



Analysis of Combinatorial Natural Products by HPLC and CE

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Natural products are among the largest and most diverse groups of plant secondary metabolites. Advances in modern genetic technology have enabled scientists to engineer biosynthetic pathways with the promise of generating novel chemical entities. In this study, the authors used an extensive database of secondary metabolite-related enzymes for cloning and testing *Catharanthus roseus* and other plant genes of interest. They discuss the use of accelerated solvent extraction, high performance liquid chromatography and capillary electrophoresis (CE) to monitor changes in combinatorial genetics-mediated secondary metabolite generation. Because of the lower sample requirement and higher sensitivity, the authors found CE to be their preferred separation method in this study.

Natural products have been proven to be the richest source of medicinal compounds. Although many drugs are made by synthetic chemistry, most of the core structures or scaffolds for synthetic chemicals are based upon natural products. The key advantage of natural products over synthetic compounds is their greater chemical diversity. Another benefit is intense biological activity as a result of natural selection. Almost one-

half of the chemical scaffolds from natural products cannot be reproduced by synthetic chemistry.¹

Modifying plant secondary metabolic pathways has been one of the earliest goals of classic phytochemistry.² Advances in tissue engineering and gene transfer technologies during the past few decades allowed the application of genetic manipulation of plant cell cultures to increase concentrations of

rate-limiting enzymes or to express gene products not normally induced in cultured cells.³ Combinatorial genomics and metabolic engineering can generate new sources of pharmaceutical compounds⁴ and augment classic breeding in the effort to develop plants with optimal natural product yield.⁵ If so, the development of an alternative system for secondary metabolite production in plant cells or tissue cultures could become a viable industrial approach. Using methods such as antisense–cosuppression technologies or overexpression, scientists could tailor medicinal plants to produce pharmaceutically important secondary metabolites by eliminating interfering metabolic steps or by introducing desired steps. For example, to accumulate more of an end product, scientists could block a side pathway that also uses the same precursor.⁶ A transgene could be introduced to accumulate a compound not normally produced in a particular plant species. An end product can be derivatized — for example, to a more soluble glycoside — by introducing a glycosyl transferase gene. Alternatively, microbes could be used to produce secondary metabolites.⁷ Short biosynthetic

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pathways can be functionally expressed in yeast or bacteria to produce a single enzyme for use in a subsequent biomimetic synthesis or even to produce the end product.

Catharanthus roseus (vinca rosea) produces widely used alkaloids such as the antihypertensive compounds ajmalicine and serpentine, as well as the anticancer agents vinblastine and vincristine.⁸ The latter compounds bind specifically to tubulin during mitosis and block the ability of the protein to polymerize into microtubules. Both normal and malignant cells exposed to these alkaloids undergo changes characteristic of apoptosis. Earlier reports about vinca rosea cell culture transformation using particle bombardment provided data about promoter activities monitored by gene expression.^{9, 10} Analysis of *C. roseus* alkaloids has been reported by various chromatographic¹¹ and electrophoretic¹² techniques.

By virtue of the importance of natural products in medicine, novel methods are being developed to alter biosynthetic pathways to produce compounds of interest or compounds that would not be produced otherwise. In this article, we report about a combinatorial natural product discovery methodology that uses a viral vector system to transfect secondary metabolite related enzymes from *C. roseus* to tobacco cell cultures in conjunction with high-resolution separation techniques of high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and mass spectrometry (MS) to analyse secondary metabolite patterns.

Experimental

Chemicals: We obtained *C. roseus* indole alkaloids of vincamine, vincristine, vindesine and vinblastine from Sigma (St. Louis, Missouri, USA); ajmalicine from ICN Biomedicals (Aurora,

Ohio, USA); and serpentine from Aldrich (Milwaukee, Wisconsin, USA). The HPLC-grade water, methanol and acetonitrile were from Aldrich. We purchased ammonium acetate, tetramethylammonium formate, sulphuric acid, sodium hydroxide, ethyl acetate and sodium phosphate from Sigma. The *C. roseus* plants were grown and the tobacco cells were cultured in-house.

Identification of genes of interest and viral vector system:

Known secondary metabolism-related genes of *C. roseus* (vinca rosea), *Lycopersicon esculentum* (tomato), *Arabidopsis thaliana* (arabidopsis) and *Oryza sativa* (rice) were used as bait to search for other secondary metabolism genes by Basic Local Alignment Search Tool (BLAST) searching.¹³ We searched the complementary deoxyribonucleic acid (cDNA) using BLAST and compared the data with the latest release of the National Center for Biotechnology Information non-redundant nucleotide database (see <http://www.ncbi.nlm.nih.gov/>) with a cutoff score of 10^{-5} . Then we designed primers to clone full-length genes of interest, which were expressed using the coat protein replacement vector derived from beet curly top geminivirus.¹⁴ The targeted genes were cloned into viral vectors and delivered into tobacco cell cultures by particle bombardment at 1100 psi using a PDS-1000 biolistic particle delivery system (Bio-Rad, Hercules, California, USA). The bombarded cells were harvested within 3–5 days for analysis. We bombarded mock sample cells with gold particles without vector constructs and used them as a negative control.

Extraction procedures: We accomplished natural product extraction from *C. roseus* leaves and overexpressed tobacco cell cultures using an ASE 100 automated accelerated solvent extraction system (Dionex Corp., Sunnyvale, California, USA). A 10 mL stainless steel extraction cell was used to process dry and wet samples in the 0.3–5.0 g tissue range. Leaves were chopped in a blender, dried overnight in petri dishes at 70 °C and homogenized in a mortar. Harvested cell cultures were kept at –80 °C before use and process-frozen to lyse cells by triturating with liquid nitrogen in a mortar. The samples were mixed with 20–50% Hydromatrix pelletized diatomaceous earth (Varian Inc., Palo Alto, California, USA) (by weight) to improve extraction efficiency and reproducibility.

We used the following procedure for automated extraction: three extraction cycles of 8 min each at 100 °C, 60% flush volume and 100 s purge time. We typically used water (adjusted with sulphuric acid to pH 3) or methanol to extract natural products from tissues. These collected extracts were centrifuged when necessary (large samples can produce turbid extraction solutions), concentrated or dried in a Büchi Rotavapor R-200 rotary vacuum evaporator (Brinkmann Instruments Inc., Westbury, New York, USA) and brought to the desired final volume by adding methanol or water.

HPLC: HPLC analysis was performed using a model 1100 HPLC system (Agilent Technologies Inc., Wilmington, Delaware, USA) equipped with a diode-array detector, a thermostated autosampler and fraction collection blocks. We used ChemStation software (Agilent) for data acquisition and analysis. We used a 150 × 4.6 mm, 5 µm d_p Zorbax SB-C8 reversed-phase analytical LC column for the separations under either isocratic or gradient elution conditions with 5 mM sodium phosphate buffer (pH 6), acetonitrile as organic modifier (20–80% range), and a 1–2 mL/min flow-rate (typically 1.5 mL/min). The separation column was thermostated at 35 °C and the autosampler at 4 °C. We

injected 5–100 μL samples. Alkaloid standards at 0.1 mg/mL concentration and 10 μL injection volume provided adequate signal at 220 nm.

CE: For CE analysis, we used a P/ACE MDQ CE instrument (Beckman Coulter, Fullerton, California, USA). UV detection at 214 nm enabled good sensitivity for the test alkaloid solutions and the natural product extracts we studied. We used 75 μm i.d. bare fused-silica capillaries (Polymicro Technologies Inc., Phoenix, Arizona, USA) with 60 cm total length and 50 cm length to the detection window for UV and 100 cm for MS detection. The capillary typically was conditioned by rinsing it with 0.5 M sodium hydroxide for 15 min, followed by a 5 min rinse with water, and a 5 min separation buffer rinse with the end of the tubing directed away from the mass spectrometer.

We used MS-compatible separation buffers of 12–50 mM ammonium acetate (pH 4.0–6.0) and 25–50 mM tetramethylammonium formate (pH 5.0–6.0) that contained 0–20% acetonitrile. The samples were injected by pressure (0.5 psi for 5–10 s) and run at 20 kV (333 V/cm) under normal polarity (anode at the injection side). We obtained excellent detection signal and separation performance using alkaloid standards in the 0.01–0.05 mg/mL concentration range.

MS: We used a P/ACE MDQ CE–MS interface block (Beckman Coulter) for on-line coupling of the separation capillaries to the LCQ DECA XP Plus mass spectrometer (Thermo Electron Corp., San Jose, California, USA). The fused-silica capillaries (Polymicro Technologies) had 100 μm inner and 375 μm outer diameters. We performed MS detection in positive ion electrospray mode. Electrospray conditions were as follows: the electrospray potential (U_{ES}) was 3 kV; the electrospray current (I_{ES}) was 0.14 μA ; the capillary temperature was 150 $^{\circ}\text{C}$; the sheath liquid was 0.1% acetic acid in 50:50 (v/v) methanol–water; and the flow-rate was 1 $\mu\text{L}/\text{min}$. Scanning was performed from m/z 300 to

m/z 1000 in 50 ms intervals. We used Excalibur software (Thermo Electron Corp.) for data acquisition.

Results and Discussion

First, we applied bioinformatics tools to identify the genes involved in important secondary metabolite pathways (Figure 1). We used known published genes from *C. roseus* as bait to BLAST against other plant species. These genes served as targets to clone the full-length cDNA. We also applied clustering methods with known secondary metabolite genes to find genes that are coregulated and assumed to be secondary-metabolite related for subsequent cloning and testing.

We established an extensive database of more than 600 genes associated with secondary metabolite production in different plant species. Of these genes, we have cloned 40 full-length genes successfully and expressed some in tobacco cell cultures.

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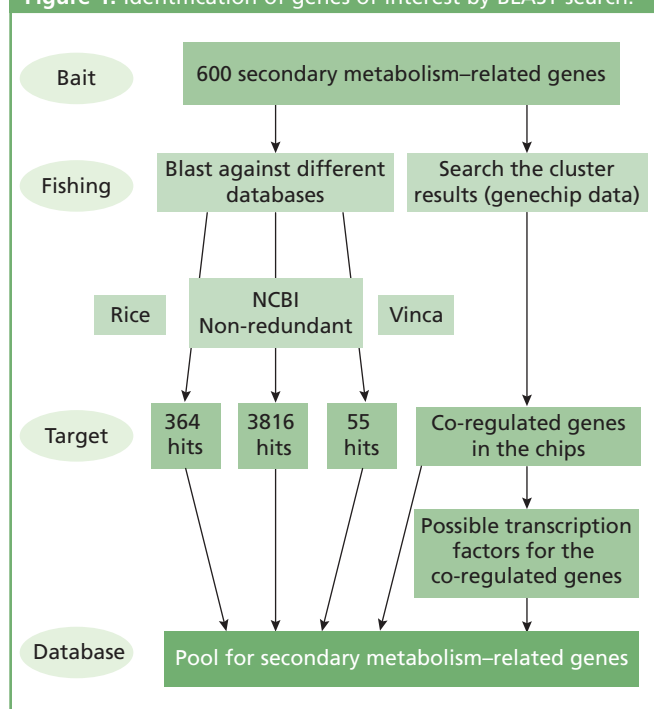
Our preliminary study suggested that high-level expression of the reporter gene β -galacturonidase was obtained by *in vivo* transient expression system of plant deoxyribonucleic acid (DNA) viral vectors that used easy-to-maintain tobacco cell cultures and an efficient particle bombardment system. More than 50% of the cells were transfected and expressed the reporter genes from the viral vector, compared with 21% of the cells expressed β -galacturonidase from the promoter- β -galacturonidase construct.

We further tested the expression levels at the condition of 1100 psi pressure with single, double and triple bombardment. Double bombardment exhibited twofold higher β -galacturonidase expression, whereas triple bombardment did not result in any further increase. We also compared expression at 3–8 days post bombardment to determine the proper harvest time, and we observed that the highest level of β -galacturonidase expression was reached in 3–5 days. The expression levels derived from the coat protein–replacement vectors increased approximately 10–40 fold.

Analytical method development: *Extraction procedures:* We evaluated the extraction process from *C. roseus* leaves and tobacco cell cultures using accelerated solvent extraction system with methanol or pH-adjusted water. In the latter instance, we also attempted liquid–liquid extraction by adding an equal amount of ethyl acetate to the aqueous phase (repeated three times) and discarding the organic phase. Next we adjusted the pH of the aqueous phase to pH 6 and performed ethyl acetate extraction. After this step, the organic phase contained the crude secondary metabolite mixture of interest. We froze tobacco cell cultures in liquid nitrogen, converted the cultures to fine powder in a mortar and performed the same extraction procedure. Although this method proved to be useful for alkaloid purification, we preferred using the accelerated solvent extraction system with methanol because of its simplicity, higher yields, speed and automation. In case of turbidity, we centrifuged the extracts and used the supernatants in subsequent separation experiments.

To estimate extraction efficiency, we added known amounts of alkaloids to dry tobacco cell culture samples, processed them

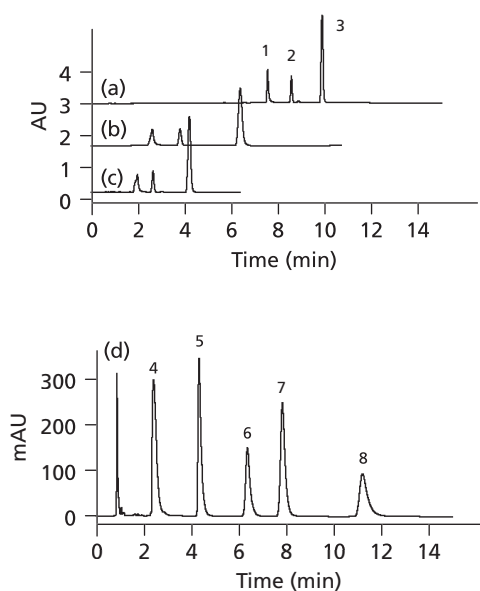
Figure 1: Identification of genes of interest by BLAST search.



as described above, and quantified them by HPLC. Spiking experiments showed higher extraction yield of standard *C. roseus* alkaloids of vinblastine, vincristine and serpentine in methanol (75–100%) than those in pH 3-adjusted water (25–50%). The actual extraction yields varied for the different alkaloid standards; the highest yields were for vinblastine and vincristine. Based upon the extraction results, we started the analysis with methanol extraction and continued it with HPLC or CE analysis. We injected undiluted extracts for HPLC analysis, but CE typically required 10-fold diluted samples.

HPLC: We evaluated and optimized several separation parameters for HPLC analysis of the extracts in both isocratic and gradient elution modes. These parameters included organic phase type and concentration, buffer composition and pH, flow-rate and temperature. Figures 2(b) and 2(c) show the separation of vindesine, vincristine and vinblastine standards under isocratic conditions using 45% and 50% acetonitrile, respectively. Figure 2(a) depicts gradient elution changing the concentration of the organic modifier from 20% to 80% in 15 min. We chose to use isocratic conditions with 45% acetonitrile because this combination was faster and required no equilibration time between the runs. We achieved optimal performance at 35 °C with 5 mM sodium phosphate buffer (pH 6.0) and a 1.5 mL/min flow-rate using 45% acetonitrile as organic modifier (Figure 2(d)). As Figure 2(d) shows, we obtained good separation of the five available *C. roseus* alkaloid standards of serpentine, vincamine, vincristine, ajmalicine and

Figure 2: Optimization of HPLC separation of alkaloid standards. Traces: (a) gradient 20–80% acetonitrile in 15 min; (b) isocratic 45% acetonitrile; (c) isocratic 50% acetonitrile; flow-rate: 2 mL/min. (d) Optimized separation with isocratic elution, 45% acetonitrile, flow-rate: 1.5 mL/min.

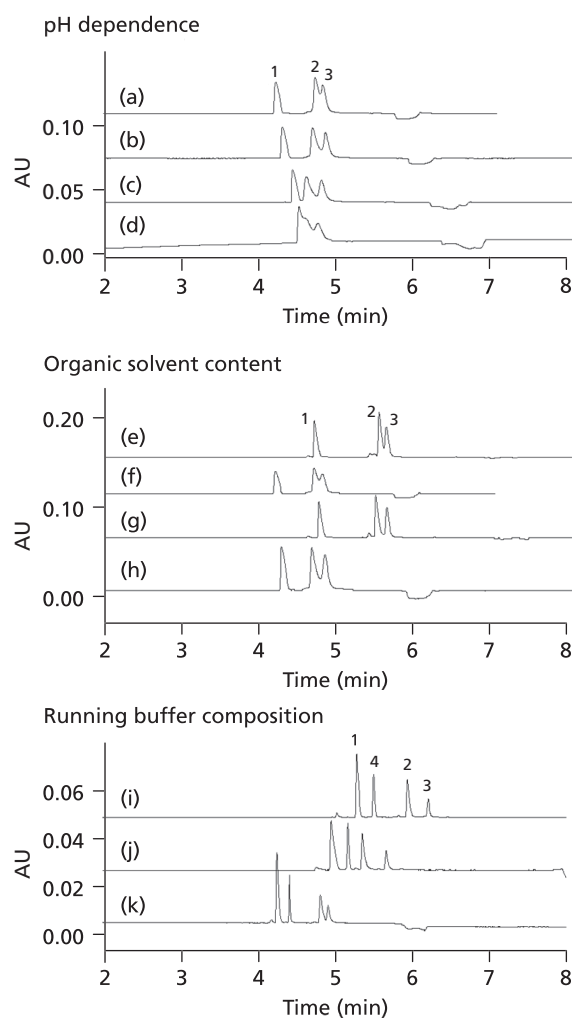


Peaks: 1 = vindesine (0.33 mg/mL), 2 = vincristine (0.33 mg/mL), 3 = vinblastine (1.67 mg/mL), 4 = serpentine, 5 = vincamine, 6 = vincristine, 7 = ajmalicine, 8 = vinblastine (0.1 mg/mL each). Separation conditions: column: ZORBAX SB-C8 at 35 °C; buffer system: 5 mM sodium phosphate (pH 6); injection volume: 10 µL; UV detection at 220 nm.

vinblastine in 12 min.

CE: We evaluated CE as a complementary separation method in combinatorial natural product analysis. In this instance, we used MS-compatible buffers of ammonium acetate (12–50 mM, pH 4.0–6.0) and tetramethylammonium formate (25–50 mM,

Figure 3: Capillary electrophoresis separation optimization with *Catharanthus roseus* alkaloid standards. pH dependence with 50 mM ammonium acetate buffer. Traces: (a) pH 6.0, (b) pH 5.6, (c) pH 5.0, (d) pH 4.75. Organic solvent dependence in 50 mM ammonium acetate buffer. Traces: (e) pH 6, 20% acetonitrile, (f) pH 6 with no organic additive, (g) pH 5.6, 20% acetonitrile, (h) pH 5.6 with no organic additive. Running buffer composition dependence. Traces: (i) 50 mM TMAF, pH 5.3, 20% acetonitrile, (j) 50 mM TMAF, pH 5.3, (k) 50 mM ammonium acetate, pH 5.6, 20% acetonitrile. Concentration of standards: serpentine, vincristine, vincamine (0.02 mg/mL each), vinblastine (0.05 mg/mL). Separation conditions: capillary length: 60 cm (50 to detection); separation voltage: 20 kV; injection: pressure 0.5 psi/5 sec; temperature: 25 °C.



Peaks: 1 = serpentine, 2 = vinblastine, 3 = vincristine, 4 = vincamine.

CE featured a lower sample requirement and enabled faster and more-sensitive analysis that exhibited changes in the migration order of the alkaloid standards compared with HPLC because of its different separation mechanism.

pH 5.0–6.0) to separate *C. roseus* alkaloid standards. We attempted using other buffers such as carbonate and low-concentration phosphates, but they failed to provide satisfactory performance. Generally, increasing buffer concentration resulted in improved resolution but yielded high currents (greater than 100 mA) and, thus, excessive heat generation.

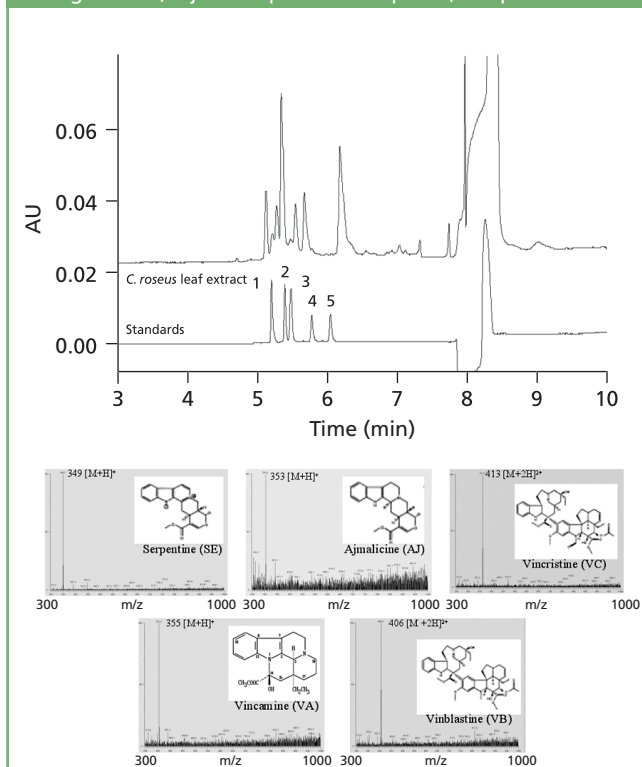
During CE method development, we evaluated parameters such as running-buffer pH, composition and organic solvent additives. Figures 3(a)–3(d) depict the pH dependence of the electrophoretic separation of a mixture of three standard

C. roseus indole alkaloids — serpentine, vinblastine and vincristine. We found pH 5.6 (Figure 3(b)) to be optimal; therefore, we used it in consequent experiments. Adding an organic modifier such as acetonitrile to the separation buffer increased separation efficiency at the expense of slightly increased analysis time. Figures 3(e)–3(h) depict the effect of acetonitrile content on the separation performance at pH 6.0 and pH 5.6 (Figures 3(e) and 3(g), respectively). We observed improved resolving power with the addition of 20% acetonitrile. Additional increase in acetonitrile concentration in the separation buffer (more than 20%) resulted in somewhat longer separation times and decreased resolution of the slower migrating peaks, probably because of the decrease in mobility differences of essentially hydrophobic molecules in the low-polarity solvent environment. Buffer composition was also very important as demonstrated in Figures 3(i)–3(k), which compare tetramethylammonium formate with (Figure 3(i)) and without (Figure 3(j)) organic modifier and ammonium acetate (Figure 3(k)). Tetramethylammonium formate provided superior resolution without any organic additives. Please note that organic additives are generally not preferred in CE applications because of possible evaporation problems.

CE featured a lower sample requirement and enabled faster and more-sensitive analysis that exhibited changes in the migration order of the alkaloid standards compared with HPLC because of its different separation mechanism. CE-based analysis also supported MS compatibility.

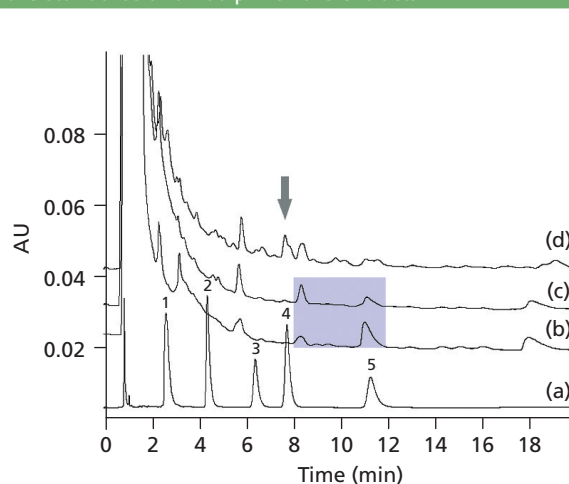
Combinatorial natural product analysis: Figure 4 compares CE analysis of a *C. roseus* leaf extract (upper trace) with the five-component test mixture of serpentine, vincamine, vincristine, ajmalicine and vinblastine (lower trace) using MS-compatible tetramethylammonium formate buffer system. The large peak migrating at 8 min probably represents all neutral components

Figure 4: Comparison of CE separation traces of *Catharanthus roseus* alkaloid standards (lower trace) and leaf extracts (upper trace). Corresponding MS traces and the structures of the standards are also shown. Dry leaf (1 g) was extracted with methanol, the final volume was adjusted to 1.5 mL and diluted 10-fold for CE analysis. Concentration of all alkaloid standards was 0.01 mg/mL. Separation conditions: buffer: 50 mM TMAF, pH 5.3, 20% acetonitrile; separation voltage: 20 kV; injection: pressure 0.5 psi/5 s; temperature: 25 °C.



Peaks: 1 = serpentine, 2 = vincamine, 3 = ajmalicine, 4 = vinblastine, 5 = vincristine.

Figure 5: HPLC analysis of combinatorial natural products. (a) *Catharanthus roseus* alkaloid standards. (b) Mock cell suspension extracts. (c) Spermidine Synthase transfected tobacco cell culture extract. (d) Germacrene C Synthase transfected tobacco cell culture extract. Separation conditions were the same as in Figure 2(d). Injection volume: 10 µL for the standards and 100 µL for the extracts.



Peaks: 1 = serpentine, 2 = vincamine, 3 = vincristine, 4 = ajmalicine, 5 = vinblastine (0.1 mg/mL each.)

in the extract carried by the electroosmotic flow. The enclosed MS panels show the corresponding mass spectra and the structures of the five standards.

Figure 5 shows a representative example of combinatorial natural product generation with the transfection of SPDE_LYCES spermidine synthase gene (terpenoid pathway) and germacrene C synthase (polyamine pathway) to demonstrate the feasibility of the proposed approach. The transfected tobacco cell lines were subjected to accelerated solvent extraction followed by HPLC analysis to determine changes in their secondary metabolite profiles. Figure 5 compares the separation of several major vinca alkaloid standards (Figure 5(a)) with a mock sample (Figure 5(b)) and with cell lines transfected with secondary metabolite genes (Figures 5(c) and 5(d)). Figure 5(c) represents the SPDE_LYCES spermidine synthase transfected cell extract and exhibits apparent peak-height ratio changes compared with the mock sample (shaded square). Figure 5(d) delineates the germacrene C synthase transfected cell extract and shows an additional new peak (arrow), which probably represents a chemical entity induced by the transfection process.

Conclusions and Outlook

Novel natural products and proprietary chemical scaffolds are in high demand by the pharmaceutical and agrochemical industries because compounds with novel chemical entities can be used to generate chemical libraries by parallel synthesis. The combinatorial natural product generation strategy described in this article enabled the transfer of biosynthetic genes from *C. roseus* to tobacco cell cultures to introduce novel pathways and to enhance diversity by possible production of novel chemical entities. State-of-the-art high-performance separation methods — HPLC and CE — revealed differences in secondary metabolite patterns. The approach can be readily automated to increase throughput when a large number of combinatorial samples need to be analysed.

A possible extension of this combinatorial genetic approach is to transfer genes involved in secondary metabolite pathways from hard-to-get or slow-growing species to industrially scalable organisms, which would enable the introduction of new enzymes to enhance diversity. We also envisage a scale-up process that uses industrial plants as low-cost renewable resources and high-throughput bioreactors to produce novel secondary metabolites.

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