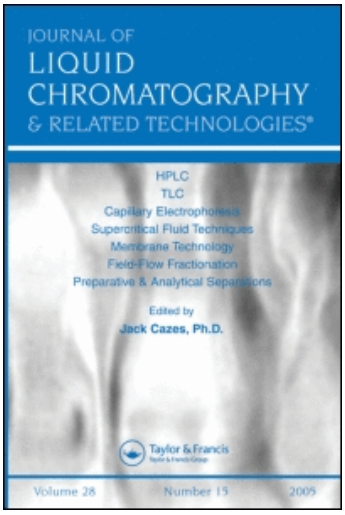

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Primary Screening of Natural Products Using Micro Fractionation Combined with a Bioassay

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ABSTRACT

An efficient off-line procedure for primary screening of the bioactivity of a complex mixture was developed. The procedure includes HPLC separation, micro-fractionation, and a bioactivity assay followed by scale up of preparative isolation of active substances. Test compounds were used to develop and validate a chemical assay based on a stable free radical, 2,2′-diphenyl-1-picrylhydrazyl (DPPH). The procedure was equally developed and validated for a cell-based bioactivity assay in which the antimicrobial activity of a test mixture was tested against *Streptococcus pyogenes*. In the procedure, the analytical-scale sample

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was separated by HPLC and fractionated into a 96-well microplate. The fractionated microplate was lyophilized and either a chemical reagent or bacterial suspension was added to the plate wells. The UV-absorbance of DPPH and the turbidity of *Str. pyogenes* were measured using a microplate reader. Three natural extracts (*Lythrum salicaria*, *Linum usitatissimum*, and *Cladina stellaris*) were analyzed by the procedure using both the chemical and cell-based assays. The analytical HPLC separation of the most bioactive components of *C. stellaris* was further scaled up using a computer assisted simulation program and preparative amounts of the active compounds were isolated and identified by UV, NMR, or MS. In most cases, the on-line screening chromatographic methods are difficult to apply to cell-based bioactivity assays. However, the off-line micro-fractionation procedure presented here was found to be a valuable tool for bioactivity screening, especially for cell-based assays.

Key Words: Microplate; Fractionation; Bioactivity test; Scaling up of separation; Screening of activity; HPLC optimization.

INTRODUCTION

Due to the need to improve drug discovery screening technology, high throughput screening (HTS) methods have become an important topic of research. A major step in developing HTS methods occurred when the microplate became available. Routine microplate applications can be used for rapid spectrophotometric measurements of chromophores, turbidity, and for fluorometric measurements.[1] Microplate-based technology allows parallel handling of multiple samples, and precise assay requires only a routine microplate reader. Modern microplate readers can also perform both endpoint and kinetic measurements, thus enabling kinetic measurements on multiple samples at considerably reduced cost. However, miniaturization to a 96-well format or further requires proper validation of the method, due to the fact that the results are not always comparable with those obtained using conventional methods.[2]

Two basic assay formats are employed in HTS. One assay format is in vitro biochemical or chemical assays, such as the methods for antioxidative agents. Enzyme inhibition and receptor-ligand binding assays are also included in this category. The reactions of chemical assays are assessed using a variety of techniques, such as fluorescence, chemiluminescence, and lipid peroxide generation.[3] Another assay format is cell-based assays, which utilize microbial or mammalian cells in screening, for example, antimicrobial agents or calcium channel activity, respectively.[4,5]

Screening for new drugs in plants implies the screening of extracts for the presence of novel compounds and an investigation of their biological activities. It has been estimated that half of all new chemical entities in the years 1981–2002 derived from natural products.[6] The path that leads from an intact plant to its pure constituents and its derivatives is long. It is currently estimated that approximately 420,000 plant species exist in nature.[7] However, although over 100,000 secondary metabolites are already known, only a small percentage of all species have been studied to some extent for the presence of secondary metabolites. It is estimated that less than 5% of known plants have been screened for one or more biological activities.[8] An important group of secondary metabolites, flavonoids, are an excellent example of the complexity involved in the discovery of active compounds from plants.[9] In addition to plants, animals, and microbes are also considerable sources of active compounds.

Testing extracts of biogenic origin is challenging due to their complexity and the presence of unknown components. There is always the possibility that one can obtain either false negative or false positive results for the tests. The usual procedure in searching for compounds from complex matrices such as extracts, involves biological screening followed by activity guided fractionation. Active fractions undergo isolation and purification until pure active principles are obtained.[10,11] It may be possible to reduce the number of false results by fractionating the extract, and then testing the activity component by component. In most cases, it is not necessary to identify all the components of the extract because identification is costly and time-consuming. In drug discovery, it is most optimal to test and identify only the active components. For industrial needs in drug discovery of biogenic materials, new automated bioactivity screening procedures need to be developed.

Most of the advanced hyphenated screening techniques are not able to combine the bioactivity detection and chemical characterization. The basic idea of LC/MS and LC-NMR based approaches is to screen for structures of compounds or extracts already known to be active.[12–14] On-line and flow-screening techniques using liquid chromatography coupled on-line to biochemical detection, as well as frontal immunoaffinity chromatography (FIC), offer a possibility to search for bioactive compounds from complex extracts and to achieve a chromatographic data simultaneously.[15–18] However, these methods are often based on a chemical or enzymatic reactions and they are very difficult to apply to cell-based assays. Although biochemical or isolated target screens each have their merits, it is highly unlikely that any lead compound will progress to drug candidate without first having demonstrated activity in an appropriate cell-based model.[19,20]

The aim of this work is to introduce a procedure for biogenic extracts, which can enhance the efficiency of the primary screening by using optimized

HPLC separation, followed by automated micro-fractionation on the microplates used for the bioassays including cell-based assays. This paper presents a fractionation procedure that can be applied to off-line bioactivity tests using microplate technology. The bioactivity tests used in the procedure are based on the addition of a chemical reagent or bacterial suspension into the fractionated microplate wells, which are then measured by UV/VIS spectroscopy. The procedure also includes the computer assisted scale up of the separation of active compounds from analytical to preparative scale. The scale up and the optimization of a preparative separation are carried out using the simulation program DryLab and the information from analytical scale separations. The scale up process facilitates the isolation of preparative amounts of active substances found from a microplate bioassay platform, for the purpose of identification by UV, NMR, or MS.

EXPERIMENTAL

Test Solutions

Two test solutions (A and B) were prepared for developing the procedure; A for the DPPH test and B for the antimicrobial activity test. The mixture used in the DPPH test consisted of rutin (Merck, Darmstadt, Germany), (+)-catechin (Sigma, St. Louis, USA), quercetin (Extrasynthése, Genay, France), isorhamnetin (Extrasynthése, Genay, France), gallic acid (Sigma, St. Louis, USA), naringin (Roth, Karlsruhe, Germany), and trolox (Aldrich, Steinhaim, Germany). The concentration of each compound was 0.05 mg/mL. Trolox (0.05 mg/mL, 20 μL), which caused full absorbance inhibition, was manually added to the plates as a reference in the DPPH test. The mixture for the antimicrobial tests consisted of apigenin (Sigma, Steinhaim, Germany) 0.4 mg/mL, penicillin G (Fluka, Steinhaim, Germany) 0.05 mg/mL, 3-(α-acetonylbenzyl)-4-hydroxycoumarin (Sigma, Steinhaim, Germany) 0.1 mg/mL, and octyl gallate (Fluka, Steinhaim, Germany) 0.2 mg/mL. Erythromycin (Sigma, Steinhaim, Germany) 0.1 mg/mL, 20 μL was added to the plates as a reference, causing full bacterial growth inhibition in the antimicrobial test.

Natural Extracts

Lythrum salicaria L. and *Linum usitatissimum* L. were dried and powdered. Ground plant material (1 g) was weighed into a test tube. Aqueous methanol [10 mL, 80% (v/v)] was added and the suspension was stirred. The tube was sonicated for 10 min and centrifuged for 10 min (2800 rpm).

The supernatant was pipetted into a 250-mL round-bottomed flask. Each plant material was extracted three times. The combined supernatants were evaporated (water bath 35°C) to a volume of about 2 mL and lyophilized to dryness. The dry residue was extracted with methanol (5 × 4 mL) into test tubes. The tubes were centrifuged, and the supernatant was evaporated under a nitrogen stream to a final volume of 2 mL, which was lyophilized to dryness. The dry extract was dissolved into the methanol as a concentration of 20 mg/mL.

Dried and powdered *Cladina stellaris* (Opiz) Brodo was extracted by weighing 80 mg of sample material into a test tube and adding 1 mL of methanol on top of the sample. The sonication and centrifugation were carried out as described above. The supernatants from three extractions were filtered with syringe driven filters, 0.22 μm (Sterile Millex, Millipore, USA). The filtrate (2.5 mg/mL) was used as a sample for fractionation.

HPLC Separation and Fractionation

The analytical HPLC separations were carried out on a Perkin Elmer Series 200 LC pump and autosampler (Norwalk, CT) with a 200 μL loop, Perkin Elmer LC 235 C Diode Array Detector (Norwalk, CT) at a wavelength of 280 nm, and PE Nelson 600 series link (Norwalk, CT). The mobile phase consisting of methanol (HPLC grade, Rathburn, Walkerburn, Scotland) and 0.1% (v/v) aqueous formic acid was used at a flow rate of 1 mL/min. Water was purified via a Millipore MilliQ filtration device and formic acid was obtained from Riedel-de Haën (pro analysis, Seelze, Germany). The column used was Supelcosil™ LC-18, 28235-05, (25 cm × 4.6 mm i.d., 5 μm, Bellefonte, USA). The injection volume for all the samples was 20 μL. The separations were performed by gradient elution, which were either optimized or full scale gradients. The gradient profile for the DPPH test solution was 30–59–70–70 (B%) for 5, 9, and 4 min, respectively. The gradient for the antimicrobial test solution was 60–92–92 (B%) for 3 and 7 min, respectively. A linear gradient 5–95 (B%) of 85 min was applied for the *L. salicaria* and *L. usitatissimum* extracts, and a linear gradient 5–95 (B%) of 20 min was applied for *C. stellaris*.

The separated samples were all fractionated on an automated fraction collector (Gilson FC 204, Middleton, WI). The fractions were collected in 96-well microplates with a volume of 330 μL/well (Nunc, Roskilde, Denmark). Fractionation was performed as a function of time, with a collection time of 0.3 min per well. The control sample, 20 μL of pure methanol, was fractionated into the first and the last rows of the plates for the test solutions. The first row in a plate was used as a blank row in which only either reagent or the bacterial suspension was added. The last row was used for positive

controls. Fractionated plates containing the control solutions were lyophilized (HETO LyoPro 3000, Heto-Holten A/S, Denmark) for 12 hr before being applied to the tests.

For the HPLC analysis of the fractions (Fig. 1), one sample (20 μL) of the test solution for the antimicrobial test was fractionated into 12 wells in the same way as for the bioactivity test. The composition of the fractions was analyzed by the HPLC with the same gradient elution as used in the fractionation and the sample volume was 40 μL.

Determination of Radical Scavenging Activity

2,2′-diphenyl-1-picrylhydrazyl (DPPH) tests are commonly used to evaluate the general radical scavenging ability of antioxidants.[21] The test solution for the DPPH tests was separated by HPLC and the separated fractions were collected in 96-well microplates. Manually added trolox (0.05 mg/mL, 20 μL) was used as a positive control and it was added in three empty wells on each fractionated plate. Lyophilized microplate wells containing either

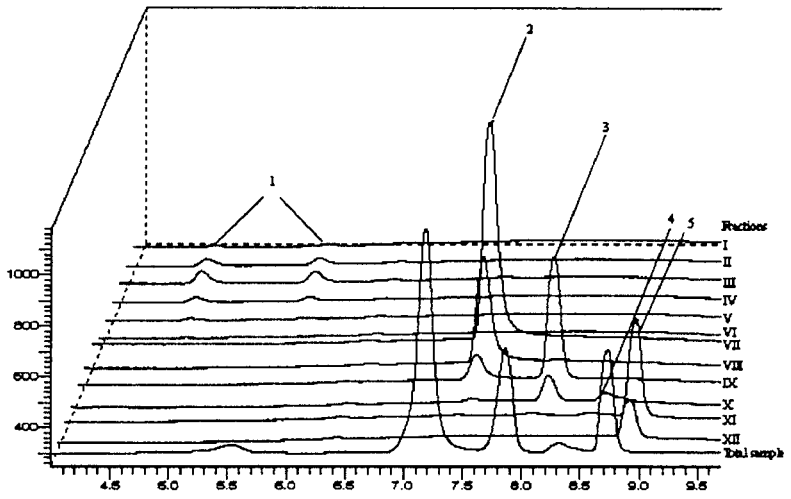

Figure 1. The analyzed fractions of a test solution for antimicrobial tests. Peaks 1, 2, 3, 4, and 5 are penicillin G, apigenin, coumarin, impurities from peaks 2 and 3, and octyl gallate, respectively. The chromatograms of the analyzed fractions (I–XII) are plotted into the same figure as the chromatogram of the total sample. The chromatograms of fractions with absorption scale of approximately a tenth of a total sample are rescaled for this figure to make the observation easier.

fractions or controls were filled with 200 μL of 15 μM DPPH solution. After addition of DPPH solution, the plates were shaken for 1 min and protected from light up until measurement at ambient temperature. The decrease in absorbance of the wells at 520 nm was determined automatically by Wallac Victor^2 V multilabel counter (Perkin Elmer Life and Analytical Sciences/ Wallac Oy, Finland) after 30 min.

Determination of Antimicrobial Activity

As in the case of the DPPH measurements, the test solution for the antimicrobial test was separated by HPLC and fractions were collected in 96-well microplates. The lyophilized microplates were tested against *Streptococcus pyogenes* ATCC 12351. The cultures had been kept at −70°C prior to use. Before the strains were used in the test, they were grown in Todd-Hewitt broth (Oxoid, CM189, In Vitro Diagnosticum, Basingstoke, Hampshire, England) at 37°C in 5% CO_2 for 24 hr. The bacterial suspension (260 μL) (10^8–10^9 CFU/mL) was added to the microplate wells, which were either empty or contained lyophilized fractions or erythromycin as a positive control. Prior to addition of the bacterial suspension, 1 μL of dimethyl sulfoxide, DMSO (Merck Eurolab, Darmstadt, Germany) was pipetted into the wells. The microplates were incubated with continuous shaking at 37°C for 6 hr. The turbidity of the wells containing fractions and suspension (A_f), the wells containing only the bacteria suspension (A_b), and the wells containing bacteria suspension and the reference compound erythromycin (A_e), was measured at 1-hr intervals on a Wallac Victor^2 V multilabel counter at 620 nm. The turbidity of the wells was determined as a measure of microbial growth.[5] The percentage growth inhibition was calculated using the equation: $PI\% = 1 - (A_f - A_e)/(A_b - A_e)$. Erythromycin totally inhibited the growth of *Str. pyogenes* and, therefore, A_e corresponds to the turbidity caused by the initial cell amount, the well itself, and the Todd-Hewitt solution used in preparing the bacterial suspension.

Scaling Up and MPLC Isolation

The separation and isolation of the two most active compounds in *C. stellaris* was carried out using preparative medium pressure liquid chromatography (MPLC). The apparatus used in MPLC was the same as in the HPLC separation, except for the column and the injector. The preparative scale column was a glass column Büchi Borosilicat 3.3, code no. 17982 (460 × 26 mm^2 i.d., Flawil, Switzerland), filled with LiChroprep RP-18

stationary phase (particle size 15–25 μm, Merck Art. 13901, E.Merck, Darmstad, Germany). A Rheodyne 7125 injector (Rheodyne, Cotati, CA) with a 4 mL loop was used as the injector.

Dried material of *C. stellaris* (480 mg) was extracted using methanol resulting in 33 mg of dried extract. The methanol solution of the extract (2 mg/mL) was fractionated with MPLC. The MPLC separation was scaled up from the analytical scale HPLC separation. The scale up procedure and the optimization of MPLC separation were carried out by using the computer assisted simulation program DryLab® 2000 (LCResources Inc., Walnut Creek, CA) as described by Wennberg et al. (2001).[22] The optimized separation was isocratic elution at flow rate 4 mL/min, B% 95, elution time 160 min, and injection volume of 4 mL. The separation was repeated four times. Two fractions were collected Fr I (8 mg) and Fr II (5 mg).

Identification

C. stellaris is known to contain usnic acid.[23] Fraction Fr I was identified to be usnic acid by comparing the UV spectra and retention times of the isolated substance and the reference compound (Serva, Heidelberg, Germany). For the identification of FR II, EI mass spectrum (EI-MS) was obtained with a Hewlett Packard HP 5970B spectrometer coupled with GC HP 5890 (Hewlett Packard). ^{1}H-NMR spectrum was obtained at 300 MHz with a Varian Mercury 300 instrument. In NMR experiments, $CDCl_3$ was used as solvent. The MS and NMR spectra of Fr II were in accordance with the report of Elix (1974)[24] for 2-*O*-methyl perlatolic acid.

RESULTS

HPLC Separation and DryLab Optimization

The separations for test solutions A and B were optimized by computer assisted simulation program DryLab® 2000. The optimization was based on two gradient elutions (B% 5–95 in 20 and 60 min). The information, retention times, and peak areas obtained from the two initial runs, as well as the column and instrument conditions, were input to DryLab. As a result of the DryLab simulation, the optimum short separation was achieved for the test solutions A and B (for example see Fig. 1). The separations for fractionation of natural extracts were carried out using full gradient (B% 5–95) elutions.

Fractionation

In this study the fractions were collected as a function of time, as seen in Fig. 4. To match the peaks in the chromatogram with the fractionated microplate wells, the dwell time from the detector to the fraction collector and the lag time caused by the injection had to be determined. As a result, there was a 0.1 min difference in our system between the collection time and appearance of the peak in a chromatogram.

The accuracy of the fractionation was studied by analyzing by HPLC the fractions of the test solution B. The chromatograms of the fractions are shown in Fig. 1. They show that fractions VII, VIII, XI, and XII were fractionated accurately, and they contained only one peak. Fraction IX contained not only the coumarin peak but also a trace of the apigenin peak. Fraction X showed two peaks, which originated from apigenin, coumarin and impurities. Fraction IX illustrates the possibility of overlapping between two adjacent peaks. In this case, the concentration of the trace peak caused by apigenin was very low compared with the main peak, and it did not show any activity. The penicillin G peak was separated into fractions I–IV. The degradation of penicillin appears as two peaks in fractions I–IV, whereas there is only one peak in the chromatogram of the total sample.

The repeatability of fractionation was studied for the DPPH test with the test solution A (Fig. 2). There were 36 DPPH activity measurements for 12 fractions. The mean and standard deviation were calculated for all the fractions. Figure 2 shows that all the measurements were between the limits of $\pm 3 \times$ standard deviation except for five individual data points, which fell outside the limits. To study within day repeatability, the relative standard deviation (RSD) of the absorbance units of individual fractions from each plate were compared. The outliers mentioned above were omitted and the RSD for the fractions varied 1– 25%, and the average RSD value was 10%.

Bioactivity Tests

Two inhibition assays were demonstrated with the fractionation procedure, a chemical assay and a cell-based assay. As a chemical assay, the antioxidative activity was tested using a stable radical, DPPH. The DPPH test was utilized particularly in developing the procedure. The activity of the fractions in the DPPH test is presented in Fig. 3. Six test compounds were fractionated into 12 wells. The peaks of both gallic acid and isorhamnetin required three wells to be fractionated. Quercetin required two wells, and trolox formed two peaks, the main peak and the minor impurity peak (trolox derivative), that were both collected in their own wells. The activity was calculated as

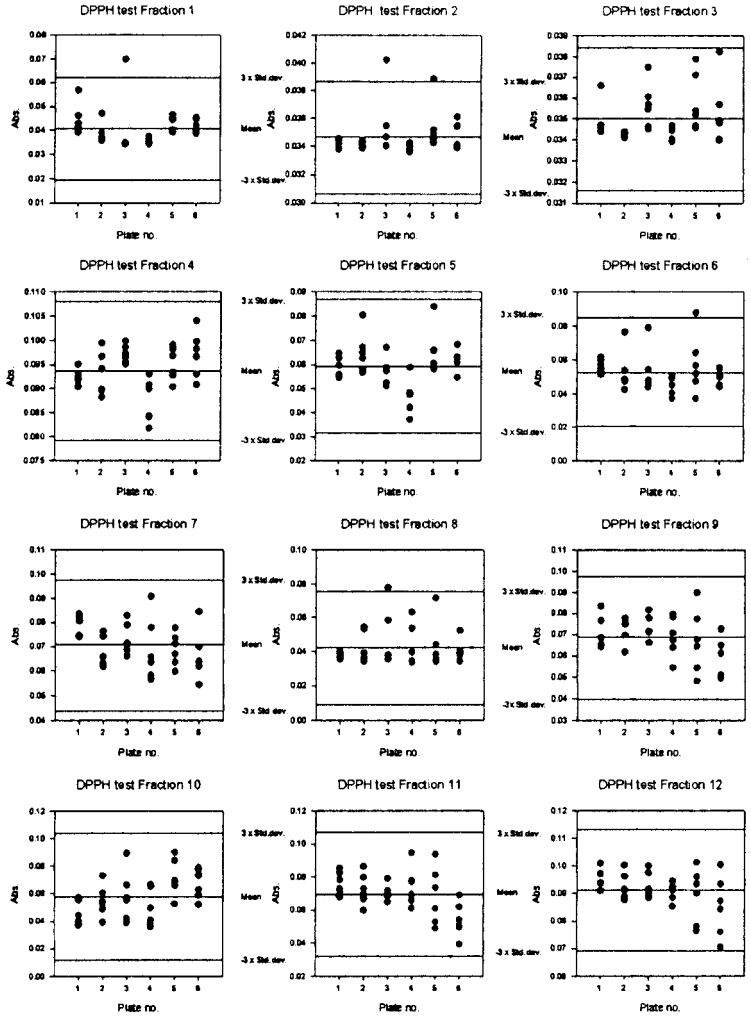

Figure 2. Repeatability data of DPPH test applied on microplate using the test solution. All the six measured absorbance values per fraction from six plates are illustrated in this figure including means and $\pm 3 \times$ SD. The DPPH test was performed on the day following fractionation.

the percentage of absorbance inhibition, $100*(A_{\mathrm{DPPH}} - A_{\mathrm{frac}})/A_{\mathrm{DPPH}}$, where A_{DPPH} was the average absorbance of the wells filled with the DPPH solution and A_{frac} was the absorbance of a well that contained both the fractionated sample and DPPH solution.

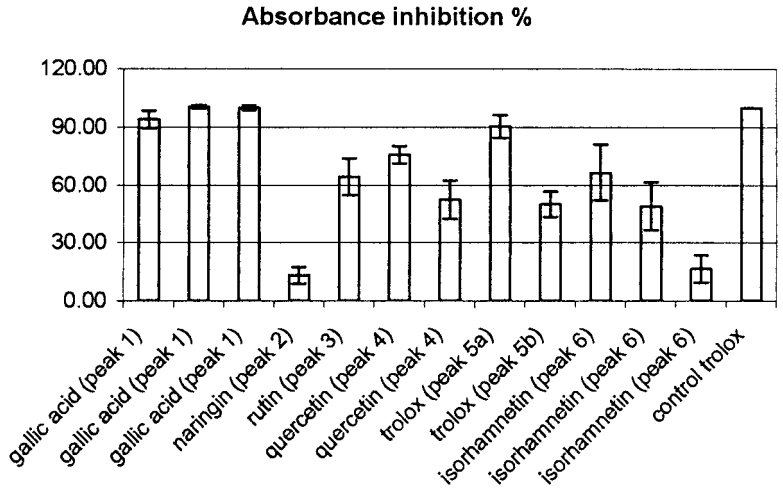

Figure 3. Distribution of activity, i.e., inhibition of absorbance over 12 wells from six peaks of fractionated test solution applied on DPPH test. All the results (mean values, $\pm$SD, $n = 36$) are related to control trolox, which caused a full inhibition.

In order to evaluate the DPPH test applied on a microplate as a primary screening tool, methanol extracts of *L. salicaria* and *L. usitatissimum* were fractionated. The results of *L. salicaria* fractions are shown in Fig. 4(A). It is evident that *L. salicaria* is a good candidate in the search for antioxidative compounds. Most of the active fractions show almost complete loss of absorbance equal to the absorbance of control trolox. *L. usitatissimum* did not show any activity.

As a cell-based assay, the antimicrobial activity of a test solution B was studied by adding the microbial cells to fractionated and lyophilized samples in the microplate wells. The antimicrobial activity was tested using *Str. pyogenes*, that has frequently produced resistance. For the fractionated test solution, one highly active antibiotic, two slightly active phenolic compounds, and one inactive coumarin were selected. The results of the antimicrobial tests are presented in Fig. 5.

Figure 5 illustrates also the high activity of penicillin. Eventhough the wide penicillin peak had to be fractionated into six wells, the fractions were therefore, highly diluted, the first five fractions caused full inhibition. If the penicillin had been fractionated into one well, the theoretical maximum concentration in the well would have been 3.3 μg/mL after addition of the bacterial suspension. A concentration of less than 0.5 μg/mL is sufficient to show the activity.

The whole procedure was proven to work as a valuable primary screening tool by searching for antimicrobial compounds from the extracts of

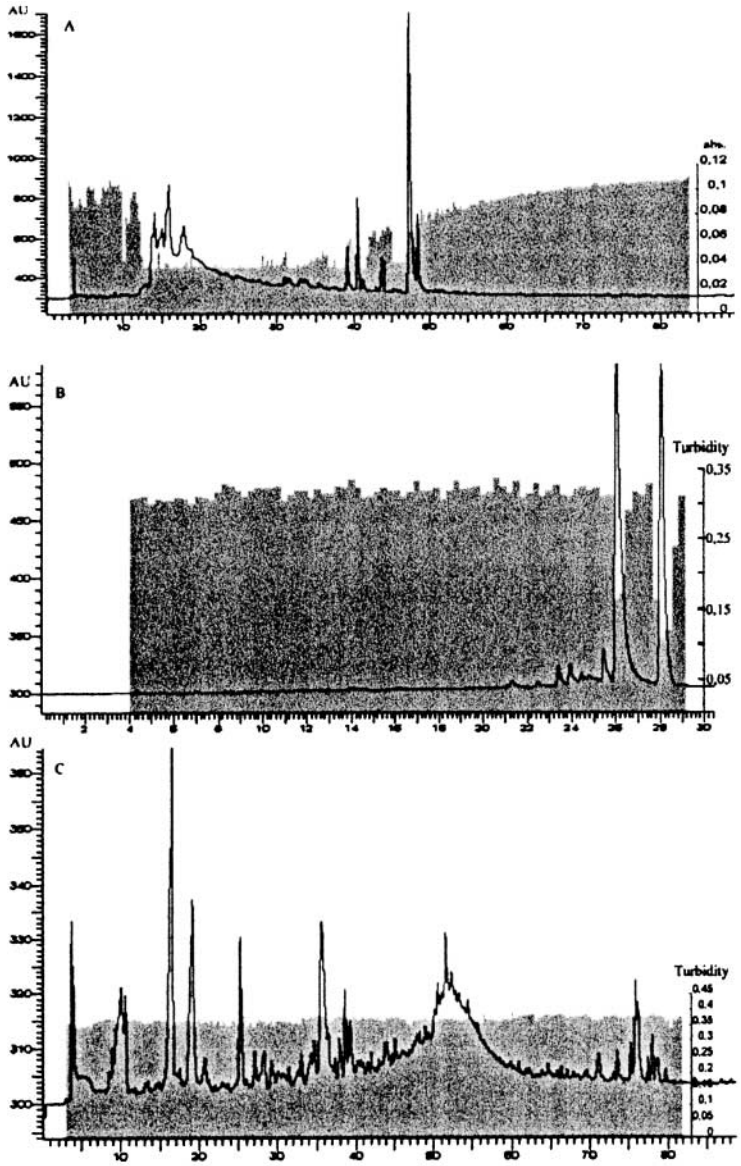

Figure 4. Fractionated plant extracts for bioassays. Chromatograms and activity diagrams (A–C) represents results from *L. salicaria*, *C. stellaris*, and *L. usitatissimum*, respectively. (A) Originates from DPPH test, and (B) and (C) show the results from antimicrobial test after 4 hr incubation.

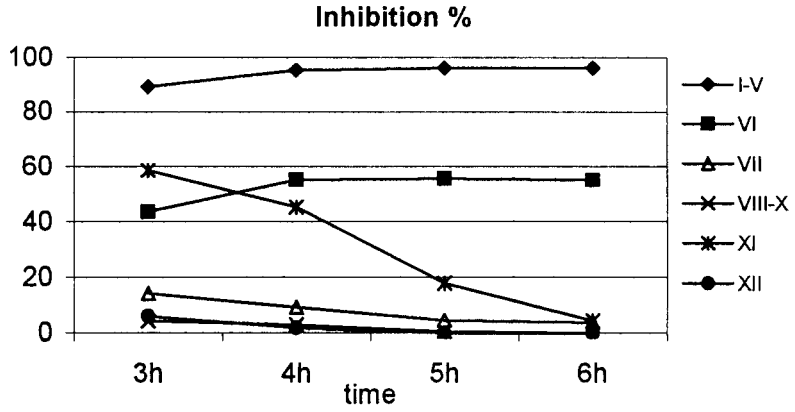

Figure 5. The growth inhibition percentage for each well of micro-fractionated test solution for antimicrobial test from 3 to 6 hr. The activity in wells I–VI was caused by fractionated penicillin and in well XI by octyl gallate. Apigenin in well VII showed also a weak activity at 3 hr. Inactive wells were second fraction from apigenin (VIII), coumarin (IX–X) and second fraction from octyl gallate (XII). To clarify the figure, the results for wells I–V and VIII–X were plotted as an average inhibition percentage.

L. usitatissimum and *C. stellaris*. The extracts were micro-fractionated, a suspension of *Str. pyogenes* was added into the plate wells and the results were measured by plate reader as described. The results at an observation time of 4 hr are presented in Fig. 4(B and C). Six active wells were found for the *C. stellaris.* The mean value was calculated for bacterial growth and the hit limit was set to $-3 \times$ SD, which corresponds to 0.285 absorbance units. In the antimicrobial test, it was possible to identify precisely two active peaks and no further fractionations or separation optimizations was subsequently required.

Scaling Up

To identify the active peaks from the antimicrobial test of *C. stellaris*, several milligrams of both compounds had to be isolated. To isolate the bioactive components, the HPLC separation was scaled up to preparative MPLC by computer assisted simulation program DryLab. For the simulation, the 20 and 60 min full scale analytical gradient separations of *C. stellaris* were carried out by HPLC. The MPLC separation was simulated on bases of the chromatographic data from the HPLC runs and on bases of MPLC column dimensions. The optimum MPLC separation for the peaks of interest was found to be isocratic elution at B% 95 in 160 min (Fig. 6).

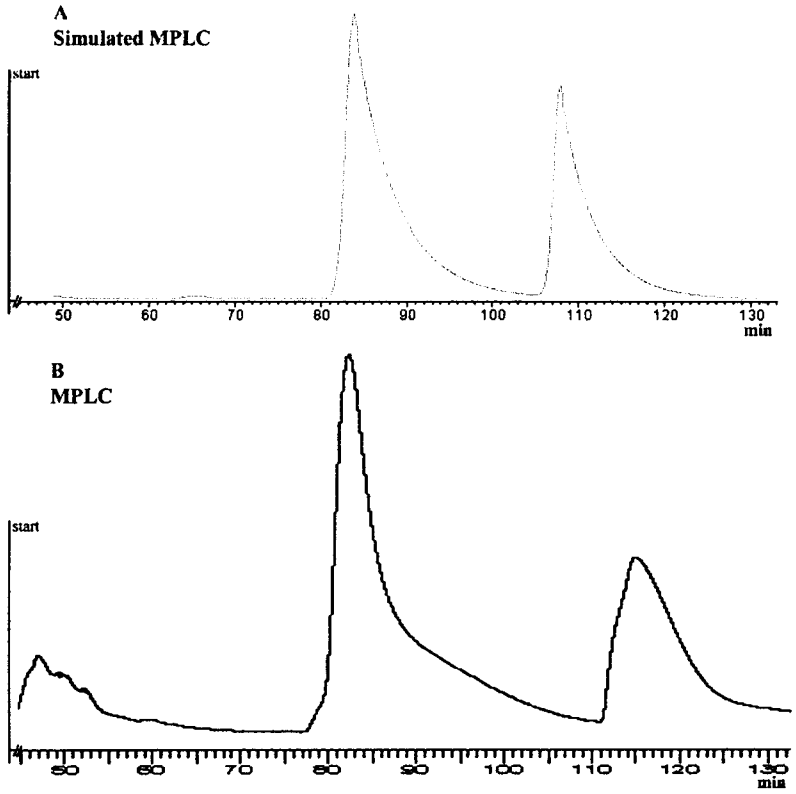

Figure 6. The DryLab simulated (A) and the actual (B) MPLC separation of the antimicrobial components of *C. stellaris*.

Isolation and Identification

Several milligrams of the fractions Fr I and Fr II were isolated by optimized MPLC separation. The Fr I was identified to be usnic acid based on the comparison with the UV-spectra and the retention times with the isolated sample and the pure substance. Fr II was found to be 2-*O*-methyl perlatolic acid based on the NMR and MS data.

DISCUSSION

The efficiency of the primary screening procedure can be improved by using DryLab either before or after the bioactivity tests. When a known

sample is to be fractionated for bioactivity tests, it is possible to save time by optimizing the separation. If the sample is of unknown composition and has many peaks, it may be best to fractionate the sample with full gradient elution (B% 5–95) and to carry out the bioactivity tests on the reasonably well separated preliminary fractions. When the active peaks and peak groups are found in the primary activity tests, it is then possible to focus the optimization of the separation only for the active fractions.

The DryLab can be used in scaling up the separation from analytical to preparative scale in the isolation of active fractions. The use of a computer assisted simulation program in developing the preparative separation saves time and effort when larger volumes of active fractions are isolated.[22] The scaling up process requires the same preliminary experiments, which would be needed if the analytical scale elution would be optimized. Also, the information from the full scale gradient separations in fractionations can be used for this purpose. In this study, the DryLab assisted scaling up procedure was found to remarkably enhance the isolation of active compounds.

The fraction collector is able to collect the samples either as a function of time or as a function of the peak signal. In primary screening, it is more reasonable to collect peaks as a function of time rather than as a function of the peak signal. In the case of complex samples, the size of the peaks of interest varies considerably, and it is difficult to adjust the settings so that the collector can recognize both the largest and the smallest peaks. There may also be a number of compounds that cannot be detected as peaks at the wavelength used. The collection as a function of the peak signal is suitable when only certain interesting peaks are to be collected.

In micro-fractionation the bioactivity in wells is linked to the peaks in the chromatogram. The detection of the bioactivity depends on the sample concentration. Collecting the maximum volume of sample per well increases the possibility of detecting the bioactivity. A highest possible sample concentration can be achieved by fractionating one peak per one well. For that purpose, the peaks should be as narrow as possible due to the low well volume in the microplate. The sample concentration can be increased by increasing the injection volume. However, increasing the injection volume spreads the peaks. The injection volume of 20 μL produced a sufficient amount of sample for the bioactivity tests. That volume did not cause disturbing peak broadening. The peaks can often be wider than can be collected in one well or they do not exactly coincide with a well. This may decrease the activity in the well compared to the actual activity of the peak. False negative results can be avoided by using the highest possible concentration of the whole sample. Fractions are not affected by the matrix and, therefore, there is a greater probability of hitting an active substance in a fraction than in the whole extract, even though the concentrations are low.

Considerable benefits of this procedure are the need for small sample amounts, and the separation of interfering colored components from other compounds. As a result, the colors do not interfere with the detection. For example, Eloff[25] was not able to determine the turbidity of the microcultures with a microplate reader because of the presence of colored compounds in non-separated samples. The problem can be overcome by using the micro-fractionation procedure presented here.

Eventhough the peak is, in some cases, divided into two or three wells, it is possible to determine the activity. For example, trolox which was fractionated into two wells showed activity in both fractions. The first fraction containing most of the trolox peak showed almost equal activity to the control trolox, causing full inhibition of absorbance. The isorhamnetin peak was tailing, which reflected the activity. As a consequence of the relatively low limit of detection in bioassays, it is possible that a non-visible amount of the active sample may be located under the non-active but much larger peak. False identification of active compounds can be difficult to detect. Special attention needs to be paid to the overlapping of active compounds, which cannot be detected by UV-detector. False positive results can be avoided by using the whole UV range in detection and/or simultaneous MS detection.

In the bioactivity assays, it is important to discriminate between the responses from active and inactive fractions. A common procedure is to set a signal window based on the standard deviation value of the negative control signal in the screening assay or to use a threshold value.[26] The DPPH test was so repeatable that the SD value was very close to zero. As a result, almost the whole sample of *L. salicaria* (Fig. 4.) would have shown activity if the limit had been set to 3 SD, which is the theoretical minimum hit limit dictated by the variability of the signal measured.[26] In the case of the chemical assay, it was found to be more appropriate to use a threshold value instead of SD. A threshold value, such as 50% of the maximum absorbance, reduced the number of active fractions found in the *L. salicaria* extract to a much more reasonable level, than when using a signal window based on the SD value.

In cell-based assays, the observation period for the results varied from 3 to 6 hr. During the first 2 hr, the cell density was not high enough to indicate reliable differences in growth between the control and wells containing samples. On the other hand, the observations were continued until bacterial growth had reached its maximum after 6 hr. The best observation times for the results were found to be after 3–4 hr. The antimicrobial activity is dependent on the sample concentration and the incubation time.[27] The decrease in activity as a function of time shown by octyl gallate corresponds with the results by Kubo et al.[28] that the amount of sample molecules needs to be increased with increasing number of viable cells.[28]

One critical issue in cell-based assays is the solubility of the fractions at the bottom of the wells. The bacterial or mammalian cell suspension is based on aqueous solutions and it is not possible to use large amounts of organic solvents to dissolve the sample. The aqueous solution is such a polar solvent that the solubility of the non-polar fractions must always be suspected. The used amount of DMSO (1 μL) that enhanced the solubility showed no effect on the growth of the used bacterial strain.

The primary screening of bioactivity is often made using traditional methods including whole extract bioassay, TLC or HPLC analysis and test tube fractionation of active extracts, bioassays of fractions, TLC or HPLC analysis, and preparative scale separation of active fractions.[25,29–31] Much of the work spent on LC separations and bioactivity assays can be avoided by using the micro-fractionated samples in bioassays. HPLC-fractionated samples can be used to identify some already known compounds in samples on the basis, for example, of UV or MS spectra, thus avoiding the unnecessary isolation of common constituents. The MS can be connected directly to the micro-fractionation system by adding a splitter before the fraction collector. It is then possible to achieve HPLC chromatogram, UV-spectrum, MS-spectrum, and fractionated micro-well plate for a bioactivity test from one HPLC separation.

CONCLUSIONS

The off-line bioactivity assays, DPPH, and antimicrobial tests presented in this paper demonstrate the suitability of biochemical and cell-based assays for the described procedure in general. Especially for cell-based bioassays, this procedure offers a solution to enhance the efficiency and throughput. Compared with traditional macro-scale fractionation and screening methods, the procedure offers direct information about which peaks, or at least which part of the chromatogram of a sample, are active. However, the procedure is not evolved for determining the concentrations of individual compounds. In the case of primary screening, it is usually inappropriate and time consuming to determine the exact concentrations of the fractions. This is especially true when there exists the possibility of false information about which compound is responsible for the activity.

According to the primary screening experiments made with the test solutions and actual natural extracts, the micro-fractionation procedure represents a valuable tool for bioactivity screening. The procedure can be applied not only to plant extracts, but also to fermentation broths and synthetic mixtures. A fractionated microplate offers an excellent platform for developing bioactivity tests based on the addition of a chemical reagent or cell suspension to the

fractionated wells. Furthermore, it is possible to facilitate the isolation process of the active substances found from bioassays by connecting the computer assisted scale up and optimization procedure to the micro-fractionation procedure in large scale isolation of active compounds.

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