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Extraction and Planar Chromatographic Separation Techniques in the Analysis of Natural Products

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ACADEMIC DISSERTATION

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1. PREFACE

This work was carried out at the Division of Pharmacognosy, Department of Pharmacy, University of Helsinki, during the years 1998-2001.

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Turku, October 2004

2. ABSTRACT

Two planar chromatographic methods were applied for the isolation and separation of bioactive constituents from different natural products. In addition, a new extraction method, namely rotation planar extraction (RPE), was implemented for the extraction of plant material and compared with medium pressure solid-liquid extraction (MPSLE) method.

In the extraction of *Ficus* leaves, both RPE and MPSLE methods were found suitable with respect to extraction efficiency. The quality of the extracts was also shown to be identical between the two methods. The extraction yields of RPE and MPSLE were of the same magnitude and they increased with decreasing plant material particle size. The results obtained in the extraction of onion revealed that the quality of the extracted material can have a significant effect on the extraction process. The high carbohydrate content of the resulting extract tended to plug the MPSLE equipment, while RPE enabled a more convenient extraction procedure with higher yields.

Given the ease of operation and significant time savings achieved, the RPE method with the ExtraChrom® separation instrument seemed to be well suited for screening purposes, in which 20-50 g of plant material at a time are to be extracted and the number of the samples is relatively large. MPSLE proved to be an exhaustive extraction method, and the possibility of scaling up the extraction process makes it a suitable method for preparative extractions.

In this study, RPE and MPSLE methods gave equivalent results in the extraction of floral stems and leaves of *G. hybrida* in terms of extraction yield and the quality of the extracts. The use of ExtraChrom® separation instrument prototype, however, permitted extraction with lower solvent consumption compared with the MPSLE method. Application of rotation planar chromatography (RPC) and medium pressure liquid chromatography (MPLC) for the isolation of gerberin and parasorboside from crude extracts enabled rapid prepurification of the extracts. This permitted an increase in the throughput of the total isolation procedure. As a result of the rapid filling procedure of the planar column, substantial time savings in the separation process were also achieved with the ExtraChrom® instrument in comparison to the MPLC method.

Suitable separation conditions for the screening of indole-3-acetic acid (IAA) and other indole derivatives by thin-layer chromatography (TLC) were obtained with the help of the PRISMA model. The advantage of the optimized conditions lay in its ability to separate the selected indole compounds from a wide range of other indole derivatives, thus making it possible to screen bacterial culture broths and other complex samples for IAA. Furthermore, the TLC method does not necessarily require any instrumentation and the determination can thus also be performed in the field, unlike the analysis with high performance liquid chromatography (HPLC). The optimum TLC solvent combination could directly be transferred to normal chamber rotation planar chromatography (N-RPC) with equally good separation, with savings in both analysis time and solvent consumption.

The suitability of a densitometer and video scanner in the detection of plant phenols by TLC was studied and the optimum performance of the methods was assessed. A mixture of phenolic acids and flavonoids, and of coumarins, were detected by UV irradiation at 254 nm and 366 nm, respectively. The methods were found to be equivalent, on the basis of repeatability, when the results from the densitometric measurements were compared with those obtained with a video scanner. The advantages and limitations of the two systems were discussed.

A method was developed to measure the radical scavenging activity of compounds separated by reversed-phase thin-layer chromatography (RP-TLC) using phenolic acids as model analytes. Thin-layer chromatographic separation was followed by derivatization of the analytes with 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol (0,04 %, w/v). The compounds possessing radical scavenging activity were detected as bright yellow bands against a purple background. A video documentation system utilizing a charged-coupled device (CCD) video camera was used for the detection of the activity. The RP-TLC-DPPH method correlated well with the widely used spectrophotometric DPPH assay. The results from the measurement of the free radical scavenging activity of rapeseed meal fractions indicated the potential of the method as a rapid alternative to some of the currently used methods in the fractionation and analysis of potential antioxidative compounds in natural extracts.

3. LIST OF ORIGINAL PUBLICATIONS

I Yrjönen, T., Vovk, I., Simonovska, B., Mousa, O., Hiltunen, R., Vuorela, H. and Vuorela, P. (2003): Comparison of medium pressure solid-liquid extraction and rotation planar extraction of *Ficus* leaves with reference to optimum operating parameters. *J. Liq. Chromatogr. Relat. Technol.* 26: 3289-3305.

II Vovk, I., Simonovska, B., Andrenšek, S., Yrjönen, T., Vuorela, P. and Vuorela, H. (2003): Rotation planar extraction and medium-pressure solid-liquid extraction of onion (*Allium cepa*). *J. Planar Chromatogr.* 16: 66-70.

III Yrjönen, T., Vuorela, P., Klika, K., Pihlaja, K., Teeri, T.H. and Vuorela, H. (2002): Application of centrifugal force to the extraction and separation of parasorboside and gerberin from *Gerbera hybrida*. *Phytochem. Anal.* 13: 349-353.

IV Yrjönen, T., Haansuu, J.P., Haahtela, K., Vuorela, H. and Vuorela, P. (2001): Rapid screening of indole-3-acetic acid and other indole derivatives in bacterial culture broths by planar chromatography. *J. Planar Chromatogr.* 14: 47-52.

V Summanen, J., Yrjönen, T., Hiltunen, R. and Vuorela, H. (1998): Influence of densitometer and video-documentation settings in the detection of plant phenolics by TLC. *J. Planar Chromatogr.* 11: 421-427.

VI Yrjönen, T., Li, P., Summanen, J., Hopia, A. and Vuorela, H. (2003): Free radical-scavenging activity of phenolics by reversed-phase TLC. *J. Am. Oil Chem. Soc.* 80: 9-14.

These publications are referred to in the text by their Roman numerals.

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4. LIST OF ABBREVIATIONS

AMD	automated multiple development
APCI-MS	atmospheric pressure chemical ionization mass spectrometry
ASE	accelerated solvent extraction
CCD	charge-coupled device
CLC	column liquid chromatography
C-RPC	column rotation planar chromatography
DMSO	dimethylsulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EPC	electro-planar chromatography
FFSLE	forced-flow solid-liquid extraction
FFPC	forced-flow planar chromatography
FTIR	fourier-transform infrared spectroscopy
HPLC	high performance liquid chromatography
HPTLC	high performance thin-layer chromatography
IAA	indole-3-acetic acid
IR	infrared
LC	liquid chromatography
MPLC	medium pressure liquid chromatography
MPSLE	medium pressure solid-liquid extraction
M-RPC	microchamber rotation planar chromatography
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
NP	normal phase
N-RPC	normal chamber rotation planar chromatography
OPLC	overpressured layer chromatography
PEC	planar electrochromatography
PHWE	pressurized hot water extraction
PLE	pressurized liquid extraction
P_S	selectivity point
R_F	retardation factor
RPC	rotation planar chromatography
RPE	rotation planar extraction
RP	reversed-phase
SFE	supercritical fluid extraction
SLE	solid-liquid extraction
S/N	signal-to-noise ratio
S-RPC	sequential rotation planar chromatography
S_T	solvent strength
SWE	subcritical/superheated water extraction
TLC	thin-layer chromatography
TLE	thin-layer electrochromatography

U-RPC	ultra-microchamber rotation planar chromatography
UV	ultraviolet
UV/VIS	ultraviolet/visible

5. INTRODUCTION

Mankind has, throughout its existence, used plant material not only as a source of nutrition but also for numerous other purposes. The knowledge of the opportunities provided by the wealth of nature has become more widely understood as human cultures and civilizations have evolved. In addition to compounds that are necessary for the growth and reproduction of plants, i.e. carbohydrates, proteins and lipids, plant cells synthesize a tremendous number of so-called secondary metabolites, which do not appear to be strictly necessary for the survival of the plant. Often these secondary metabolites are produced as a response to external stimuli, e.g. infection or nutritional or climatic changes, and they may be accumulated in only certain parts of the plant (VERPOORTE 1999).

Indigenous cultures have learnt to exploit the properties of secondary metabolites in many ways, e.g. specific plants or parts of them have been used as poisons, analgesics, stimulants, preservatives, colorants, tanning agents for tanning leather etc. (DE PASQUALE 1984). As our understanding of chemistry and other natural sciences has increased, the active chemical compounds of these traditionally used plants have been successfully isolated and identified. Nowadays, instead of using e.g. pastes or crude extracts prepared from plant material, the tendency is to use pure compounds, irrespective of whether the intended use be analgesia or for coloring fabrics. Of course, there are also many exceptions to this tendency, e.g. refreshments such as coffee and tea are consumed because of their refreshing effect caused mainly by caffeine. Nevertheless, the general trend has been towards the use of pure, and often synthetic, compounds (GRABLEY and THIERICKE 2000, RASKIN et al. 2002).

The first step in the process of obtaining secondary metabolites from biogenic materials is to release them from the matrix by means of extraction (CANNELL 1998). Due to the often very complex composition of the material and the minute amounts of some of the constituents present, the choice of extraction method is of great importance. Obviously, an incorrect choice will cause the entire isolation process to fail if some or all of the desired components of the material cannot be released satisfactorily from the matrix.

The initial crude extract is usually a more or less complex mixture. Quite often there are certain target compounds or compound groups of interest. A logical next step in the isolation process is to separate the target compounds from the crude extract. This can be achieved e.g. by liquid-liquid partition or by some low-resolution chromatographic isolation. The aim of these steps is to concentrate the desired components and make the sample amenable to the final purification steps.

The third step in the isolation process usually involves some type of high-resolution method to separate the compounds of interest from the other compounds still remaining in the extract. As the undesired components of the mixture are likely to bear some resemblance to the target compounds, this stage usually involves optimization of the separation method to achieve sufficient resolution in the final preparative isolation. Often the final isolation step involves

liquid chromatography, especially HPLC or TLC, although other separation methods have been successfully applied (CANNELL 1998).

6. REVIEW OF THE LITERATURE

6.1. Extraction of plant material

Most of the bulk of the biomass, irrespective of whether it is plants or microbes, exists as fairly inert, insoluble, and often polymeric material, such as cellulose of plants or fungi and the microbial cell wall (CANNELL 1998). The first step of the extraction is therefore to release and solubilize the smaller secondary metabolites in the matrix, resulting in the initial extract.

In liquid extractions the choice of extraction solvent or solvents provides the first and most obvious means of sample preparation (HOSTETTMANN et al. 1998). Initial extraction with low-polarity solvents yields the more lipophilic components, while alcohols isolate a broader spectrum of apolar and polar compounds from the material. In addition to the choice of extraction solvent, there are also different approaches to the actual extraction procedure. While stirring or mechanical agitation are the most common methods, percolation or even pressurized solid-liquid extraction are possible. The most commonly used extraction methods are reviewed in the following chapter.

6.1.1. Selection of extraction method

The most widely used extraction processes have traditionally been based either on different liquid extraction methods or on vapor-phase extraction methods (STARMANS and NIJHUIS 1996). A more recent method whose application has steadily increased is supercritical fluid extraction (SFE), which is based on the properties of gases compressed and heated to a state above their critical pressure and temperature, at which no distinction between the gas and liquid phases can be discerned (TSERVISTAS et al. 2000).

At the present time, there are also a number of non-conventional extraction methods in use that are all, in principle, solid-liquid extractions (SLE) but which introduce some form of additional energy to the process in order to facilitate the transfer of analytes from sample to solvent. These methods include ultrasonic extraction, microwave-assisted extraction and pressurized liquid extraction (HUIE 2002, ZYGMUNT and NAMIESNIK 2003), as well as vortical (turbo) extraction. Even extraction by electrical energy has been studied (VINATORU 2001). Forced-flow solid-liquid extraction (FFSLE) techniques, such as medium-pressure solid-liquid extraction (MPSLE) and rotation planar extraction (RPE), are methods in which the extraction solvent is forced through the sample bed either by means of pressure or by centrifugal force, respectively, thus increasing the efficiency of the extraction process. (NYIREDY 2001a). The main advantage of these non-conventional methods compared to conventional SLE methods is the increased extraction efficiency, which leads to increased yields and/or shorter extraction times.

The simplest method of extraction, however, needs no extraction medium. Mechanical pressing has been traditionally applied to the extraction of oils from oilseeds (ABU-ARABI et al. 2000, VINATORU 2001). This process may be combined with some form of pretreatment such as cleaning, dehulling, crushing or flaking before the extraction but, in general, the only equipment needed is a hydraulic press. Despite the simple operating principle, there are several operating parameters that need to be controlled in order to obtain a sufficient extraction rate and yield. The most important parameters affecting the yield of the extraction procedure are the moisture content of the seeds and temperature (ZHENG et al. 2004).

Traditional extraction processes may be classified as follows: extraction with organic solvents: percolation, maceration, and extraction using a Soxhlet apparatus; and extraction with water: infusion, decoction, and steam distillation (SILVA et al. 1998). An old method also worth mentioning is extraction with cold fat, called enfleurage, used mainly for the extraction of fragrances from flowers (STARMANS and NIJHUIS 1996, VINATORU 2001).

Percolation is one of the most widespread methods employed in plant extraction since it does not require much sample manipulation or long pre-treatment times (SILVA et al. 1998). The only equipment required is a conical glass container with a tap at the base used to set the rate of solvent elution. Percolation is a continuous process in which the saturated solvent is constantly displaced by fresh solvent, but normally the sample is steeped in solvent in the percolator for 24 hours for up to three times, and the extracts are then collected and pooled.

In maceration the sample is placed in a stoppered container and is in contact with the solvent. This allows the solvent to penetrate into the cellular structure in order to dissolve the soluble compounds (SILVA et al. 1998). Its efficiency may be increased by occasionally shaking the container or by using a mechanical or magnetic stirrer to homogenize the final solution and saturate the solvent. As maceration is a discontinuous method, the solvent should be renewed until the plant material is exhausted; this requires filtration steps that may result in the loss of solvent, analytes, and/or plant material.

Soxhlet extraction is a very old, clean-up method, but it is still relatively widely used in plant analysis (ZYGMUNT and NAMIESNIK 2003). It is used mainly with one solvent at a time due to the fact that individual solvents may distill off at different temperatures, with the result that the mixture in the chamber containing the drug becomes enriched in the solvent of lower boiling point (SILVA et al. 1998). The main advantages of the Soxhlet technique are that it is an automatic and continuous method that does not require much manipulation. It has also been shown to be very effective in terms of extraction yield and therefore often used as a reference method for newer extraction methods. One disadvantage is that the extractives are heated during extraction at the boiling point of the solvent employed and thermally labile compounds may hydrolyze, decompose, or produce artifacts.

Infusion and decoction are simple methods for extraction with water (SILVA et al. 1998). In the infusion technique, boiling or cold water is added to the milled sample; in decoction the

sample is boiled for about 15 minutes in water. Extraction with pure water, however, is seldom used for plant material as hydrophilic compounds are usually extracted with methanol-water or ethanol-water mixtures.

Steam distillation is an old extraction method that is primarily used to obtain essential oils from plant material. In this method, a packed bed of plant material is continuously flushed with steam and the volatile organic compounds present in the material are taken up by the vapor phase due to their low partial vapor pressure (STARMANS and NIJHUIS 1996). Compounds carried by the vapor stream are then separated after decreasing the temperature of the vapor by forced condensation.

Ultrasonic extraction takes advantage of the very high effective temperatures (which increase solubility and diffusivity) and pressures (which favor penetration and transport) at the interphase between the solvent solution subjected to ultrasonic energy and a solid matrix, combined with the oxidative energy of radicals created during sonolysis, resulting in high extractive power (LUQUE-GARCIA and LUQUE DE CASTRO 2003). Ultrasonically assisted extraction methods have been employed for a great number of different plant materials, e.g. *Salvia officinalis* L., *Valeriana officinalis* L., *Calendula officinalis* L., *Gentiana lutea* L., *Hibiscus tiliaeus* L., and chrysanthemum flowers to name a few (SALISOVA et al. 1997, VINATORU et al. 1997, HROMADKOVA et al. 1999, OTTERBACH and WENCLAWIAK 1999, VALACHOVIC et al. 2001, VINATORU 2001, MELECCHI et al. 2002). Compound groups that have been obtained by ultrasonic extraction include polysaccharides, volatile oils, fatty acids and their esters, stigmasterol derivatives, and pyrethrins.

Another way of increasing the efficiency of conventional extraction methods is to use microwave irradiation. Microwave-assisted extraction consists of heating the solvent in contact with the sample by means of microwave energy. The process involves disruption of hydrogen bonds, as a result of microwave-induced dipole rotation of molecules, and migration of the ions, which enhance penetration of the solvent into the matrix, allowing dissolution of the components to be extracted (HUDAIB et al. 2003). The main advantages of microwave-assisted extraction over the conventional extraction techniques are reduced solvent consumption, shorter operational times, moderately high recoveries, good reproducibility and minimal sample manipulation for extraction process (GARCIA-AYUSO and LUQUE de CASTRO 1999, PAN et al. 2000, GARCIA-AYUSO and LUQUE DE CASTRO 2001, BRACHET et al. 2002, HUDAIB et al. 2003). Microwave-assisted extraction methods have been applied to the extraction of oil from olive seeds, pigments from paprika powders, glycyrrhizic acid from liquorice root, lipids from several oleaginous seeds, cocaine and benzoylecgonine from coca leaves, and alkamides from *Echinacea purpurea* L. roots (GARCIA-AYUSO and LUQUE de CASTRO 1999, CSIKTUSNADI KISS et al. 2000, PAN et al. 2000, GARCIA-AYUSO and LUQUE DE CASTRO 2001, BRACHET et al. 2002, HUDAIB et al. 2003).

Pressurized liquid extraction (PLE, also commonly known as accelerated solvent extraction; ASE) works according to the principle of static extraction with superheated liquids (BENTHIN et al. 1999). The method uses an organic solvent at high pressures and temperature above the boiling point (ONG et al. 2000). The main reasons for the enhanced performance of PLE are the higher solubility of analytes in solvent at higher temperatures, higher diffusion rate as a result of higher temperatures, and disruption of the strong solute-matrix interaction caused by van der Waals forces, hydrogen bonding and dipole-dipole attractions between solute molecules and active sites on the matrix. The PLE technique is well suited for the extraction of various types of compound from different plant materials because parameters other than temperature can be varied and the polarity of the extraction solvent can be chosen from a wide range and adapted to the respective matrix. PLE has been reported to have been applied to e.g. the extraction of dianthrons from *Hypericum perforatum* L., deacylsaponins from *Aesculus hippocastanum* L., silybin from *Silybum marianum* L., curcumin from *Curcuma xanthorrhiza*, thymol from *Thymus vulgaris* L., flavanones and xanthones from *Maclura pomifera*, aristolochic acids from *Radix aristolochiae*, berberine from *Coptidis rhizoma* and oxysterols from whole egg powder and egg-containing foods (BENTHIN et al. 1999, da COSTA et al. 1999, ONG et al. 2000, BOSELLI et al. 2001).

Subcritical water extraction (SWE, also called pressurized hot water extraction, PHWE, or superheated water extraction) is based on the unique solvent properties of water, namely its disproportionately high boiling point for its mass, a high dielectric constant and high polarity (SMITH 2002). The method involves heating water above its boiling point but below its critical point (i.e. 374°C) under elevated pressure so that the water remains in a liquid state. As the temperature rises there is a marked and systematic decrease in permittivity, an increase in the diffusion rate and a decrease in the viscosity and surface tension. SWE has been found to be an efficient extraction method and a potential alternative to steam distillation and solvent extraction in the extraction of essential oils from plant material. Satisfactory results have been reported for SWE of essential oils from marjoram (*Thymus mastichina*), clove (*Syzygium aromaticum*), fennel (*Foeniculum vulgare*) and sage (*Salvia officinalis*) (JIMENEZ-CARMONA et al. 1999, ROVIO et al. 1999, GAMIZ-GRACIA and LUQUE de CASTRO 2000, OLLANKETO et al. 2001). Besides essential oils, the method has also been applied to the extraction of lactones from kava root (*Piper methysticum*) and iridoid glycosides from *Veronica longifolia* leaves (SUOMI et al. 2000, KUBATOVA et al. 2001).

In recent years, the extraction method that has received increasing attention and many industrial applications in the isolation of natural products is supercritical fluid extraction (SFE). SFE has several advantages over the conventional liquid-liquid and solid-liquid extraction techniques, e.g. the elimination of most of the organic solvents that may pose a safety risk during extraction, elimination of carry-over of the more or less toxic solvents in the final extracts, and the possibility of avoiding the detrimental effects of these solvents on the environment (STUMPF et al. 1992, JARVIS and MORGAN 1997, HOSTETTMANN et al. 1998, TSERVISTAS et al. 2000, LANG and WAI 2001). The disadvantages of SFE include the low polarity of the most commonly used fluid, i.e. carbon dioxide, possible problems

caused by the presence of water, unpredictability of the matrix effect and the need for specialized/expensive equipment (VENKAT and KOTHANDARAMAN 1998).

A number of compounds have been tested as supercritical fluids, including pentane, nitrous oxide, ammonia and Freon® fluorocarbons but, on the grounds of cost and safety, carbon dioxide either alone or modified with methanol or some other polar solvent is by far the most widely used supercritical extraction solvent. It has also the advantage of a low critical temperature and pressure, which enable pressurization of the gas into a supercritical fluid. The practical aspects of SFE and its applications have been recently reviewed (LANG and WAI 2001). Due to the low polarity of carbon dioxide it is best suited for the extraction of non-polar compounds. SFE has been successfully applied to e.g. the extraction of essential oil from *Angelica archangelica* L. roots, as well as from *Matricaria chamomilla* flowerheads, and the extraction of lycopene from tomato skins (DONEANU and ANITESCU 1998, OLLANKETO et al. 2001). However, by varying the pressure and temperature of the supercritical carbon dioxide and the amount of polar modifiers, the method has also been found suitable for the extraction of more polar compounds such as flavanones and xanthones from *Maclura pomifera*, flavonoids from *Scutellaria baicalensis* roots and apigenin from *Matricaria chamomilla* (da COSTA et al. 1999, LIN et al. 1999, SCALIA et al. 1999). A more thorough review on the application of SFE to the isolation of plant products has been published by JARVIS and MORGAN (1997).

6.1.2. Selection of extraction solvent in solid-liquid extraction (SLE)

Although the choice of extraction method may have a significant effect on the quality of the extract, the solvent used provides the most obvious means of influencing the qualitative composition of the extract. Despite this, relatively little attention has been paid to the selection of an appropriate solvent or solvent system for solid-liquid extractions and the selection has been generally based on trial and error (NYIREDY 2000b).

When it comes to characterizing different solvents, the classification system of SNYDER (1978) forms the basis of most approaches to solvent selection. SNYDER calculated the "polarity" or chromatographic strength and selectivity of more than 80 solvents and, on the basis of these values, the solvents were classified into eight groups according to their selectivity, i.e. according to their properties as proton acceptors (x_e) and donors (x_d), and their dipole interactions (x_n). A solid-liquid extraction (SLE) strategy based on SNYDER's solvent characterization has recently been proposed (NYIREDY 2000b).

FITZPATRICK and DEAN (2002) outlined a method for the prediction of a suitable solvent for the extraction of pesticides. Their procedure is based on the Hildebrand solubility parameter (δ_t), which is a measure of the internal energy of cohesion in the solvent/solute and can be divided into three components, namely hydrogen-bonding ability, dispersion coefficient, and polarity contributions. This method differs fundamentally from that suggested by NYIREDY (2000b) as the analyte or analytes to be extracted should be known beforehand

in order to calculate the suitability of the possible extraction solvents, whereas the latter is actually a structured trial-and-error process requiring no prior knowledge of the compounds to be extracted.

The effect of the physical properties of the solvents on the extraction procedure has also been studied in the extraction of coumarins from *Angelica archangelica* L. roots by HÄRMÄLÄ et al. (1992c). The results suggested that the physical properties of the solvents, namely density, viscosity and surface tension, do not appear to make any major contribution to the extraction abilities of the solvents. Some of the chemical properties of the solvents, i.e. number of carbon atoms, molecular connectivity indices, selectivity interaction values and solvent strength, on the other hand seemed to be of some importance in the extraction procedure (HÄRMÄLÄ et al. 1992c).

Some of the general properties of the solvent that should be considered when selecting the most appropriate extraction solvent include the ability of the solvent to dissolve the compounds of interest, ease of removal, inertness, toxicity and flammability (SILVA et al. 1998). As expected, the matrix and the target compounds have perhaps the most significant effect on the selection of suitable extraction solvent. Low-polarity solvents yield more lipophilic compounds, whereas alcohols extract both apolar and polar compounds, and water extracts only polar components from the sample.

If the intention is to screen a very large number of natural products, the ease of subsequent treatment of the extracts becomes a significant factor. For instance, dimethylsulfoxide (DMSO) has a high boiling point 189°C and its evaporation is an extremely tedious and inconvenient task (ELOFF 1998). If the extracts are to be subjected to bioassays, the toxicity of the solvent may also be critical as the solvent, even in trace amounts, should not inhibit the bioassay procedure. Attention should also be paid to possible interactions between the solvent and solutes as the solvent may react with certain compounds to produce artifacts or cause decomposition, dehydration, or isomerization of these compounds. Examples include methylation or esterification of target compounds by methanol, the formation of acetonides by acetone, and epimerization and hydrolysis of glycosides in acidic conditions (SILVA et al. 1998).

Several criteria have been used in evaluating the effectiveness of the extraction method and the suitability of a solvent for a particular extraction procedure. The most commonly encountered criterion is extraction yield, i.e. the total yield or the yield of a certain target compound or compounds (e.g. HÄRMÄLÄ et al. 1992c, BOURGAUD et al. 1994, KALLITHRAKA et al. 1995, NYGAARD JOHANSEN et al. 1996, ELOFF 1998, REVILLA et al. 1998, ABBOTT et al. 1999, NYIREDY 2000b, FITZPATRICK and DEAN 2002, SHI et al. 2004). In taxonomic studies and general screening purposes, however, the qualitative composition of the extract may be the decisive factor over yield in selecting the solvent of choice. Yet another important and emerging criterion for solvent selection is based on bioassays, i.e. the extracts are subjected to biological tests including lower organisms, isolated

subcellular or cellular systems, isolated organs of vertebrates and whole animals (VLIETINCK 1999). Based on the responses in the bioassays, the solvent giving the highest recoveries is chosen and the extract is further purified to isolate the active component.

The term solvent extraction has traditionally been interpreted as meaning extraction using organic solvents, water or their mixtures. In addition, extractions can alternatively be carried out using water to which a surfactant has been added to improve the solubilization properties of pure water. This method, named micelle-mediated extraction, has been applied to e.g. the extraction of ginsenosides from American ginseng root and the extraction of tanshinones from *Salvia miltiorrhiza bunge* (FANG et al. 2000, SHI et al. 2004). The use of different terpenes (e.g. terpineol, D-limonene, α-pinene, β-pinene) and plant oils (e.g. rosemary oil, lavender oil) and their mixtures for the extraction of insecticidal and bactericidal compounds from plants of the *Chrysanthemum* and *Helianthus* families has also been suggested (PISACANE 2001). Extraction yield can also be increased by enzymatic pretreatment of the material, which causes degradation of the cell walls and thus improves the penetration of the solvent into the matrix (SANTAMARIA et al. 2000).

6.1.3. Medium pressure solid-liquid extraction (MPSLE)

Medium pressure solid-liquid extraction (MPSLE), introduced by NYIREDY et al. (1990a) as a new preparative separation method for laboratory purposes, is an extraction technique based on the principles of the diffusion-dissolving processes of parametric pumping. Changes in temperature, pressure, pH or electrical field, result in a reversible differential alteration of the distribution of components between the solid and the liquid phases. In MPSLE, the extraction column, i.e. a medium pressure liquid chromatographic (MPLC) column, is filled with fine-powdered plant material, and the extraction solvent is pumped through the stationary bed. This method constitutes the relative counter-current extraction, and results in exhaustive and rapid extraction. The method can be used for the rapid extraction of various substance classes occurring as complex solid matrices (HÄRMÄLÄ et al. 1992a, RAUHA et al. 2001). The exhaustive extraction of 100 - 3000 g of finely powdered plant material of can be performed with automated equipment within a few hours. The environmentally friendly process performed in a closed system enables operation with a relatively low volume of extractant. The same principals as in column chromatography, e.g. the geometry of the column, physico-chemical properties of the solvent, flow rate and amount of solvent, pressure, equilibrium time, sample particle size, compactness and amount of extracted material, are valid in MPSLE (NYIREDY et al. 1990a).

6.1.4. Rotation planar extraction (RPE)

Rotation planar extraction (RPE) is a forced-flow solid-liquid extraction (FFSLE) technique, in which the extraction solvent migrates mainly through the action of centrifugal force (MESZAROS et al. 1987, NYIREDY 2001a). A novel, multi-functional separation instrument prototype ExtraChrom® enables the rotation planar extraction of complex matrices because a

planar column can be attached to it and filled with the material to be extracted. Factors affecting the RPE process are basically the same as in MPSLE. Using the ExtraChrom® instrument, solid-liquid extraction and off-line analytical and micropreparative, as well as on-line preparative solid-liquid chromatographic methods, can be carried out with complex matrices (VOVK et al. 2003). The instrument enables extraction of materials of small particle size, resulting in particle-free extracts. The extraction takes place in a closed chamber, and it is possible to extract the material successively with solvents of different polarity (ANDRENSEK et al. 2004). In the implementation of solid-liquid extraction and extraction strategy as delineated by NYIREDY (2001c) and HÄRMÄLÄ et al. (1992a), the RPE method seems to be well suited for screening purposes when the number of biogenic samples is fairly high and 20-50 g of the material are to be extracted at a time. One disadvantage of ExtraChrom® compared to MPSLE extraction is that it lacks the possibility of scaling up the procedure, and therefore the two methods can be regarded as complementary.

6.2. Thin-layer chromatography (TLC)

6.2.1. Principles of TLC

Thin-layer chromatography is, in essence, liquid chromatography performed on a stationary phase present as a sheet or layer of solid particles immobilized on a planar support, or a layer of polymerized substance (GEISS 1987, BARISKA et al. 1999). Currently, the different variants of TLC form the basis of planar chromatographic methods and, since the 1950s, have almost entirely displaced their predecessor paper chromatography.

In contrast to e.g. column liquid chromatography (CLC), TLC is usually performed in a manually operated, and often to some extent uncontrollable, system. This creates additional challenges for the analyst as a larger number of parameters affect the separation compared to CLC. GEISS (1987) has listed a total of 26 parameters influencing the separation, the most important ones being: sorbent, solvent, chamber type, preadsorption of a solvent mixture, chamber and layer saturation, and particle diameter.

The most common sorbent of choice in TLC is still by far silica, whereas e.g. in HPLC analyses nonpolar reversed-phase (RP) sorbents have almost entirely replaced silica and other normal-phase (NP) sorbents. The mean particle size and particle size distribution of the silica gel used as adsorbent depends on the nature of the separation task: for high performance thin-layer chromatography (HPTLC) plates, the mean particle size is approximately 5 μm with a narrow particle size distribution, and for TLC it is approximately 12 μm and the particle size distribution is wider. The pore diameter for both is approximately 60 Å and the surface area approximately 500 m^2/g (GOCAN 2002).

Other relatively frequently used polar TLC sorbents include alumina, cellulose, chitin and chitosan, and polar chemically silica bonded phases such as aminopropyl, cyanopropyl and diol phases (GEISS 1987, GOCAN 2002). The latter three sorbents can also be used in RP

separations, although RP separations are most often carried out using chemically bonded non-polar phases, i.e. sorbents derivatized with various alkyl groups (e.g. C_2, C_8 and C_{18}). In the analysis of enantiomers, cellulose, modified cellulose, and cyclodextrin-modified stationary phases, among others, have been applied (GOCAN 2002).
By design, and being an off-line technique, there is practically no limitation in the composition of mobile phase that can be used (SHAH and REICH 1999). This means that the analyst does not need to consider e.g. the possibly detrimental effects of solvents on the detection method when selecting the optimum solvent combination, but can concentrate on maximizing the selectivity of the separation. The selection of a suitable stationary phase, vapor phase, and developing solvent combination is discussed in more detail in chapter 6.2.2.

Solvent flow in TLC is traditionally achieved as a result of capillary forces, i.e. weak forces arise from the decrease in free energy of the solvent as it enters the porous structure of the layer (POOLE 2003). This mode of action has several consequences for the separation process in TLC. First of all, in the case of fine particle layers capillary forces are unable to generate sufficient flow to minimize zone broadening. In addition to this, the mobile phase velocity varies as a function of time and elution distance, i.e. the mobile phase velocity declines as the solvent front migrates further, and the mobile phase velocity is determined by the system variables and is otherwise beyond experimental control. As a result of the inherent properties of the capillary flow, a constant and optimum mobile phase velocity cannot be achieved, thus leading to broad separated zones and limited separation capacity.

In order to overcome the deficiencies of capillary flow separations, a number of forced-flow planar chromatographic (FFPC) methods have been developed and these have been recently reviewed by NUROK (2000) and NYIREDY (2003). A common goal with all these methods is to achieve a sufficient mobile phase velocity, in other words to increase the velocity compared to capillary flow and, if possible, the velocity should be constant at this optimum.

The earliest of these FFPC methods is the application of centrifugal force, which was first mentioned by HOPF in 1947 (NYIREDY 2003). The term rotation planar chromatography (RPC) describes all such separations, both analytical and preparative, in which centrifugal force is used to drive the mobile phase through the stationary phase from the center to the periphery of the plate. It should, however, be noted that terminology in this area is not entirely consistent as the method is sometimes also called centrifugal TLC (HOSTETTMANN et al. 1980, STAHL and MULLER 1982, HOSTETTMANN et al. 1998, GUPTA et al. 2001). The fundamentals and applications of analytical and preparative RPC will be covered in chapter 6.3.

The FFPC technique that has perhaps received the most interest in recent years, and which has also found the greatest number of practical applications, is overpressured layer chromatography (OPLC). The method, introduced by TYIHAK et al. (1979), makes use of overpressure subjected on the TLC plate and a pump to deliver the mobile phase through the sorbent layer. In OPLC, the vapor phase is completely eliminated and, in that sense, it

resembles high performance liquid chromatography. The main advantage of OPLC over other modes of TLC is the possibility to achieve optimum mobile phase velocity over almost the whole separation distance without loss of resolution (NYIREDY 2001b).

OPLC can be used in various off-line and on-line modes, i.e. as a fully off-line process, with off-line sample application and on-line separation and detection, with on-line sample application and separation and off-line detection, and as a fully on-line process (NYIREDY 2001b). The fully on-line process is in fact a form of HPLC performed on a planar column. Special techniques such as multi-layer OPLC and long-distance OPLC have also been developed.

The suitability of OPLC is not limited to analytical separations only as it can also be applied to micropreparative and preparative separations either in an on-line or off-line mode, as reviewed by NYIREDY (2001b, 2001c). The separation of six to eight compounds in amounts up to 300 mg can be achieved using linear on-line OPLC on 20×20 cm preparative plates.

Planar electrochromatography (PEC), also sometimes called thin-layer electrochromatography (TLE) or electro-planar chromatography (EPC), constitutes another means of achieving a constant optimum mobile phase velocity and, additionally, a plug-like flow profile using electroosmotic flow (POOLE 1999, 2003, NUROK 2000, NYIREDY 2003). The first results on the application of electroosmotic flow in both column and planar chromatography were published in 1974 by PRETORIUS et al., but it took approximately 20 years before the next report on electrochromatography on planar layers was published by PUKL et al. (1994).

Since then, interest in PEC has gradually increased and some of the aspects of the theory and practical considerations of PEC have been studied in recent years (POOLE and WILSON 1997, NUROK et al. 1998, 2000, 2003, 2004, SHAFIK et al. 1999, HOWARD et al. 1999, MALINOWSKA 2000a, b). At present, however, no practical applications have been reported with the exception of separations of some simple model mixtures.

In addition to the previously mentioned forced-flow methods, instrumental methods have also been developed to control the environmental factors and enhance the separation efficiency in capillary TLC. Automated multiple development (AMD) is performed using a specially designed apparatus that permits stepwise gradient elution on a TLC plate. The method was developed in the mid-eighties and has some significant advantages over traditional capillary TLC (BURGER and TENGLER 1986, POOLE and BELAY 1991). First of all, the development is carried out in a controlled atmosphere thus enabling the achievement of more reproducible results. Secondly, as the plate is dried in a vacuum between successive runs and the developments are carried out under a nitrogen atmosphere, oxidation of the analytes can be avoided during the chromatographic separation. Moreover, as stated earlier, AMD permits gradient elution. The high separation performance of the method compared to traditional TLC and HPTLC, as well as the effect of the large number of parameters on the results, have been extensively studied by several research groups (e.g.

LODI et al. 1991, GOCAN et al. 1996, ESSIG and KOVAR 1997, SUMMANEN et al. 1998, SZABADY et al. 1999, POTHIER et al. 2001, GALAND et al. 2002).

6.2.2. Method development in TLC

Method development for TLC generally starts with the selection of the basic separation conditions. Besides the choice of stationary phase, the chosen developing technique, the size and type of developing chamber and the vapor space have a pronounced effect on the separation (GEISS 1987, NYIREDY 2002). In practice, however, only little attention is usually devoted to this initial, yet important step.

Unlike the case in HPLC, the most important stationary phase in TLC is by far silica and it is usually selected as the sorbent to start method development with. However, a general approach to stationary phase selection has also been proposed based on the properties of the sample components (POOLE and DIAS 2000). The various method development procedures for TLC presented in the literature usually involve only the optimization of the mobile phase using the previously chosen separation conditions. In contrast, the PRISMA optimization system also includes the selection of these basic parameters and appropriate development mode and operating conditions (NYIREDY 2002).

Most of the published literature on TLC method development focus on optimization of the composition of the mobile phase as it is the second most important factor after sorbent affecting the quality of the separation (GEISS 1987). Generally, the methods rely on empirical data and therefore some experimental runs are required.

The only recently proposed method that does not involve any preliminary experiments is the LSChrom software, which automatically calculates the recommended solvent strength of the mobile phase based on the functional groups of the analytes, and suggests several solvent combinations of this particular solvent strength (PALAMAREVA et al. 2003). It should, however, be noted that the proposed solvent combinations do not take into account the optimum separation of the analyte mixture, the software only suggesting mobile phases which ensure that the retention of all the analytes is in the range of $0 < R_F < 1$.

The retardation factor R_F describes numerically the position of the analyte on the TLC plate. It is the ratio of the distance travelled by the analyte in relation to the distance travelled by the solvent front on the plate, and is described by the following equation:

$$R_F = z_x/z_f - z_o$$

where z_x is the distance travelled by the analyte from the starting position, z_f is the distance travelled by the solvent front starting from the bottom of the plate, and z_o is the distance of the start position of the analyte from the bottom of the plate.

New or improved mobile phase optimization methods for TLC have frequently been proposed in the literature. Some of them are based e.g. on the adaptation of window diagrams (COSTANZO 1997), while others rely on mathematical models and numerical methods (CIMPOIU et al. 1999, MALES and MEDIC-SARIC 2001). Several extensive reviews discussing the different optimization methods and their benefits and drawbacks have been published in recent years (SIOUFFI 1991, REICH and GEORGE 1997, ROZYLO and SIEMBIDA 1997, POOLE and DIAS 2000, GOCAN and CIMPAN 2004).

Perhaps the most widely used optimization method in TLC today, however, is the PRISMA optimization system (NYIREDY et al. 1985, 1988, NYIREDY 2002). The system is a structured trial and error method consisting of three parts. In the first part the basic conditions, i.e. stationary phase, vapor phase and individual solvents for the optimization process, are selected. The second part is the optimization of the mobile phase composition using the previously selected solvents, and the third part involves the selection of development mode and chromatographic technique, and the transfer of the optimized TLC mobile phase to the various analytical and/or preparative planar and column liquid chromatographic techniques. A computer program employing the desirability function technique combined with the PRISMA model has also been developed (PELANDER et al. 1999).

6.2.3. Preparative TLC

Preparative TLC is one of the isolation methods requiring the least financial outlay and using the most basic equipment. The technique is suitable for the fractionation and/or isolation of up to 1000 mg of sample depending on the sample composition, layer thickness and chosen mode of action (NYIREDY 2000a). As in analytical TLC, preparative TLC can also be divided into classical capillary-driven preparative TLC and forced-flow preparative TLC, i.e. preparative OPLC and RPC methods.

The main differences between analytical and preparative TLC lie in the layer thickness and particle size of the stationary phase, and the amount of sample applied to the plate. The layers used in preparative planar chromatographic separations are generally 20 cm × 20 cm or 20 cm × 40 cm in size, with a sorbent thickness varying between 0.5 mm and 2 mm. Their particle size is coarse (approx. 25 μm) and particle size distribution wide, usually between 5 and 40 μm (HOSTETTMANN et al. 1998, NYIREDY 2000a). In micropreparative TLC separations, typically up to 10 mg of crude or purified plant extract can be applied to a single TLC plate, whereas preparative forced-flow planar separations allow the application of sample amounts of up to 1000 mg if the sample contains less than five substances.

For a successful preparative TLC separation, an optimized mobile phase is essential as the separation is generally inferior to analytical TLC. This is mainly caused by two factors: the often larger particle size and particle size distribution of the sorbent, and the overloading of the plate with the sample (NYIREDY 2000a). Overloading can be performed by increasing the sample volume (volume overloading) or by increasing the sample concentration

(concentration overloading), which is more advantageous if sample solubility allows it (WAKSMUNDZKA-HAJNOS and WAWRZYNOWICZ 2002). In the first case the eluted bands are significantly broadened, whereas in the latter case the bands become asymmetric.

The loading capacity of preparative layers increases with the square root of the thickness and therefore, as a rule of thumb, the loading capacity of a 2 mm layer is approximately twice that of a 0.5 mm layer (NYIREDY 2000a). It should, however, be noted that higher resolution can generally be achieved on thinner layers, while the resolution is more limited on thicker layers.

Of the various preparative TLC methods, traditional capillary TLC is suitable if there are no more than five compounds to be separated and they are distributed over the whole R_F range in fairly equal amounts. The total amount of sample should be less than 150 mg. Online OPLC can be used for the separation of five to seven compounds in amounts up to 300 mg, and the appropriate modes of RPC for the isolation of up to ten compounds in amounts up to 500 mg. The aspects of preparative RPC will be further reviewed in chapter 6.3.1.

6.2.4. TLC as a pilot method for RPC and MPLC

All TLC methods, with the exception of OPLC, differ from column liquid chromatography (CLC) in the sense that they are performed in non-equilibrated conditions; CLC is generally a system equilibrated with the mobile phase (NYIREDY et al. 1990b). Using a multi-component mobile phase system in a non-equilibrated system results in the formation of multiple fronts and this phenomenon is called solvent demixing (GEISS 1987). In practice, this means that the TLC system is generally equilibrated only close to the solvent reservoir, i.e. below $R_F = 0.3$.

Transfer of an optimized TLC mobile phase to different modes of RPC is fairly straightforward as long as attention is paid to the saturation grade of the TLC chamber during mobile phase optimization (NYIREDY et al. 1992, NYIREDY 2002). If the TLC method was optimized using nonsaturated chromatographic chambers, the mobile phase can be directly transferred to ultra-microchamber rotation planar chromatography (U-RPC) and column rotation planar chromatography (C-RPC) without altering its composition. The method can also be directly transferred from analytical U-RPC to preparative U-RPC with the same mobile phase. If the TLC mobile phase was optimized in saturated chambers, the microchamber rotation planar chromatography (M-RPC) technique can be used without altering the mobile phase, whether it be for analytical or preparative separations. The properties of the different modes of RPC are described in chapter 6.3.

Unlike the case with RPC, the transfer of an optimized TLC mobile phase to CLC generally involves more possible pitfalls. Solvent demixing poses the greatest difficulty and may lead to entirely different selectivity in column chromatography, even though the solvent systems used are identical. Other factors that may affect the separation include the quality of the sorbent, especially the binders present in TLC layers, but also the different degree of coverage of

bonded phases, and the presence of water on the surface of the layer and in the solvent system (GEISS 1987).

Some reports have been published demonstrating similar retention behavior of closely related coumarins in NP-TLC, NP-OPLC and NP-HPLC, thus suggesting that in these cases NP-TLC is suitable as a preassay for NP-HPLC and can be used to optimize and predict the separation in CLC (HÄRMÄLÄ et al. 1992b, VUORELA et al. 1994). VALKO et al. (1990) studied the retention behavior of some benzodiazepine derivatives by RP-TLC and RP-HPLC and came to the conclusion that RP-TLC can be used for predicting the RP-HPLC retention behavior of these compounds, although the predictive power of TLC was found to be limited. A clear correlation has also been reported for the retention behavior of deoxyuridine derivatives on alumina TLC layers and HPLC using an aluminum column, both in reversed phase and in normal phase modes with different eluents (VALKO et al. 1991).

As the direct transfer of a mobile phase from TLC to various CLC methods, including MPLC, can be generally expected to produce similar separation only when neat solvents are used or the TLC separation is achieved below $R_F = 0.3$, the use of OPLC as a bridge between TLC and CLC (e.g. MPLC and HPLC) has been suggested (NYIREDY et al. 1990b, NYIREDY 2001c). According to this strategy, the optimized TLC mobile phase is transferred either directly after a prerun, or after reduction of the solvent strength to analytical OPLC. Based on the results of analytical OPLC, the MPLC separation may be carried out starting with a dry column, equilibrating it with the optimized TLC/OPLC mobile phase, or with the solvent used for the prerun in analytical OPLC. The major advantage of this mobile phase transfer strategy is that the whole R_F range can be used during the optimization of the TLC separation, and the prediction of the MPLC separation can also be improved.

6.3. Rotation planar chromatography (RPC)

Rotation planar chromatography is a forced-flow planar chromatographic technique utilizing, in addition to capillary action, centrifugal force to drive the mobile phase through the stationary phase from the center to the periphery of the plate (NYIREDY et al. 1989, ERDELMEIER and KÖNIG 1991, MAZUREK and WITKIEWICZ 1998, NYIREDY 2003). The technique can be applied in its various forms to analytical, off-line micro-preparative and on-line preparative separations.

The size of the vapor space is an essential criterion in RPC methods and, based on this, the methods are classified into four basic techniques, namely normal chamber RPC (N-RPC), microchamber RPC (M-RPC), ultra-microchamber RPC (U-RPC), and column RPC (C-RPC). Sequential RPC (S-RPC) is a special technique in which circular and anticircular development modes are carried out sequentially in a normal chamber.

In N-RPC the layer rotates in a stationary chromatographic chamber, whereas in M-RPC a co-rotating chromatographic chamber is used and the vapor space reduced. When using U-RPC,

a quartz glass cover plate is placed directly on top of the layer which almost completely eliminates the vapor space. All these methods are suitable for preparative separations, and M-RPC and U-RPC can also be applied for analytical purposes.

C-RPC differs from the previous three methods in that the stationary phase is placed in a closed circular chamber (planar column) and hence there is no vapor space. Due to the special geometric design of the column, the volume of the stationary phase remains constant along the entire separation distance and the flow is accelerated linearly as in column chromatography (NYIREDY et al. 1986). The primary advantage of this design is the elimination of the extreme band broadening normally observed in all circular development techniques. As a result of its operating principle C-RPC is only used for preparative separations.

For difficult separations the S-RPC technique utilizing a combination of circular and anticircular development can be used. Since the mobile phase can be introduced onto the plate at any desired point, it is possible to start the separation as in N-RPC in the circular mode, and then use the anticircular mode for pushing the substance zones back towards the center of the plate with a strong solvent ready to be re-separated again after drying the plate. In this technique the separation pathway becomes theoretically unlimited.

Although several prototype instruments for RPC had been developed since the introduction of the method by HOPF in 1947, it was not until the introduction of two commercially available instruments, the Chromatotron (HARRISON 1977) and the Hitachi CLC-5 Centrifugal Chromatograph, that RPC really became of more general interest (HOSTETTMANN et al. 1998). Since then, two other commercial instruments, Rotachrom® and Cyclograph™, have also been introduced for RPC, as well as the versatile prototype instrument ExtraChrom® for RPC and RPE (MESZAROS et al. 1987, NYIREDY et al. 1989, GUPTA et al. 2001, NYIREDY 2001a). Of the previously mentioned instruments, Chromatotron, Hitachi CLC-5 and Cyclograph can only be used for preparative separations. In contrast, Rotachrom® and ExtraChrom® are suitable for both analytical and preparative purposes. At present, despite some of its shortcomings, Chromatotron seems to be the instrument that has found the most practical applications both as an intermediate purification step in the isolation of various natural products and also in the isolation of pure substances (HOSTETTMANN et al. 1998).

6.3.1. Preparative RPC

RPC is mainly used for preparative separations and all modes of RPC are well suited for this purpose (NYIREDY et al. 1989, MAZUREK and WITKIEWICZ 1998). In the early 1980's most publications dealt with the applications of Chromatotron as a tool for preparative separations. HOSTETTMANN et al. (1980) evaluated Chromatotron for its suitability to achieve rapid preparative separations of various classes of natural products, and came to the conclusion that it is a simple, rapid and economical method for the purpose. However, they found that the resolution was limited and the choice of stationary phases restricted. STAHL

and MULLER (1982) investigated the effect of various flow rates and rotational speeds on the separation performance of Chromatotron. According to their results, maximum resolution was achieved at medium speeds and flow rates and they found the method to be superior to conventional preparative TLC. The construction of the solvent collection system, however, was found to be inadequate as the zones which had already been separated in the layer were partially remixed in the collection system. In a comparison of Chromatotron and preparative liquid column chromatography, MAITRE et al. (1986) found that the resolving power of Chromatotron was superior to that of preparative LC and they concluded that centrifugal TLC was a a rapid, efficient and cost-effective technique for the decigram-scale separation of diastereomers. For larger scale separations preparative LC had advantages. RODRIGO et al. (1999) reported the use of Chromatotron in the preparative separation of a benzothiazinone from other reaction products and found it to be a simple, effective and inexpensive means of separating reaction products in a mixture when other methods fail. Recently, PINTO et al. (2000) reported the successful isolation of peridinin and β-carotene from the marine alga *Gonyaulax polyedra* in a one-step purification protocol using Chromatotron. The published applications of RPC in natural product isolation have been reviewed by e.g. HOSTETTMANN et al. (1998) and MAZUREK and WITKIEWICZ (1998).

The construction and operating modes of Rotachrom® model P rotation planar chromatograph, as well as some of its preparative applications, have been extensively reviewed by NYIREDY et al. (1989). The instrument had been successfully applied to the micro-preparative separation of coumarins and saponin glycosides, as well as for the preparative separation of a test dye mixture and coumarin-containing plant extract. The methods investigated included M-RPC, U-RPC and C-RPC. The versatility of the instrument was seen as one of its main advantages, whereas the main disadvantage was considered to be the fact that some experience is necessary for a successful separation process. Since sample application is one of the most critical steps in planar chromatography, a solid phase sample application method especially suitable for C-RPC was proposed by BOTZ et al. (1990a). The performance of the method was investigated in C-RPC separation of a furocoumarin-containing plant extract and was shown to enable the application of a large amount of sample with better resolution.

At the present time, relatively few results have been published on the Cyclograph Centrifugal Chromatography System. The instrument has been applied to the fractionation of moderate molecular weight polysiloxanes by GUPTA et al. (2001). The separation time was typically 30 – 60 min and the solvent consumption 600 – 900 ml. The method was found effective for the fractionation of 1 – 2 g of polysiloxanes with a molecular weight less than 50 000 Dalton. The on-line coupling of Cyclograph with athmospheric pressure chemical ionization mass spectrometry (APCI-MS) as a detection method has been recently studied (VAN BERKEL et al. 2004). Using this arrangement, the eluting components were successfully detected and characterized by the mass spectrometer in parallel with the fraction collection process.

In this thesis the performance of the new prototype separation instrument ExtraChrom® in the isolation of constituents of various natural products was investigated. In addition to the publications included in this study, the suitability of ExtraChrom® has been studied in the isolation of antimicrobial and antioxidative compounds from oak bark (VOVK et al. 2003, ANDRENSEK et al. 2004). C-RPC fractionation of 840 mg of crude oak bark extract yielded 6.7 mg of pure (+)-catechin in one run. The advantages of the method were considered to be the easy and rapid filling of the planar column and the possibility to use adsorbent material of small particle size.

6.3.2. Analytical RPC

The analytical applications of RPC are not as numerous as preparative ones, although some results have been published on the topic. U-RPC separation of iridoid glycosides has been compared to TLC, HPTLC and OPLC, and the resolution was found to be better than with TLC and HPTLC methods (DALLENBACH-TOELKE et al. 1987). The best resolution was obtained using linear OPLC, but the U-RPC method was preferred when a large number of samples had to be analyzed. VUORELA et al. (1988a) achieved a good separation of six main coumarins isolated from the roots of *Peucedanum palustre* (L.) Moench using U-RPC and concluded that radial U-RPC yielded better results than linear elution. In the field of enantiomeric separations, U-RPC has been found suitable for the quantitative analysis of glycyl-*D*, *L*-valine and *D*, *L*-α-methylserine on chiral plates (NYIREDY et al. 1989). When comparing HPTLC, OPLC and U-RPC in the separation of ergot alkaloids, BOTZ et al. (1990b) found U-RPC to be the most favorable method because, due to the presence of a certain amount of vapor space, the multi-front effect does not occur. BOTZ and colleagues also investigated the applicability of Empore TLC sheets in forced-flow chromatography (1990c). The sheets, which are prepared from silica entrapped in a an inert matrix of polytetrafluoroethylene microfibrils, were found to be unsuitable for M-RPC because of their distortion due to the applied centrifugal force. However, when U-RPC was used it was possible to achieve the optimum flow rate, thus leading to rapid separations with good resolutions. In the analysis of oak bark extract using the ExtraChrom® instrument in U-RPC mode, (+)-catechin and (–)-epicatechin have been successfully separated on a cellulose layer using water as developing solvent (VOVK et al. 2003). The most significant advantages of the method were concluded to be the possibility to use normal, commercially available TLC plates, the adjustable vapor phase, and the possibility of mobile phase optimization using conventional capillary-driven TLC.

6.4. Medium pressure liquid chromatography (MPLC)

MPLC is one variant of pressure liquid chromatography as opposed to conventional gravity-driven column chromatography (HOSTETTMANN et al. 1998). The application of pressure to force the mobile phase through the column has two effects: first of all it increases the flow rate of the eluent leading to faster separations, and secondly packing material of finer particle size can be used, thus giving higher resolution. The different preparative pressure liquid

chromatographic methods are usually classified in four categories according to the pressure employed for the separation, namely flash chromatography (pressure approx. 2 bar), low-pressure liquid chromatography (< 5 bar), medium-pressure liquid chromatography (5 – 20 bar), and high-pressure (high-performance) liquid chromatography (> 20 bar). It should, however, be noted that considerable overlap exists between the last three classes.

The construction of an MPLC system is fairly straightforward and it can be assembled using various commercially available components, such as pumps, columns, detectors and fraction collectors, although complete MPLC systems are also commercially available from e.g. Büchi and Labomatic (HOSTETTMANN et al. 1998). The construction and assembly of an MPLC system have been discussed e.g. by LEUTERT and VON ARX (1984) and HWU et al. (1987). In contrast to contemporary HPLC, MPLC columns are filled by the user. This can be performed either using a slurry-packing or dry-filling technique, but for bonded phases the slurry method is always used. It has been shown that a higher packing density can be achieved by dry-filling methods compared to slurry packing, and dry-filling is also suitable for silica of 15 μm average particle size (ZOGG et al. 1989a).

Besides the column filling method and particle size of the sorbent, other parameters affecting MPLC separation are the solvent strength, sample load, and column dimensions. Generally, reduction of solvent strength will increase resolution, although at the cost of longer separation time (ZOGG et al. 1989b). It was, however, noted that when *n*-hexane was used to reduce solvent strength, the viscosity of the mobile phase decreased enabling higher flow rates and thus compensating for the slower migration of the sample components in the column. Sample loads of 3 – 10 mg sample/g support were found applicable for the isolation of even closely related compounds. The use of a long column with small internal diameter rather than a shorter column with the same amount of stationary phase and a larger internal diameter was shown to significantly increase resolution.

MPLC has been successfully applied to numerous preparative isolations in the field of natural product chemistry. The method has been used e.g. for the isolation of several coumarins in pure form from the roots of *Peucedanum palustre* (VUORELA et al. 1988b), *Heracleum sphondylium* (ZOGG et al. 1989c), and *Angelica archangelica* (HÄRMÄLÄ et al. 1992a). Other examples of successful MPLC separations include the isolation of flavonoid glycosides from *Calluna vulgaris* (ALLAIS et al. 1995), glycoalkaloids from *Solanum tuberosum* (SOULE et al. 1997), and sesquiterpenes from *Petasites hybridus* (SIEGENTHALER and NEUENSCHWANDER 1997). A more comprehensive review of the applications of MPLC in natural product isolation has been compiled by HOSTETTMANN et al. (1998).

6.5. Detection methods

In TLC and other modes of planar chromatography the chromatographic separation and detection of the analytes are in most cases performed separately and the processes are independent of each other (JORK et al. 1990, SOMSEN 1995). Exceptions to this general rule

include on-line OPLC and C-RPC, which may be combined with on-line detection methods (NYIREDY 2003). As most forms of TLC are performed off-line, the analyst has more flexibility in the detection of analytes on the TLC plate compared to column liquid chromatography. Since detection takes place in the absence of a mobile phase, possible detrimental effects of the solvent system can be avoided. Detection is also free of time constraints and allows the use of several sequential detection techniques, of course provided that they are nondestructive (POOLE 1999, SHAH and REICH 1999).

Detection methods can be divided into three main categories, namely physical methods (e.g. UV/VIS, IR), microchemical methods (pre- or postchromatographic derivatization with universal or specific reagents), and biological-physiological methods, which are based on the biological activity of the separated components independent of their physical or chemical properties (JORK et al. 1990).

The most commonly employed physical detection methods are based on the absorption or emission of electromagnetic radiation, which is detected by suitable detectors (JORK et al. 1990). If the analyte absorbs light in the visible wavelength range, i.e. the compound is colored, it can also be detected visually by the analyst. In other cases, e.g. in the UV, IR and radiofrequency range, specific detectors have to be employed. As these detection methods are generally nondestructive, several of them can be used sequentially and they can be followed by microchemical and/or biological-physiological detection methods. Currently, detection methods such as mass spectrometry, MS (see e.g. WILSON 1999), fourier-transform infrared spectroscopy, FTIR (e.g. STAHLMANN 1999), and radioactivity detection (e.g. MOROVJAN et al. 2002) have found numerous applications in the field of TLC, and even the suitability of nuclear magnetic resonance spectroscopy (NMR) as a detection method for TLC has been investigated (WILSON et al. 1997). Several extensive reviews have been published on the combination of TLC and the various hyphenated techniques (e.g. SOMSEN et al. 1995, CSERHATI and FORGACS 1997, 1998, POOLE 1999). Table 1 summarizes some of the physical detection techniques that can be combined with TLC.

Table 1 Detection techniques used in TLC.

Detection technique	Principal features of the technique
UV-VIS / densitometry	Free choice of excitation and detection wavelength, possibility to capture UV-VIS spectra of the analytes directly on the plate, fluorescence detection possible, sequential detection of the analytes
UV-VIS / video densitometry	Limited number of detection wavelengths, simultaneous detection of all the analytes on the plate, enables a more convenient analysis of two-dimensional chromatograms
MS	Several ionization methods available, provides structural information about the analytes
FTIR	Can be performed directly on the plate or after elution of the analyte from the sorbent, provides structural information about the analytes
Raman spectroscopy	Sensitivity can be considerably increased by treating the plates with silver solution (surface-enhanced resonance Raman spectroscopy), provides structural information about the analytes
Radioactivity-based methods	Applied both for on-line and off-line detection, several methods available, mostly used for metabolic studies
NMR	Cannot be performed directly on the TLC plate, provides structural information about the analytes
FID (flame ionization detection)	Separation and detection are performed on a specific sorbent-coated quartz rod, suitable for compounds lacking chromophores
SWASV (square-wave stripping voltammetry)	Electrochemical detection technique, detection directly on the plate, sensitive
Photothermal methods	Several methods available, also provides information about the analytes distributed vertically inside the depth of the layer
AAS (atomic absorption spectroscopy)	Analytes are eluted from the layer before detection, used for the determination of metals

The dominant method of documenting the separation on the TLC plate in the UV-visible range is slit-scanning densitometry (POOLE 2003). Current instruments do not only enable the qualitative detection of the separated substances, but also the characterization of the substance by recording the spectrum and quantitative determinations. The technology is relatively mature and major changes in operation and performance of slit-scanning densitometry seem unlikely.

In recent years image analysis, also known as video densitometry, has received considerable attention as a detection method complementary to traditional slit-scanning densitometry. In video densitometry, optical scanning takes place electronically, using a computer with video digitizer, light source, monochromators and appropriate optics to illuminate the plate and focus the image onto a charge-coupled device (CCD) video camera (POOLE 2003). The main attractions of video densitometry are fast and simultaneous data acquisition from the whole plate, a simple instrument design without moving parts, increase in sensitivity by using longer acquisition times, and compatibility with data analysis of two-dimensional chromatograms. At present, however, video densitometry cannot compete with slit-scanning densitometry in terms of sensitivity, resolution and available wavelength measuring range. Because the technology in video densitometry is still rapidly evolving, research in the field has been very active in recent years (see e.g. VOVK and PROSEK 1997, VOVK et al. 1997, ESSIG and KOVAR 1999, PETROVIC et al. 1999, 2000, EBEL and HENKEL 2000, CAMPBELL and SHERMA 2003, HOPKALA et al. 2003).

In microchemical detection methods the TLC plate is sprayed with or dipped into a suitable reagent to form absorbing or fluorescent derivatives with the analytes which can then be detected. Derivatization can take place either before or after the TLC separation, and the terms pre- and postchromatographic derivatization are used to separate these two options. A great number of different universal and specific, i.e. group-characterizing reagents which react with a specific functional group, have been developed and new variants continue to be published (e.g. STAHL 1967, JORK et al. 1990, 1994, TOUCHSTONE 1992).

Biological detection of separated substances on the TLC plate in situ has been applied to the screening of various extracts for e.g. antimicrobial and antioxidant activity or general toxicity (EBERZ et al. 1996, WEINS and JORK 1996, HOSTETTMANN et al. 1997). These methods are especially suitable for screening purposes as the fractions or compounds with most pronounced activity can be detected directly on the TLC plate and subjected to further purification and/or identification steps.

7. AIMS OF THE STUDY

The general aim of the study was to investigate the applicability of different modes of planar chromatography in the isolation and analysis of complex mixtures of natural origin.

The specific aims were:

- to study the effect of some operating parameters of MPSLE and RPE extraction methods, and to compare the performance of the methods (**I, II, III**)

- to develop an isolation method for 2-pyrone derivatives from *Gerbera hybrida* (**III**)

- to develop a planar chromatographic screening method for indole derivatives (**IV**)

- to evaluate the performance of a video scanner and traditional densitometer in the detection of plant phenolics (**V**)

- to establish a rapid assay for the determination of the free radical scavenging activity of phenolic compounds using planar chromatography (**VI**)

8. EXPERIMENTAL

A detailed presentation of the materials and methods can be found in the original publications.

8.1. Materials

8.1.1. Standard compounds

The standard compounds used in the individual studies are presented in Table 2. The indole derivatives (**IV**) were used as 0.5 mg/ml solutions in methanol. The phenolic acids (**V**) and flavonoids (**V**) were used as 0.5 mg/ml solutions in methanol, except for (±)-catechin (**V**) which was used as a 1.0 mg/ml solution and ellagic acid (**V**) which was used as an 0.2 mg/ml solution, both in methanol. Of the three coumarins studied, herniarin (**V**) was used as a 50 μg/ml solution, scopoletin (**V**) as a 25 μg/ml solution and umbelliferone (**V**) as a 10 μg/ml solution in methanol. For the measurement of the free radical scavenging activity the standard solutions of the phenolic compounds were prepared to a concentration of 20 mM in methanol, except for ellagic acid which was dissolved in DMSO (**VI**).

8.1.2. Plant material

Ficus sycomorus L. and *F. bengalensis* L. were grown in the fields of the Faculty of Agriculture, Cairo University, Cairo, Egypt. The material was identified by B. El-Dewan (senior research scientist, Ministry of Agriculture, Cairo, Egypt). The leaves were air-dried and kept in a paper sack in a dry, dark and cool place and then transferred to the Division of Pharmacognosy, Department of Pharmacy, University of Helsinki (**I**). The ground and air-dried onion, *Allium cepa* L., was imported from California, USA by Paulig Group Ltd., Helsinki (**II**). Floral stems and leaves of *Gerbera hybrida* were collected from a greenhouse in Viikki, Helsinki. The plant material was freeze-dried and stored in plastic bags at -20ºC. The material was ground and sieved prior to extraction (**III**). Leaves of *Phyllanthus emblica* L. were collected from a wild tree close to the beach of Teluk Bahang, Penang Island, Malaysia. The plant was identified by Dr. Mashhor Mansor (School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia), and a voucher specimen was deposited in the herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The leaves with their petioles were oven dried at 40°C for 48 hours and kept in paper sacks in dry and dark conditions at room temperature until used (**V**). The rapeseed meal used in the radical scavenging activity tests was obtained from the companies Mildola Ltd. and Raisio Group Ltd (**VI**).

Table 2 Sources of the standard compounds used in this study.

Compound	Source
2-Pyrones	
5,6-dihydro-6-methyl-4-hydroxy-2-pyrone	Aldrich Chemical Co., WI, USA
Indole derivatives	
Indole-3-acetic acid	Sigma Chemical Co., MO, USA
Indole-3-acetaldehyde	Sigma Chemical Co., MO, USA
Indole-3-acetamide	Sigma Chemical Co., MO, USA
Indole-3-acetone	Sigma Chemical Co., MO, USA
Indole-3-aldehyde	Sigma Chemical Co., MO, USA
Indole-3-acetonitrile	Sigma Chemical Co., MO, USA
Indole-3-butyric acid	Sigma Chemical Co., MO, USA
Indole-3-carboxylic acid	Sigma Chemical Co., MO, USA
Indole-3-lactic acid	Sigma Chemical Co., MO, USA
Indole-3-methanol	Sigma Chemical Co., MO, USA
Indole-3-propionic acid	Sigma Chemical Co., MO, USA
Indole-3-pyruvic acid	Sigma Chemical Co., MO, USA
Indole-3-ethanol	Sigma Chemical Co., MO, USA
5-Hydroxy-indole-3-acetic acid	Sigma Chemical Co., MO, USA
5-Hydroxy-indole-3-acetamide	Sigma Chemical Co., MO, USA
Tryptamine	Sigma Chemical Co., MO, USA
L-Tryptophan	Fluka Chemie AG, Switzerland
3-Hydroxyanthranilic acid	Sigma Chemical Co., MO, USA
Flavonoids	
(±)-Catechin	Carl Roth GmbH & Co, Germany
Kaempferol	Sigma Chemical Co., MO, USA
Rutin	Sigma Chemical Co., MO, USA
Quercetin	Sigma Chemical Co., MO, USA
Phenolic acids and derivatives	
Caffeic acid	Extrasynthese S.A., France
Chlorogenic acid	Sigma Chemical Co., MO, USA
3-Coumaric acid	Extrasynthese S.A., France
p-Coumaric acid	Sigma Chemical Co., MO, USA
2,3-Dihydroxybenzoic acid	Extrasynthese S.A., France
Ellagic acid	Carl Roth GmbH & Co, Germany; Sigma Chemical Co., MO, USA
Ferulic acid	Extrasynthese S.A., France
Gallic acid	Carl Roth GmbH & Co, Germany; Sigma Chemical Co., MO, USA
3-Hydroxybenzoic acid	Extrasynthese S.A., France
Methyl gallate	Carl Roth GmbH & Co, Germany
n-Propyl gallate	Sigma Chemical Co., MO, USA
Protocatechuic acid	Sigma Chemical Co., MO, USA
Sinapic acid	Extrasynthese S.A., France
Syringic acid	Sigma Chemical Co., MO, USA
Vanillic acid	Extrasynthese S.A., France
Coumarins	
Herniarin	Sigma Chemical Co., MO, USA
Scopoletin	Sigma Chemical Co., MO, USA
Umbelliferone	Sigma Chemical Co., MO, USA
Miscellaneous compounds	
L-Ascorbic acid	Aldrich Chemical Co., WI, USA
α-Tocopherol	Merck, Germany

8.1.3. Instrumentation

The instrumentation used in the respective studies is presented in Table 3.

Table 3 Instrumentation used in this study.

Method	Instrument	Manufacturer	Publication
MPSLE	Waters A-45 HPLC pump	Waters, Milford, MA, USA	**I**
	Waters M-600E Chromatography pump	Waters, Milford, MA, USA	**I, II**
	Waters M-6000A Chromatography pump	Waters, Milford, MA, USA	**I, III**
	LKB UV detector	LKB Ltd., Sweden	**I**
	Shimadzu C-R1B integrator	Shimadzu Corp., Japan	**I**
RPE	ExtraChrom® separation instrument	—*	**I, II, III, IV**
MPLC	Waters M-6000A Chromatography pump	Waters, Milford, MA, USA	**III**
	Hitachi L-5200 fraction collector	Hitachi Corp., Tokyo, Japan	**III**
RPC	ExtraChrom® separation instrument	—*	**III, IV**
	Hitachi L-5200 fraction collector	Hitachi Corp., Tokyo, Japan	**III**
TLC	Linomat IV TLC applicator	Camag, Muttenz, Switzerland	**I, II, III, IV, V, VI**
	Chromatogram Immersion Device II	Camag, Muttenz, Switzerland	**V**
	Chromatogram Immersion Device III	Camag, Muttenz, Switzerland	**III, VI**
	Shimadzu CS-9001 PC scanner	Shimadzu Corp., Japan	**I, V**
	Desaga CD 60 densitometer	Desaga, Wiesloch, Germany	**I, IV**
	Camag Video Documentation System	Camag, Muttenz, Switzerland	**II, III, IV, V, VI**
Spectrophotometry	Perkin-Elmer Lambda 11 UV/VIS spectrophotometer	Perkin-Elmer, Germany	**VI**

* A prototype, not commercially available.

8.1.4 TLC plates

The TLC plates used in the respective studies were:

Silica gel 60 F_{254} TLC plate (Merck, Darmstadt, Germany; Art. No. 1.05715) (**I, III, IV**)
Silica gel 60 F_{254} TLC plate (Merck, Darmstadt, Germany; Art. No. 5554) (**II, III, V**)
Silica gel 60 F_{254} HPTLC plate (Merck, Darmstadt, Germany; Art. No. 5642) (**II, V**)
RP-18 F_{254s} TLC plate (Merck, Darmstadt, Germany; Art. No. 5559) (**V, VI**)

8.1.5. Columns and sorbents

The columns and sorbents used in the extraction and fractionation studies were:

Büchi borosilicate column No. 19674 (230 mm × 26 mm i.d., Büchi Laboratoriums-Technik AG, Flawil, Switzerland) (**I, III**).
Büchi borosilicate column No. 17982 (460 mm × 26 mm i.d., Büchi Laboratoriums-Technik AG, Flawil, Switzerland) (**I**).
Büchi borosilicate column No. 17981 (460 mm × 36 mm i.d., Büchi Laboratoriums-Technik AG, Flawil, Switzerland) (**I**).
Büchi borosilicate column No. 17980 (460 mm × 49 mm i.d., Büchi Laboratoriums-Technik AG, Flawil, Switzerland) (**I**).
Büchi borosilicate column No. 19675 (230 mm × 36 mm i.d., Büchi Laboratoriums-Technik AG, Flawil, Switzerland) (**II**).

Silica gel 60 HF_{254}, mean particle size 15 μm (Merck, Darmstadt, Germany; Art. No. 17739) (**III**).

8.2. Methods

8.2.1. Extraction of plant material by RPE and MPSLE (**I, II, III**)

In the RPE method the planar chamber of the ExtraChrom® separation instrument prototype was filled with plant material using a rotational speed of 1700 rpm. The plant material was wetted with a sufficient volume of extraction solvent and left to equilibrate for the desired time. After the equilibrium time the extracts were collected using a sufficient rotational speed and the procedure repeated up to five times if needed. The extracts were evaporated to dryness under reduced pressure and the dry weights of the residues were determined. MPSLE experiments were carried out by filling a column with the plant material and wetting it by pumping a predetermined volume of solvent into the column. The column was left to equilibrate for a predetermined time before pumping the solvent out of the column. The procedure was repeated up to seven times if needed. The extracts were evaporated to dryness under reduced pressure, and the dry weights of the residues were determined. The two methods were evaluated by comparing the extraction yield as well as the quality of the extracts by TLC analysis.

8.2.2. Preparative purification of extracts by RPC and MPLC (**III**)

The rotation planar chromatographic purification of the extracts was carried out by filling the planar chamber of the ExtraChrom® separation instrument prototype with approximately 50 g of dry silica using a rotational speed of 2000 rpm. The sample was dissolved in a small volume of solvent and applied to the centre of the chamber using a low rotational speed. The rotational speed was adjusted to the desired value and the solvent flow was initiated. The

solvent composition was optimised using TLC as a pilot method. A step gradient with increasing solvent strength was applied. MPLC was performed by filling the column first with approximately 50 g of dry silica and conditioning it with the elution solvent for the required time. The sample was dissolved in a small volume of solvent, applied on top of the column and the solvent flow was initiated. A similar solvent combination to that used in RPC was applied with a step gradient of increasing solvent strength. Fractions were collected using an automatic fraction collector.

8.2.3. Screening of indole derivatives by TLC and RPC (**IV**)

For the TLC analysis the standards and samples were applied to the TLC silica plates as 2 mm-wide bands. 3 µL of standard and 3 to 5 µL of sample were applied at an application rate of 10 s μL^{-1}. The plates were developed in ascending mode in an unsaturated 10 cm × 20 cm twin trough chamber at ambient temperature and protected from direct daylight. The development distance was 8 cm. The developed plates were dried at room temperature and protected from light, and scanned at λ = 280 nm. The images of the plates were acquired with the Video Documentation System at λ = 254 nm. For the RPC analysis the standards and samples were applied to the TLC silica plates in the form of a circle with a 500 µL syringe at a point ca. 2 cm from the center of the plate (Hamilton, Bonaduz, Switzerland). The applied volume was 50 µL or 100 µL. The speed of rotation was 1700 rpm (57 Hz) and the solvent flow rate was varied between 0.6 mL/min and 0.9 mL/min. The mobile phase was identical with that used in the TLC procedure. Different development distances were tested, the development time with these settings varying between 9 min to 19 min. All the separations were performed by normal chamber RPC (N-RPC), i.e. the vapor space was not eliminated. The developed plates were dried, scanned and documented as described for the TLC analysis.

8.2.4. Comparison of densitometer and video scanner in quantitative TLC (**V**)

The standards and samples were applied as 6 mm bands on silica HPTLC plates and RP-18 TLC plates, and the rate of application was 10 s/µl. The mobile phases were optimized by means of the PRISMA system (NYIREDY et al. 1985, 1988). The separations were performed either in an unsaturated twin trough chamber for 10 cm × 20 cm plates, or in an unsaturated horizontal developing chamber for 10 cm × 20 cm plates. The analytes were detected by UV irradiation at 254 nm for flavonoids and phenolic acids, and by UV irradiation at 366 nm for coumarins. The influence of the densitometer and video scanner instrument settings on the quantitative results was studied and the two detection methods were compared.

8.2.5. Assay for radical scavenging activity of phenolics by RP-TLC (**VI**)

The standards were applied as 9 mm-wide bands on RP-18 TLC plates using an application rate of 15 s/µl. The solvent system used was optimized for the separation of rapeseed meal extract according to the PRISMA model (NYIREDY et al. 1985, 1988). The plates were

developed in an unsaturated 10 cm x 20 cm twin trough chamber to a distance of 75 mm. After development, the plates were air-dried for 15 min and the dried plates subsequently dipped in a 0.04 % (w/v) solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol. The plates were then placed in the center of the imaging cabinet and the images were obtained under visible light 120 seconds after dipping. The spectrophotometric DPPH assay was used as a reference method.

9. RESULTS AND DISCUSSION

9.1. Extraction of plant material by RPE and MPSLE (I, II, III)

RPE is a solid-liquid extraction method in which the accelerated flow of extractant is produced by the action of centrifugal force (MESZAROS et al. 1987). In this study ExtraChrom®, a multi-functional separation instrument prototype with an extraction chamber, was used for the rotation planar extraction of different plant materials, namely *Ficus* leaves, oak bark, dried and ground onion, and *Gerbera hybrida* floral stems and leaves. The RPE method was compared with the MPSLE technique, which is a rapid and easy to perform extraction method, and can be performed in an MPLC system.

A factorial experimental design with three levels was applied for studying the MPSLE process and for predicting the influence of the operating variables, *i.e.* medium particle size of the material to be extracted, equilibrium time and the volume of solvent, on the extraction yield and the quality of the extracts (**I**). The dried and ground leaves of *Ficus sycomorus* L. served as plant material. For RPE the studied variables were the medium particle size of the material to be extracted and the volume of solvent, and the dried and ground leaves of *F. bengalensis* L. were used as plant material. The optimum results achieved by the methods were compared with each other, and other factors such as the ease of operation, solvent consumption and extraction time were also taken into account.

The results obtained from the MPSLE experiments with *Ficus* leaves showed that the medium particle size of the plant material clearly affected the extraction yield and the amount of nonpolar compounds detected in the extract by TLC (see Table 2 in **I**). A pronounced increase in the extraction yield, as well as in the peak area of the nonpolar compounds present in the extract, was observed with the smallest medium particle size compared to the other two medium particle sizes. An increase in the equilibrium time increased the extraction yield only slightly, and the other parameters did not reveal any significant changes either. Rotated factor analysis indicated that the most important factor affecting the extraction efficiency was the volume of the solvent (see Table 3 in **I**). Also cluster analysis revealed a strong linkage between the volume of solvent and the extraction yield (see Fig. 4 in **I**). The influence of equilibrium time and medium particle size on the extraction process was less pronounced.

The dependence of the extraction yield on the three independent operating variables was studied using multilinear stepwise regression analysis. The regression model had the following form:

$$Y = 699.3\ P^2 - 1144\ P + 0.98\ V + 23.3\ T + 980$$

where Y is the extraction yield, P is the medium particle size of the plant material, V is the volume of solvent and T is the equilibrium time. The multiple regression coefficient R for the experimental and calculated residue yield was 0.89.

Choice of the most suitable operating variables for the efficient extraction of the nonpolar coumarin-type compounds from *Ficus sycomorus* L. leaves was evaluated on the basis of extraction yields and the peak areas of polar and nonpolar compounds in the extracts and the lower segments of the column by TLC at 300 nm. According to these criteria, a medium particle size of 0.40 mm, a solvent volume of 90 ml and an equilibrium time of 2 hours were selected as the most efficient conditions for the extraction process.

The influence of the medium particle size of the plant material on the extraction efficiency using RPE differed to some extent from that in the MPSLE method. The highest yield was obtained using a medium particle size of 0.67 mm. The amount of nonpolar compounds in the extract on the other hand was the highest using a medium particle size of 0.40 mm. A direct dependence was observed between the medium particle size and the amount of nonpolar compounds extracted from the outer segment of the planar column, i.e. decreasing the medium particle size of the plant material resulted in a decrease in the amount of nonpolar compounds left in the outer segment of the column, indicating a more efficient extraction. The two extraction methods seemed equal in terms of the volume of solvent used for the extraction. Increasing the volume of solvent led to a slight increase in the extraction yield but, at the same time, the amount of nonpolar components in the extract decreased.

Based on the same criteria as with MPSLE, the optimum extraction efficiency was achieved with a medium particle size of 0.40 mm and a solvent volume of 90 ml, i.e. the same values that were chosen for MPSLE.

In the extraction of onion, the quality of the extracts presented a great challenge, as they were very sticky due to the presence of substantial amounts of carbohydrates (**II**). This significantly limited the suitability of MPSLE in this work. The flow of extract could only be established by flushing the column with a fourfold larger volume of the extraction solvent and thus quantitative values of extraction yields could not be obtained. With RPE, however, extractions could be successfully carried out despite the viscous nature of the extract.

In RPE, the equilibration time $t_{equil.}$ = 0 min produced markedly higher yields than an equilibration time of 60 min (see Table 2 in **II**). This was probably due to the partial blockage of the extraction equipment and the fact that the very viscous extract remained inside the extraction chamber.

The results obtained for the effect of sample particle size depended inversely on the equilibrium time. For the equilibration time $t_{equil.}$ = 0 min, the highest yield of extract was obtained with the smallest particle size, and the lowest yield with the largest particle size, i.e. the yield of extract increased as the surface area of the material to be extracted increased. With the longer equilibrium time $t_{equil.}$ = 60 min, the results were exactly the opposite and the highest yield of extract was obtained for the largest sample particle size. The reason for this was probably the previously mentioned partial blockage of the extraction equipment and the high viscosity of the resulting extract.

The qualitative composition of the extracts obtained by MPSLE and RPE was found to be very similar. TLC analysis showed the presence of fructose, glucose, and saccharose, and the presence of oligofructans was also confirmed by TLC (see Figs. 2 and 3 in **II**). In HPLC-MS, oligofructans with a degree of polymerization up to 12 were detected as the main components of the onion extract (Fig. 4 in **II**).

In the extraction of *Gerbera* floral stems and leaves, the choice of extraction solvent was based on preliminary antimicrobial assays in which only the methanol extract showed clear inhibition zones against plant pathogenic bacteria and fungi (**III**). The volume of solvent per cycle, equilibrium time and the number of extraction cycles were chosen in order to achieve a satisfactory compromise between extraction time, solvent consumption and extraction yield, i.e. a total extraction time of approximately 12 hours and a solvent consumption of less than 500 mL were preferred.

A total extraction yield of 41.3% (calculated on the basis of the dry weight of freeze-dried plant material) was obtained with the RPE method from this plant material (see Table 2 in **III**). The total extraction yield was 45.1% using MPSLE, which was used as a reference method as it has been shown to be an exhaustive extraction technique (MOUSA, 1995). Although the total extraction yield obtained by the RPE method was less than that with the MPSLE method, the difference was not really significant and may be partly due to the different solvent flow rates. Qualitative TLC analysis revealed no differences in the composition of the extracts obtained with RPE and with MPSLE (see Fig. 2 in **III**). The extraction yields obtained by the two methods in this study are summarized in Table 4.

Table 4 Comparison of extraction yields by the RPE and MPSLE methods.

Original publication	Extraction yield/plant material (mg/g)	
	RPE	MPSLE
I[a]	22	19
II[b]	113	31
III[c]	413	451

[a] Extracted material *Ficus* leaves, extraction solvent chloroform, optimum operating variables.
[b] Extracted material onion, extraction solvent methanol-water (80:20, v/v), equilibrium time 60 min, medium particle size 1.5 mm (the only conditions that permitted MPSLE of the material due to the stickiness of the extracts).
[c] Extracted material *Gerbera* stems and leaves, extraction solvent methanol.

The differences between the two extraction methods are primarily due to the way the solvent flow is created in the column, i.e. in RPE by centrifugal force, and in MPSLE by a pump, and in the geometry of the column. However, RPE does have one significant advantage over MPSLE in that centrifugal force is used to produce the accelerated solvent flow. With RPE it is possible to drive the extraction solvent out of the column without pumping more solvent into the column, unlike the case with MPSLE where the solvent has to be forced out of the column by displacing it with an equal volume of new solvent. This provides a practical means of reducing solvent consumption, which is of both environmental and economical benefit. An additional advantage of the ExtraChrom® instrument lay in the possibility to use raw material of very small particle size in the extraction process. Because of the construction of the

instrument, there is no risk of plugging the system, whereas with MPSLE finely pulverised materials sometimes cause problems, namely increased back pressure and plugging of the lower frit of the column.

9.2. Purification of 2-pyrone derivatives from *Gerbera hybrida* (III)

When optimising the mobile phase for the separation of gerberin and parasorboside, the best results were achieved in TLC using the solvent system consisting of methanol, ethyl acetate and tetrahydrofuran at selectivity point P_S = 118. However, due to solubility problems with this solvent combination, selectivity point P_S = 111 was chosen for RPC and MPLC. The optimised step gradients (see Table 1 in **III**) enabled the complete elimination of chlorophylls and other non-polar compounds from the extract by both RPC and MPLC. Gerberin and parasorboside, on the other hand, were not completely separated. Therefore the fractions containing either gerberin or parasorboside, or a mixture of these two compounds, were combined and the resulting mixture was subjected to further purification and subsequent isolation of the pure compounds by TLC.

The problems arising from insufficient resolution affected both RPC and MPLC. However, they were more problematic in the RPC method. This may have been partly due to manual application of the extract into the column, and also to remixing of the solvent in the collecting channel before reaching the output opening. These problems had the greatest effect on compounds that elute close to each other, thus limiting the use of the current ExtraChrom® separation instrument prototype in difficult separations, i.e. the instrument can, at its present stage, be applied for fast preliminary separations where the purity of the fractions is not critical. The prototype could, however, be improved, e.g. by adding more output openings to the collecting channel in order to reduce any remixing of the solvent (MESZAROS et al., 1987), and by developing a more reproducible application procedure. In any case the RPC method is superior to MPLC in the rapidity of the separation process, especially as regards filling of column; it takes approximately 10 min for RPC and 12-24 hours for MPLC (ZOGG et al., 1989a).

The optimised TLC method proved to be a useful and simple procedure for isolating gerberin and parasorboside. A total of 35 mg of the purified extract was applied onto silica TLC plates and separated, using methanol–ethyl acetate–tetrahydrofuran–*n*-hexane–formic acid (8:9:80:3:1, v/v/v/v) as the eluent, to give 16.1 mg of gerberin and 9.7 mg of parasorboside. According to TLC analysis, the purity of gerberin was > 94%, but parasorboside contained slightly more impurities (purity ~ 92%), mainly gerberin and an unknown polar impurity (see Table 3 in **III**).

The identification of gerberin and parasorboside was based on the MS and NMR studies. LC/MS analysis yielded small, but diagnostic [M+1] ions of 291 and 293 amu for gerberin and parasorboside, respectively. The identification of gerberin was readily straightforward on the basis of the ^{13}C NMR and ^{1}H NMR spectra; the carbon spectrum was sufficiently

consistent with the literature values (NAGUMO et al., 1989) despite the different solvent (in this work d_6-DMSO was used instead of d_5-pyridine). The identification of parasorboside was more problematic due to the presence of some minor impurities whose signals overlapped with several of the parasorboside sugar-ring proton resonances. In addition to the standard techniques, selective 1-D TOCSY analyses were used to confirm the overlapped assignments from the COSY analyses, and to identify the multiplet peaks in the 1-D ^{1}H NMR spectra in order to enable extraction of the chemical shifts and coupling constants. Both the ^{1}H NMR and ^{13}C NMR spectra were sufficiently consistent with the literature values (NUMATA et al., 1990) despite the different solvent (in this work d_6-DMSO was used instead of d_4-methanol). The spectral assignments for both compounds are listed in Tables 5 and 6.

Table 5 ^{13}C-NMR chemical shifts of gerberin and parasorboside in d_6-DMSO.

	Compound	
C-atom	Gerberin	Parasorboside
1	166.13	169.55
2	93.06	35.33
3	170.28	69.67
4	33.15	35.08
5	71.71	72.10
6	20.12	21.02
1’	98.77	101.63
2’	72.73	76.54
3’	76.12	73.28
4’	69.27	70.02
5’	77.18	76.77
6’	60.35	61.06

Table 6 ^{1}H-NMR data of gerberin and parasorboside in d_6-DMSO.

	Chemical shift[a]		**Multiplicity[b]**		**H,H coupling (*J*)[c]**	
H-atom	Gerberin	Parasorboside	Gerberin	Parasorboside	Gerberin	Parasorboside
2	5.304	—	s	—	—	—
2α	—	2.562	—	d(AB)dd	—	1.7,3.1,17.7
2β	—	2.719	—	d(AB)d	—	4.9,17.6
3β	—	~4.21	—	m	—	—
4α	2.470	2.090	d	dm	7.4	14.3
4β	2.470	1.674	d	ddd	7.4	2.9,11.4,14.3
5	4.507	4.728	tq	dqd	6.3,7.6	2.8,5.9,12.8
6	1.331	1.267	d	d	6.3	6.4
1’	4.940	4.940	d	d	7.8	7.8
2’	~3.17	~3.16	d(AB)d(AB)	m (o’lapped)	7.8,8.9	—[d]
3’	~3.24	2.928	t(2×AB)	t	8.9	8.4
4’	~3.15	3.018	d(AB)d(AB)	t	8.9,9.6	9.2
5’	3.356	~3.11	ddd	m (o’lapped)	2.0,5.5,9.6	—[d]
6’	3.660	~3.65	dd	dd (o’lapped)	1.8,11.9	1.8,~10
6’’	3.453	~3.39	dd	dd (o’lapped)	5.6,11.9	~5,~10

[a] δ_H in ppm.
[b] Key to abbreviations: s – singlet, d – doublet, t – triplet, q – quartet, m - multiplet
[c] *J* in Hz.
[d] Not determined due to signal overlap.

9.3. Screening of indole derivatives in bacterial culture broths (IV)

The PRISMA system (NYIREDY et al. 1985, 1988) was used in the pre-assays to optimize the solvent composition. On the basis of preliminary experiments, acetic acid (solvent strength S_{HOAc} = 6.0 according to SNYDER [1978]), ethyl acetate (S_{EtOAc} = 4.4) and toluene (S_{Tol} = 2.4) were selected for further optimization at different selectivity points (P_S). The optimal separation was achieved at selectivity point P_S 127, and the solvent strength was adjusted to S_T = 2.4 with *n*-hexane. This gave a solvent composition of acetic acid–ethyl acetate–toluene–*n*-hexane, (4:11:70:15, v/v/v/v). The chosen solvent composition later referred to as solvent system 1, was then applied for TLC and RPC without modifications.

The optimized solvent system was compared with the TLC system published by HAAHTELA et al. (1990). One of the mobile phases they used to detect indole compounds produced by bacteria consisted of chloroform–ethyl acetate–formic acid, (5:4:1, v/v/v). The solvent combination gives a moderate separation and even L-tryptophan migrates from the application position. However, the R_F values of the majority of the indole compounds lie very close to each other, between 0.6 and 0.9. As a result, several compounds including IAA comigrate and cannot be separated.

Using a mixture of chloroform–ethyl acetate–formic acid, (8:1:1, v/v/v), optimized on the basis of the system by HAAHTELA et al. (1990), the R_F values of the compounds were more evenly spread over a wider range. This can be considered an improvement, but IAA could still not be separated from all the other indole derivatives. The system gave a better separation over the lower R_F range than the system optimized with the help of the PRISMA model and was therefore used as a complementary method in TLC. This mobile phase is later referred to as solvent system 2, and the system published by HAAHTELA et al. (1990) as solvent system 3. The solvent systems are presented in Table 7.

Table 7 Solvent systems used in the screening of indole derivatives.

Solvent system	Composition	Reference
1	acetic acid–ethyl acetate–toluene–n-hexane, (4:11:70:15, v/v/v/v)	**IV**
2	chloroform–ethyl acetate–formic acid, (8:1:1, v/v/v)	**IV**
3	chloroform–ethyl acetate–formic acid, (5:4:1, v/v/v)	HAAHTELA et al. 1990

IAA could be separated from all the other compounds with solvent system 1 (see Fig. 5a in **IV**). In addition to the compounds that remained at, or very close to the application position and therefore could not be separated, 3-hydroxyanthranilic acid and indole-3-ethanol formed a critical pair that was not separable with this system. Indole-3-acetone, indole-3-butyric acid, indole-3-carboxylic acid and indole-3-propionic acid also comigrated and formed a broad peak in the densitogram. This was considered to be acceptable because the main objective was to screen the bacterial culture broths for IAA, and the other studied solvent systems that could not separate IAA from all the other compounds could be used to complement the TLC analysis.

The TLC method with solvent system 1 was applied for screening IAA from the bacterial culture broths. Identification of the compounds was based on their R_F values and UV spectra measured directly on the TLC plate. The culture broths of *Bacillus sp.* strain KMNL2 and *Pseudomonas fluorescens* strain KNCNK13 were found to contain only traces of IAA. The culture broth of *Klebsiella pneumoniae* strain As contained indole-3-aldehyde as the main indole derivative, but also a prominent peak corresponding to IAA and, in contrast to the other cultures, indole-3-methanol as a minor constituent (see Fig. 6a in **IV**). Both indole-3-aldehyde and indole-3-methanol are known to be degradation products of IAA produced by oxidative decarboxylation (FUCHS and SPITELLER 1997).

The unmodified solvent system 1 was applied for the N-RPC separation of the indole compounds. The TLC and RPC methods correlated very well (see Figs. 2-5 in **IV**). Indole-3-carboxylic acid and indole-3-propionic acid, which migrated together with the TLC method, were partially separated by RPC. This was to some extent due to the higher R_F values obtained for the compounds less strongly retained by RPC. The higher R_F values obtained by RPC meant that shorter separation distances could be used, resulting in savings in analysis time and solvent consumption. Development over the distance of 5 cm took 9 min by RPC vs. 20 min by TLC with a migration distance of 8 cm. The solvent consumption of one 9-min RPC analysis with a flow rate of 0.9 mL/min was approximately 8 mL.

Solvent systems 2 and 3 were unsuitable for N-RPC and a satisfactory separation was not achieved. This was probably due to the formic acid concentration in the solvent systems, as well as to the large vapor space of the N-RPC system. The proportion of formic acid, which is a relatively non-volatile compound, increased at the expense of the more volatile compounds (chloroform and ethyl acetate) as the solvent front migrated further, thus leading to the formation of a solvent mixture of entirely different composition. As a result, the analytes could not be separated and detected in a satisfactory manner.

The N-RPC method with solvent system 1 was applied for the screening of IAA from the culture broth of *Bacillus sp.* strain KMNL2, known to contain IAA. The results were in agreement with those obtained by the TLC method (see Fig. 6 in **IV**).

9.4. Comparison of densitometer and video scanner in quantitative TLC (V)

In the densitometric detection the slit size of 1.0 mm × 5.0 mm provided optimum results and was therefore chosen for the study. The effect of data accumulation function values of 1 and 4 was studied. Since no significant differences were observed and the higher value markedly increased the analysis time, the data accumulation function was set to the value of 1.

The settings optimized in the video scanner detection included DTL, frame accumulation, integration period and aperture (see Table 1 in **V**). The DTL setting affects the resolution of the image. For the quantitative measurements the off mode yielded the best results and all comparisons were made with DTL set off. The frame accumulation is used to accumulate

several images of the TLC plate, which in turn increases the possibility to capture faint spots on the layer. For the phenolic acids and flavonoids better results were achieved with the frame accumulation turned off, whereas the coumarins could be better detected with the frame accumulation on. This was most probably due to the different detection wavelengths used for the mixtures. Four different integration periods were tested, i.e. exposure times from 40 ms to 200 ms were applied. For the phenolic acids and flavonoids an exposure time of 40 ms was chosen, and for the coumarins the optimal exposure time was 200 ms. Large aperture settings proved to be the most suitable for detection and f = 2.0 was chosen for phenolic acids and flavonoids and f = 2.8 for coumarins.

The repeatability of the methods was evaluated using methyl gallate (λ = 254 nm) and umbelliferone (λ = 366 nm) as standards. The tracks were detected six consecutive times by both densitometer and video scanner. For methyl gallate the relative standard deviations of the peak area using the densitometer were 0.4% for NP-TLC and 0.9% for RP-TLC. The repeatability of video scanner detection was not quite as good, the relative standard deviations being 3.8% and 1.5% for NP-TLC and RP-TLC, respectively. When detecting umbelliferone at λ = 366 nm the video scanner on the other hand performed better than the densitometer. The relative standard deviations of the peak area for the video scanner were 1.5% for NP-TLC and 0.5% for RP-TLC, whereas the relative standard deviations of the densitometer were 1.9% for both NP-TLC and RP-TLC.

The two detection methods were also compared in detecting an unidentified gallic acid derivative in the *Phyllanthus emblica* L. ethyl acetate fraction (chromatograms are presented in Fig. 2 in **V**). The quantitation was based on the calibration data for methyl gallate. The quantitative results obtained with the densitometer were approximately equal with the densitometer and the video scanner, i.e. the calculated amount of gallic acid derivative in the sample was 0.51 μg with the densitometer and 0.46 μg with the video scanner (see Table 4 in **V**). In terms of repeatability the densitometer performed better with a relative standard deviation of 6.8% vs. 14.2% for the video scanner.

9.5. Radical scavenging activity of phenolics by RP-TLC (VI)

For silica plates the purple background produced by the DPPH radical after spraying the plate with DPPH solution has been shown to be relatively stable, thus enabling the examination of radical scavenging activity after a period of 30 minutes (CUENDET et al. 1997, CAVIN et al. 1998). In our study the same was found to be true for dipping the silica plates in DPPH solution. However, this was not the case when using reversed-phase thin-layer chromatography plates. The developing colour proved to be very unstable and started to fade in approximately 3 min after dipping. The fading background led to poor peak shapes, as well as to decreased peak heights and areas. Increased interference from the background also made it necessary to use manual integration, thus adding uncertainty to the analysis. Since conventional slit-scanning densitometers operate serially, i.e. measure the response lane by lane, which is time consuming and results in poor accuracy and repeatability with this assay,

they could not be applied for the quantitative determination of radical scavenging activity directly from the RP-TLC plate.

The problem of declining intensity of the forming colour can, however, be circumvented by using an image analysis system that obtains the image of the whole plate simultaneously with a CCD video camera. Several systems are commercially available and they have been shown to perform almost as well as slit-scanning densitometers in terms of accuracy, precision and robustness (VOVK and PROŠEK 1997, VOVK et al. 1997, PETROVIC et al. 1999, 2000).

Measurement of the radical scavenging ability of the tested compounds on RP-TLC plates was based on the peak areas produced with a video scanning system 2 minutes after dipping. Polynomial second degree calibration equations calculated for the standards were found to give satisfactory correlation with regression coefficients of between $r^2 = 0.947$ and $r^2 = 0.996$ for protocatechuic acid and caffeic acid, respectively (see Table 1 in **VI**).

With the RP-TLC-DPPH method, gallic acid, ellagic acid, L-ascorbic acid and 2,3-dihydroxybenzoic acid were the most active DPPH radical scavengers depending on the amount applied to the plate (see Fig. 2 in **VI**). Coumaric acid, *p*-coumaric acid, 3-hydroxybenzoic acid and vanillic acid, on the other hand, showed no radical scavenging activity. With this method gallic acid showed significantly higher radical scavenging activity than *n*-propyl gallate. The non-polar nature of the RP-TLC sorbent may account for this difference. The dimethoxylated phenolic acids, syringic acid and sinapic acid, scavenged DPPH radicals more effectively than the diphenolic acids protocatechuic acid and caffeic acid with this method, in contradiction to the results of the spectrophotometric assay.

Detection limits were determined for all the tested compounds based on a signal-to-noise ratio (S/N) of 3 after dipping the plates in DPPH solution. The detection limits varied between 40 ng and 689 ng for syringic acid and α-tocopherol, respectively (see Table 2 in **VI**).

Both methods were compared by performing regression and correlation analyses. Outliers were tested by F approximation. The two methods seemed to be relatively similar. However, a few exceptions were detected. Syringic acid, ascorbic acid and *n*-propyl gallate were shown to be outliers in the regression analyses with $p>6/N$ resulting in $r^2 = 0.545$. After excluding these three compounds a value of $r^2 = 0.923$ was obtained.

The RP-TLC-DPPH method was applied for measuring the free radical scavenging activity of rapeseed meal fractions separated by RP-TLC. A total of 10 separate bands with free radical scavenging activity were detected, with R_F values ranging from 0.04 to 0.85 (see Fig. 3 in **VI**). The most active fraction observed was the one with an R_F value of 0.41 that possessed 38% of the total radical scavenging activity.

10. CONCLUSIONS

Both the RPE and MPSLE methods were shown to yield satisfactory results in the preparative scale extraction of secondary metabolites from natural products. The optimum values of the studied operating parameters, determined for the extraction of *Ficus* leaves, were found to be identical between the two methods. The methods were also found to be roughly equivalent in terms of extraction efficiency and quality of the extracts. For difficult matrices such as onion, which contains substantial amounts of carbohydrates, the use of RPE was found to be advantageous, as the high carbohydrate content made the extract very sticky and it tended to plug the MPSLE equipment.

The main advantages of RPE over MPSLE were the simple and rapid operation of the instrument resulting in significant time savings, and reduced solvent consumption, because extraction solvent could be driven out of the extraction chamber without the need to displace it with the same volume of fresh solvent. RPE, however, lacks the possibility of scaling up the extraction process and therefore, if larger amounts of material need to be extracted, MPSLE outperforms RPE.

In the preparative isolation of gerberin and parasorboside from the extracts of *G. hybrida*, both the RPC and MPLC methods allowed rapid prepurification of the extracts, although the compounds could not be completely separated in one run. As a result of the very rapid filling procedure of the planar column, substantial time savings were achieved by using RPC in comparison to MPLC.

The PRISMA model was successfully applied to the optimization of the TLC mobile phase for various indole derivatives. The optimized mobile phase separated IAA from a wide range of other indole derivatives, thus enabling screening of bacterial culture broths and other complex samples for IAA. The optimized TLC mobile phase was directly transferred to N-RPC without any loss in resolution, but this resulted in shorter analysis times and reduced solvent consumption.

The comparison of the performance of a densitometer and video scanner in the quantitative analysis of plant phenolics by UV irradiation at 254 nm and 366 nm confirmed that the methods can be considered approximately equal in terms of repeatability and recovery. One of the main advantages of densitometry is the free choice of detection wavelength, whereas video scanners provide faster detection and the possibility to archive the captured images in digital form.

RP-TLC combined with dipping the plate in DPPH solution afforded a quick and simple method for identifying compounds with radical scavenging activity from plant extracts. As the developing color was very unstable, a video scanner was used for the simultaneous detection and quantitation of the activity. The developed RP-TLC-DPPH method was shown to correlate well with the widely used spectrophotometric DPPH assay. The results from the

measurement of the free radical scavenging activity of rapeseed meal fractions showed that the method can be efficiently used for the fractionation and analysis of potential antioxidative compounds in natural extracts.

In conclusion, the results obtained with the extraction techniques and planar chromatographic methods indicated their potential as rapid and simple tools in the isolation and analysis of various natural products. It is, however, evident that further experiments are still needed in the studied areas, especially with regard to the application of the ExtraChrom® prototype separation instrument, in order to reach their full potential in natural product chemistry.

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