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Full Length Research Paper

Comparison of pyrethrins extraction methods efficiencies

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Extraction efficiency of insecticidal active compounds from dried Dalmatian pyrethrum flowers (*Chrysanthemum cinerariaefolium* /Trevir/Vis) was tested using different techniques and solvents. The research included six treatments which are the combination of two techniques (soxtec and ultrasound) and three different solvents (hexane, ethanol and petroleum ether). Dalmatian pyrethrum is a perennial herb native to Croatia. Its powder prepared from dried flower heads has been used as natural insecticide for centuries in traditional Croatian farming systems. It has no toxicity to man and animals but possesses ecological benefits that have led to increasing worldwide production of this natural insecticide. Nowadays, it is cultivated mainly at higher altitudes in tropical countries such as Kenya, Tanzania and Rwanda. The present investigation was directed in identifying a simple and reliable extraction treatment using solvents with lower cost and toxicity and an adequate method for the identification and separation of active compounds (pyrethrins) with possible application in enterprises or industry. Best developed method was used for determination of pyrethrin content in three different natural populations of Chrysanthemum. The results revealed high content of total pyrethrins in populations grown in Croatia. Developed method and good quality product give a possibility for this culture to become again an exporting and economically valid product for Croatia.

Key words: *Chrysanthemum cinerariaefolium*, extraction, pyrethrum, reversed phase-high performance liquid chromatography, soxtec, ultrasound.

INTRODUCTION

Pyrethrum is a natural insecticide produced from Dalmatian Chrysanthemum (*Chrysanthemum cinerariae-folium*) flowers. There are many areas of application of pyrethrum. The most important use is probably in mosquito control for both rural and urban areas where human safety is of prime considerations (US Environmental Protection Agency Office of Pesticide Programs, 2009). It is applied as an insecticide in home gardens and in organic farming. Pyrethrum is already included in most lists of approved insecticides for organic production throughout the world and has become the dominant insecticide (Glynne-Jones, 2001). The term “pyrethrum” referres to the plant, flower or flower extract, with the active insecticidal components of pyrethrum known as “pyrethrins” (Morris et al., 2006). Pyrethrins are esters of chrysanthemic (pyrethrins I, PI) and pyrethric (pyrethrins II, PII) acid. Thus, “total pyrethrins” refers to sum of pyrethrins I and pyrethrins II esters (Casida and Quistad, 1995). Pyrethrins I group include pyrethrin I, cinerin I, and jasmolin I, while pyrethrins II group consists of pyrethrin II, cinerin II and jasmolin II (Essig and

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Abbreviations: SFE, Super critical fluid extraction; **RP-HPLC,** reversed phase-high performance liquid chromatographic; **NP-HPLC,** normal phase- high performance liquid chromatography; **GC,** gas-liquid chromatography; **CEC,** capillary electro chromatography; **HPLC,** high performance liquid chromatography; **RSD,** relative standard deviation.

Zhao, 2001a). Among these compounds, pyrethrin I and pyrethrin II are the most predominant and active (Casida and Quistad, 1995). Pyrethrins content can vary from 0.9 to 1.3% by weight of dried flowers in native populations (Kolak et al., 1999; Casida and Quistad, 1995). Tasmanian commercial varieties contain 1.8 - 2.5% (Morris et al., 2006), while 3.0% of pyrethrin was reported in flowers of clones and breeding lines from breeding programs in Australia, Kenya, and USA (Casida and Quistad, 1995). The extraction yields of each pyrethrin ester depend on extraction conditions (temperature, solvent) but their relative proportions do not vary significantly (EU project, 2002). Because of separation difficulties and lack of absolute standards for each compound, the content of pyrethrum extract is not usually reported nor analysed for the individual pyrethrins, but for total pyrethrins or total pyrethrins I and total pyrethrins II.

There are many organic solvents and extraction methods which are not sufficiently effective or are too expensive for large scale profitable pyrethrum production. Among methods of pyrethrin extraction, the classic organic solvent extraction methods are still the most commonly used in industry and laboratories. Ultrasound (Kasaj et al., 1999), soxtec (Otterbach and Wenclawiack, 1999) and recently the super critical fluid extraction (SFE) methods (Pan et al., 1995; Wynn et al., 1995; Otterbach and Wenclawiack, 1999; Della Porta and Reverchon, 2002; EU project, 2002; Pol and Wenclawiack, 2003; Reverchon and De Marco, 2006) have been widely investigated. Different solvents have been tested for their efficiency in pyrethrins extraction. These include n-hexane (Pan et al., 1995; Kasaj et al., 1999; EU project, 2002), methanol, ethanol, propanol (EU project, 2002), dichloromethane (Kasaj et al., 1999; EU project, 2002), and petroleum ether (Della Porta and Reverchon, 2002).

Among the separation techniques, many have been reported and compared: reversed phase-high performance liquid chromatographic (RP-HPLC) (Pan et al., 1995; Wang et al., 1997; Kasaj et al., 1999; EU project, 2002), normal phase-high performance liquid chromatography (NP-HPLC) (Essig and Zhao, 2001a; Essig and Zhao, 2001b), gas-liquid chromatography (GC) (Nguyen et al., 1998; Della Porta and Reverchon, 2002), and some others, e.g. high-performance capillary electrophoresis (HPCE) (Henry III et al., 1999) and capillary electro chromatography (CEC) (Henry III et al., 2001) with the official AOAC titration method used as a referent method (Casida and Quistad, 1995). The pyrethrins are light (especially UV), oxygen, water, and elevated temperature sensitive (Casida and Quistad, 1995). Termolabile pyrethrins could be extracted without decomposition in the temperature range of 20 to 40°C (Della and Reverchon, 2002) and therefore the HPLC to the GC technique for analysing pyrethrins was preferred. The advantage of reversed-phase HPLC over normal-phase HPLC methods is the very low level of interferences in the chromatography (Wang et al., 1997). Chrysanthemum is a native plant in Croatia (Kolak et al., 1999) and the world need of natural insecticides has increased considerably recently. A study on methods of extraction, seperation and identification of pyrethrum active compounds is of particular interest for potential industrial or home production. Extraction efficiency of six different treatments (combinations of two extraction techniques and three solvents) for isolation of natural pyrethrins was investigated. The extraction recovery and repeatability, cost for eventual commercial extraction and the lowest toxicity of solvents used were considered. Developed method was used for determination of pyrethrin content in three native populations of *C. cinerariaefolium.*

MATERIALS AND METHODS

Chemicals and reagents

Pyrethrum extract containing 25% pyrethrin I + pyrethrin II, 4'methoxyflavanone and acetonitrile HPLC grade were obtained from Fluka (Buchs, Switzerland). Ethanol, methanol, hexane and petroleum ether were p.a. grade, purchased from Kemika (Croatia). Pure water was obtained from an Ellix 3 purification system (Millipore, USA).

Plant materials

The seed of native pyrethrum plants *C. cinerariaefolium* /Trevir./Vis was collected in their natural habitat near Split, Croatia. Seed was sown and one month old transplants were planted at experimental field of the Institute of Agriculture and Tourism-Poreč (Croatia). During the growing season, common cultural practices was applied. Flowers were hand harvested in 2004 at optimum maturity and the flowers were spread in a thin layer on wooden pallets. Prior analysis, flowers were dried for two months under dry, cool and dark conditions. Dry flowers (91.4% dry matter) were pulverised (with an electric mixer) and stored at 4°C in a dark well tapped glass. Dry samples were dusted in a dark room and extracted at the lowest possible temperature. They were preserved at -18°C in well closed flasks protected with parafilm and Al-folium and the time between extraction and analysis was as short as possible.

Treatments

The research included six treatments which were the combination of two techniques (soxtec and ultrasound) and four solvents (ethanol, hexane, methanol and petroleum ether).

Instrumentation

The extractions were made on a soxtec avanti 2055 manual system (Foss, Sweden) and in the ultrasound bath (Branson, The Netherlands). To achieve better and uniform elution of pyrethrins from crude oleoresin, a laboratory stirrer 3005 (GFL, Germany) was used. Solvent was evaporated on a rotary evaporator, Laborota 4000, comprising a Rotavac vacuum pump (Heildorph, Germany).

HPLC analyses were performed on a Varian Pro Star HPLC system comprising a Pro Star 230 solvent delivery module, Pro Star UV-Vis detector and manual 7725I Rheodine injector with a 20 µl sample loop. Separation of compounds was achieved using a Chrompack Omnisphere C18 column (250 × 4.6 mm, 5 µm particle size). Monitoring, pump control and data processing were performed by means of Star LC Workstation Version 5.5 software.

The spectra of individual esters were obtained using a Varian Pro Star HPLC system including: Pro Star 230 pump, Pro Star 330 UV-Vis Photodiode Array Detector, Pinaclle C18 column (250 × 4.6 mm, 5 µm particle size) and LC Workstation Version 6.20 software.

Soxtec extraction

Pulverised material (1 g) was extracted in soxtec apparatus with each solvent at a recommended temperature and duration (155°C/ 85 min for hexane, 200°C/ 110 min for ethanol, 135°C/ 80 min for petroleum ether). The evaporation of the solvent and its recovery was automated. Dried pyrethrum extract was collected in Al-vessels. The extraction was repeated on the already extracted sample of the pulverised material. The obtained crude extract was then eluted with acetonitrile (25 ml in two portions) using an electric laboratory stirrer for 10 min at 200 RPM. The elutes were collected with Pasteur pipettes in 25 ml graduated flasks and kept at - 18°C prior to analysis. The elution of the same crude extract was made five times in the preliminary studies, and two times for the analysed samples.

Ultrasound extraction

Pulverised material (1 g) and 15 ml of solvent were transferred into 25 ml flasks and sonicated for 1 h. The extraction of the same sample was repeated 5 times in the preliminary studies, and four times for the analysed samples. After filtration, the combined extracts of the first three extractions were collected into 25 ml graduated flasks and then the solvent was evaporated on a Rotavac (30°C, vacuum, 150 RPM). Preliminary studies included the fourth and fifth extraction (collected and analysed separately). To get a purified extract of the residue, the elution of pyrethrins was performed the same way as described in the soxtec extraction method. Results are expressed as g/100 g (or %) of dried flowers for each ester and calculated as a mean value of three replicates.

Chromatographic conditions (RP-HPLC)

RP-HPLC method proved to be in good correlation with the standard AOAC method and has been successfully used to separate the pyrethrins from the pyrethrum extract (Kasaj et al., 1999). By modifying the HPLC conditions (Table 1), excellent separation and resolution of all six compounds and the internal standard was achieved (Figure 1). The mobile phase components used were acetonitrile (solvent A) and water (solvent B). The flow rate was 1 ml/ min. The pyrethrins were detected at 230 nm. The same gradient program was used to obtain spectra for each ester with the use of (diode array detection) DAD scanning over a wavelength range from 200 to 400 nm (Table 1).

Standard solutions

Stock solutions containing pyrethrin I and pyrethrin II (3 mg/ml) and 4'-methoxyflavanone (internal standard; 2.417 mg/ml) in acetonitrile were used to prepare 9 standard pyrethrin mixtures containing 0.147 - 4.705 ml of the stock solution and 1 ml of the 4'-methoxyflavanone solution in 25 ml graduated flasks.

Data analysis

For comparing the efficiency of each combination of techniques (ultrasound, soxtec) and solvents (ethanol, hexane, and petroleum ether), two-way ANOVA was used. We used t-test to determine the difference between populations *of C. cinerariaefolium.*

Table 1. HPLC condition as listed in literature (Kasaj et al., 1999) and modified in our study.

Acetonitrile (Solvent A, %)	Time (min)	
	Kasaj et al.	Modification
58	0 - 5	0 - 5
58 - 75	5 - 35	5 - 50
75 - 100	35 - 36	50 - 51

Table 2. Separation factor [$\alpha = t'_R(B) / t'_R(A)$; $t'_R = t_R - t_R(i.st.)$] of individual successive pyrethrin esters (A, B) for validated (Kasaj et al., 1999) and our modified method.

B/A	α*	
	Kasaj et al. (1999)	Modified
Pyrethrin II/ CinerinII	1.127	1.112
Jasmoline I/ Pyrethrin II	1.437	1.442
Cinerin I/ Jasmoline II	1.565	1.645
Pyrethrin I/ Cinerin I	1.044	1.036
Jasmoline I/ Pyrethrin I	1.250	1.152
Cinerin II/ Jasmoline I	0.302	0.317

*α = Separation factor, $\alpha = t'_R(B) / t'_R(A)$; $t'_R = t_R - t_R(i.st.)$; A, B = individual successive pyrethrin esters.

RESULTS AND DISCUSSION

Identification and quantification

Individual esters were identified by matching the separation factors (α) from our analysed samples to those calculated from the relative retention times of the esters reported in the work of Kasaj et al. (1999) as shown in Table 2. The identification was confirmed comparing the UV spectra of each ester from the analysed sample with those from the standard solution. The found UV maximum matched those reported in the literature as listed in Table 3. The individual pyrethrum esters are unavailable, thus most HPLC quantification methods use a commercial pyrethrum mixture with an estimated amount of 25% of total pyrethrins as a standard solution. The amount of total pyrethrins in the assayed sample was estimated by calculating the sum of measured peak areas of individual pyrethrins. Figure 1 shows the chromatogram overlaying for a prepared standard mixture and for an analysed sample. The calibrating curves for total pyrethrins and for each pyrethrin were obtained from the prepared standard mixtures. The calibrating intervals covered the range of occurrence of all six compounds in the analysed sample. These calibrating curves were used to determine the amounts of total pyrethrins, pyrethrins I, pyrethrins II, as well as the amounts of each pyrethrin ester in the assay and their percent in dried flowers. The same detector response for all six esters based on their very similar chemical structure was assumed (Figure 2).

Table 3. Absorbance maximum wavelengths (λ_{max}, nm) of the pyrethrin compounds as referred in the literature (Casida and Quistad, 1995) and found in our study.

Pyrethrin compounds	λ_{max}, nm (literature)	λ_{max}, nm (found)
Pyrethrin I	226	224.31
Cinerin I	226	225.87
Jasmoline I	226	225.89
Pyrethrin II	229	228.13
Cinerin II	234	233.70
Jasmoline II	234	233.49

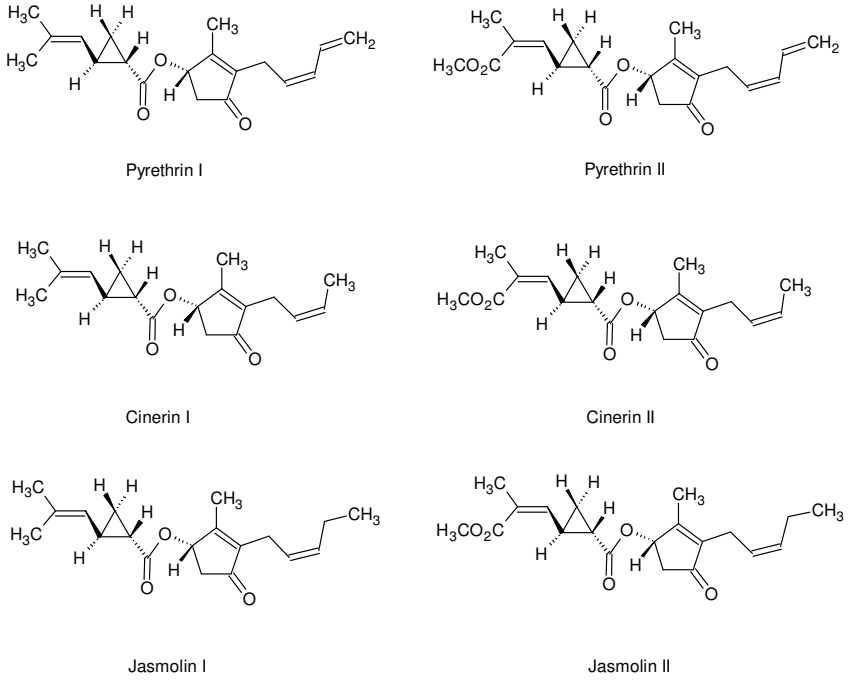

Figure 1. Chemical structure of individual pyrethrins.

Linearity of acetonitril standard solutions and detection limits

For all assays, the relationship between the signal (peak area normalised by the internal standard) and the concentration was linear, and the regression coefficient was higher than 0.999. Detection limits were estimated by the analysis of standard solutions and real samples. The obtained figures corresponded to the concentration at which the signal-to-noise ratio became 3. The estimated detection limits for pyrethrins I ranged from 0.025 to 0.028 mg/L and for pyrethrins II from 0.034 to 0.038 mg/L.

Repeatability of results

Table 4 shows the relative standard deviation (RSD) for all treatments. Comparing the extraction techniques, soxtec extraction showed lower repeatability than ultrasound extraction, probably because of the low content of the fatty material, and for only one gram of dried flowers taken into extraction. For the soxtec system used, the more sample taken into extraction, the more the reliabliability of the results (Table 4).

Extraction recovery

Both techniques of extraction (ultrasound and soxtec) and the elution of obtained crude extract with acetonitrile were repeated several times to achieve exhaustive extraction of the plant material. According to HPLC analysis, the second soxtec and the forth and fifth ultrasound extraction did not yield pyrethrins. The first acetonitrile elute contained more than 99% of pyrethrins

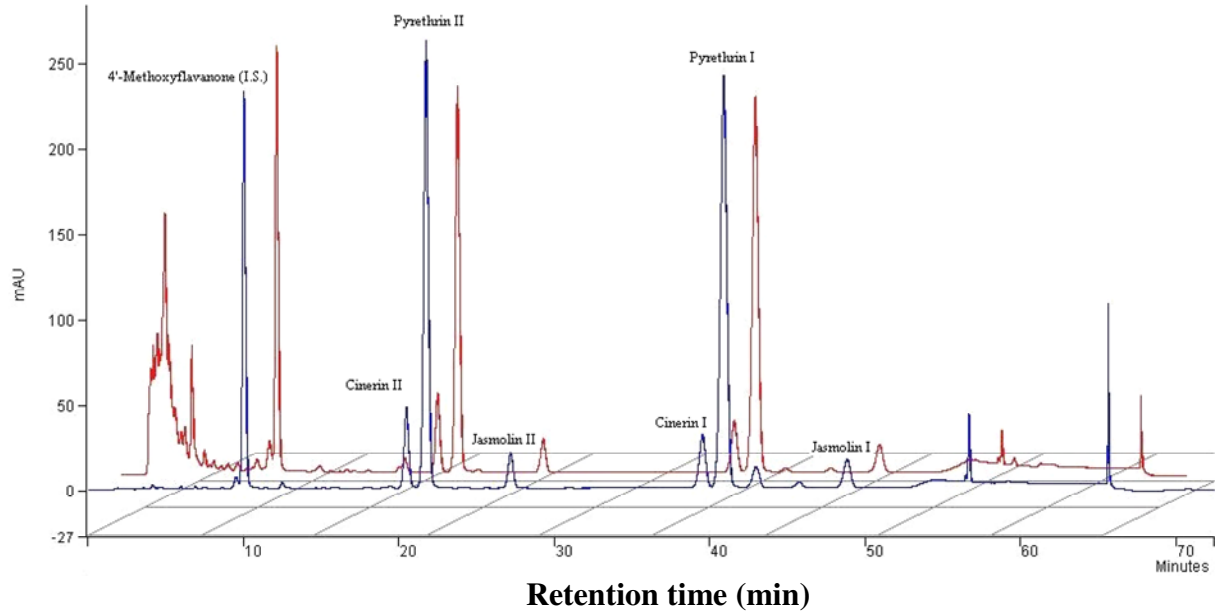

Figure 2. Reversed-phase HPLC chromatogram of standard (blue line) and sample (red line) of pyrethrum extract. (Mobile phase flow rate 1 mL min^{-1}, injection volume 20 µL, UV detector at 230 nm)

Table 4. Relative standard deviation (RSD) for each pyrethrin compounds obtained with ultrasound or soxtec extraction using ethanol, petroleum ether or hexane as a solvent.

Pyrethrin compounds	RSD					
	Ultrasound			Soxtec		
	Ethanol	Petroleum ether	Hexane	Ethanol	Petroleum ether	Hexane
Pyrethrin I	0.78	11.30	7.57	51.64	15.55	13.72
Cinerin I	3.40	10.59	5.54	42.50	12.25	2.85
Jasmoline I	1.54	9.21	1.49	9.68	8.61	11.95
Pyrethrin II	1.14	10.59	5.17	21.21	17.50	16.20
Cinerin II	0.81	9.64	5.37	10.49	12.37	2.74
Jasmoline II	1.69	10.58	5.12	6.25	12.58	3.53

and the second one contained the rest, whereas the third, forth and fifth elution did not contain pyrethrins. Therefore, the results for each pyrethrin were calculated summing the peak areas obtained, analysing the first and second elution.

Methanol was included at the beginning of the study but soon rejected because of low extraction efficiency even though it was reported to be the most effective among tested solvents: ethanol, methanol, propanol and acetonitrile (EU project, 2002). Moreover, methanol is very toxic and as such not suitable for commercial extraction.

The influence of the extraction technique and the solvent on the extraction efficiency

The extraction efficiency for these treatments on the amounts of pyrethrins I and II, and total pyrethrins was observed since their content depends on the amount of predominant singular compounds of pyrethrin 1 and pyrethrin 2 (Table 5), the ones being the most active too (Casida and Quistad, 1995; EU project, 2002). Guided by that fact, and in order to clarify the differences between treatments results in Table 6 that shows the percent of TP extracted with each treatment were summarized. For the pyrethrins (p1 and p2), as well as for PI, PII and TP, the ultrasound-ethanol treatment showed the best extraction efficiency. Next treatments, in decreasing order of efficiency that also showed good results are the soxtec-petroleum ether, ultrasound-petroleum ether, and ultrasound-hexane, but soxtec-petroleum ether showed the worse repeatability. All three ultrasound treatments and the soxtec-petroleum ether treatment did not show significant difference in extracting pyrethrins ($P > 0.01$). Ethanol as a solvent was less hazardous than the other

Table 5. Treatments with related order based on extraction efficiency of pyrethrin compounds (p1, c1, j1, p2, c2, j2), the groups of pyrethrins I (PI) and II (PII), and for total pyrethrins (TP).

Pyrethrin compounds	Ultrasound			Soxtec		
	Ethanol	Hexane	Petroleum ether	Ethanol	Hexane	Petroleum ether
TP	1	4	3	6	5	2
PI	1	4	3	6	5	2
PII	1	4	3	6	5	2
p1	1	3	2	5	4	2
c1	3	4	5	6	1	2.
j1	4	4	2	5	3	1
p2	1	4	2	6	5	3
c2	3	4	4	5	1	2
j2	3	4	5	4	1	2

Table 6. Percentage of total pyrethrins extracted by two techniques and tree solvents.

Technique	Solvent	Total pyrethrins* (%, flower dry weight)
Ultrasound	Ethanol	1.207 a
	Hexane	0.967 b
	Petroleum ether	1.020 ab
Soxtec	Ethanol	0.530 c
	Hexane	0.743 c
	Petroleum ether	1.100 ab

*The results are expressed as a mean of three measurements. Means within a column followed by the same letter are not significantly different at P ≥ 0.01 by LSD test.

Table 7. Content of total extracted pyrethrins for three populations of *Chrysanthemum cineraraefolium* originating from Dalmatia, Croatia.

Population	Total pyrethrins (g/100 g dried flowers)	RSD (%)
I	1.25 ab*	5.32
II	1.16 b	1.08
III	1.30 a	1.99

*Means within a column followed by the same letter are not significantly different at P ≥ 0.01 by LSD test.

compared solvents (OJEC, 2001), has lower cost than hexane, the most frequently used in laboratories and referred as the most effective (Casida and Quistad, 1995; Kasaj et al., 1999; EU project, 2002). These assumptions make it a preferable solvent for potential commercial use. The treatments soxtec-hexane, and soxtec-ethanol gave the worse results, without significant difference between them (P > 0.01).

It is clear that the extraction efficiency does not depend on the use of a particular solvent, nor a particular method of extraction, but it depends on the use of a proper solvent for a particular method, for example, ethanol used with ultrasound gave the best results, while used with soxtec, the worse, or the most suitable solvent for ultrasound is ethanol, and for soxtec is petroleum ether (Table 5). We cannot say that the polarity of the solvents affects the extraction efficiency, probably due to bipolar character of pyrethrin compounds. Further investigations should be carried out on the combination of solvents. Even though it has been reported that the degradation of pyrethrins starts from 40°C (Della Porta and Reverchon, 2002), the soxtec treatment soxtec-petroleum ether, gave as good results as the best treatment ultrasound-ethanol. The conditions were 135°C/80 min. Among all other soxtec treatments, this was the mildest.

Considering the results so far, and after the method was validated, the ultrasound-ethanol treatment which showed the best efficiency and repeatability, as well as the lowest cost and toxicity, was chosen for isolation of active ingredients in three *Chrysanthemum* populations (Table 7). The average pyrethrins content in wild populations collected from Dalmatia (Croatia) and planted in Kenya was 0.89% ranging from 0.75 to 1.04% (Casida and Quistad, 1995). The present results with values higher than these, confirm that the Croatian costal area is very suitable for pyrethrum growing, not surprising since this plant is native to Dalmatia (Croatia). In further researches, population III should be included due to significantly (P ≥ 0.01) highest amount of total pyrethrins. New investigations embracing the production technology development, clonal selection and pyrethrum product development are in course. Selection and introduction of commercial clones in local plantings would make pyrethrum crops grow on economically profitable agricultural activity.

Conclusion

A method that is efficient, reliable and simple, with low

toxicity and cost for routine analyses of pyrethrins was described. Two extraction techniques (ultrasound and soxtec) were tested with no difference in efficiency. However, soxtec was less reliable, even though it requires less sample manipulation. To our knowledge this is the first report on using soxtec for pyrethrin extraction.

For the first time, the results demostrated that ethanol could be more effective in extraction of pyrethrins than hexane or methanol. Considering its lower cost and toxicity, it is being recommended as the optimal solvent for laboratory and industrial scale purposes. The content of total pyrethrins in three different natural populations of *C. cinerariaefolium* grown in Croatia demonstrates that Croatian coast is a very suitable place for growing and commercialising of this culture.

REFERENCES

Casida JE, Quistad GB (1995). Pyrethrum flowers Production, Chemistry, Toxicology, and Uses, Oxford University Press, New York. 1-350.

Della Porta G, Reverchon E (2002). Supercritical Fluids Extraction and Fractionation of Pyrethrins from Pyrethrum-s. In: Symposium Proceedings of 4th International Symposium on high pressure process technology and chemical engineering, Venice, Italy.

Essig K, Zhao ZJ (2001a). Preparation and characterisation of a Pyrethrum extract standard. LC/GC. 19. 7: 722-730.

Essig K, Zhao Z (2001b). Method development and validation of a high-performance liquid chromatographic method for pyrethrum extract. J. Chromatogr. Sci. 39(4): 473-480; 488.

EU project (FAIR Programme, Agriculture and Fisheries) No 1436 "Improvement of efficiency and reduction of application rates of preferable naturally grown biocides by complexing with gamma-cyclodextrin", 1999-2002, Available: http://www.biomatnet.org/secure/Fair/S486.htm.

Glynne-Jones A (2001). Pyrethrum, Pesticide Outlook-Biopesticides, 195-198.

Henry III CW, McCaroll EM, Warner IM (2001). Separation of the insecticidal pyrethrin esters by capillary electrochromatography. J. Chromatogr. A. 905: 319-327.

Henry III CW, Shamsi SA, Warner IM (1999). Separation of natural pyrethrum extracts using micellar electrokinetic chromatography. J. Chromatogr. A. 863: 89-103.

Kasaj D, Rieder A, Krenn L, Kopp B (1999). Separation and Quantitative analysis of Natural Pyrethrins by High Performance Liquid Chromatography. Chromatographia. 50. 9/10: 607-610.

Kolak I, Šatović Z, Rukavina H, Filipaj B (1999). Dalmatinski buhač. Sjemenarstvo, 16, 5: 425-440 (in Croatian with English abstract).

Morris SE, Davies NW, Brown PH, Groom T (2006). Effect of drying conditions on pyrethrins content, Ind. Crop. Prod. 23(1): 9-14.

Nguyen KT, Moorman R, Kuykendall V (1998). Determination of *N*-Octyl Bycicloheptene Dicarboximide, Pyrethrins, and Butylcarbityl 6-Propylpiperonyl Ether in Technical Materials, Concentrates, and Finished Products by Capillary Gas Chromatography: Colaborative Study. J. AOAC Int. 81. 3: 503-512.

OJEC (Official Journal of the European Communities), Commission directive 2001/59/EC of 6 August 2001.

Otterbach A, Wenclawiak BW (1999). Ultrasonic/Soxhlet/supercritical fluid extraction kinetics of pyrethrins from flowers and allethrin from paper strips. Fresenius J. Anal. Chem. 365(5): 472-474.

Pan WHT, Chang C, Su T, Lee F, Fuh MS (1995). Preparative supercritical Fluid Extraction of pyrethrin I and II from Pyrethrum Flower. Talanta 42: 1745-1749.

Pol J, Wenclawiak BW (2003). Direct On-Line Continuous Supercritical Fluid Extraction and HPLC of Aqueous Pyrethrins Solutions. Anal. Chem. 75: 1430-1435.

Reverchon E, De Marco I (2006). Supercritical fluid extraction and fractionation of natural matter. The Journal of Supercritical Fluids. 38/2: 146-166.

US Environmental Protection Agency Office of Pesticide Programs (2009). A review of the Relationship between Pyrethrins, Pyrethroid Exposure and Asthma and Allergies, September 2009, Available: http://www.epa.gov/oppsrrd1/reevaluation/pyrethrins-pyrethroids-asthma-allergy-9-18-09.pdf.

Wang IH, Subramanian V, Moorman R, Burleson J, Ko J (1997). Direct determination of pyrethrins in pyrethrum extracts by reversed-phase high-performance liquid chromatography with diode-array detection. J. Chromatogr. A. 766: 277-281.

Wynn HTP, Cheng-Chin Chang, Tien-Tsu Su, Fong Lee and Ming-Ren Steve F (1995). Preparative supercritical fluid extraction of pyrethrin I and II from pyrethrum flower. Talanta. 42(11): 1745-1749.