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A simple and rapid HPLC-DAD method for simultaneously monitoring the accumulation of alkaloids and precursors in different parts and different developmental stages of *Catharanthus roseus* plants

Qifang Pan[a,*], Mohd Zuwairi Saiman[c], Natali Rianika Mustafa[b], Robert Verpoorte[b], Kexuan Tang[a]

[a] Plant Biotechnology Research Center, SJTU-Cornell Institute of Sustainable Agriculture and Biotechnology, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 200240, PR China
[b] Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72, Leiden 2333 BE, The Netherlands
[c] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

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ABSTRACT

A rapid and simple reversed phase liquid chromatographic system has been developed for simultaneous analysis of terpenoid indole alkaloids (TIAs) and their precursors. This method allowed separation of 11 compounds consisting of eight TIAs (ajmalicine, serpentine, catharanthine, vindoline, vindolinine, vincristine, vinblastine, and anhydrovinblastine) and three related precursors i.e., tryptophan, tryptamine and loganin. The system has been applied for screening the TIAs and precursors in *Catharanthus roseus* plant extracts. In this study, different organs i.e., flowers, leaves, stems, and roots of *C. roseus* were investigated. The results indicate that TIAs and precursor accumulation varies qualitatively and quantitatively in different organs of *C. roseus*. The precursors showed much lower levels than TIAs in all organs. Leaves and flowers accumulate higher level of vindoline, catharanthine and anhydrovinblastine while roots have higher level of ajmalicine, vindolinine and serpentine. Moreover, the alkaloid profiles of leaves harvested at different ages and different growth stages were studied. The results show that the levels of monoindole alkaloids decreased while bisindole alkaloids increased with leaf aging and upon plant growth. The HPLC method has been successfully applied to detect TIAs and precursors in different types of *C. roseus* samples to facilitate further study of the TIA pathway and its regulation in *C. roseus* plants.

1. Introduction

Terpenoid indole alkaloids (TIAs) are a group of important secondary metabolites of medical bioactivity produced in *Catharanthus roseus* (L.) G. Don. Among them, monoindole alkaloids catharanthine, vindoline and vindolinine could lower blood sugar levels to ease the symptoms of diabetes; ajmalicine and serpentine are used for their sedative activity in the treatment of hypertensia, as well as their antidiabetic activity [1–3]. Through the coupling of catharanthine and vindoline, anhydrovinblastine is the first bisindole alkaloids product and further converted into the well-known antitumor compounds vinblastine and vincristine, which are prescribed for the treatment of various types of cancer [4]. Their trace amounts but high value urgent us to investigate the pattern of these TIAs accumulation and biosynthesis. These useful TIAs all derive from two precursor pathways, the indole pathway and the iridoid pathway. Tryptophan and tryptamine are important intermediates from the indole pathway while loganin, the precursor of secologanin, comes from the iridoid pathway. Production of either the indole or the iridoid precursors could affect the biosynthesis of TIAs [5,6]. TIAs accumulation was enhanced by addition of loganin, indicating the limitation by the steps in the iridoid pathway [7]. Moreover, the biosynthesis of TIAs and precursors has a tight relationship with tissue differentiation and cell compartmentation, which is limiting the use of *C. roseus* tissue cultures (cells and hairy roots) in the industrial production of valuable alkaloids. Bisindoles, such as vincristine and vinblastine, are synthesized in aerial parts of the plant, mainly in leaves [8], whereas vingramine and methylvingramine have been reported to be detectable only in seeds of *C. roseus* [9]. Ajmalicine, serpentine, hörhammericine, lochnericine, 19-hydroxytabersonine, 19-*O*-acetyl-hörhammericine and

* Corresponding author at: Shanghai Jiaotong University, 800 Dongchuan Road, Shanghai, China.
E-mail address: panqf@sjtu.edu.cn (Q. Pan).

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echitovenine are mostly present in roots [10–12], and are rarely detected in cell cultures [13]. Vindoline, one of the two precursors of the bisindole alkaloids, is produced in the green parts of the plant and does not exist in the roots, hairy roots or cell cultures [14]. The activity of key enzymes LAMT and 16-hydroxytabersonine by *O*-methyltransferase (16OMT) showed a negative relation with leaves age [15]. Previous publications reported that the alkaloids content changed coinciding with the different developmental stages of *Catharanthus pusillus* plants [16]. However, *C. pusillus* does not produce the bisindoles vinblastine and vincristine. To better understanding the regulation of TIA biosynthesis, it is crucial to determinate both TIAs and its related precursors qualitatively and quantitatively in an accurate and efficient way that could be used in different tested matrix of *C. roseus*.

Various analytical techniques and methods have been developed to screen and analyze the profiles of alkaloids in *C. roseus* plants, cell cultures and hairy roots. Thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) are general separation techniques coupled with different types of detectors, such as fluorescence, UV-detection (photo diode array), ESI-MS, MS–MS, NMR and CD, which are useful in determining different types of TIAs and precursors [14,17–20]. HPLC-UV is a favored technique that can separate, identify and quantify TIAs due to the efficiency, reproducibility, availability in most laboratories, simple sample preparation, and ability of both qualitative and quantitative analysis. However, the capacity of TIAs separated in one run by one HPLC/UPLC method is always limited from two to seven compounds in the TIA or precursor pathways [21]. Due to different characteristics of alkaloids and precursors, different HPLC methods were used to separate and analyze TIAs and their precursors [21–24], which caused the inefficiency in determining the TIAs production. However, there are no studies reporting a single method to separate both precursors and TIAs in a sample extract. It would be better to simultaneously measure both TIAs and precursors in the complex matrix of *C. roseus* plant extracts by one simple method.

In this study, an optimal HPLC-UV method was developed to simultaneously analyze eight TIAs (vindoline, catharanthine, ajmalicine, serpentine, vindolinine, anhydrovinblastine, vinblastine and vincristine) and three precursors (tryptophan, tryptamine and loganin) in *C. roseus* plant materials. The contents of TIAs and precursors were analyzed and compared among flowers, young leaves, old leaves, stems and roots of *C. roseus*. In addition, the metabolic profile of TIAs and precursors in leaves of different ages and growth stages were studied in order to evaluate the accumulation and distribution of TIAs in space and through time. This work improved the coverage of TIA related compounds tested by HPLC-UV, which provided a simple and rapid analytical method to simultaneously investigate the production of TIAs, indole and iridoid precursors in all types of *C. roseus* samples for future study.

2. Materials and methods

2.1. Chemicals

The HPLC grade methanol was bought from Carl Roth GmbH (Karlsruhe, Germany) and Biosolve B.V. (Valkenswaard, The Netherlands), respectively. Methanol and ethanol were of analytical reagent (AR) grade from Biosolve B.V. Strictosidine and secologanin were from Phytoconsult B.V. (Leiden, The Netherlands). Loganic acid, loganin, and vindoline were bought from PhytoLab, Vestenbergsgreuth, Germany. Tryptamine was purchased from Aldrich Chemical, Milwaukee, WI, USA. Tryptophan, vindolinine and ajmalicine were purchased from Sigma–Aldrich, St. Louis, MO, USA. Serpentine was purchased from Carl Roth GmbH. Catharanthine, anhydrovinblastine, vinblastine and vincristine were from Pierre Fabre, Gaillac, France. $NaClO_2$ was purchased from Sigma–Aldrich.

2.2. HPLC conditions

The chromatographic system was an Agilent Technologies 1200 series, consisting of a G1322A vacuum degasser, a G1310A pump, a G1329A auto-sampler, and a G1315D diode array detector (Agilent Techologies Inc.). The column was an Agilent Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm particle size) (Agilent Techologies Inc., Santa Clara, CA, USA), coupled with a SecurityGuard™ column (Phenomenex, Torrance, CA, USA).

The mobile phase consisted of a mixture of 5 mM Na_2HPO_4 (pH adjusted to 6 with HCl) (solvent A) and methanol (solvent B) at a flow rate of 1.5 ml per min. The eluent profile (solvent A/solvent B) was set to a linear gradient from 86:14 to 14:86 at 0–26 min, an isocratic mode (14:86 v/v at 26–30 min), a linear gradient from 14:86 to 86:14 at 30–35 min, an isocratic elution with 14:86 (v/v) at 35–37 min. The injection volume was 30 μl.

2.3. Identification and quantitation

Standard solutions were prepared following Tikhomiroff and Jolicoeur publication [24]. The quantification was performed using six levels of external standards. Level 6 consisted of a dilution (1:50 v/v) of stock solution (5 mg/ml) in methanol. Levels 5–1 were obtained by dilution of level 6 by a factor 2, 4, 8, 16 and 32 respectively. The ranges obtained were 2–4 μg/ml to 64–128 μg/ml depending on the concentration of each compounds' stock solution. Identification of alkaloids from the plant extracts was performed by comparison of the UV spectra and retention time with those of authentic standards. Each calibration curve was obtained by making each concentration in triplicates and measuring them. Test samples were analyzed in triplicate for quantification using the calibration curves of the standards. The limit of detection and recovery was measured as described the previous publication [24,25].

2.4. Plant materials

Seeds of *C. roseus* (cv. Pacific Cherry Red) were purchased from PanAmerican Seed Company (West Chicago, IL, USA). The seeds were surface sterilized in 75% (v/v) ethanol for 2 min and 5% (v/v) $NaClO_2$ for another 5 min. Subsequently, seeds were washed five times with sterile distilled water and germinated on Petri dishes containing MS (Murashige and Skoog) basal medium. Cultures were grown under 16 h light and 8 h dark photoperiod at 25 ± 2 °C. After germination for 2 weeks, seedlings were transplanted into soil and cultivated in the green house (the air temperature and relative humidlity were set at 25 °C and 55%). When blooming, flowers, leaves, stems and roots of the plants were separately harvested and directly frozen in liquid nitrogen for the following experiments. Some plants of another batch were collected at 4:00 pm of the 30th, 44th, 62th, 79th and 99th days after germination in order to observe the change of alkaloid content during the growth stages. Samples of each observation group were measured in triplicate.

2.5. Sample preparation

Freshly collected sample materials were ground in liquid nitrogen using mortar and pestle. Subsequently, the samples were lyophilized for 72 h. The dried sample (30 mg) was put in a micro-tube and extracted with 1 ml methanol by vortexing and a sonication (30 min) in an Ultrasonic bath (DL-60D). Then, samples were centrifuged at 12,000 rpm for 10 min at room temperature

and the supernatant was filtered with 0.45 μm needle type PTFE membrane filter (from Sigma–Aldrich) prior to HPLC analysis.

2.6. Data statistical analysis

All experiments were conducted with three replicates. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range (DMRT) test. The values are mean ± SD for three samples in each group. p values ≤0.05 were considered as significant. The ANOVA for all the data was performed by SPSS (version 17.0, Chicago, IL, USA).

3. Results and discussion

3.1. Separation of standards

To improve the efficiency and capacity of TIA analysis by HPLC, our study developed one method for simultaneous analysis of eight TIAs and three precursors. To obtain an optimal eluent profile, five gradient proportions of potassium phosphate buffer and methanol (80:20, 82:18, 84:16, 86:14, 88:12, 90:10 v/v) were tested with two time procedures. One took 25 min to transpose the proportion of the buffer and methanol, and the other took 20 min. The results showed that the transposing process took time longer, the separation of tested compounds was better. The methanol content in the mobile phase has great effect on the retention time of compounds [26]. When the proportion of potassium phosphate buffer and methanol was 80:20 (v/v), the signal of vinblastine was totally overlapped with ajmalicine signal. With the proportion changing from 80:20 to 90:10 (v/v), vinblastine signal was separated from ajmalicine. But when the proportion was 90:10 (v/v), the signals of catharanthine, vinblastine and ajmalicine tended to be clustered and could not separate well. The proportion of potassium phosphate buffer and methanol at 86:14 (v/v) provided a good separation of eight TIAs (serpentine, vindoline, vincristine, catharanthine, vinblastine, ajmalicine and anhydrovinblastine) and three precursors i.e. tryptophan, tryptamine and loganin. Fig. 1 shows the chromatogram of the mixture of the reference compounds. Retention time, UV wavelength, and quantification range of standard compounds are presented in Table 1.

Under the described HPLC conditions, a linear relationship between the concentration of precursors and TIAs and their UV absorbance at 254 nm was obtained. The correlation coefficient for the standard curves (r^2) exceeded 0.99 (Table 2). Based on the standard deviation (SD) of the response and the slope (S) of the calibration curves, the limit of detection (LOD) was calculated according to the formula [27]: LOD = 3.3 (SD/S) and the limit of quantification (LOQ) was calculated following the formula: LOQ = 10(SD/S). The SD of the response was determined based on the y-intercepts of regression lines. Results are shown in Table 2.

Repeatability was checked by analyzing five times the same sample, by the same analyst, within the same day (Table 1). Relative standard deviation (RSD) varying between 1.19% and 2.98% indicated that the repeatability of the procedure was good. Intermediate precision determined by different analysts on three separate days was also found satisfactory (RSD ranging from 1.58% to 3.10%).

The recovery was measured to validate the extraction method for each studied compound and shown to be dependent on the compound (Table 1). The recoveries of most compounds were higher than two direct HPLC analytical methods for alkaloids and precursors, indicating that the method was appropriate for screening of both TIA and precursors in *C. roseus* plants.

By adjusting the gradient timing and the proportion of phosphate buffer and methanol, the validated method not only improved the efficiency but also increased the coverage of HPLC analysis, which could simultaneously measure eleven TIA related compounds. Compared with the previous works using two direct HPLC methods to analyze less than ten TIAs or precursors [21,24], the present method has the advantage in determining more TIAs and precursors in one direct HPLC run.

3.2. The profile of alkaloids and precursors in different organs

The extraction and the chromatography method were evaluated by extraction of different organs i.e., flower, leaf, stem, and root of *C. roseus* with methanol and analyzing the extracts using the HPLC system. Fig. 1 shows the chromatograms of crude extracts of *C. roseus* different organs. Compounds in crude extracts were identified based on both its retention time and UV absorbance compared to the standards [21]. The signals at the retention time of tryptophan, tryptamine and serpentine in the flower chromatogram were not identified because their UV absorbance were not the same as the three standards (Supplemental Fig. 1). Only vindolinine, vindoline, catharanthine, ajmalicine, and anhydrovinblastine were identified in the flowers. Most of the alkaloids were detected in the leaves, where also the precursors tryptophan and loganin are found. Higher content of loganin indicated that the steps in the iridoid pathway might not limit the TIA biosynthesis. The leaf TIAs included ajmalicine, serpentine, vindoline, catharanthine, as well as the bisindole alkaloids of anhydrovinblastine and vinblastine. In contrast, no bisindole alkaloids were detected in *C. roseus* stems, but tryptophan, serpentine, vindoline and catharanthine were identified in this organ. Similarly, no bisindole alkaloids were found in the roots, but serpentine, vindolinine, catharanthine and ajmalicine were clearly detected. Furthermore, quantitative analysis was performed to evaluate the levels of precursors and TIAs in different organs (Table 3). Our results suggested that leaves were the main place for the biosynthesis of bisindole alkaloids anhydrovinblastine and vinblastine because of the high accumulation of vindoline and catharanthine. Interestingly, anhydrovinblastine content was much higher than vinblastine, which might be an alternative substrate for the semi-synthesis of vinblastine. Roots produced more vindolinine, ajmalicine and serpentine than other organs, implying roots or hairy roots could be optimal materials for the industrial production of these alkaloids. Both flowers and stems contained a relatively low level of TIAs and precursors. For the different organs that have been analyzed, vinblastine was only detected in the leaves, while tryptamine and vincristine were not detected in any organ. Transcriptome analysis showed a significant difference of gene expression pattern in *C. roseus* leaves and roots [28]. It was reported that complex alkaloid fluctuations were recorded from organ to organ in *C. pusillus*, which also belongs to the genus *Catharanthus* [16].

3.3. Alkaloids and precursors in leaves of different age

Leaves at different positions along the stem are of different age. Younger leaves are in the upper-3-layers from the top and the older ones are in the lower-3-layers from the bottom [29]. The leaves in the middle layers (excluded from upper- and lower- layer leaves) are at an age in between. In this study, the levels of TIAs and precursors were measured at the three different layers of leaves along the stem of *C. roseus* plants (Fig. 2), which represents different leaf ages. The levels of TIAs (serpentine, vindolinine, vindoline, catharanthine and ajmalicine) decreased while the precursors loganin and tryptophan increased as *C. roseus* leaves were aging. Bisindole alkaloids anhydrovinblastine and vinblastine showed the highest level in the middle-layer leaves with a fluctuating pattern of accumulation. Statistical analysis showed that the levels of monoindole alkaloids were significantly higher in upper-layer leaves, and precursor lev-

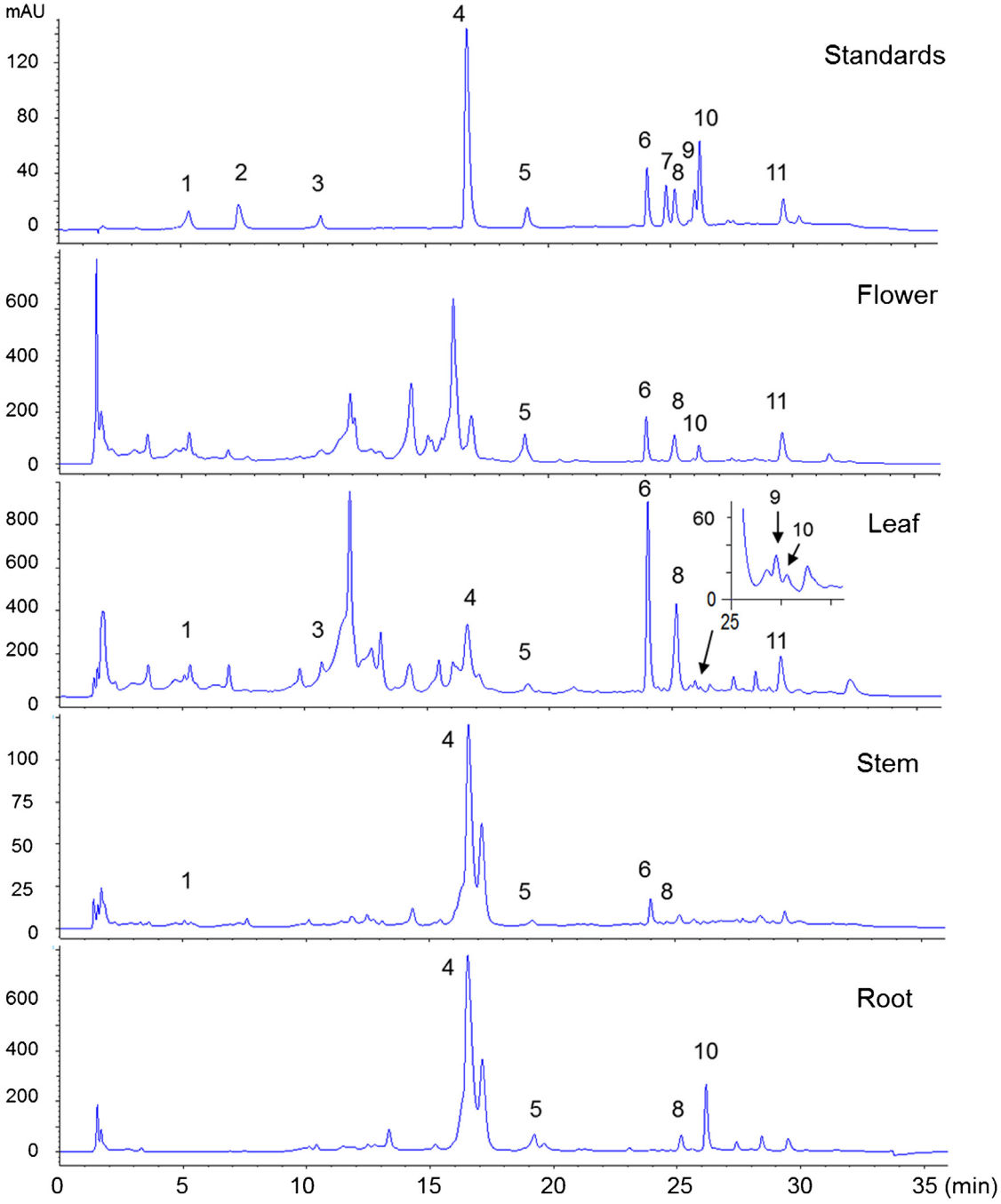

Fig. 1. HPLC-DAD chromatograms of the standard mixtures (TIAs and precursors) and methanol crude extracts of flower, leaf, stem and root of *Catharanthus roseus* at wavelength of 254 nm. (1) Tryptophan; (2) tryptamine; (3) loganin; (4) serpentine; (5) vindolinine; (6) vindoline; (7) vincristine; (8) catharanthine; (9) vinblastine; (10) ajmalicine; (11) anhydrovinblastine.

els were significantly higher in lower-layer leaves, while the levels of bisindole alkaloids were significantly higher in middle-layer leaves. The difference of accumulation pattern between monoindole and bisindole alkaloids indicates that their levels were not directly correlated to each other. Previous studies reported that the biosynthesis of vinblastine is related with the age of leaves [30]. Moreover, it was revealed that the activity of biosynthetic enzymes (NMT, 16-OMT and LAMT) decreased as the leaf age of *C. roseus* increased [15]. Our results indicate that when leaves are getting older, the alkaloid precursors are accumulating but seem not to be available for the biosynthesis of monoindole alkaloids as they show a reduction of their level. Bisindole alkaloids accumulated in aging leaves. The production of defense compounds is highest in younger leaves as these leaves have the highest potential for photosynthesis for the plant [31–34]. This results thus in the highest levels in the youngest leaves. But the total absolute amount of alkaloid in a leave is more or less the same for young and old leaves [32]. The experiments done support this scenario for alkaloid levels.

3.4. *Alkaloids and precursors in the growth stage of* C. roseus *leaves*

The *C. roseus* plants got mature and began to flower at around 65–75 days after sowing. The flowering stage lasted for at least 3

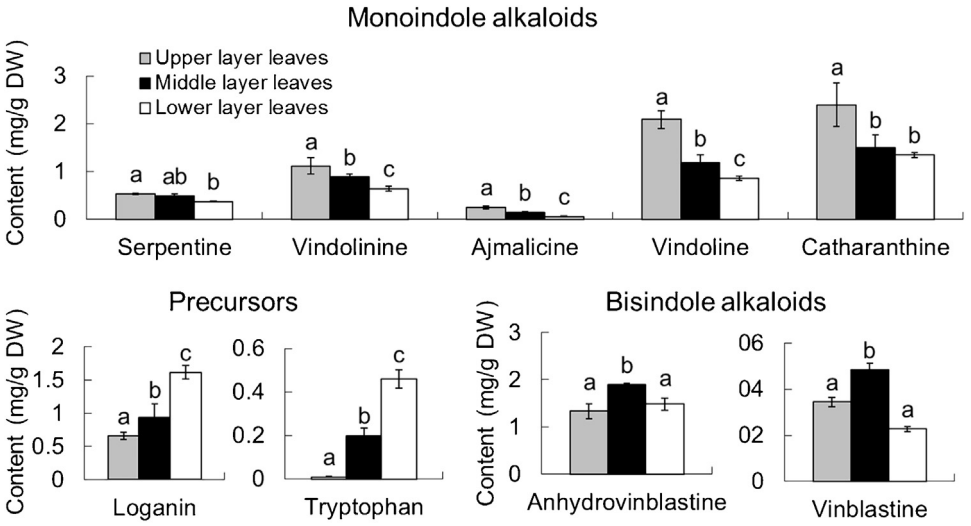

Fig. 2. Terpenoid indole alkaloids and precursors content in *Catharanthus roseus* leaves at different ages. Layer from upper to lower represents leaves getting old with aging. Different letters indicate significant difference ($p < 0.05$ by ANOVA).

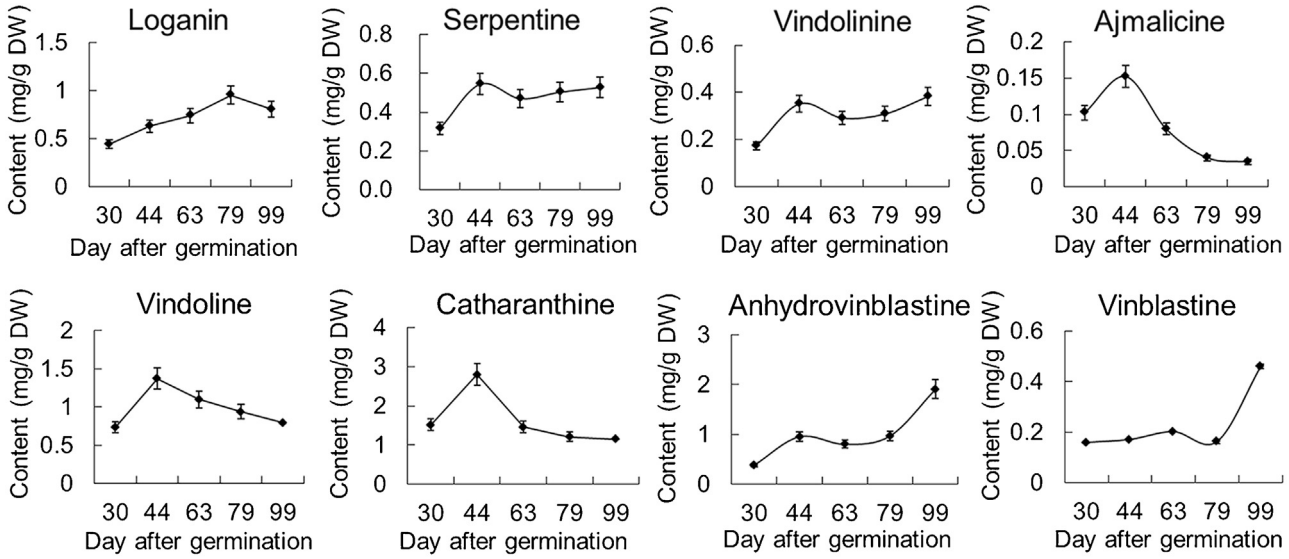

Fig. 3. Levels of TIAs and loganin in *Catharanthus roseus* leaves during growth stage.

Table 1
Detection parameters of indole alkaloids and precursors.

No.	Metabolite	Retention time (min)	Wavelength (nm)	Testrange (μg/ml)	Recovery (%)[a]	RSD (%)	Repeatability RSD (%)	Intermediate precision RSD(%)
1	Tryptophan	5.21	270	6.07–109.99	74.06%	1.94%	2.49%	1.40%
2	Tryptamine	7.65	270	1.43–94.84	103.77%	0.62%	2.98%	2.93%
3	Loganin	10.33	238	4.28–102.12	91.51%	0.47%	1.26%	1.23%
4	Serpentine	17.55	306	2.95–83.17	57.19%	0.31%	2.42%	3.09%
5	Vindolinine	19.75	220	3.04–85.39	58.75%	2.07%	1.58%	1.87%
6	Vindoline	24.00	220	3.20–99.94	92.38%	1.00%	1.19%	1.65%
7	Vincristine	24.93	220	3.62–95.68	92.02%	0.32%	1.91%	2.74%
8	Catharanthine	25.45	220	1.46–99.60	106.56%	0.87%	2.55%	3.10%
9	Vinblastine	26.07	220	0.28–33.51	80.05%	0.62%	2.62%	2.99%
10	Ajmalicine	26.36	270	0.17–56.10	99.31%	1.03%	1.70%	2.20%
11	Anhydrovinblastine	29.78	220	3.77–98.38	93.64%	0.54%	1.55%	1.59%

[a] Intervals are standard deviations ($n = 3$).

Table 2
Equations for regression lines, correlation coefficients, limit of detection (LOD) and limit of quantification (LOQ) of the method for alkaloids.

No.	Compounds	Equation	r^2	LOD (μg/ml)	LOQ (μg/ml)
1	Tryptophan	$y = 357409x - 59.649$	0.9953	0.55	1.67
2	Tryptamine	$y = 322821x + 41.738$	0.9943	0.43	1.29
3	Loganin	$y = 203553x - 24.251$	0.9987	0.39	1.19
4	Serpentine	$y = 3000000x - 531.15$	0.9993	0.58	1.77
5	Vindolinine	$y = 98946x - 11.484$	0.9934	0.38	1.16
6	Vindoline	$y = 704511x + 25.892$	0.9997	0.12	0.37
7	Vincristine	$y = 157734x - 8.3234$	0.9989	0.17	0.53
8	Catharanthine	$y = 257481x + 104.35$	0.9998	1.34	4.05
9	Vinblastine	$y = 418103x + 91.45$	0.9992	0.72	2.19
10	Ajmalicine	$y = 1000000x - 1283.9$	0.992	4.24	12.84
11	Anhydrovinblastine	$y = 382040x + 40.505$	0.9982	0.35	1.06

Table 3
The levels of TIAs and precursors in flower, leaf, stem and root of *Catharanthus roseus* during flowering.

No.	Compound(mg/g)	Flower	Leaf	Stem	Root
1	Tryptophan	–	[a]0.23 ± 0.01	[a]0.03 ± 0.01	–
2	Tryptamine	–	–	–	–
3	Loganin	–	1.14 ± 0.08	–	–
4	Serpentine	–	[a]0.40 ± 0.09	[a]0.16 ± 0.02	[a]0.73 ± 0.25
5	Vindolinine	[a]0.67 ± 0.32	[a]0.51 ± 0.23	[a]0.23 ± 0.04	[b]3.38 ± 0.61
6	Vindoline	[a]0.22 ± 0.09	[b]1.29 ± 0.36	[a]0.06 ± 0.01	–
7	Vincristine	–	–	–	–
8	Catharanthine	[a]0.16 ± 0.08	[c]1.54 ± 0.49	[a]0.03 ± 0.01	[b]0.81 ± 0.25
9	Vinblastine	–	0.05 ± 0.02	–	–
10	Ajmalicine	[a]0.09 ± 0.02	[a]0.04 ± 0.02	–	[b]0.30 ± 0.06
11	Anhydrovinblastine	[a]0.49 ± 0.10	[a]1.30 ± 0.51	–	–

[a,b,c]Significant difference ($p < 0.05$ by ANOVA).

months. In this study, we observed the TIA levels in the leaves from the seedling stage to flowering stage (Fig. 3).

The major two TIAs in *C. roseus* leaves were vindoline and catharanthine, which are the precursors of bisindole alkaloids. Accumulation of vindoline and catharanthine at the seedling stage, tended to increase and reached the highest levels at day 44 (1.37 mg/g DW and 2.79 mg/g DW) before flowering, and subsequently declined with the flowering time. Ajmalicine levels had a similar pattern with that of vindoline and catharanthine, reaching the highest level at day 44 (0.15 mg/g DW). In *C. pusillus*, samples of the cotyledons had the highest value of ajmalicine [16]. Other TIAs, such as serpentine and vindolinine, showed different patterns. The contents of serpentine and vindolinine increased to the value of 0.54 mg/g DW and 0.35 mg/g DW respectively at the seedling stage and remained similar to the flowering stage. The accumulation patterns of the bisindole alkaloids anhydrovinblastine and vinblastine were totally different from their precursors. Both of them accumulated at the flowering time and displayed the highest levels (1.90 mg/g DW and 0.46 mg/g DW) at day 99 at the flowering stage. The alkaloid yields depend on the developmental stage of plants [16,35]. These results indicate that before flowering the *C. roseus* plants already actively accumulate various important TIAs possibly as defence for the growing plant [32,36,37]. Since bisindole alkaloids increased as leaves aged [29], the mature and flowering plants produced more bisindole alkaloids than the seedlings. So the flowering period might be an optimal timing to harvest *C. roseus* leaves for the industrial production of the valuable bisindole alkaloids.

4. Conclusion

A simple and rapid HPLC method was developed and validated for simultaneously determination of eight TIAs and three precursors in *C. roseus*. The validated method improved the coverage of HPLC analysis for TIA related compounds, which made the investigation of TIAs accumulation more efficient and convenient in *C. roseus*. The observations of both TIAs and precursors accumulation in different organs, aging leaves and developmental stages uncovered the pattern of metabolic fluxes from upstream to downstream of the TIA pathway, which facilitated to elucidate the regulation of TIA biosynthesis, and provide a guidance to the industrial production of these compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2016.01.034.

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