extraction techniques was performed by the Bland-Altman analysis, revealing good agreement between the three methods.

Key words

Dalmatian pyrethrum, MSPD, UAE, maceration, optimization, pyrethrin, Tanacetum cinerariifolium

1 University of Zagreb, Faculty of Agriculture, Department of Seed Science and Technology, Svetošimunska cesta 25, 10000 Zagreb, Croatia
2 Centre of Excellence for Biodiversity and Molecular Plant Breeding (CoE CroP-BioDiv), Svetošimunska cesta 25, 10000 Zagreb, Croatia
3 Institute for Adriatic Crops and Karst Reclamation, Put Duilova 11, 21000 Split, Croatia
4 University of Zagreb, Faculty of Chemical Engineering and Technology, Department of Analytical Chemistry, Marulićev trg 19, 10000 Zagreb, Croatia

✉ Corresponding author: mgrdisa@agr.hr

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INTRODUCTION

Pyrethrin is a naturally occurring substance that is produced by Dalmatian pyrethrum (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.), a plant species that is endemic to the east Adriatic coastal region. As an agricultural crop, it is grown worldwide, namely Tanzania, Ruanda, Papua New Guinea, Italy, and Kenya (FAO, 2018). Pyrethrin is composed of six distinct compounds: pyrethrin I and II, cinerin I and II, and jasmolin I and II (Crombie, 1995; Boller and Silverstein, 2009) (Fig. 1) and it exhibits a potent insecticidal effect. The exceptional insecticidal performance of pyrethrin is the reason for its use in households and traditional agricultural systems since ancient times and the justification for the increasing cultivation in the world (Grdiša et al., 2009). Apart from being used against numerous crop pests, pyrethrin based products are successfully used in preserving public health by controlling many species of mosquitoes (*Anopheles* spp., *Aedes* spp., *Culex* spp.) responsible for malaria transmission (Boyce et al. 2007; Duchon et al. 2009). The insecticidal activity of pyrethrin is focused on the interruption of the normal functioning of the insect’s nervous system, causing a quick knockdown effect, hyperactivity, and convulsions (Urkude et al., 2019). Pyrethrin has low persistence in the environment as the exposure to higher temperatures, oxygen, sunlight, and moisture accelerate its degradation, which makes it ideally suitable for the use in food processing and handling facilities (Peckman and Arthur, 2006).

Previous investigations on the extraction of pyrethrin from Dalmatian pyrethrum have mainly involved the comparison of several extraction methods and the achievement of optimal extraction conditions, using methods such as Soxhlet extraction (Ban et al., 2010), ultrasound-assisted extraction (Babić et al., 2012), supercritical fluid extraction (Kiriamiti et al., 2003a, 2003b), etc. More recently matrix solid phase dispersion method has been optimized and successfully applied in the extraction of pyrethrin (Biošić et al., 2020). Gallo et al. (2017) compared the efficiency of supercritical fluid extraction, maceration, and rapid solid-liquid dynamic extraction of pyrethrin. The extraction efficiency of different extraction techniques (percolation, agitation with heat, sonication, and Soxhlet) was evaluated by Nagar et al. (2015).

As the easiest and the simplest extraction method, maceration has successfully been used in the extraction of numerous natural ingredients (Čujić et al., 2016; Monton et al., 2019; Cvetanović et al., 2020). This conventional extraction technique has many advantages. The most prominent advantage is the efficient extraction of different natural compounds at room temperatures, which is of great importance for those compounds that are heat sensitive. Additionally, maceration does not require laborious preparations or expensive equipment and it offers the possibility of using various solvents (Uysal et al., 2019). To the best of our knowledge, factors affecting maceration extraction of pyrethrin from Dalmatian pyrethrum flowers have never been evaluated, even though this method has traditionally been used in the extraction of many natural compounds, including pyrethrin.

Ultrasound-assisted extraction (UAE) is another extraction method that has been widely employed in the last two decades to extract bioactive compounds from various plant materials (Zhong et al., 2010; Bimakr et al., 2017; Chemat et al., 2017; Giacometti et al., 2018). The mechanism of UAE is based on the mechanical effect caused by the implosion of the cavitation bubbles, resulting in the disruption of the cell membranes and subsequently enhanced penetration of the solvent into the cell that facilitates the release of the cell content (Romero et al., 2010; Veillet et al., 2010; Hossain et al., 2012; Dent et al., 2015). Because of this effect, UAE has the advantage of considerably reducing the extraction time and increasing the extraction yield in comparison to the conventional extraction methods. The method favors the dissolution of the targeted compounds with a lesser amount of solvents used (Luque de Castro and Priego-Capote, 2007), and enables the extraction of heat-sensitive ingredients at lower temperatures (Vilkhu et al., 2008; Esclapez et al., 2011).

![Figure 1. Structural formulas of six pyrethrin components](image)
Matrix solid phase dispersion (MSPD) is a somewhat newer extraction method that represents a fast and safe alternative to traditional solvent extraction. This method proved to be very effective in the extraction of various active substances from the plant, animal and human tissues, food products, and soil samples (Dawidowicz and Wianowska, 2009; Rubert et al., 2011; Mutavdžić Pavlović et al., 2012; Souza et al., 2013; Tao et al., 2014; Rashidipour et al., 2015). In comparison to many other conventional extraction methods, it combines sample homogenization, disruption of the sample architecture, extraction and purification of the sample in just one step, thus reducing the required amount of solvent used by approximately 95% and time by 90% (Wei et al., 2011; Rallis et al., 2012). Several factors affect MSPD extraction, the most crucial is the type of sorbent and solvents used (Dassanayake et al., 2009).

This study aimed to optimize the maceration extraction conditions for dried Dalmatian pyrethrum flowers to achieve the highest pyrethrin levels, based on the choice of the most suitable solvent and its volume, the rotational speed of the stirrer, and extraction time. Therefore, extraction conditions were carefully selected to achieve maximal recovery of all six distinct compounds. The extraction efficiency of maceration was compared to that of UAE and MSPD, previously optimized for the pyrethrin extraction.

**MATERIALS AND METHODS**

**Chemicals and standards**

The solvents (acetone, ethanol, ethyl acetate, methanol, and n-hexane) used in this investigation were of HPLC grade and were purchased from Kemika (Zagreb, Croatia). The water distilled with the Millipore Simplicity A10 water system (Millipore Corporation, Billerica, MA, USA) was used. Pyrethrin standard was obtained from Sigma-Aldrich (Steinheim, Germany). The mass fractions in pyrethrin standard Pestanal were: 2.06% for cinerin II, 15.97% for pyrethrin II, 1.26% for jasmolin II, 2.57% for cinerin I, 26.09% for pyrethrin I, and 1.73% for jasmolin I. Florisil was purchased from Agilent (USA), formic acid from T. T. T. (Sveta, Nedjelja, Croatia), and acetonitrile from Sigma-Aldrich (Steinheim, Germany).

**Plant material**

The flower heads of three natural Dalmatian pyrethrum populations (Krk, Mt. Kozjak, and Senj) were sampled in June 2018, from the field trial set at the Institute of Adriatic Crops and Karst Reclamation in Split, Croatia. Sampling was carried out at the stage when approximately 3/4 of the disc flowers were opened. The samples were air-dried to the final moisture content of 10-12% and stored in a dark and dry place until further analysis. Before the analysis, the flowers were pulverized with Microtron and transferred to Erlenmeyer flasks and the selected solvent was added. The sealed flasks were transferred to a magnetic stirrer (RO 10, IKA, Staufen, Germany) and kept in a dark chamber at room temperature. The experiments were performed at different extraction conditions. The first step involved testing the extraction efficiency of different solvents (acetone, ethanol, and ethyl acetate), followed by examining the efficiency of different extraction times (0.5, 1, 2, and 3 h), the rotational speed of the stirrer (200, 300, 400, and 500 rpm) and solvent volume (5, 7, 9, and 11 mL). Before the high-performance liquid chromatography analysis (HPLC-DAD), the obtained extracts were filtered through 0.45 μm filter paper (HPLC certified, Pall Life Sciences, Port Washington, NY, USA).

**Ultrasound-assisted extraction (UAE)**

Ultrasound-assisted extraction was performed under the optimum conditions described in the paper by Babić et al. (2012). Pulverized Dalmatian pyrethrum flowers (0.25 g) were placed in 100 mL plastic vessels and 5 mL of acetone was added. The vessels were sealed and placed in an ultrasonic bath (Sonorex Digital 10P, BANDELIN, Berlin, Germany), with the power adjusted to 1200 W and ultrasonic frequency of 35 kHz, at 50°C for 60 minutes. The obtained extracts were filtered through 0.45 μm pore-size nylon membrane disc filters (HPLC certified, Pall Life Sciences, Port Washington, NY).

**Matrix solid phase dispersion (MSPD)**

Matrix solid phase dispersion extraction of pyrethrins was performed according to Biošić et al. (2020). Pulverized flowers (0.25 g) were mixed with 0.50 g of florisil and 0.40 g of NaSO₄. Before use, florisil was activated at 160°C and washed with n-hexane and methanol. The mixture was homogenized in a mortar using a glass pestle and transferred into a polypropylene column to which a polyethylene (PE) frit was previously placed on the bottom. The PE frit was also placed on top of the column to prevent the solvent from evaporating rapidly. The 1 mL of elution solvent (acetone and ethyl acetate; 1:1, v/v) was added to the column and left in contact with the mixture for five minutes. The columns were placed on the SPE (solid phase extraction), vacuum manifold (Visiprep™, Supelco, Sigma-Aldrich, Steinheim, Germany) and after the expiration of five minutes, the solvent was passed through a column. The extracts (5 mL) were collected in a cuvette, evaporated to dryness, dissolved in 1 mL of acetonitrile, and transferred to vials.

**Chromatographic analysis**

Extracts obtained with maceration, UAE, and MSPD were stored in a cool and dark place until further analysis. The recoveries (%) were calculated as the ratio of the amount of extracted component (mg g⁻¹) and the highest detected amount (mg g⁻¹) for each component in each set of extraction experiments. Each experiment was repeated three times and the results were expressed as average values. The content and composition of extracted pyrethrins were estimated with HPLC-DAD (Varian Pro Star 500 system, Walnut Creek, CA, USA), on 250 x 4.6 mm Luna C18 column, particle size 5 μm (Phenomenex, CA, USA) by using the method developed and validated by Biošić et al. (2020).
Statistical analysis

Statistical analysis was performed using the SAS statistical software version 9.3 (SAS Institute, 2011). Averages of total pyrethrin content obtained by the three methods were calculated to assess the mean difference (bias) between the three methods. Pearson correlation coefficients between the methods were calculated and tested based on the total pyrethrin content values. Bland-Altman analysis (Bland and Altman, 1986) was used to describe the agreement between the three extraction methods (maceration, UAE, and MSPD) based on total pyrethrin content. The mean difference (bias) and the limits of agreement (LoA) of the differences were calculated. The population data obtained by the three methods were combined into all possible combinations \(3^3 = 27\) to evaluate the interchangeability of the methods in population diversity analysis. Analysis of variance (ANOVA) between three populations was conducted using the PROC GLM function in SAS for each of the combinations. Post hoc comparisons were conducted using Tukey’s HSD test.

RESULTS AND DISCUSSION

Maceration optimization

The applicability of different extraction methods and extraction conditions should be examined to understand the extractive selectivity of desirable active compounds (Azmir et al., 2013). The present study was designed to determine the optimal conditions for maximizing pyrethrin extraction with maceration and to compare the extraction efficiency of maceration with the efficiency of two other extraction methods: UAE and MSPD. Selecting the most effective extraction method is the crucial step in increasing the yield and the bioactivity of the obtained extract.

The maceration extraction parameters that were optimized in this study were the type and volume of the solvent, extraction time, and rotational speed of the magnetic stirrer. The intent was to achieve maximum extraction efficiency with a minimum solvent volume used and the minimum extraction time. The experiments were conducted at room temperature as the maceration extraction method provides this possibility. The possibility of performing maceration without the application of high temperatures is the main advantage of this method, especially for the extraction of thermolabile components (Zhang et al., 2018). Pyrethrins fit into this category as it has been reported that they degrade rapidly at higher temperatures (Dickinson, 1982; Allan and Miller, 1990). The initial step of the maceration optimization process was to select an appropriate extraction solvent for pyrethrin extraction. In addition to achieving the highest possible extraction yield, in selecting the appropriate solvent other factors such as environmental safety, human toxicity, and financial affordability should be considered (Nedović et al., 2015). The solvent should have good solubility of the target compound and low solubility of potentially interfering molecules. Among various optimization studies for enhancing extraction efficiency, the solvent type is recognized as the most influential parameter (Azwanida, 2015). Therefore, in this investigation, three solvent systems with different polarities (acetone, ethyl acetate, and ethanol) were tested. The empirical parameter of solvent polarity \(E\) for ethanol is 0.654, for ethyl acetate 0.228, and 0.355 for acetone (Reichardt and Welton, 2011).

The highest recovery rate (100%) of all six pyrethrin components was obtained by using acetone. Ethyl acetate was the second-best solvent, with a recovery rate ranging from 87 to 94%, while the lowest recovery rate values were achieved with ethanol (82 - 88%) (Fig. 2). In the investigation of Babić et al. (2012) acetone has also proven to be the most efficient solvent in the ultrasound-assisted extraction of pyrethrin.

**Figure 2.** Relative recoveries of six pyrethrin components using different extraction solvents \((V_{\text{solute}} = 5 \text{ mL}, t = 3 \text{ h}, m_{\text{sample}} = 0.25 \text{ g}, \text{rpm} = 400\)
Following the determination of the optimal solvent type, the effects of different maceration times were tested. Different extraction times (0.5, 1, 2, and 3 h) influenced the chemical composition of the obtained extracts. The extraction time of 3 h was the most efficient, resulting in the recovery rate of 100% of all six pyrethrin components (Fig. 3). The requirement for longer extraction time has been reported as one of the disadvantages of maceration extraction (Zhang et al., 2018), which might lead to degradation of thermolabile active ingredients (Monton and Luprasong, 2019).

To determine the effects of the different rotational speeds of the stirrer, the extractions were performed at 200 rpm, 300 rpm, 400 rpm, and 500 rpm. At 400 rpm the 100% recovery rate of all active components was obtained. The lowest relative recovery values (81 - 89%) were achieved at 200 rpm (Fig. 4).

The final step of the optimization was to select the appropriate solvent volume. Four different volumes of acetone were tested: 5 mL, 7 mL, 9 mL, and 11 mL. The maceration with 5 mL of the solvent resulted in a recovery rate of 100%. Using 7 mL of solvent, relative recoveries ranging from 80 - 90% were obtained, while the lowest relative recovery values of six pyrethrin compounds were achieved using 9 mL of acetone (77 - 83%), indicating that the treatment with the lowest amount of solvent volume improves the extraction of pyrethrin (Fig. 5).

As stated in previous research, one of the disadvantages of using maceration for the extraction of natural compounds is the need of utilizing large amounts of solvents (Azwanida, 2015). However, this was not the case in our investigation, as the lowest solvent volume resulted in the highest recovery values. This fact is certainly an advantage since lower volumes of solvents are favorable for the personnel performing the extraction, and from an environmental point of view. Additionally, higher solvent volumes require higher waste management costs, which ultimately increases the costs of the entire extraction process (Babić et al., 2012).

Comparison of extraction techniques

The extraction efficiency of maceration, UAE, and MSPD based on the total pyrethrin content (% in dry flower mass) in three natural Dalmatian pyrethrum populations was determined by HPLC analysis (Fig. 6).

On average, the highest extraction efficiency was recorded for MSPD (0.52%) followed by UAE (0.48%) and maceration (0.45%). In the Krk population the highest extraction efficiency was achieved with maceration (0.62%), while MSPD (0.59%) and UAE (0.49%) yielded lower values (Fig. 7). The highest total pyrethrin content obtained in Mt Kozjak population from was with UAE (0.46%), while slightly lower values were obtained when applying MSPD and maceration (0.45 and 0.43). In the Senj population, MSPD yielded the highest total pyrethrin yield (0.54%), followed by UAE (0.49%) and maceration (0.29 %). The MSPD and maceration appeared to be moderately correlated (r = 0.41), while MSPD and UAE displayed low correlation (r = 0.15). Maceration and UAE appeared to be uncorrelated (r = 0.04). All the correlations were statistically non-significant.

To describe the extent of agreement in extraction efficiency of pyrethrin between the evaluated extraction methods Bland-Altman plot (Bland-Altman, 1983) was constructed. The Bland-Altman analysis computes the agreement between two quantitative measurements by evaluating the mean difference and constructing limits of agreement (Giavarina, 2015).

![Figure 3. Relative recoveries of six pyrethrin components at different extraction times using acetone as extraction solvent (V\textsubscript{solvent} = 5 mL, m\textsubscript{sample} = 0.25 g, rpm = 400)](image-url)
Figure 4. Relative recoveries of six pyrethrin components at the various rotational speed of the stirrer using acetone as extraction solvent ($V_{\text{solvent}} = 5 \text{ mL}$, $m_{\text{sample}} = 0.25 \text{ g}$, $t = 3 \text{ h}$).

Figure 5. Relative recoveries of six pyrethrin components at different acetone volumes ($m_{\text{sample}} = 0.25 \text{ g}$, $t = 3 \text{ h}$, rpm = 400).
Figure 6. HPLC-DAD chromatograms of Dalmatian pyrethrum flower extracts: population Senj (a), population Mt Kozjak (b), and population Krk (c); IS-internal standard, 1-cinerin II, 2-pyrethrin II, 3-jasmolin II, 4-cinerin I, 5-pyrethrin I, and 6-jasmolin I.

Figure 7. Pyrethrin profiles of Dalmatian pyrethrum populations obtained with three extraction methods.
Bland-Altman plots (Fig. 8 a, b, and c) show that there is a good agreement between the three methods compared with all points scattered well within the lines of agreement. A comparison of different measuring methods is common across different industries and scientific disciplines when trying to update and innovate the measuring techniques or devices used (Downie, 2015). If the techniques or devices used for measuring the same characteristics are in a good agreement, that can potentially increase the extent of obtainable data (from both different measuring techniques and institutions).

The three populations (Krk, Mt. Kozjak, and Senj) were selected based on previous research by Grdiša et al. (2013) due to different total pyrethrin content recorded in each of them (high total pyrethrin content in Krk population, intermediate in Senj population, and low in Mt. Kozjak population). The results obtained with MSPD were the only one consistent with previous research. ANOVA revealed significant differences between populations in 77% of the combinations created (Table 1). In 33% of the combinations highly significant differences between all three populations were revealed. Although Bland-Altman analysis showed that there is a good agreement between the three extraction methods, the results of ANOVA conducted on combinations of population data may indicate differently. Both results should be taken with caution due to high chemical variability within the natural populations.

**Figure 8.** The Bland-Altman comparison plots of paired differences against the mean total pyrethrin content (% in dry flower weight) obtained by three extraction methods: (a) MSPD and UAE; (b) MSPD and maceration; (c) UAE and maceration. The solid lines represent the mean differences (bias) and the dashed lines represent the limits of agreement.
Table 1. Results of ANOVA showing the variation between sample means in three populations of Dalmatian pyrethrum based on different combinations of extraction methods

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ns - non-significant;
* - significant at \(P<0.05\); ** - significant at \(P<0.01\); *** - significant at \(P<0.001\)

Values in the Tukey HSD test columns were marked by the same letter when there was no observed significant difference in the Tukey test.
CONCLUSION

The optimal maceration conditions obtained in this investigation might be used as a guide for the extraction of pyrethrins with an inexpensive method, to obtain the high extraction yield. The determination of the most effective extraction method is justified as different methods employed on the same plant material show significant variations in extraction efficiency. The good methods agreement determined by comparison of the results obtained by MSPD, UAE, and maceration indicates that the extraction techniques tested in this investigation could be used interchangeably in the extraction of pyrethrins. The differences between the results of ANOVA should be taken into consideration considering the intrapopulation variability of the plants used as source material. The main common advantage of all three extraction methods is that they all use moderate temperatures that is beneficial for pyrethrin extraction, since pyrethrin is a heat-sensitive compound. Due to the simplicity, the extraction of pyrethrin by maceration can be carried out without sophisticated and expensive laboratory equipment and does not require special skills. When compared to maceration, the MSPD method is somewhat more labor-intensive, while UAE demands moderately expensive equipment. In comparison to the other tested methods maceration is more time-consuming, which could be considered as a disadvantage in the choice of the extraction method, however, it could be widely used for small scale extraction.

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