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Extraction of iridoid glycosides and their determination by micellar electrokinetic capillary chromatography

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Abstract

Several methods for the extraction of two iridoid glycosides, catalpol and aucubin, from the plant matrix (*Veronica longifolia* leaves) were compared. Pressurized hot water extraction and hot water extraction were the most efficient isolation techniques for both. Pressurized liquid extraction and maceration with various organic solvents were also tested. Relative to the amounts extracted with hot water, ethanol extracted only 22% of catalpol and 25% of aucubin and pressurized hot water extracted 83% of catalpol and 92% of aucubin. The lowest relative standard deviations, 22% for catalpol and 8% for aucubin, were achieved with hot water extraction (13 repetitions), and the highest relative standard deviations, 76% for catalpol and 73% for aucubin, with pressurized liquid extraction (five repetitions). A fast capillary electrophoretic method was developed for the quantitative determination of catalpol and aucubin.

Keywords: Pressurized liquid extraction; Pressurized hot water extraction; Hot water extraction; Extraction methods; *Veronica longifolia*; Plant materials; Iridoid glycosides; Glycosides; Catalpol; Aucubin; Monoterpenes; Terpenes

1. Introduction

Iridoids are cyclopentanoid monoterpene derivatives that fall into four distinct groups: iridoid glycosides, aglycone or non glycosidic iridoids, secoiridoids and bisiridoids. Recently, they have been shown to possess therapeutic effects [1,2]. Because of their bitter taste, iridoids are used by some plants and insects as defensive compounds [3,4].

A typical feature of iridoids is partly hydrogenated *cis* fused cyclopenta-[*c*]pyran system, which arises from the intramolecular acetalization of a 1,5-cyclopentandialdehyde moiety and usually is stabilized by acetalization or esterification [5]. Since iridoids are hydrolysed and possibly rearranged under even mildly acidic conditions, [6] all analyses must be performed in basic buffer solutions. Basic conditions are an advantage in capillary electrophoresis because the electro-osmotic flow increases and thus the migration times of the analytes as well as the total analysis time decrease.

The most common method for separating iridoids from the sample matrix is extraction. Methanol and ethanol are the usual extraction solvents [7–10]. Both dissolve iridoids efficiently and both are cheap and easily available. The last few years have seen the development of new extraction methods, such as pressurized liquid extraction (PLE, also known under

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the Dionex trade name Accerelated Solvent Extraction), supercritical fluid extraction and pressurized hot water extraction (PHWE), which are more environmentally friendly. Not only is much less solvent needed in these new methods but they are also more efficient.

The need to avoid burdening the environment with solvents has encouraged interest in water as a medium for extraction. Supercritical water is too corrosive to be a good extraction fluid, but water at high pressure and temperature promises to find good use in analytical chemistry. The dielectric constant, surface tension and viscosity of water change as a function of temperature, so that with a mere adjustment of the extraction conditions water can be made to resemble different organic solvents. In addition, enhanced vapour pressures, thermal desorption and diffusion rates of compounds will be obtained by increasing the water temperature.

PHWE has previously been used for extracting polychlorinated biphenyls [11], soil organic matter [12], organics from hydrocarbon wastes [13] and other aromatic organic compounds [14]. Our earlier confirmation that PHWE can also be used for extracting compounds from plant material [15] led us to study the suitability of the method for the current work. The preliminary results indicate that PHWE is indeed suitable for the extraction of iridoid glycosides [16]. Hot water has not been used earlier for the extraction of iridoids.

A few reports have been published on the capillary electrophoretic analysis of iridoid glycosides. Wu et al. [17] used capillary zone electrophoresis for the analysis of nine iridoids, including catalpol and aucubin. However, although the separation of the other iridoids was good, the two compounds could not be separated under the conditions used (borate buffer and 2,6-di-*O*-methyl-β-cyclodextrin). According to our earlier study [16], several iridoids can be successfully separated by micellar electrokinetic capillary chromatography (MECC).

We studied the suitability of four methods for the extraction of catalpol and aucubin from *Veronica longifolia*. This work is part of a project focusing on the determination of catalpol and aucubin from the host plants of the Glanville fritillary, *Melitaea cinxia*. The aim was to find an extraction method that would be efficient, environmentally friendly and reliable for this difficult biological matrix. We also wanted to develop a simple and fast method for the identification and quantification of these two iridoid glycosides from plant extracts.

2. Experimental

2.1. Chemicals

Catalpol and aucubin standards were donated by Sören Rosendal Jensen (Department of Organic Chemistry, Technical University, Lyngby, Denmark). The compounds had been extracted from plant material with ethanol, purified by RP–HPLC and identified by UV detection at wavelengths 206 and 254 nm. The structures of the compounds are shown in Fig. 1.

Borate ($Na_2B_4O_7 \cdot 10H_2O$) and a standard solution of NaOH were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) was

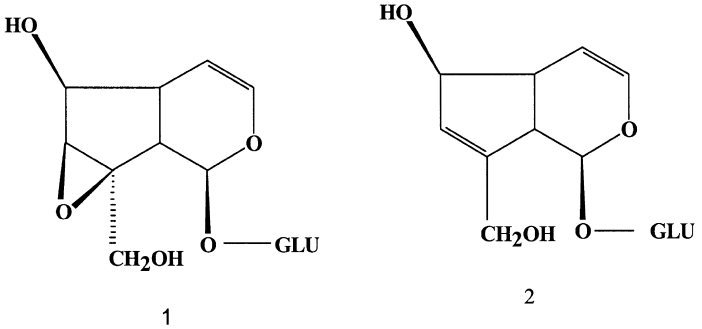

Fig. 1. Catalpol (1) and aucubin (2). GLU=Glycosidic residue, which is usually β-D-glucose.

supplied by two manufacturers: SDS from Sigma (St. Louis, USA) was 95% pure and SDS from BDH (Poole, UK) was 99% pure. The differences in the purity did not affect the resolution. The water for the experiments was first distilled and then purified further with a Water-I instrument (Gelman Sciences, Ann Arbor, MI, USA) until its resistance was 18 mΩ.

2.2. Apparatus

PHWE was performed using the self-constructed apparatus shown in Fig. 2. The system consisted of two Jasco PU-980 HPLC pumps (Jasco, Tokyo, Japan), one to pressurize the water and the other to pump the flushing solvent (methanol), a Fractovap series 2150 GC oven (Carlo Erba, Milan, Italy) to heat the 2.2 ml high-temperature extraction vessel (Keystone Scientific, Bellefonte, PA, USA), a 30-15 HF4-HT high-temperature three-way valve (High Pressure Equipments, Erie, PA, USA), a manually adjustable pressure restrictor (Jasco) and a 50-ml measuring flask for the collection of the extract. On/off valves were type 15-11AF1 from High Pressure Equipments. Approximately 3 m of 1/16 in. stainless steel tubing (0.02 in. I.D.; 1 in.=2.54 cm) was used for the preheating coil. The same tubing was used for all the other connections between different parts, except for the cooling coil, where tubing of ca. 1 m with larger I. D. (0.03 in.) was used.

PLE was performed with a Dionex ASE 200 Accelerated Solvent Extractor (Dionex, Sunnyvale, CA, USA). The volume of the extraction vessel in PLE was 11 ml.

The capillary electrophoresis system was a Hewlett-Packard 3DCE (Hewlett-Packard, Avondale, PA, USA). The total length of the silica capillary (Composite Metal Services, The Chase, Hallow, UK) was 33.5 cm (25 cm to the diode array detector) and the diameter was 50 μm I.D.×375 μm O.D.

2.3. Preparation of electrolyte and standard solutions

Different compositions of the electrolyte solution were tested for optimization. The borate concentration was between 25 and 65 m*M* and the SDS concentration was between 35 and 200 m*M*.

The final composition of the electrolyte solution was 50 m*M* borate, 180 m*M* SDS, pH 9.35. The pH was not adjusted when Sigma's SDS was used but, probably due to the greater purity, the pH had to be adjusted with 0.1 *M* NaOH to 9.35 when BDH's SDS was used. The pH was determined with a MeterLab PHM 220 laboratory pH meter (Radiometer, Copenhagen, Denmark) using a combination electrode, which was calibrated before use with standard solutions of pH 7.00 and 10.01 (FF-Chemicals Ab, Yli-Ii, Finland).

Standard solutions of catalpol and aucubin were prepared by adding water to the standard compounds.

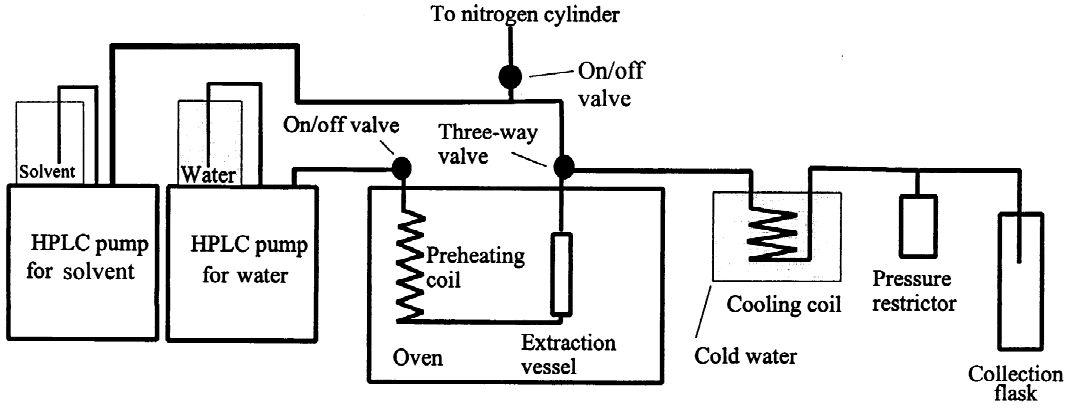

Fig. 2. Diagram of the pressurized hot water extraction (PHWE) system.

2.4. Samples

Veronica longifolia was collected from Joutseno (Southeastern Finland) on 21 August, 1996 by Niklas Wahlberg (Department of Ecology and Systematics, University of Helsinki, Finland). The plant leaves were air dried and kept in a freezer until prepared for extraction.

The leaf material was crushed by hand and weighed to the nearest 0.1 mg. Some of the samples were ground in a mortar with sea sand in order to break down the cells. Since no significant differences were noticed in the results, however, most of the samples were crushed by hand. After extraction with the methods and conditions described in Table 1, the samples were evaporated to dryness and then partitioned between water and diethyl ether. The water phase was filtered through a 0.2-μm filter and water was added up to a specific volume before injection to the capillary electrophoresis equipment.

Before injection to the capillary electrophoresis equipment the samples and electrolyte solutions were filtered through Gelman's Supor Acrodisc 25 sterile filters, pore size 0.2 μm (Gelman Sciences, Ann Arbor, MI, USA).

2.5. CE conditions

The applied separation voltage was +10 kV (current 90 μA). The analytes were injected at a

Table 1
Extraction methods and conditions used[a]

Extraction method	Solvent	Temperature (°C)	Pressure (atm)	Extraction time
Solvent extraction	Methanol	25	1	Overnight
	Methanol	**50**	**1**	**Overnight or 4 h**
	Ethanol	50	1	Overnight
	CH_2Cl_2	25	1	Overnight
	Diethyl ether	25	1	Overnight
	2-Propanol	50	1	Overnight
Microwave extraction	Water	Not measured	~1	1–2 min in a microwave oven (328 W)
HWE	Water	50	1	Overnight or 4 h
	Water	60–90	1	Overnight
	Water	**100**	**1**	**1 h after standing at 25°C for 40 min**
PLE	**Water**	**100**	**103 (1500 p.s.i)**	**5+5 min**
	Water	100	103	10+10 min
	Water	150	103	5+5 min
	Water	100 or 150	69 (1000 p.s.i)	5+5 min
	Water	100	137 (2000 p.s.i) or	5+5 min
			172 (2500 p.s.i)	5+5 min
	Water	150	137 or 172	5+5 min
	Water	80	103 or 69	5+5 min
PHWE	**Water**	**100**	**145 (150 kg/cm^2)**	**30 min**
	Water	150	<5	30 min
	Water	150	97 (100 kg/cm^2)	30 min
	Water	80	97	30 min
	Water	100	73 (75 kg/cm^2)	30 min
	Water	200	97	30 min
	Water	150	77 (80 kg/cm^2)	30 min
	Water	150	145	30 min
	Water	100	193 (200 kg/cm^2)	30 min
	Water	150	193	30 min

[a] The conditions printed in bold gave the best results for each method.

pressure of 50 mbar×5 s (1 bar=14.504 p.s.i) and the temperature of the cassette was kept at 25°C. The separation time was 10 min. Compounds were detected with a diode array detector at 200 nm with a reference wavelength of 250 nm and they were identified by their spectra. Between analyses the capillary was rinsed with the electrolyte solution for 8 min, and after three successive analyses it was rinsed with 0.1 *M* NaOH (2 min), water (5 min) and electrolyte solution (2 min). This was done to avoid blockage. Each morning the capillary was regenerated by rinsing it with 0.1 *M* NaOH (10 min), water (15 min) and electrolyte solution (15 min). This was also done when the capillary was changed.

2.6. General extraction conditions: PLE, hot water extraction (HWE) and PHWE

The pressurized liquid extractions were static and the extraction cycle (5–10 min) was repeated twice. The dead volume was minimized by filling the extraction vessel with an inert hydromatrix (Varian, Walnut Creek, CA, USA) after introduction of the sample.

HWE was performed as shown in the flow-chart shown in Fig. 3. The extraction was static: the sample was merely kept in the hot water for 60 min. Longer extraction times (2–3 h) were tested, but the results did not improve.

PHWE was dynamic and took 30 min in all experiments. After the extractions the capillaries were flushed with 10 ml of methanol and dried with nitrogen. The methanol was collected into the same flask as the extract to minimize the loss of iridoids. Pressure and temperature were checked frequently during the extractions to ensure there was no deviation from the set values. The maximum variation in pressure and temperature during the extractions was 3%.

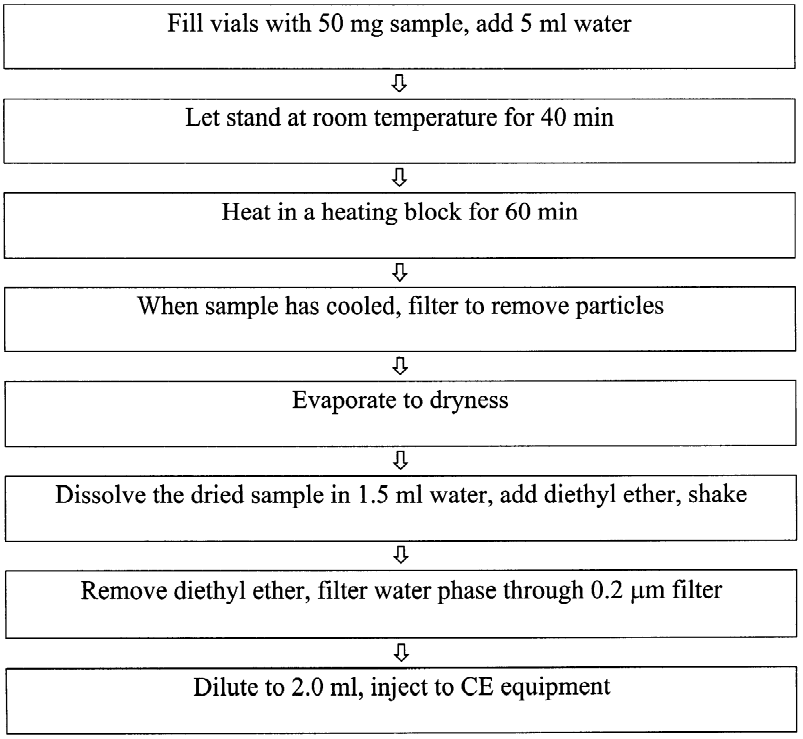

Fig. 3. Flow chart of the hot water extraction procedure and sample preparation. From evaporation onwards the procedure is the same for all the extracts.

3. Results and discussion

Our preliminary study showed that ten iridoid glycosides could be separated by MECC [16]. In the present study the aim was to develop a fast separation method for catalpol and aucubin. Previous work suggested that extraction with pressurized hot water (subcritical water) was a good method for the separation of iridoid glycosides in plant matrices. The PHWE conditions as well as the conditions for other extraction methods were studied in detail.

3.1. CE method development

To shorten the analysis times, we used as short a capillary as possible. The shortest capillary that could be used in the HP 3DCE instrument was 33.5 cm (25 cm to the detector). Different compositions and pH values of the buffer solution were tested. All buffers were basic in order to avoid hydrolysis and rearrangement of the iridoids as well as to speed up the electro-osmotic flow. Solutions of the standard compounds were used in the optimization of the CE separation.

The separation improved slightly when the borate concentration increased, and more markedly when the SDS concentration increased. Lowering the pH from 9.35 to 9.1 decreased the resolution. Baseline separation of the two compounds was achieved using a buffer with 50 m*M* borate and 180 m*M* SDS at pH 9.35. The voltage was +10 kV and the current 90 μA. The migration times were less than 5 min. The identification was confirmed by the UV spectra shown in Fig. 4.

For quantitative measurements, linearity curves were calculated for both iridoids. Detection limits ($S/N=2$) were 35 mg/l for catalpol and 25 mg/l for aucubin. The high levels were due to background noise at 200 nm. Both curves were linear from the detection limit to 400 mg/l with $R^2=0.9922$ for catalpol and $R^2=0.9876$ for aucubin. The concentrations of iridoids in the extracts were calculated from the peak heights by the external standard method.

3.2. Extraction methods

Maceration with methanol was performed under different conditions, as shown in Table 1. Methanol at 50°C extracted the iridoid glycosides more effi-

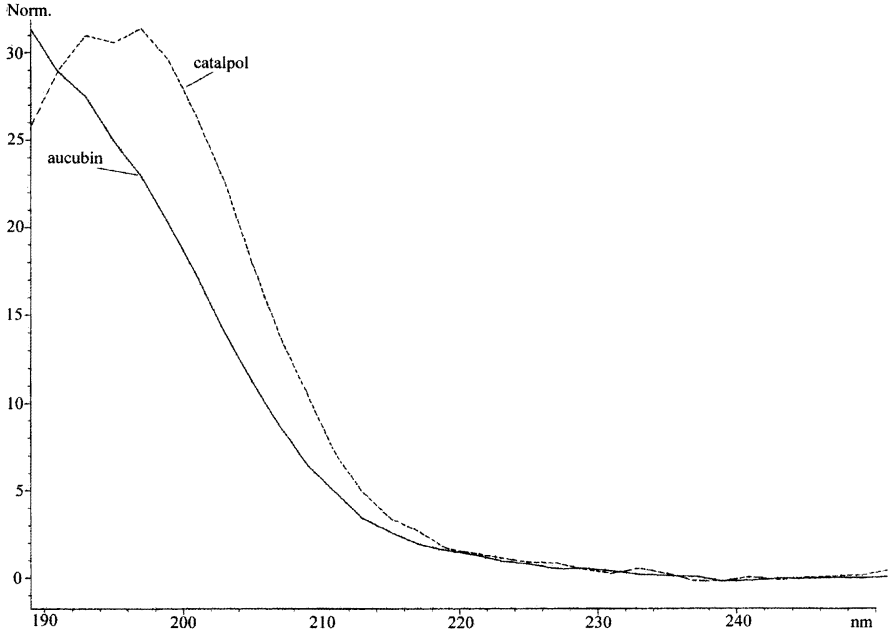

Fig. 4. Spectra of aucubin and catalpol. Spectra were measured in the separation buffer (50 m*M* borate, 180 m*M* SDS, pH 9.35).

ciently than methanol at room temperature because the solubility of the analytes was better at higher temperatures. We also noticed that an increase in the extraction time with methanol at 50°C did not significantly improve the results. Ethanol extracted the iridoids less efficiently than methanol did and 2-propanol was even less efficient than ethanol. This behaviour was expected, since the iridoid glycosides are highly polar compounds and the polarity of alcohols decreases as the carbon chain lengthens. Predictably, diethyl ether did not extract the compounds at all.

Extraction was also studied in a microwave oven. The oven was a commercial Siemens microwave oven for the preparation of food. The samples were put into closed glass vials with water and warmed for 1–2 min at 328 W. Some promising results were obtained. The amount of extracted iridoids was about half of the amount that was extracted with HWE, but the microwave extractions were poorly repeatable and twice the vial was too tightly shut and broke when warmed. The microwave project was abandoned as a result of this unpredictability. With proper equipment, however, microwave extraction might be a quick and useful extraction method for iridoids.

HWE, in which the samples were treated as shown in the flow-chart displayed in Fig. 3, was quick and repeatable. The best results were obtained at 100°C and atmospheric pressure. The recovery of the standard compounds with this method was 89% for catalpol and 124% for aucubin (Fig. 5). Relative standard deviations were 6.5% for catalpol and 7.7% for aucubin in six successive extractions and 22% for catalpol and 8% for aucubin in 13 extractions. Fig. 5 also shows the recoveries of the spiked standards with PLE and PHWE. The recovery of aucubin was best and the recovery of catalpol second best with HWE. Extraction of the spiked standards gave us some information about the solubilities, extraction and collection efficiencies, and of the possible losses during the sample handling. However, it was impossible to say whether the poor recovery was due to a particular step or not. Usually, the recoveries were worse with real samples than with the spiked samples. The values in Fig. 5 represent the maximum values for the different methods and conditions.

The efficiency of the optimized methods was determined by calculating the relative concentrations of the iridoid glycosides in the dry *Veronica longifolia* sample. The true concentrations were not known. HWE proved to be the most effective

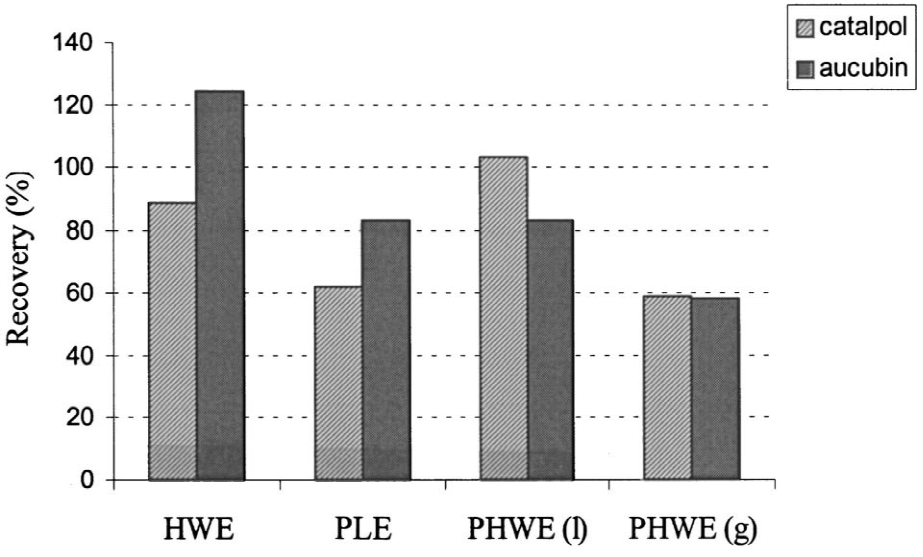

Fig. 5. Recoveries of the standard compounds extracted by the different methods. PHWE (l) was performed at 100°C and 150 kg/cm^2 (145 atm) and PHWE (g) at 150°C and 5 kg/cm^2. HWE was performed at 100°C and atmospheric pressure. PLE was performed at 100°C and 1500 p.s.i (103 atm) with two 5-min extraction cycles.

method for the extraction of catalpol and aucubin and its repeatability was excellent.

Several different pressure and temperature combinations for PHWE were tested, as shown in Fig. 6. Up to a certain point, the amount of iridoid glycosides extracted increased when the temperature was raised at constant pressure and it increased even more when the pressure was raised; however, above 100°C, 150 kg/cm^2 (1 kg/cm^2=0.967 atm) the results slowly began to deteriorate. This poor extraction efficiency at the highest temperature was probably due to decomposition of the iridoids. Extraction with steam at 150°C and less than 5 atm was also studied, but the recovery was clearly poorer than when the compounds were extracted at 100°C and 150 kg/cm^2. These PHWE conditions were selected for further studies.

The suitability of PLE for iridoids was also tested. Since the solvent was water, the method resembled PHWE. However, the PLE extraction was static, whereas the PHWE was dynamic. In the beginning, extractions with PLE were efficient, but the repeatability was poor. The reason for the poor repeatability was not discovered, but there may have been some blockage in the equipment during the later extractions, or the vessel may have been leaking. Occasionally there were problems with the hydrocarbon sensor as well. The recovery of the standard compounds was 62% for catalpol and 83% for aucubin (Fig. 5). A clear advantage of the PLE system compared with other methods was the automated instrumentation.

The second best efficiencies were achieved with the PHWE apparatus, as shown in Fig. 7. The repeatability of the results was not as good as with HWE, but better than with PLE. The extractions performed with steam were more repeatable than those done with liquid water: the relative standard deviation of catalpol was 37% with liquid water and 14% with steam, and the relative standard deviation of aucubin was 65% with liquid water and 22% with steam. Both PHWE and HWE are suitable extraction methods for the analysis of catalpol and aucubin. Both are environmentally friendly and easy to use.

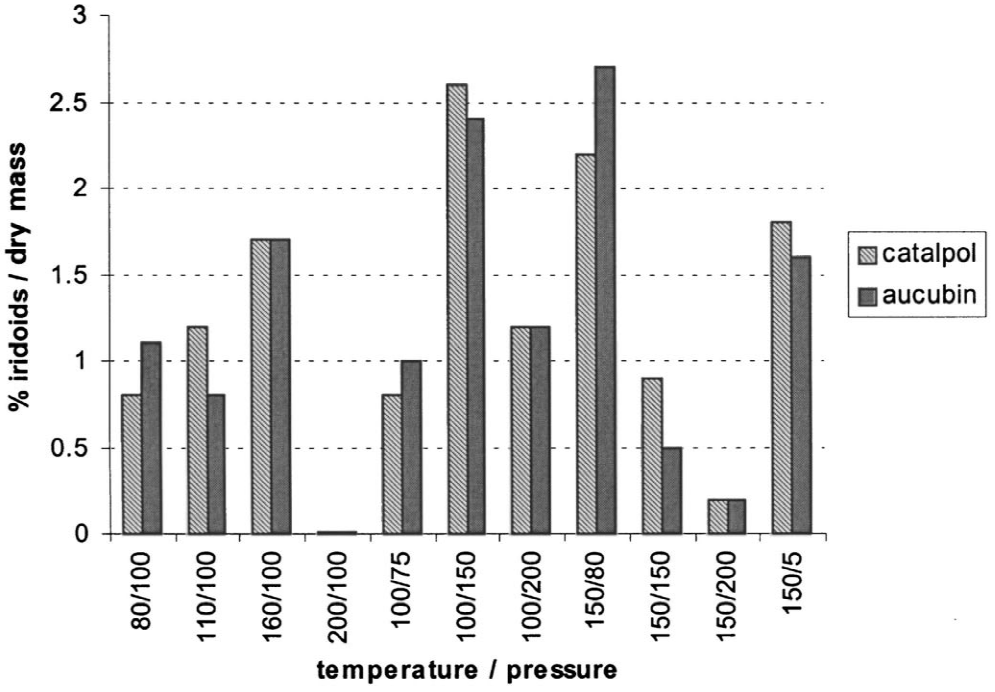

Fig. 6. PHWE extraction conditions and the results obtained. The temperature is given in °C and the pressure in kg/cm^2. The water was in the liquid phase except in the last experiment (pressure 5 kg/cm^2), where it was in the gas phase.

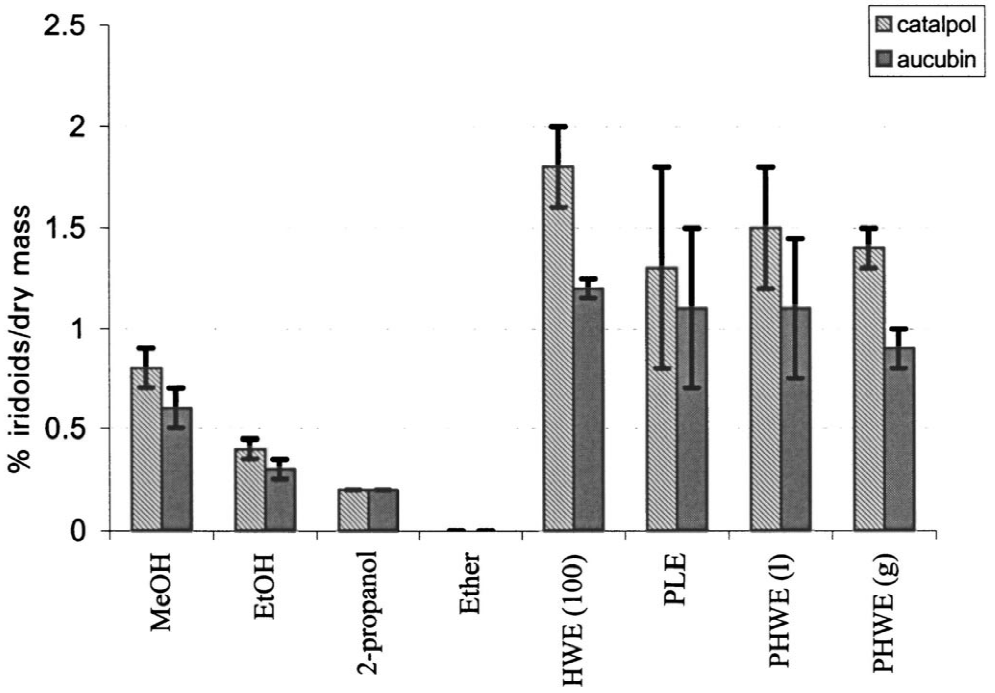

Fig. 7. Catalpol and aucubin concentrations (%) in *Veronica longifolia* as determined by the different extraction methods. Standard deviations are marked with lines. HWE (100) is hot water extraction at 100°C and atmospheric pressure (13 repetitions). PLE was performed at 100°C and 103 atm (five repetitions). PHWE (l) was performed at 100°C and 150 kg/cm^2 (seven repetitions) and PHWE (g) at 150°C and 5 kg/cm^2 (six repetitions). The least successful extractions (2-propanol and diethyl ether) were not repeated. The methanol (MeOH) and ethanol (EtOH) extractions were performed at room temperature.

Extraction of the two compounds from the plant matrix was slightly more effective by HWE than by PHWE. HWE also exhibited better repeatability and the extractions were faster because several samples could be extracted at the same time and the smaller volumes of extract made the evaporation to dryness quicker.

Fig. 8 shows the electropherograms of the hot water and pressurized hot water extracts of *Veronica longifolia*. There are notable differences in the profile of the electropherograms, revealing the different selectivities of the two methods used for the plant matrix extraction.

As indicated in Fig. 7, the methods differed in their efficiency for extracting catalpol and aucubin. Moreover, almost all of the methods were more selective to catalpol. The solubility (polarity) and thermal properties (diffusivity, vapour pressure) of the two compounds can explain most of the differences.

The dielectric constant of water decreases as the temperature is raised, and it is clearly smaller in vapour phase than in liquid phase at the same temperature. Comparing the recoveries in Fig. 5 we can see that the greatest recovery of aucubin relative to catalpol was obtained with HWE and the lowest with PHWE (l). Owing to the lower pressure, the polarity of water was presumably lower in HWE than in PHWE (l) at the same temperature, which would suggest that catalpol is more polar than aucubin.

A comparison of Figs. 5 and 7 shows that the relative recoveries of catalpol and aucubin were similar when the methods were applied to the plant matrix but different when they were applied to the spiked analytes. This can be explained by the diffusion restricted extraction through the plant cells where differences in compound vapour pressures and solubilities do not play as significant a role as with the spiked samples.

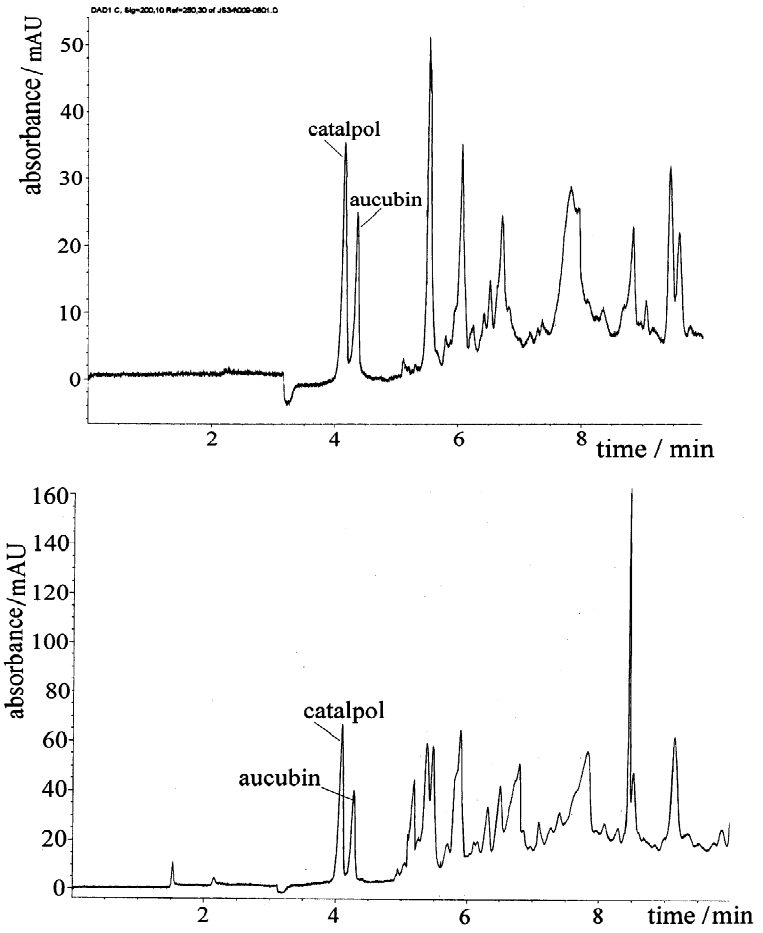

Fig. 8. Electropherogram of a hot water extract (above) and a pressurized hot water extract (below) of *Veronica longifolia*. MECC conditions: 50 m*M* borate, 180 m*M* SDS, pH 9.35. Voltage: +10 kV, current: ca. 90 μA, capillary: 33.5 cm(25 cm to detector)×50 μm I.D.×375 μm O.D. Detected at 200 nm, reference at 250 nm. HWE was one of the HWE(100) repetitions and PHWE was one of the PHWE(l) repetitions. (See Fig. 7.)

4. Conclusions

Two environmentally friendly and efficient methods were developed for the extraction of catalpol and aucubin from *Veronica longifolia* leaves. PHWE extracted catalpol and aucubin from the plant matrix efficiently at high pressure and 100°C and slightly less efficiently but more repeatably with steam. HWE was the most efficient of the methods tested; it was quicker than PHWE and also the repeatability was better. Although both methods were superior to solvent extraction methods, HWE is to be preferred since several extractions can be done at the same time.

The MECC profiles of the HWE and PHWE extracts were different, which suggests that in addition to the iridoid glycosides studied in this work, the two methods extract different compounds or different amounts of the same compounds from the plant matrix. Optimizing of the extraction conditions would presumably increase the selectivity still further.

In addition, a quick and reliable capillary electrophoretic method was developed for the determination

of catalpol and aucubin. The two iridoids could be baseline separated in 5 min with good repeatability. Detection limits were 35 mg/l for catalpol and 25 mg/l for aucubin at 200 nm.

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