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Journal of Chromatography A, 755 (1996) 281–288

JOURNAL OF CHROMATOGRAPHY A

Quantification of vincristine and vinblastine in *Catharanthus roseus* plants by capillary zone electrophoresis

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Received 11 March 1996; revised 2 July 1996; accepted 2 July 1996

Abstract

A systematic and comprehensive study of the separation and quantification of two dimeric catharanthus alkaloids, vincristine (VC) and vinblastine (VB), in *Catharanthus* leaves by capillary zone electrophoresis (CZE) was conducted. Various separation parameters such as buffer concentration and pH, column internal diameter and applied voltage were studied. Due to the complexity of the samples, the optimum conditions for separating VC and VB from other alkaloids that co-exist in the plant samples are matrix-dependent. We found the following conditions, in general, gave the best results in terms of resolution and analysis time: column, 72 cm (57 cm effective length)×75 μm I.D.; buffer, 0.2 *M* ammonium acetate solution, pH 6.2 and an applied voltage of 10 kV. Although the separation of VB and VC was the primary focus, the separation parameters determined in this study can be applied to the separation of other alkaloids as well. Separation of many of the other alkaloids in the plant samples was observed under conditions presented in this paper. A secondary objective of this study was to develop a method with experimental conditions which could be applied to electrophoresis–mass spectrometry. For this reason, ammonium acetate buffers, which are more compatible with mass spectrometry than the widely used phosphate buffers, were used exclusively. Also, methanol–water–acetic acid was used as external buffer for the same reason. The effects of this buffer and its compositions on the resolving power of CZE for the VB/VC pair are discussed.

Keywords: *Catharanthus roseus*; Vincristine; Vinblastine; Alkaloids

1. Introduction:

Vincristine (VC) and vinblastine (VB) are dimeric catharanthus alkaloids isolated from the plant *Catharanthus roseus* (Fig. 1). Both alkaloids are therapeutically proven to be effective in the treatment of various neoplastic diseases [1–4]. Consequently, the determination of these compounds in plant samples, as well as biological fluids, are of interest to many scientists. Many gas (GC) and high-performance liquid chromatographic (HPLC) and mass spectrometric (MS) methods [2,5–15] have been developed for the determination of VC and VB in either plant samples or biological systems. The potential use of information-rich detectors such as mass spectrometry (MS) with capillary zone electrophoresis (CZE) has made this a more attractive separation method. However, to the best of our knowledge, CZE has not been used for the determination of these types of dimeric alkaloids in biological matrices.

One of the greatest challenges in the determination

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0021-9673/96/$15.00
PII S0021-9673(96)00614-0

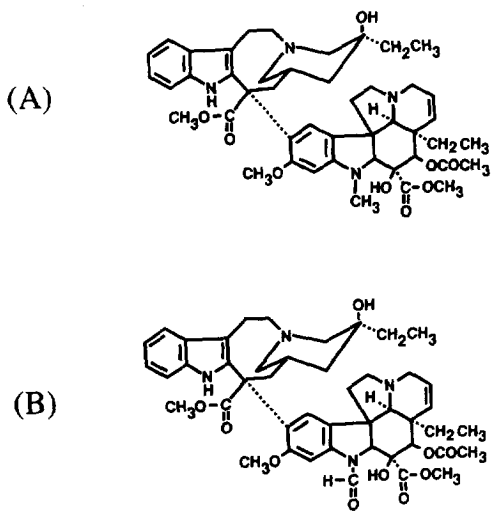


Fig. 1. Chemical structures of two dimeric indole alkaloids, vinblastine (A) and vincristine (B).

of VB and VC by CZE is presented by their similarity in both structure and pK_a values [16]. In this study, we systematically determined the effects of applied voltage, buffer composition, pH and sample concentration on the separation and quantification of VB and VC in the *Catharanthus roseus* plant. Optimal conditions in terms of resolution and analysis time for the direct application of this method to the CZE–MS using the MS compatible ammonium acetate buffer were established and used.

2. Experimental

2.1. Reagents and materials

Plant samples were obtained from Goldsmith Seeds (Gilroy, CA, USA). Vincristine sulfate, vinblastine sulfate and ammonium acetate were purchased from Aldrich (Milwaukee, WI, USA). Palmatine chloride was purchased from Sigma (St. Louis, MO, USA). Methanol (HPLC grade) and cyclohexane, (spectroscopy grade) were purchased from Baxter Scientific (McGaw Park, IL, USA) and water (HPLC grade) was obtained from Burdick and Jackson (Muskegon, MI, USA). Glacial acetic acid was obtained from Fisher Scientific (Fair Lawn, NJ, USA). All chemicals were used without further purification.

Glass fiber Acrodisc, 25 mm, 0.45 μm (Gelman Science, Ann Arbor, MI, USA) and glass fiber syringe filters, 13 mm, 0.45 μm (Whatman Lab Division, Clifton, NJ, USA) were used for filtration.

2.2. Standard preparation

Vincristine sulfate and vinblastine sulfate were prepared in de-ionized water at concentrations of 2.0, 5.0, 10, 20 and 50 μg/ml by the appropriate dilution of a 100 μg/ml stock solution, following sonication for 1 min. The internal standard solution (palmatine, 100 mg/ml) was mixed 1:1 with each analytical standard, as were the plant extract samples.

2.3. Sample preparation

The sample preparation procedures were previously described in detail [15]. Briefly, accurately weighed *Catharanthus roseus* leaves (0.6 g/sample) were placed in glass scintillation vials. Isopropyl alcohol (4 ml) was added and the vials were shaken for 15 min on a wrist-action shaker. The extracts were filtered through 25-mm glass fiber Acrodiscs into 2-ml LC autosampler vials. The samples were then dried under a stream of nitrogen and re-dissolved in 1.0 ml of 0.01 *M* acetic acid. The acid solutions were extracted 3 times with 1.0 ml of cyclohexane. The organic layers were discarded and the acid fraction was taken to dryness under a stream of nitrogen at room temperature. The samples were finally reconstituted in 0.2 ml of 0.01 *M* acetic acid and sonicated for 1 min prior to a final filtration through 13-mm glass fiber syringe filters.

Since no VC was detected in the extracts of these particular *Catharanthus roseus* plants by HPLC in a previous study [15] all of the samples analyzed in this study were spiked with 20 ppm each of VB and VC.

2.4. Capillary electrophoresis

Capillary electrophoresis experiments were carried out with a ATI (Boston, MA, USA) Crystal CZE system equipped with a Unicam 4225 UV detector at λ=254 nm. Both 50 μm I.D. and 75 μm I.D. uncoated fused-silica capillary columns (Supelco) were tested in our investigations. Ammonium acetate

at various concentrations, ranging from 0.04 to 0.25 *M* were used (running buffer) to determine buffer concentration effects on theoretical plate number (N) and resolution (R_s) for the VB/VC pair in both water and a water–methanol mixture [17]. Buffer solution was not pH adjusted, unless otherwise indicated. Other experimental parameters, such as applied voltage, column I.D. and buffer pH were also tested. Plant samples and standards were dissolved in a 1:50 dilution of running buffer for better sample stacking effect [18,19]. Positive identification of alkaloid compounds of interest was made by both spiking the sample with standards and by comparing the relative migration time between the internal standard and the compounds under investigation. When relative migration times were used to locate the VB and VC peaks, the migration times at the middle of the peak, rather than at the apex (average of peak start/end times) were used to give more accurate identifications.

All of the solutions were filtered through a 0.45-μm filter and sonicated for 5 min before use. Pretreatment of a new column prior to analyses included flushing the column with 0.1 *M* sodium hydroxide solution followed by de-ionized water and running buffer, each at 2000 mbar for 5 min. Subsequently, a voltage of 20 kV was applied to the system for 10 min and the column was allowed to equilibrate overnight. No subsequent NaOH wash was performed after the initial column treatment procedure, especially when acidic buffer solution was used, due to the long equilibration time required [20]. When buffer pH was changed, a 1000 mbar rinse with the new buffer followed by a 20 kV run for 10 min was performed. The same rinse steps were repeated twice before sample analysis and a 2 min wash with running buffer was performed on the CZE column prior to each analysis.

2.5. Calibration curves

Five-point calibration curves were generated for VC and VB to determine the relationship between peak area and the concentration of samples. Standard solutions were prepared in 0.01 *M* acetic acid ranging from 2 to 50 ppm for both VB and VC. Observed detection limits were estimated to be approximately 5 and 1 ppm for the 50 and 75 μm I.D. columns, respectively. Since the migration mobility changes with buffer pH [21] the different residence times for the solutes in the detection region artificially affect the peak area. Consequently, it was necessary to adjust the peak area of palmatine, VB and VC by dividing their peak areas by their migration times to obtain more reproducible data. The adjusted peak areas of VB and VC were then normalized against the peak area of palmatine [21]. Calibration curves were generated by plotting the normalized peak areas versus the standard concentrations used (Table 1). Each standard was analyzed at least three times on two different days and the average of those data were plotted as the standard intensity.

Table 1
Slopes and linear regression data obtained for VB, VC calibration curves by CZE[a]

	Slope	Interceptions	R^2
VB	0.011	−0.050	0.995
VC	0.023	−0.006	0.997

[a] Data obtained by normalizing peak areas of VB and VC against that of palmatine.

3. Results and discussion

The resolution (R_s) for the VB/VC pair and the theoretical plate number (N) for both analytes were calculated with standard chromatographic equations [22]. Five plant extract solutions were studied after optimum conditions for standard compounds were established.

Since the goal of this research was to determine the optimum conditions for the quantification of VB and VC, analyses were terminated after VB and VC were detected. Analytes with slower mobility than that of VB and VC were not studied. The effects of relative surface position of running buffer and external buffer, buffer concentration and pH, applied voltage and organic modifier were determined.

3.1. Effect of capillary internal diameter

The relative surface positions of the two buffer solutions were determined by varying the surface level of external buffer solution while keeping that of

the running buffer constant. The external buffer volume that gave the optimum resolution for VB/VC pair was used in all experiments. Two commonly used capillary internal diameters, 50 and 75 μm, were tested for the determination of VB and VC in the plant sample. The 50 μm I.D. column provided better heat dissipation, column efficiency and resolution for the VB/VC pair than the 75 μm I.D. column at the expense of lower sample capacity as predicted by Eq. (1) [23]

$$\sigma_S^2 = \frac{S^2}{12v^2\pi^2(d_c/2)^4} \quad (1)$$

where σ_S^2 is the total variance contributed by the sample volume, S is the sample volume, v is the electrophoretic velocity of the analyte and d_c is the internal diameter of a cylindrical capillary. From Eq. (1) it is clear that, assuming v is the same in both columns, a 75 μm I.D. column can accommodate about 5 times more sample than that of a 50 μm I.D. column to yield the same σ_S^2. In this study, the 75 μm I.D. capillary usually provided 2–3 times lower detection limit than that of the 50 μm I.D. column.

3.2. Effect of buffer concentration

Seven different ammonium acetate concentrations (0.05 to 0.25 M) were prepared to investigate the effect of buffer strength on the separation of VB and VC. The migration time (t_m) for both VB and VC decreases with decreasing buffer concentration as one might predict [24–28]. The counter effect of a reduction in t_m, however, is the loss of resolution [23,24]. At a constant applied voltage, the resolution increases as buffer concentration increases as has been shown earlier [29]. Therefore, 0.25 M ammonium acetate buffer gives the highest resolution but increases the analysis time (t_m for VC is about 30 min) and the 0.05 M buffer provides the fastest analysis for the VC/VB pair (t_m for VC <10 min) but has the worst resolution for the pair. Buffer concentrations outside the range were not studied due to either severe joule heating (buffer concentration >0.25 M) or co-migration of the samples of interest (buffer concentration <0.05 M). In this study, a buffer concentration ~ 0.2 M was used routinely to separate VB and VC from other close-migrating components in the plant samples.

3.3. Effect of applied voltage on resolution

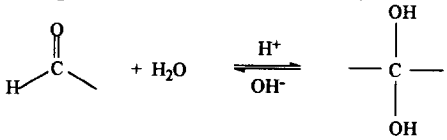
At a given applied voltage, the current increases as buffer concentration increases. For example, at an applied voltage of 10 kV, the current was 25, 60, 100, 140 and 180 μA for 0.05, 0.10, 0.15, 0.20 and 0.25 M buffer concentrations, respectively. As a result, the maximum applicable voltage (V_{max}), the voltage where no joule heating is occurring, for each buffer concentration, decreases as the buffer concentration increases. Except for the 0.1 M buffer, at a constant buffer concentration, both N and R_s increase initially with increasing voltage, but drop at high voltage due to the effect of joule heating. The highest N and R_s in these experiments always occur at an applied voltage where joule heating exists (as indicated by the non-linear increase in current with voltage). Apparently, the zone broadening effect caused by joule heating was overcome by the increase in both N and R_s when the applied voltage is slightly greater than the V_{max}. At applied voltage much higher than V_{max}, the effect of joule heating has become too large to be compensated by the effect of increasing voltage, so that both N and R_s decrease. The use of applied voltages slightly above V_{max} for CE analyses has another advantage which is the reduction in analysis time. Migration time of the sample is inversely proportional to the applied voltage, therefore, the use of applied voltage higher than that of V_{max} can reduce the analysis time. This is important especially when a large number of samples need to be processed. This approach, however, has a limitation. Because of the insufficient heat dissipation, thermal decomposition of the sample occurred when samples were re-analyzed several times. For this reason, when the quantity of the sample or thermal stability of the sample is a concern, using applied voltage less than or equal to V_{max} is recommended.

3.4. Effect of buffer pH on resolution

Buffer solution pH is one of the key parameters in CZE separations. Electroosmotic flow (EOF), solute charged state and analysis time are all affected when the buffer pH changes [21]. In this study, the effects

of buffer pH on the separation of VC and VB in the plant samples were investigated over the pH range 5 to 8. The suggested pH range for acetate buffer is 3.8 to 5.8 [22]; however, a buffer pH<5 was found to be be useless in our studies due to the interference of other analytes in the extracts. Buffer pH values >5.8 are outside the capacity of the ammonium acetate but were found to be useful for the separation of compounds migrating faster than VB and VC (probably monomeric alkaloids) in this study. That is the main reason for the pH range selected.

Resolution factors calculated for the VB/VC pair at various buffer pH values are listed in Table 2. The data clearly indicate that resolution increases as buffer pH decreases due to the longer migration times for VB and VC at lower buffer pH (lower EOF) and the zone broadening effect in higher buffer pH because of joule heating (Fig. 2). Solvent effects is another factor that can account for the pH dependent resolution for the VB/VC pair. VC has a similar pK_a and structure as VB [15]. The only difference between these two compounds is the replacement of an aldehyde (–CHO) in VC with a methyl ($-CH_3$) group in VB. An aldehyde, at lower pH, will react with H_2O to form a hydrate or a *gem*-diol [30].



This reaction will increase the apparent mass and, hence, the hydrodynamic radius of VC. The degree of conversion is pH dependent, which might explain the relative electrophoretic mobility (μ_{EF}) change between VB and VC as pH varies. Even though the resolution for the VC/VB pair increases with decreasing buffer pH, co-migration of VB or VC with other analytes that become highly charged at buffer

Table 2
Buffer pH effects on ($R_s \pm$S.D.; $n=3$) for VB/VC pair

Buffer pH	R_s
7.937	1.0±0.05
7.301	1.1±0.2
6.956	1.3±0.2
6.180	2.3±0.5
5.580	4.4±0.5

Buffer: 0.2 *M* ammonium acetate; column: 67 cm (injector to detector)×50 μm I.D.; applied voltage: 12 kV.

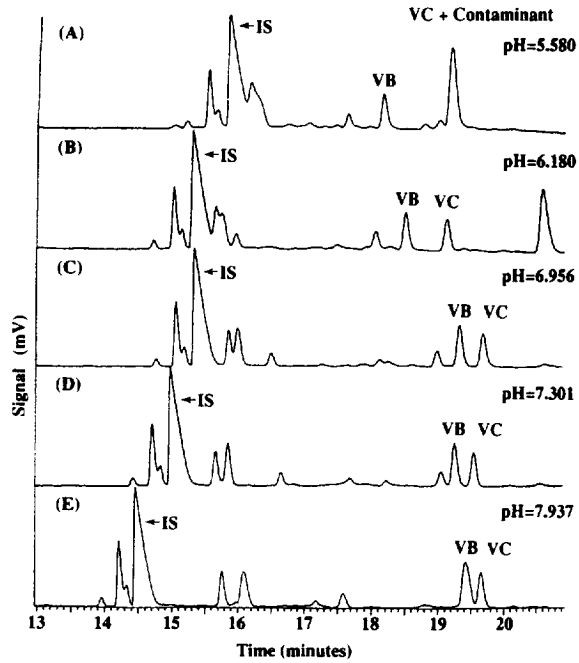


Fig. 2. Electropherograms of plant extract analyzed under various buffer pH conditions. Buffer: 0.2 *M* ammonium acetate; column: 75 cm (60 cm injector to detector)×50 μm I.D.; applied voltage 10 kV.

pH<5.6 (Fig. 2A) was occurring. For this reason, a buffer pH of 6.2 was selected for all analyses.

Also shown in Fig. 2, a buffer at pH of approx. 7.3 provides the largest difference in relative mobilities of analytes which migrate faster than that of VB and VC. The different behaviors between the VB/VC pair and other alkaloids is believed to be the result of their different degrees of dissociation under the buffer pH used [21]. However, more studies are needed to confirm this theory.

3.5. Effect of organic modifier

An experiment was conducted to investigate the effects of organic modifier on the resolution and migration time of the VB/VC pair. A running buffer with 15% methanol was used in this study and the results were compared with that obtained by using pure buffer solution. The migration times (Fig. 3) of both VB and VC are longer in 15% methanol solution, which is probably due to a combination of reduction in the electroosmotic flow and an increase

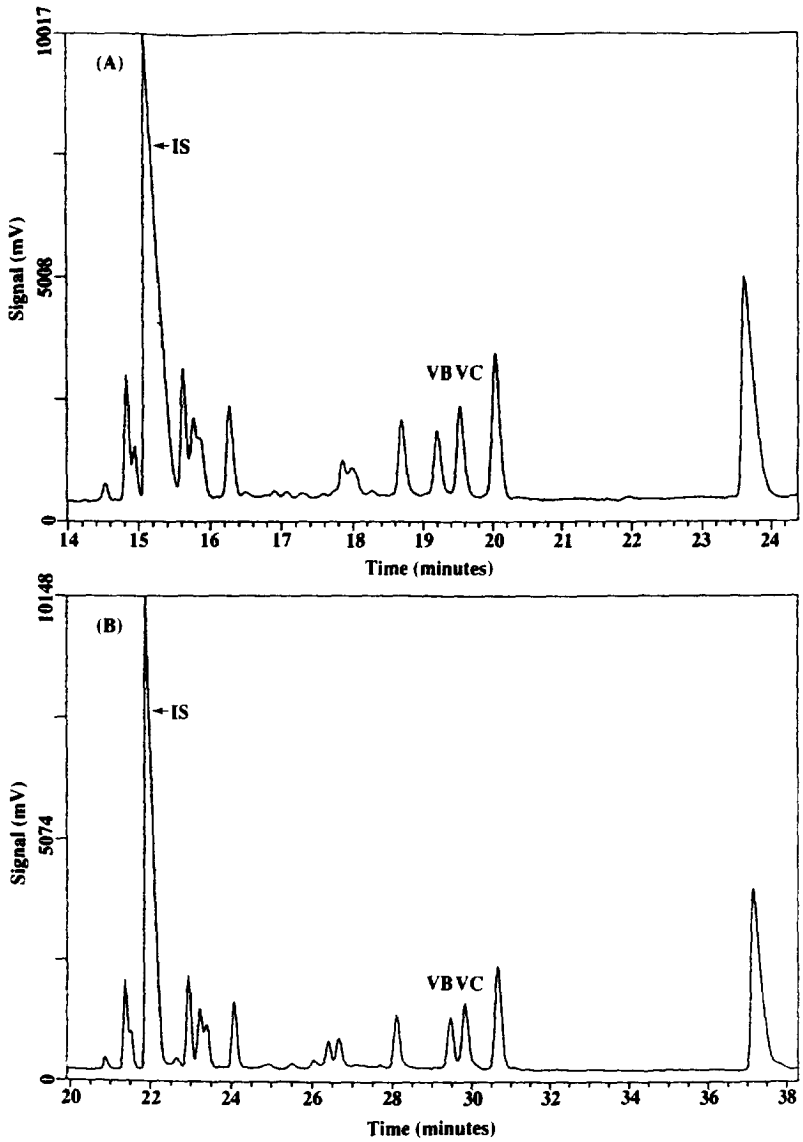


Fig. 3. Electropherograms of plant extracts. Buffer: (A) 0.2 *M* ammonium acetate and (B) 85% 0.2 *M* ammonium acetate + 15% methanol. Column: 72 cm (57 cm effective length) × 75 mm I.D.; applied voltage 10 kV, buffer pH = 6.2.

in viscosity [31,32]. Also shown in Fig. 3, especially for compounds with higher mobility than VB and VC, peak intensities increase and peak widths decrease with the addition of methanol. Consequently, the resolution for the higher mobility solutes increases in the modified buffer. On the other hand, resolution of VB/VC is reduced by one-half when methanol is added to the solution (Table 3). A buffer which contains 15% ethanol shows the same effect on VB and VC as methanol. This reduced resolution in 15% methanol solution could be, at least partially, due to the impact of the decreased apparent mobilities on the diffusion [33] and change in the degree of relative solvation between these two analytes [31,34]. VC which has one more polar group (–CHO) than VB (Fig. 1) is subject to a higher degree of interaction with polar molecules (H_2O) and/or buffer ions (NH_4^+). These interactions will

Table 3
Effect of methanol on the column efficiency (N) and resolution ($R_s \pm$S.D.; $n=3$)

Buffer	N		R_s
	Vinblastine	Vincristine	
0.2 M ammonium acetate	73 333±568	66 762±732	2.22±0.08
85% 0.2 M ammonium+ 15% methanol	205 405±1139	213 307±1526	1.11±0.09

Buffer and column as in Table 2.

certainly increase the effective mass and r of VC. This increase in r reduces the relative mobility of VC to that of VB and hence, a better separation between these two compounds. Upon the addition of methanol, the solvation of VC decreases due to the weaker interaction between the less polar solvent, methanol, and VC. This results in a reduction of hydrodynamic radius of VC and an increase in its effective mobility so that it migrates closer to VB.

3.6. Effects of external buffer compositions

When different types of buffer anions were used in the running buffer and in the external buffer (liquid sheath in CE–MS), the anions of the external buffer reservoir will migrate toward the instrument's injector under the influence of applied voltage. These anions, which are moving against the sample components, might destroy the boundaries and change the pH of the sample zones [35]. For this reason, acetic acid was mixed with the external buffer solution to reduce the effects of external buffer on the separation of VB and VC. Little or no change in resolution was observed for the 0.2 M ammonium acetate buffer and those with varying MeOH concentration.

3.7. Comparison with HPLC

The elution sequence of the two dimeric alkaloids in CE is the reverse of that observed in reversed-phase HPLC [15] which is consistent with the separation mechanisms regarding these two techniques. HPLC separations are based on the interactions between analytes with the stationary phase and the mobile phase. VC, which has more polar groups than that of VB, elutes faster in reversed-phase HPLC than VB. On the other hand, CE analyses are based on different mobility of solute in the buffer solution under the influence of high voltage. VB, which has a smaller molecular mass and lower charge density than that of VC, moves faster than VC under a constant applied voltage and elutes from the column faster. Thus, a small molecular mass change ($M_r = 14$) combined with the difference in charge density have a dramatic effect on CE mobility.

Under the HPLC conditions used in a previous study [15], the retention time difference for the VB/VC pair was about 5 min compared to approximately 1 min in CZE, under optimum conditions, as determined in this study. These results suggested that the resolution for HPLC could be better than that of CZE for the VB/VC pair. Quantification of VB and VC by HPLC suffered from interferences with other closely eluting components such that the detection limit was far greater than that observed in CZE [15]. Although no information-rich detection was used in the CZE analyses to determine co-elution, it appears to have a lower detection limit than HPLC. This result, plus the fact that CZE analysis is easier to conduct, requires less solvents and less sample, employs a relatively inexpensive column, and generates less environmentally hazardous waste, make CZE a more attractive tool than HPLC for future studies.

4. Conclusion

We have reported a systematic and comprehensive study of CZE analysis of VB and VC in the plant *Catharanthus roseus*. A 0.2 M ammonium acetate buffer solution with a 75 μm I.D. capillary and an applied voltage of 10 kV was found to provide the best combination of detection limit, analysis time and resolution for the quantification of these two alkaloids. A buffer pH of approximately 6.2 resulted in the best resolution of the VB/VC pair. On the

other hand, a buffer with pH 7 was found to be better for separation of alkaloids eluting earlier than the two analytes of interest (Fig. 2). The addition of methanol as an organic modifier increased the column efficiency, but reduced the resolution for the VB/VC pair and therefore, it is not recommended. However, for the separation of other alkaloids, resolution was improved dramatically by the addition of organic modifiers (Fig. 3), particularly the monomeric alkaloids as observed earlier by Liu et al. [17]. The use of water–methanol–acetic acid mixture as external buffer solution provided similar resolution as 0.2 *M* ammonium acetate buffer. CZE conditions which are compatible with mass spectrometry were achieved in this study with optimum resolution and column efficiency for the determination of VB and VC in plant samples. The optimum buffer concentration (0.2 *M*) in this study is too high to be applied to the CZE–MS system without post-column mixing with a high liquid sheath (LS) flow. Buffers with lower concentration and/or lower pH can not provide the same separation efficiency due to interferences of other analytes in the sample solution. Fortunately, the capability of the mass spectrometer to selectively monitor the ions of interest [15] make it possible to perform accurate quantification on analytes which are partially resolved by the CZE. For this reason, less concentrated buffer could be used in the CZE–MS analysis of dimeric alkaloids, which would result in improvements in both the detection limit (lower LS flow required) and analysis time.

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