Parameters affecting microwave-assisted extraction of withanolides. Phytochem Anal

ARTICLE *in* PHYTOCHEMICAL ANALYSIS · SEPTEMBER 2001

Impact Factor: 2.34 · DOI: 10.1002/pca.599 · Source: PubMed

3 AUTHORS:

Béatrice Kaufmann
University of Geneva
11 PUBLICATIONS **329** CITATIONS
SEE PROFILE

Philippe Christen
University of Geneva
141 PUBLICATIONS **2,285** CITATIONS
SEE PROFILE

Jean-Luc Veuthey
University of Geneva
326 PUBLICATIONS **8,938** CITATIONS
SEE PROFILE

PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **12**, 327–331 (2001)
DOI: 10.1002/pca.599

Parameters Affecting Microwave-assisted Extraction of Withanolides

Béatrice Kaufmann, Philippe Christen* and Jean-Luc Veuthey
University of Geneva, Laboratory of Pharmaceutical Analytical Chemistry, 20 bd d'Yvoy, CH-1211 Geneva 4, Switzerland

Focused microwave-assisted extraction was applied to the extraction of three main withanolides from air-dried leaves of *Iochroma gesnerioides*, namely, withaferin A, iochromolide and withacnistin. Six extraction variables, i.e. nature and volume of extracting solvent, sample moisture, extraction time, power of irradiation and particle size, were investigated with respect to the recovery of withanolides. The most favourable conditions were obtained by using powdered plant material (<220 μm), previously impregnated with water for 15 min, and extracted with methanol for 40 s at 25 W. The results obtained using the optimised method were compared to those achievable with Soxhlet extraction. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: Microwave-assisted extraction; Soxhlet extraction; comparison of extraction methods; steroids; withanolides.

INTRODUCTION

Withanolides are steroidal lactones derived from an ergostane-type skeleton occurring almost exclusively in the Solanaceae family. A characteristic feature of this skeleton is the α,β-unsaturated δ-lactone ring formed in the side chain. These compounds are mainly localised in leaves, and their concentration usually ranges from 0.001 to 0.5% dry weight (Tursunova *et al*., 1977; Christen, 1986; Alfonso *et al*., 1993; Alfonso and Kapetanidis, 1994). Withanolides possess various pharmacological properties (Christen, 1986, 1989; Alfonso *et al*., 1993; Alfonso and Kapetanidis, 1994; Fügner, 1997; Habtemariam, 1997) including anti-bacterial and virostatic activity, and they also act as immunomodulators and ecdysteroid antagonists (Dinan *et al*., 1996).

Liquid–solid extraction with methanol is the conventional method used to recover withanolides from plant material (Tschesche *et al*., 1966; Kirson *et al*., 1970, 1972); however, this procedure needs several purification steps, is time consuming, and requires a large volume of solvent. With the increasing demand for more environmentally friendly methods, new techniques, such as supercritical fluid extraction (King and Bott, 1995), accelerated or pressurised solvent extraction (Richter *et al*., 1996) or microwave-assisted extraction (MAE; Jassie *et al*., 1997) have been developed recently.

With the wide availability of appropriate microwave ovens, this type of heating system has been introduced into many analytical laboratories (Neas and Collins, 1988). Use of microwave heating for organic extraction was first reported by Ganzler *et al*. in 1986. Since then, numerous laboratories have investigated the analytical possibilities of this new extraction technique (Sinquin *et al*., 1993). Disruption of hydrogen bonds, resulting from dipole rotation of molecules, and migration of dissolved ions facilitate the penetration of solvent molecules into the matrix and allow the solvation of components to be extracted (Ganzler *et al*., 1990). MAE seems particularly promising for the extraction of compounds with medium to high polarity, in solid matrices.

This paper describes the development of a rapid, reliable and sensitive method of extraction, using a focused microwave oven, of three withanolides from *Iochroma gesnerioides* leaves. The method is compared with Soxhlet extraction, and various experimental factors have been investigated with respect to their effect on extraction efficiency.

* Correspondence to: P. Christen, University of Geneva, Laboratory of Pharmaceutical Analytical Chemistry, 20 bd d'Yvoy, CH-1211 Geneva 4, Switzerland.
Email: philippe.christen@pharm.unige.ch

EXPERIMENTAL

Materials and reagents. Seeds of *Iochroma gesnerioides* (Kunth) Miers (Syn. *I. coccineum* Scheidweiler; Solanaceae), originating from South America, were provided by the Jardim Botanico da Universidade de Lisboa (Portugal). Plants were grown at the Station Fédérale de Recherches Agronomiques (Centre des Fougères, Conthey, Wallis, Switzerland), and a voucher specimen is deposited in our laboratory. Air-dried leaves were thoroughly ground in a domestic mixer and sieved. Two particle sizes were selected: particles less than 220 μm and particles between 1500 and 2000 μm.

All chemicals were of analytical grade and were purchased from Amman Tech (Kölliken, Switzerland). Ultra-pure water was provided by a Milli-Q RG unit (Millipore, Bedford, MA, USA). Withaferin A (**1**), iochromolide (**2**) and withacnistin (**3**) were isolated from *I. gesnerioides* by Alfonso *et al*. (1991).

Soxhlet extraction. A sample of 1g of powdered plant material (<220 μm) was placed into an extraction thimble (80 × 22 mm; Schleicher and Schüll, Dassel, Germany), and impregnated with 6 mL water. After

Received 2 November 2000
Revised 13 February 2001
Accepted 20 February 2001

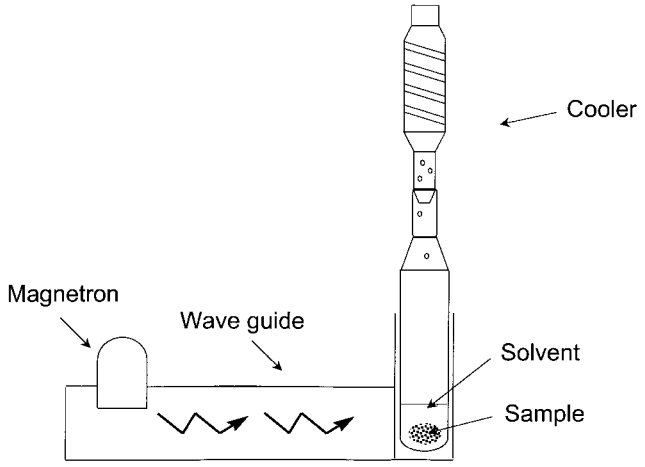
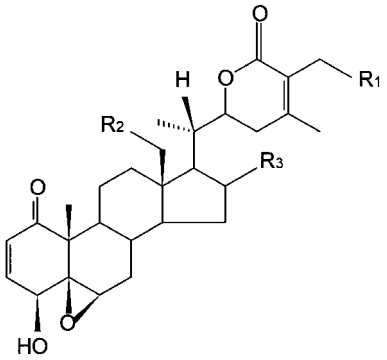


Figure 1. Diagram of a focused microwave system.

15 min, Soxhlet extraction was performed for 6 h with 100 mL methanol. The solvent was then evaporated, the residue dissolved in 20.0 mL methanol and 1 mL of this solution was centrifuged at 8000 × *g* for 10 min at 20°C (Biofuge model 17RS, Heraeus Sepatech, Osterode, Germany). An aliquot (300 µL) of the supernatant was analysed by HPLC-UV using an external calibration curve.

Microwave-assisted extraction. A sample (100 mg) of dried, powdered plant material was placed into the cell of a focused MAE apparatus (Soxwave, Prolabo, Fontenay-Sous-Bois, France), working at atmospheric pressure (Fig. 1), with the desired amount of solvent (5–30 mL). A condenser was placed above the extraction cell in order to avoid solvent evaporation, and power (25–250 W) was applied for different time periods (40 s to 10 min). After extraction, each sample was transferred to a conical glass tube and centrifuged for 5 min at 8000 × *g*. The residue was rinsed twice with 1.5 mL of the corresponding solvent and centrifuged. The organic phase was collected and evaporated to dryness under a nitrogen flow at 40°C. The residue was redissolved in 500 µL methanol and centrifuged at 8000 × *g* for 10 min at 20°C. An aliquot (300 µL) of the supernatant was analysed by HPLC-UV using an external calibration curve.

HPLC analysis. The chromatographic system consisted of a Waters (Milford, MA, USA) model 600 E Multi-solvent delivery system equipped with a Waters 700 Satellite Wisp auto-injector coupled with a Waters 484 variable wavelength UV detector. A Nucleosil (Macherey-Nagel, Oensingen, Switzerland) 100-5 C_8 column (125 × 4 mm i.d.) was used. A linear binary gradient was applied, using 5% acetonitrile in water (solvent A) and 5% water in acetonitrile (solvent B). The elution profile was: 0–5 min, isocratic mode with A:B 70:30; 5–15 min, linear gradient from A:B 70:30 to 65:35; followed by isocratic elution with A:B 65:35 for 15 min. HPLC analyses were performed at room temperature; the injection volume was 20 µL and the flow rate was set to 1 mL/min. UV absorbance was monitored at $\lambda = 215$ nm.



1. R_1 = OH, R_2=R_3 = H
2. R_1=R_3 = H, R_2 = OAc
3. R_1=R_2 = H, R_3 = OAc

RESULTS AND DISCUSSION

Soxhlet extraction

Although pure methanol is usually used for withanolide extraction, it has been reported that addition of water can benefit the extraction of this class of compounds (Alfonso *et al*., 1991). Therefore, samples were thoroughly moistened for 15 min with 6 mL of water prior to Soxhlet extraction with 100 mL methanol for 6 h. This procedure allowed the recovery of 0.41% withaferin A, 0.81% iochromolide and 0.38% withacnistin (by dry weight). Increasing the extraction time to 24 h did not improve the recovery of withanolide. These values will be considered as reference values in the following discussion.

Table 1. Effect of solvent on the recovery of withanolides from leaves of *Iochroma gesnerioides*: comparison with Soxhlet extraction

		Withanolide recovered (% dry weight)[a]		
Extraction method	Solvent	Withaferin A	Iochromolide	Withacnistin
Soxhlet	Methanol:water	0.41 ± 0.04	0.81 ± 0.06	0.38 ± 0.03
MAE	Water	0.33 ± 0.08	0.20 ± 0.13	0.10 ± 0.10
	Methanol	0.12 ± 0.10	0.34 ± 0.07	0.12 ± 0.10
	Methanol:water	0.48 ± 0.04	0.85 ± 0.05	0.39 ± 0.01
	Ethanol	0.11 ± 0.11	0.34 ± 0.06	0.14 ± 0.09
	Dichloromethane	0.09 ± 0.07	0.27 ± 0.05	0.09 ± 0.10
	Dichloromethane:water	0.25 ± 0.10	0.43 ± 0.07	0.24 ± 0.07
	Hexane	nd[b]	nd	nd

[a] Mean value $\pm t * \sigma/\sqrt{n}$.
[b] nd, not detected.

Table 2. Influence of water impregnation time on recovery of withanolides from leaves of *Iochroma gesnerioides*[a]

	Withanolide recovered (% dry weight)[b] Impregnation time		
	0 min	15 min	60 min
Withaferin A	$0.18 \pm 0.07^{*}$	0.38 ± 0.05	0.43 ± 0.07
Iochromolide	0.34 ± 0.10	0.79 ± 0.05	0.81 ± 0.05
Withacnistin	0.20 ± 0.05	0.38 ± 0.07	0.40 ± 0.05

[a] MAE conditions: sample impregnated with 600 μL of water for time indicated and extracted with 15 mL of methanol at 250 W for 40 s.
[b] Mean value $\pm\ t^{*}\sigma/\sqrt{n}$.

Table 4. Influence of extraction time on recovery of withanolides from leaves of *Iochroma gesnerioides*[a]

	Withanolide recovered (% dry weight)[b] Extraction time	
	40 s	10 min
Withaferin A	0.38 ± 0.09	0.42 ± 0.04
Iochromolide	0.79 ± 0.06	0.75 ± 0.05
Withacnistin	0.38 ± 0.13	0.41 ± 0.07

[a] MAE conditions: sample impregnated with 600 μL of water for 15 min and extracted with 5 mL of methanol at 250 W for time indicated.
[b] Mean value $\pm\ t^{*}\sigma/\sqrt{n}$.

Microwave-assisted extraction

The effect on the efficiency of extraction resulting from the alteration of various parameters, including the nature and volume of the extracting solvent, sample moisture, extraction time, power of irradiation and particle size, was studied.

Nature of the extracting solvent. Solvents of different polarity were tested, ranging from hexane to water (Table 1). The power of the apparatus was set at 75 W (30% of maximal value). The significant formation of foam/emulsion with pure water precluded the use of this solvent for the exhaustive extraction of withanolides. Extraction using pure methanol and ethanol gave low efficiencies of extraction of withanolides. In all the tested conditions (data not shown), recovery did not exceed 40% of that obtained with Soxhlet, which suggests that pure alcoholic solvents are not able to overcome interactions between analytes and matrix since the solubility of withanolides in these solvents is sufficient (greater than 1 mg/mL). Dichloromethane, which possesses a lower dielectric constant than methanol and ethanol, gave similar recoveries (Student *t*-test). Hexane was not suitable as a solvent as none of the investigated withanolides could be extracted.

Sample moisture. It has been reported that preliminary impregnation of matrices with water is beneficial for high recovery microwave-assisted extraction (Paré, 1990; Onuska and Terry, 1993; Paré *et al*., 1994; Majors, 1998). Therefore, an attempt was made to impregnate the plant material with water prior to extraction. Water (600 μL) was added to the sample (100 mg) followed by extraction with 15 mL methanol or dichloromethane for 15 min (Table 1). In both cases, recovery was significantly improved compared to that obtained using dry plant material under identical extraction conditions. This is probably because water absorbs microwave energy and promotes cell disruption by internal superheating, facilitating desorption of analytes from the matrix (Jassie *et al*., 1997). The phenomenon was particularly important when the moisturised sample was extracted with methanol, and under these conditions a recovery similar to that from the Soxhlet extraction was obtained. In the light of these preliminary experiments, methanol, with water as moisturising agent, was used for further investigations.

Impregnation time. The influence of water impregnation time on the recovery of withanolides was studied. Water (600 μL) was added immediately, 15 or 60 min prior to methanol extraction (15 mL; 250 W; 40 s). As shown in Table 2, impregnation for 15 min is necessary and sufficient for a complete extraction: a longer impregnation time did not significantly improve the extraction efficiency. These results suggest that, because it is the solvent which absorbs the most microwave energy, the presence of water has a beneficial effect and allows faster extractions than with organic solvent alone.

Volume of extracting solvent. When the volume of methanol used for the extraction was varied from 5 to

Table 3. Influence of water volume on recovery of withanolides from leaves of *Iochroma gesnerioides*[a]

	Withanolide recovered Impregnation volume			
	200 μL water		600 μL water	
	Percentage dry weight	RSD (%)	Percentage dry weight	RSD (%)
Withaferin A	0.36 ± 0.08[b]	8.8	$0.48 \pm .04$	3.6
Iochromolide	0.75 ± 0.15	7.8	0.85 ± 0.05	2.4
Withacnistin	0.34 ± 0.08	9.1	0.39 ± 0.004	0.4

[a] MAE conditions: sample impregnated with water as indicated for 15 min and extracted with 5 mL of methanol at 25 W for 40 s.
[b] Mean value $\pm\ t^{*}\sigma/\sqrt{n}$.

Table 5. Influence of irradiation power on recovery of withanolides from leaves of *Iochroma gesnerioides*[a]

	Withanolide recovered (% dry weight)[b] Irradiation power	
	25 W	250 W
Withaferin A	0.40 ± 0.02	0.38 ± 0.09
Iochromolide	0.74 ± 0.17	0.79 ± 0.06
Withacnistin	0.38 ± 0.10	0.38 ± 0.13

[a] MAE conditions: sample impregnated with 600 μL of water for 15 min and extracted with 5 mL of methanol at power indicated for 40 s.
[b] Mean value $\pm t * \sigma/\sqrt{n}$.

Table 6. Influence of particle size on recovery of withanolides from leaves of *Iochroma gesnerioides*[a]

	Withanolide recovered (% dry weight)[b] Particle size	
	<220 μm	1500–2000 μm
Withaferin A	0.48 ± 0.04	0.33 ± 0.02
Iochromolide	0.85 ± 0.05	0.74 ± 0.05
Withacnistin	0.39 ± 0.01	0.30 ± 0.01

[a] MAE conditions: sample impregnated with 600 μL of water for 15 min and extracted with 5 mL of methanol at 25 W for 40 s.
[b] Mean value $\pm t * \sigma/\sqrt{n}$.

30 mL, no influence on recovery could be observed confirming that solubility is not a limiting factor in the investigated interval. Therefore, in all further analyses, 5 mL methanol was used.

Volume of water. The effect of altering the volume of water used as moisturising agent was also investigated. For practical reasons, the interval range was 200–600 μL of water added to the dry sample (100 mg) prior to extraction. Recovery was slightly improved when 600 μL water was added (Table 3), but the major effect was a better repeatability with 600 μL water, probably due to a more complete sample moisturising, leading to a more homogeneous irradiation.

Extraction time. Similar experiments were carried out, applying microwave heating for 40 s or 10 min. Table 4 shows that a period of 40s is sufficient to bring about the quantitative extraction of withanolides. These results confirm that microwave energy excitation of a wet matrix is a very fast process.

Power of irradiation. Chen and Spiro (1994) demonstrated that an increase of irradiation power can lead to a better extraction efficiency. Thus, extractions were performed at 25 and 250 W, corresponding to the minimal and maximal power values of the apparatus employed. As shown in Table 5, the power of irradiation had no influence on withanolide recovery and a power of 25 W was sufficient to extract quantitatively the investigated analytes.

Particle size. Letellier and Budzinski demonstrated the influence of sediment particle size on the efficiency of microwave extraction of polycyclic aromatic hydrocarbons (Letellier and Budzinski, 1999). In the present study, two different particle sizes were selected, namely, particles less than 220 μm (fine fraction) and particles between 1500 and 2000 μm (coarse fraction). Each fraction (100 mg) was submitted to MAE as described above (600 μL water; 5 mL methanol; 25 W; 40 s). As shown in Table 6, fine samples gave a significantly higher withanolide recovery, indicating that the limiting step of the extraction is mainly the diffusion of analytes out of the matrix.

Chemical stability of withanolides under the applied conditions. In order to assess the stability of withanolides under the extraction conditions described above, a standard solution of the three selected compounds was exposed to the optimised MAE treatment outlined above: no degradation occurred and recoveries ranged from 97 to 102%.

The present study shows the main advantages of MAE over Soxhlet extraction. They are mainly associated with the drastic reduction in organic solvent consumption (5 vs 100 mL) and extraction time (40 s vs 6 h) as shown in Table 7. Moreover, MAE gave similar or even better precision data.

Table 7. General comparison between Soxhlet and MAE for the extraction of withanolides from leaves of *Iochroma gesnerioides*

	Soxhlet extraction	MAE
Sample weight	1 g	100 mg
Extraction time	6 h	40 s
Total handling time	9 h	40 min
Solvent volume	100 mL methanol + 6 mL water	5 mL methanol + 0.6 mL water
Precision (RSD %)[a]		
Withaferin A	4.1	3.6
Iochromolide	3.1	2.4
Withacnistin	3.6	0.4

[a] $n = 3$.

REFERENCES

Alfonso D, Kapetanidis I. 1994. Withanolides from *Iochroma gesnerioides*. *Phytochemistry* **36**: 179–183.

Alfonso D, Kapetanidis I, Bernardinelli G. 1991. Iochromolide: a new acetylated withanolide from *Iochroma coccineum*. *J Nat Prod* **54**: 1576–1582.

Alfonso D, Bernardinelli G, Kapetanidis I. 1993. Withanolides from *Iochroma coccineum*. *Phytochemistry* **34**: 517–521.

Chen SS, Spiro M. 1994. Study of microwave extraction of essential oil constituents from plant materials. *J Microwave Electromagn Energy* **29**: 231–241.

Christen P. 1986. Les withanolides, stéroïdes végétaux à structure originale. *Pharm Acta Helv* **61**: 242–246.

Christen P. 1989. Withanolide: Naturstoffe mit vielversprechendem Wirkungsspektrum. *Pharm Zeit* **5**: 129–139.

Dinan L, Whiting P, Alfonso D, Kapetanidis I. 1996. Certain withanolides from *Iochroma gesnerioides* antagonise ecdysteroid action in a *Drosophila melanogaster* cell line. *Entomol Exp Appl* **80**: 415–420.

Fügner A. 1997. Hemmung immunologisch bedingter Entzündungen durch das Pflanzensteroid Withaferin A. *Arzneim-forsch* **23**: 932.

Ganzler K, Salgò A, Valkó K. 1986. Microwave extraction. A novel sample preparation method for chromatography. *J Chromatogr* **371**: 299–306.

Ganzler K, Szinai I, Salgò A. 1990. Effective sample preparation method for extracting biologically active compounds from different matrices by a microwave technique. *J Chromatogr* **520**: 257–262.

Habtemariam S. 1997. Cytotoxicity and immunosuppressive activity of withanolides from *Discopodium penninervium*. *Planta Med* **63**: 15–17.

Jassie L, Revesz R, Kierstead T, Hasty E, Matz S. 1997. Microwave-assisted Solvent extraction. In *Microwave-Enhanced Chemistry. Fundamentals, Sample Preparation, and Applications*, Kingston HMS, Haswell SJ (eds). American Chemical Society: Washington, DC; 569–609.

King MB, Bott TR. 1995. *Extraction of Natural Products using Near-critical Solvents*. Blackie: Glasgow.

Kirson I, Glotter E, Abraham A, Lavie D. 1970. Constituents of *Withania somnifera* Dun.—XI. The structure of three new withanolides. *Tetrahedron* **26**: 2209–2219.

Kirson I, Lavie D, Subramanian SS, Sethi PD, Glotter E. 1972. Withanicandrin, a ring-c substituted withanolide from *Nicandra physaloides* (Solanaceae). *J Chem Soc Perkin Trans 1* **P1**: 2109–2111.

Letellier M, Budzinski H. 1999. Influence of sediment grain size on the efficiency of focused microwave extraction of polycyclic aromatic hydrocarbons. *Analyst* **124**: 5–14.

Majors RE. 1998. Sample preparation perspectives. New approaches to sample preparation. *LC-CG Int* **8**: 128–133.

Neas ED, Collins MJ. 1988. *Microwave Heating. Theoretical Aspects and Equipment Design*. American Chemical Society: Washington, DC.

Onuska FI, Terry KA. 1993. Extraction of pesticides from sediments using a microwave technique. *Chromatographia* **36**: 191–194.

Paré JRJ. 1990. Microwave extraction of volatile oils and apparatus therefor. Patent no. 90250286.3 (0 485 668 A1).

Paré JRJ, Bélanger JMR, Stafford SS. 1994. Microwave-assisted process (MAP): a new tool for the analytical laboratory. *TRAC* **13**: 176–184.

Richter BE, Jones BA, Ezzel JL, Porter NL, Avdalovic N, Pohl C. 1996. Accelerated solvent extraction: a technique for sample preparation. *Anal Chem* **68**: 1033–1039.

Sinquin A, Görner T, Dellacherie E. 1993. L'utilisation des micro-ondes en chimie analytique. *Analusis* **21**: 1–10.

Tschesche R, Schwang H, Fehlhaber HW, Snatzke G. 1966. Inhaltstoffe aus *Jaborosa integrifolia* Lam.— II. Strukturermittlung der Jaborosalactone A und B. *Tetrahedron* **22**: 1129–1138.

Tursunova RN, Maslennikova VA, Abubakirov NK. 1977. Withanolides in the vegetable kingdom. *Chem Nat Compd (English Trans)* **13**: 131–138.

