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Pressurized liquid extraction of medicinal plants

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Abstract

The suitability of pressurized liquid extraction (PLE) in medicinal plant analysis was investigated. PLE extracts from a selection of representative herbs were compared with extracts obtained according to Pharmacopoeia monographs with respect to yield of relevant plant constituents, extraction time and solvent consumption. In all cases a significant economy in time and solvents was realized, while extraction yields of the analytes were equivalent or higher.

Keywords: Pressurized liquid extraction; Extraction methods; Medicinal plants

1. Introduction

The first step in the qualitative and quantitative analysis of medicinal plant constituents is extraction, that is the separation of compounds to be analysed from the cellular matrix. Ideally, an extraction procedure should be exhaustive with respect to the constituents to be analysed, rapid, simple, inexpensive and – for routine analysis – amenable to automation. A broad spectrum of extraction procedures are currently being used. Pharmacopoeia monographs [1–4], which serve as official standards for the quality control of many medicinal plants, employ a broad range of methods such as Soxhlet extraction, percolation, maceration, digestion, extraction under reflux and steam distillation. Non-Pharmacopoeia methods reported in the literature are by ultrasonication or turboextraction. From a technical viewpoint, most of these methods are relatively simple. Unfortunately, they suffer, to varying degrees, from disadvantages such as long extraction time, labour intensive manual procedures, relatively high solvent consumption and unsatisfactory reproducibility.

With the advent of laboratory automation and fast quantitative assays for crude drug analysis, conventional extraction technologies are increasingly becoming the bottleneck in routine analysis. Instrumental extraction methods requiring minimal sample handling are thus highly desirable. Pressurized liquid extraction (PLE), a recently developed extraction technique could in principle overcome some of the drawbacks of the extraction methods currently in use. PLE works according to the principle of static extraction with superheated liquids. Enhanced diffusivity of the solvent leads to an increase in extraction speed and efficiency [5,6]. At its origin, PLE was developed for sample preparation in environmental analysis to serve as a substitute for Soxhlet extraction, supercritical fluid extraction (SFE) and other methods [7–9].

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Since the development of a first commercial PLE instrument, this extraction technique has been successfully employed for extraction of agrochemicals and various other pollutants, pharmaceuticals, lipids from diverse of matrices [10,11]. Surprisingly however, no data have been published as yet on a possible application of PLE in medicinal herb analysis. A single note on secondary metabolite extraction from yew has been published recently [12]. We therefore carried out a comparative study to evaluate PLE as a possible alternative to current extraction methods like Soxhlet, maceration, percolation, reflux, with regard to extraction time, solvent consumption, extraction yields and reproducibility. These investigations were carried using a selected number of representative medicinal plants containing important classes of secondary metabolites.

2. Experimental

2.1. Samples

Dried medicinal plants were purchased from Caelo (Hilden, Germany), Galke (Gittelde, Germany) and Klenk (Schwebheim, Germany) and were of Pharmacopoeia quality. The samples were ground with an ultracentrifugal mill ZM 100 (Retsch, Haan, Germany) and sieved according to the respective Pharmacopoeia monographs. Details are given in Tables 1 and 2.

2.2. Equipment

PLE was performed with a Dionex ASE 200 instrument equipped with a solvent controller for ASE 200 (Dionex, Sunnyvale, CA, USA). The system consists of a solvent module, pump, thermostatted extraction cell containing the material to be extracted, an electrovalve and an extract collection device. The extraction cells and sample collection vials are mounted on carousels. Solvent selection and extraction conditions are individually programmable for each sample. Extraction vessels of 11, 22 and 33 ml were used (Table 1).

Spectrophotometric assays were carried out with a UV-120-01 spectrophotometer (Shimadzu, Kyoto, Japan). The high-performance liquid chromatography (HLPC) system consisted of a HP 1050 Series pump, HP 1100 Series autosampler, HP 1040M Series II DAD detector and a HP Chemstation (Hewlett-Packard, Waldbronn, Germany). The densitometric determinations were performed with a CD60 densitometer; a AS30 thin-layer chromatography (TLC) spotter was used for sample application (Desaga, Heidelberg, Germany). Gas chromatography (GC) was carried out with a GC 14B system equipped with a split injector and a C-R 5a Chromatopac integrator (Shimadzu).

2.3. Extraction

Conditions employed for PLE are listed in Table 1. Reference extracts were prepared according to the

Table 1
Extraction conditions for PLE

Sample	St. John's wort herb	Horse chestnut seed	Milk thistle fruit	Turmeric rhizome	Thyme herb
Amount (sieve size, μm)	2 g (710)	1 g (710)	5 g (710)	1 g (710)	10 g (315)
Extraction cell	11 ml	11 ml	22 ml	11 ml	33 ml
Extraction pressure	140 bar	140 bar	140 bar	140 bar	140 bar
Extraction time 1	5 min	2×5 min	5 min	3×6 min	5 min
Solvent 1	CH_2Cl_2 (20 ml)	CH_2Cl_2 (2×20 ml)	Hexane (20 ml)	MeOH (3×20 ml)	Hexane (35 ml)
Extraction temperature 1	100°C	100°C	100°C	50–100°C	50°C
Extraction time 2	3×5 min	2×6 min	5 min		5 min
Solvent 2	MeOH (3×20 ml)	MeOH (2×20 ml)	MeOH (20 ml)		CH_2Cl_2 (35 ml)
Extraction temperature 2	50–100°C	100°C	100°C		50°C

Table 2
Extraction conditions for methods according to Pharmacopoeia monographs

Sample	St. John's wort herb	Horse chestnut seed	Milk thistle fruit	Turmeric rhizome	Thyme herb
Extraction according to	DAC 86	DAB 9	DAB 9	DAB 1997	Ph. Eur. 1997
Amount (sieve size, μm)	2 g (710)	2 g (710)	5 g (710)	0.1 g (710)	20 g (315)
Extraction time 1	3 h	3 h	4 h	1 h	2 h
Solvent 1	CH_2Cl_2 (100 ml)	CH_2Cl_2 (70 ml)	Petrol (100 ml)	MeOH (50 ml)	Water (250 ml)
Method 1	Soxhlet extraction	Soxhlet extraction	Soxhlet extraction	Reflux extraction	Steam distillation (2–3 ml/min)
Extraction time 2	35 h	3.5 h	5 h		
Solvent 2	Acetone (150ml)	MeOH (100 ml)	MeOH (100 ml)		
Method 2	Maceration	Maceration	Soxhlet extraction		
Extraction time 3		0.5 h			
Solvent 3		MeOH			
Method 3		Reflux extraction			

respective Pharmacopoeia monographs. Conditions are given in Table 2.

2.4. Quantitative analysis

Quantitative determinations were performed according to the methods of the respective pharmacopoeia monographs or other methods. All analysis were carried out in triplicate.

The determination of dianthrons in St. John's wort (Hyperici herba) was performed according to the DAC 86 [3]. The extracts obtained according to DAC were evaporated to dryness, dissolved in 25 ml of MeOH and filtered. After five-fold dilution, the absorption at 590 nm was determined with MeOH as reference. Total dianthrons were expressed as hypericin.

The escin content of horse chestnut seed (Hippocastani semen) was determined after extraction according to DAB 9 [1], using a HPLC method developed by Wagner et al. [13]. According to this method, the complex mixture of over 30 structurally very similar saponin esters is submitted to basic hydrolysis to afford a much simplified pattern of deacylsaponins which are then quantified in reference to an escin standard submitted to identical treatment. After extraction of the crude drug, extracts were evaporated to dryness and dissolved in 60% aqueous methanol (MeOH) (PLE extracts 50 ml; DAB extracts 100 ml). Of these solutions, 10 ml each were submitted to alkaline hydrolysis (10 ml of 0.1 *M* KOH, reflux for 15 min). After hydrolysis, the extracts were concentrated to 5 ml and applied onto a C_{18} solid-phase extraction (SPE) cartridge (J.T. Baker, Griesheim, Germany). The cartridge was washed with 30 ml water, followed by elution of deacylsaponins with 30 ml MeOH. The MeOH solution was evaporated in vacuo. MeOH (4.0 ml) and 1 ml of a solution of the internal standard (20 mg *p*-hydroxybenzoic acid in 100.0 ml MeOH) were added. The HPLC conditions were: LiChrospher 100 RP18 (5 μm, 150×4 mm I.D.) (Merck, Darmstadt, Germany); 22–28% aqueous MeCN (containing 10 ml 0.1 *M* phosphoric acid per liter) over 10 min; flow-rate: 1 ml/min; detection: 200 nm; injection volume 5 μl.

The silybin content of milk thistle fruit (Cardui mariae fructus) was determined by a densitometric method developed in our laboratory. The extracts obtained were evaporated in vacuo, redissolved in 10 ml MeOH (sample solution). A reference stock solution was prepared by dissolving 1.00 mg of silybin (Roth, Karlsruhe, Germany) in 1.00 ml MeOH. High-performance (HP) TLC separation was carried out as follows: 20×20 cm twin-trough chamber (Camag, Berlin, Germany); saturation for 60 min at r.t.; 20× 20 cm silica gel 60 HPTLC plates (Merck, Darmstadt, Germany); chloroform–acetone–formic acid (75:16.5:8.5) [14]; development: 11 cm; sample and reference applied as 10 mm bands;

sample volumes: 1, 3, 5 μl of the reference solution and 2 μl of the sample solution; densitometric measurement at 288 nm.

The determination of the curcuminoid content in turmeric rhizome (Curcumae rhizoma) was carried out according to the DAB 1997 [2]. Extracts were evaporated to dryness and redissolved in 60 ml of glacial acetic acid. After addition of 2.0 g of boric acid and 2.0 g oxalic acid, the solutions were kept in a water bath at 90°C for 60 min. The assay solutions were diluted with glacial acetic acid to 100.0 ml (dilution factor for PLE extracts 1:100, for DAB extracts 1:10) and total curcuminoid content established by spectrophotometric determination of rubrocurcuminoids at 530 nm.

The essential oil of thyme (Thymi herba) was obtained by steam distillation according to the method described in the Ph. Eur. 1997 [4] (distillation time was 2 h at 2–3 ml min^{-1}). After separation of the essential oil from the water phase, 10 μl of oil were diluted with 4.0 ml of hexane. In the PLE experiments thyme oil was contained in hexane or dichloromethane. These solutions were diluted to 100.0 ml with the corresponding solvent. GC analysis was performed as follows: capillary column FS-SE54-CB (30 m×0.25 mm I.D.) (J&W Scientific, Folsom, CA, USA); injection volume (standard and sample solution): 1 μl; temperature programme: 40–240°C (3°C min^{-1}); mobile phase: helium; detection by flame ionization detection (FID). A calibration curve for thymol was established with standard solutions containing 0.5, 0.75, 1.0, 1.5 and 2.0 mg thymol ml^{-1} in hexane.

3. Results and discussion

The following officinal herbs were used for the investigation: St. John's wort herb (Hyperici herba; *Hypericum perforatum*), horse chestnut seed (Hippocastani semen; *Aesculus hippocastanum*), milk thistle fruit (Cardui mariae fructus; *Silybum marianum*), turmeric rhizome (Curcumae xanthorrhizae rhizoma; *Curcuma xanthorrhiza*); thyme herb (Thymi herba; *Thymus vulgaris*). These crude drugs contain structurally diverse metabolites of varying polarity and solubility, including essential oil constituents such as thymol (**1**) (Thymi herba), polycyclic phenols such as hypericin (**2**) (Hyperici herba), phenylpropane derivates like the curcuminoids (**3**) (Curcumae xanthorrhizae rhizoma), flavonolignans such as silybin (**4**) (Cardui mariae fructus) and triterpene saponins such as escin (**5**) (Hippocastani semen) (Fig. 1).

All experiments involving conventional extraction or PLE were carried out at a scale indicated by the respective Pharmacopoeia monographs. First PLE experiments served for the optimization of extraction parameters with respect to temperature, extraction time and number of extraction cycles required for exhaustive extraction of compounds of interest. For the optimization of extraction time, samples were submitted to several static extraction cycles at a given temperature. Extracts from each cycle were collected in separate vials and assayed individually. An example for PLE of turmeric is shown in Fig. 2. Three extraction cycles of 6 min each with MeOH at 80°C were carried out. The quantitative assay of total curcuminoids according to the DAB 1997 [2] revealed that >98% of curcuminoids were extracted by the first cycle already, whereas the second and third step afforded only minimal additional amounts of pungent principles. Interestingly, the extraction according to a conventional method afforded significantly lower curcuminoid content and thus was apparently not exhaustive.

Not all plant constituents of interest are as easily soluble as the curcuminoids. Dianthrons (**2**) (Fig. 1), for instance, the characteristic red pigments in St. John's wort, show poor solubility in many organic solvents. We therefore investigated their extraction in respect to temperature. After removal of undesirable lipophilic constituents by one extraction cycle with CH_2Cl_2 at 100°C, dianthrons were extracted by three cycles of 5 min each with MeOH at temperatures ranging from 50 to 100°C. The hypericin content of the individual extracts is shown in Fig. 3. In contrast to the previous example, extraction cycles 2 and 3 still afforded further hypericin, albeit at significantly decreasing yields. Surprisingly, the temperature increase did not lead to an appreciable improvement of hypericin extraction in the first extraction cycle. The extraction kinetics appears to be mainly governed by the poor solubility of hypericin. In this preliminary study we did not take into account additional factors affecting the extraction behaviour, such as particle

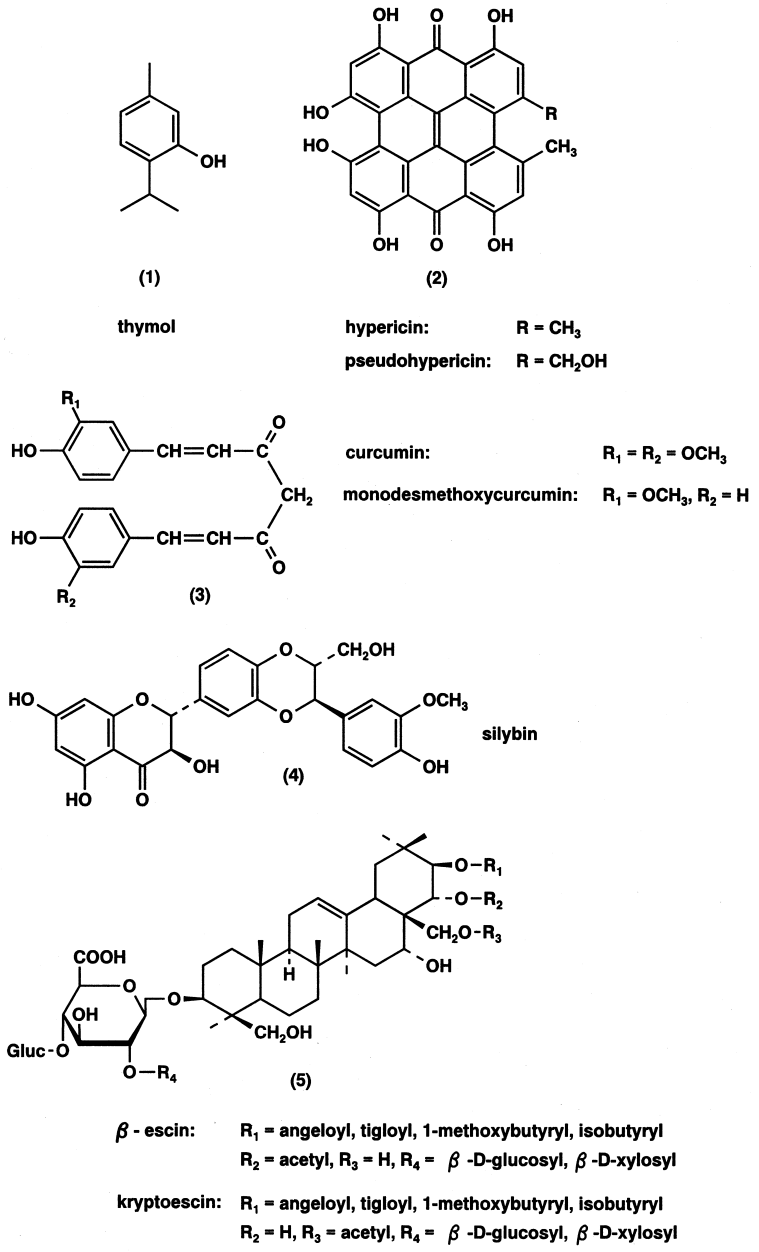

Fig. 1. Structures of compounds analyzed: thymol (**1**); hypericin (**2**); curcuminoids (**3**); silybin (**4**); escin (**5**).

size of the herb and dissolution rates of analytes. More detailed investigations are certainly warranted. Interestingly, the yield of total dianthrons was again significantly higher in the PLE than in the extracts obtained by the DAC method [3] (see Table 3).

In summary, these first experiments indicated that reasonably soluble plant constituents could be extracted from suitably powdered plant material within one to three extraction cycles of 5 to 6 min each at temperatures slightly above the boiling point of the extraction medium. These findings are comparable with data reported for other matrices such as soil,

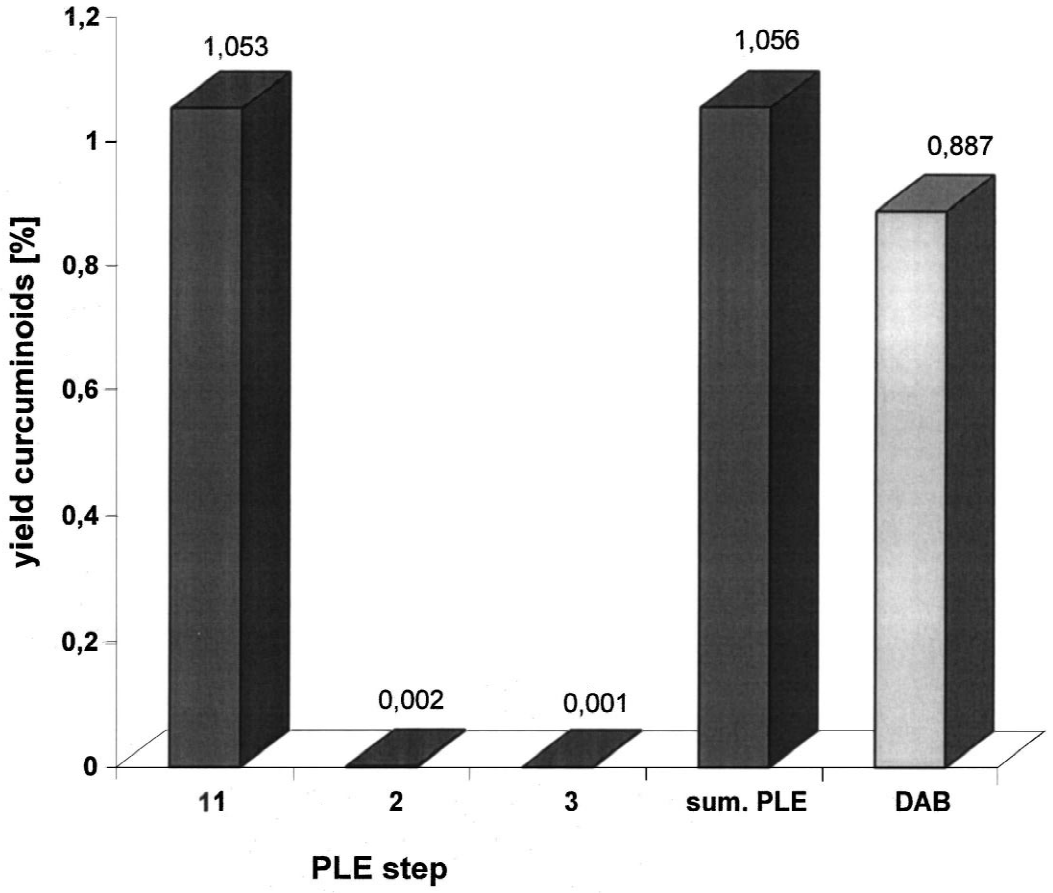

Fig. 2. Extraction of turmeric (Curcumae xanthorrhizae rhizoma). Curcuminoid content in PLE extracts and in extracts prepared according to the DAB monograph.

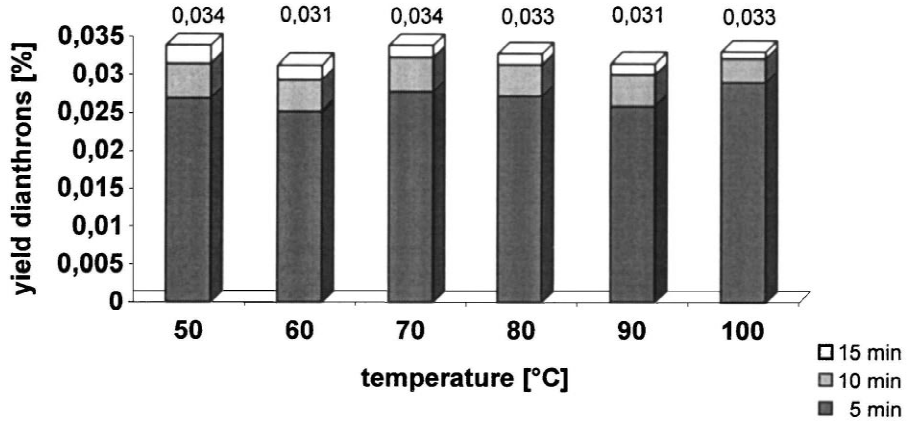

Fig. 3. PLE of St. John's wort. Effect of extraction temperature on yields of total dianthrons. Extraction with MeOH in three cycles of 5 min each.

Table 3
Quantitative analysis of extracts obtained according to Pharmacopoeia monographs and by PLE

Sample	St. John's wort herb	Horse chestnut seed	Milk thistle fruit	Turmeric rhizome	Thyme herb
Compound(s)	Dianthrons (calculated as hypericin)	Deacylsaponins (calculated as escin)	Silybin	Curcumin	Thymol
Pharmacopoeia extracts[a]	0.028 (±0.002)%	2.6 (±0.3)%	1.13 (±0.04)%	0.89 (±0.02)%	1.15 (±0.08)%
PLE extracts[a]	0.035 (±0.001)%	3.7 (±0.2)%	1.16 (±0.04)%	1.06 (±0.01)%	1.17 (±0.04)%

[a] All assays in triplicate.

solid wastes, foods and pharmaceuticals [7,9,11]. In the following, we employed the above mentioned PLE conditions for extraction of additional medicinal plants of importance.

Horse chestnut extracts and the saponin mixture escin (**5**) (Fig. 1) derived from the seeds are the active ingredient in numerous phytopharmaceuticals used in the adjuvant therapy of a chronic insufficiency of veins. The extraction procedure according to DAB 9 [1] comprises 3 h of defatting with CH_2Cl_2 in a Soxhlet apparatus, followed by maceration with MeOH 65% for 3.5 h at room temperature and 30 min of refluxing (Table 2). The PLE of horse chestnut was performed in analogy to that procedure: defatting with CH_2Cl_2 at 100°C (two cycles of 5 min each), followed by an extraction of the saponins with MeOH 65% at 100°C (two cycles of 6 min each) (Table 1). The escin content was determined by a quantitative assay involving RP-HPLC of a simplified mixture of deacylsaponins obtained upon alkaline hydrolysis [13]. The extracts according to DAB 9 contained 2.63% deacylsaponins, calculated as escin, whereas the escin content found by PLE was 3.73% (Table 3).

Milk thistle contains hepatoprotective flavonolignans such as silybin (**4**) (Fig. 1). Extracts are used for treatment of functional disorders of liver and gall bladder, whereas purified flavonolignan fractions serve as hepatoprotectant and antidote against various hepatotoxic agents. Extraction according to the DAB 9 monograph [1] includes defatting of the fruits with 100 ml petroleum ether in a Soxhlet apparatus for 4 h, and subsequent Soxhlet extraction of the flavonolignans with 100 ml MeOH (Table 2). The PLE protocol for milk thistle consisted of a single extraction cycle with 20 ml hexane for defatting, followed by extraction with 20 ml MeOH. A single 5 min extraction cycle was sufficient for a virtually exhaustive extraction of flavonolignans. The silybin content, determined by HPTLC–densitometry at 288 nm (Fig. 4), was comparable for the two extraction protocols (Table 3). The solvent consumption for PLE was five times lower than for the extraction according to DAB 9.

Essential oil plants play a major role as herbal teas and as ingredients in numerous phytopharmaceuticals. Thyme was selected for a comparative evaluation of PLE extraction and obtenation of essential oil by steam distillation according to the Ph. Eur. 1997 monograph [4] for Thymi herba. PLE was carried out with one 5 min cycle of extraction with hexane at 50°C, followed by a 5 min cycle with CH_2Cl_2 at the same temperature (Table 1). Steam distillation over 2 h with the apparatus according to Ph. Eur. was required for exhaustive extraction of essential oil. Quantitative determination of thymol (**1**) (Fig. 1), the main component in thyme oil, gave comparable results (Table 3).

4. Conclusions

The results presented here are, to our knowledge, the first published data on a comparative evaluation of PLE and classical extraction methods in medicinal plant analysis. Given the limited number of examples treated, a thorough evaluation of all parameters affecting the extraction was not possible. Nonetheless, the usefulness of PLE in the quantitative analysis of medicinal herbs becomes obvious. The optimization of extraction protocols was fast and straightforward. For finely ground plant material as prescribed by the respective pharmacopoeia monographs, the number of extraction cycles needed for exhaustive extraction was apparently mainly dependent on the solubility of the analytes. Our experi-

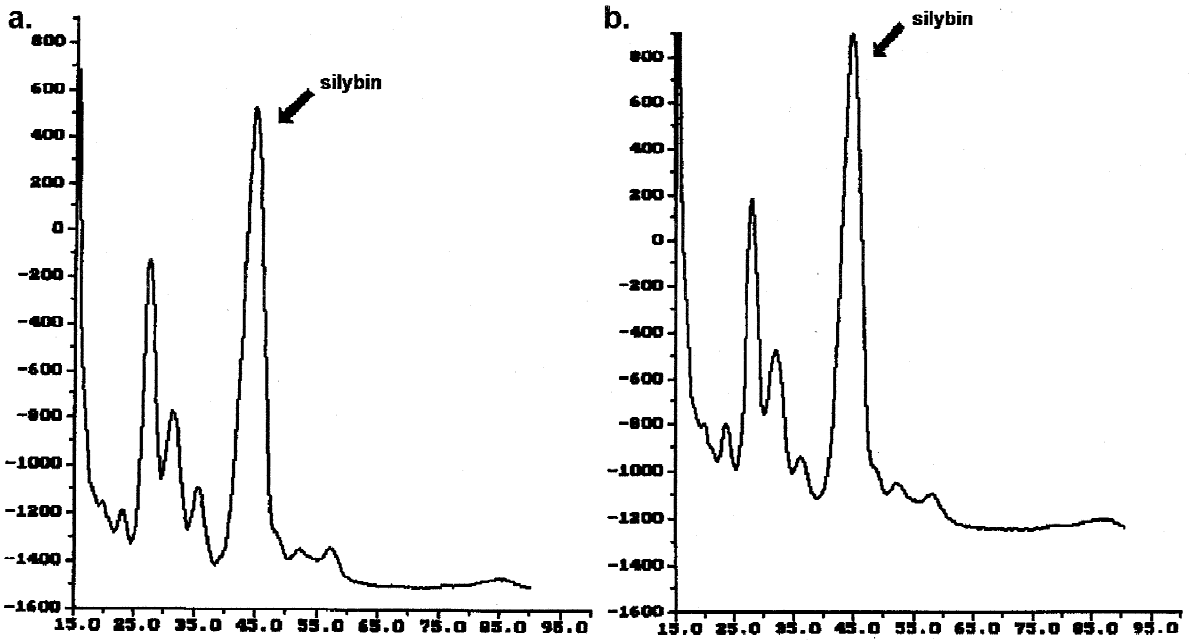

Fig. 4. Densitograms of milk thistle extracts. (a) DAB extract; (b) PLE extract. Conditions: silica gel 60 HPTLC plates; chloroform–acetone–formic acid (75:16.5:8.5); detection at 288 nm.

ments showed that one to three extraction cycles of 5 to 6 min at temperatures somewhat above the boiling point of the respective solvent afforded exhaustive or almost exhaustive extraction. In PLE, the extraction yield for compounds to be assayed was at least comparable, if not superior in most cases, to the extraction protocols of the respective pharmacopoeia monographs. Reproducibility of results was generally better with PLE (Table 3), a fact that is likely due to the minimal need for sample handling during the extraction steps.

Reduced solvent consumption and shorter extraction times have been emphasized as a major advantage of PLE [5,6]. For the medicinal plants investigated in this study, solvent use was reduced by a factor of two (e.g., horse chestnut seed) to five (e.g., milk thistle) (see Tables 1 and 2). Time savings were significant, especially in cases where the plant material required consecutive extraction with solvents of increasing polarity (see Tables 1 and 2). Extraction of St. John's wort, for example, was achieved within less than 80 min instead of 38 h according to the DAC method. Changing of solvents during an extraction programme (for example defatting of the drug and subsequent extraction of the constituents of interest with another solvent) was possible thanks to the programming capabilities of commercial equipment.

Obviously, an PLE protocol needs to be optimized for each particular drug and validated against the respective pharmacopoeia method. For analytical laboratories handling large sample numbers, the high purchase price of commercial PLE equipment is offset by savings in time, solvents and labor cost. Another possible field of application for PLE could be in high-throughput screening programmes for natural products lead discovery, where large numbers of small scale extractions have to be carried out in an efficient and reproducible manner. In summary, our preliminary findings demonstrate that PLE has to be considered as an interesting and often superior alternative to extraction methods currently used in crude drug analysis. Further studies, however, are needed, in particular a systematic investigation of the various parameters affecting extraction behaviour of constituents from the complex herbal matrix.

Note added in proof:

A poster about PLE of natural products (cap-

saicinoids from cayenne fruit, hypericin from St. John's wort, alkaloids from golden seal root and rutin from elderberry flowers) was presented by Peng et al. at the 111th AOAC International Annual Meeting and Exposition San Diego, CA, September 1997.

Acknowledgements

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