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Isolation of the active constituents in natural materials by 'heart-cutting' isocratic reversed-phase two-dimensional liquid chromatography

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Abstract

A multidimensional heart-cutting reversed-phase HPLC separation approach, where two columns were operated independently via two six-port two-position switching valves, was employed in the isolation of a major bioactive found in the ethanol–water (80:20) crude extract of *Clerodendrum floribundum*. In this mode of operation, the specific productivity of the multidimensional approach under overload conditions was twice that of conventional gradient methods with the same loading factor. Isolated sample purities were greater than 99% with recoveries of 95%. The independent operation of each of the two columns employed in the multidimensional approach allowed the cycle time to be less than 7 min, compared to 23 min in the gradient elution single-dimension mode of operation.
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1. Introduction

While analytical-scale HPLC aims to accomplish fast separations with maximum resolution under linear separation conditions for the purpose of identification and determination of mixture composition, the objective behind preparative-scale liquid chromatography is very different and highly dependent upon individual user requirements. The notion of preparative chromatography for some, is a small laboratory-scale process, while others aim for pilot plant-scale separation. Consequently, optimisation of separation conditions across this vast range of requirements is difficult since the important aspects of optimisation for one group may differ for the other. Regardless, there usually remains a commonality between each of these processes irrespective of the scale of separation. This commonality is the production rate, where the resolution is optimised to yield a product with the appropriate purity (usually 99%) and recovery such that sample throughput per unit time is maximised [1–4]. In this respect non-linear or overload separation conditions are the norm. The production rate of component i (Pr_i) may be determined according to Eq. (1) [1].

$$\mathrm{Pr}_i = \frac{V_s C_i^0 Y_i}{\varepsilon S_a \Delta t_c} \quad (1)$$

where V_s is the sample volume; C_i^0, the injected concentration of sample constituent i; ε, the total porosity; S_a, the cross-sectional surface area; Y_i, the recovery yield and Δt_c is the cycle time.

There are a number of different preparative-scale processes that may be employed, some of these offer continuous sample feed, while in others the sample feed is applied in a batch process. One of the most commonly employed forms of continuous separation is simulated moving bed (SMB), which has become the technique of choice for the separation and purification of two-component or racemic mixtures of enantiomers [5]. However, the SMB process is complicated and is limited to applications containing only two components, except in specialised academic applications. Another example of the continuous separation process is preparative continuous annular chromatography (P-CAC) [6–8].

A limitation of the continuous mode of separation is their inability to handle complex samples. In many instances, the sample complexity may be decreased through additional pre-chromatographic solvent extraction processes and this

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may allow continuous modes of operation. However, solvent extraction processes may be detrimental to the integrity of the sample, which is often the case when dealing with natural products. Furthermore, the cost of these extraction processes may be relatively high and routinely involve the removal and disposal of large quantities of environmentally and physiologically harmful organic solvents. Consequently, as the complexity increases batch type separation processes become necessary, at the expense of the production rate. Batch type processes typically involve periodic injection of sample feed onto a chromatographic column. Elution may be isocratic in the case of simple sample matrices, and here its advantage is simplicity. However, for more complex samples, gradient elution or displacement techniques may be necessary. Applications of gradient or displacement modes are also favoured when the retention factor increases [9]. Provided a column regeneration step is not required to flush strongly retained contaminants from the column, isocratic conditions are usually sought since this speeds the production rate. The advantage of gradient or displacement methods over isocratic methods is the former methods increase the product concentration, especially in the displacement mode of operation [9]. Regardless, all processes operated in a batch mode lead to a dilution effect in comparison to the continuous mode of operation.

Another form of batch type process involves the application of multidimensional chromatography. In this form of separation the peak capacity is increased by virtue of separations being performed in two or more separate separation stages or dimensions. Theoretically, the peak capacity of a two-dimensional system in which each dimension is chromatographically orthogonal is the product of the individual peak capacities of each dimension [10]. However, if there is correlation between each of the dimensions, then the usable separation space [10,11] is reduced. Hence, separation environments based on fundamentally different retention processes are usually sought so as to maximise the separation space. Although multidimensional methods of separation have been employed for decades in open column work for example, it is only in the last decade that appropriate advancements have been made in instrumentation that have enabled the routine application of coupled multidimensional separation methods. When coupling systems separation environments based on fundamentally different retention processes are usually sought so as to maximise the separation space. Consequently, techniques such as size-exclusion chromatography (SEC) and reversed-phase or normal-phase chromatographies are commonly coupled as too are ion-exchange and adsorption methods. However, in some instances, such as natural products, where the sample composition is largely unknown, employing SEC may not be useful if the range in molecular masses is small. Likewise, ion-exchange chromatography assumes the presence of ionisable species, which may not be the case, or at least the separation may be difficult to optimise. Coupling reversed-phase with normal-phase chromatographies is usually difficult due to the incompatibility of solvent environments. In comparison, the coupling of two similar phases such as reversed-phase with reversed-phase, may be experimentally simple, however, the correlation between the retention mechanisms is usually high and hence the available separation space (peak capacity) decreases due to the correlation in the systems [11]. Other factors such as band broadening, the physical limitations of the operation of switching valves, and dead volumes in real chromatographic systems may further decrease the separation of components using multidimensional systems compared to theoretical calculations [12–14]. Nevertheless, when the objective of the separation process is the isolation and purification of one component from a complex mixture, some correlation and reduction in peak capacity can be tolerated. Under these circumstances the technique approaches that of a coupled-column system where the objective of the first dimension is to allow only the components of interest to be transported to the second column, where the separation may be tuned to resolve only a few components from a simple sample matrix. This allows the application of isocratic conditions where otherwise gradients would be necessary.

In the present study our aim is to illustrate the application of multidimensional chromatography in the isolation of an active constituent from *Clerodendrum floribundum*, a native Australian shrub located in the northern tropical regions of continental Australia. In doing so our objective function is to maximise the production rate. The production rate defined in Eq. (1) was developed with one-dimensional (1D) systems in mind. However, Eq. (1) is also directly applicable to two-dimensional systems, albeit with appropriate argument. In a two-dimensional system, the column located in the first dimension is subjected to overload conditions. It is this column that dictates the solute loading factor. The column in the second dimension is subjected to only a fraction of the sample that passes through the first dimension and hence often would behave under linear chromatographic conditions, despite an overload effect in the first dimension. Consequently, the cross-sectional surface area of the second column is irrelevant to the discussion. In many systems the cross-sectional surface area would be the same as that in the first dimension. However, as we shall see in the development of this work, the diameter of the column in the second dimension may necessarily be larger than that of the column in the first dimension. Likewise, the porosity of the column in the first dimension is important, but not so for the second dimension. Regardless, the porosity of each of two well-packed columns should essentially be equal and not differ significantly from the porosity of the system. The yield, however, in two-dimensional chromatography is a function of the recovery associated with the heart cutting in the first dimension and also with the collection of the desired fraction eluting from the second dimension. This is discussed in further detail later in the text. The definition of the cycle time in two-dimensional chromatography is exactly the same as that in one-dimensional chromatography, which is

measured according to the period required between successive sample loading into the chromatographic system. In this regard, in order to minimise the cycle time each of the columns employed in the multidimensional system should operate in parallel, rather than sequentially [15]. The following discussion compares the multidimensional approach to gradient elution in a single dimension.

2. Experimental

2.1. Chemicals and chromatography columns

HPLC-grade acetonitrile (ACN) was obtained from Mallinckrodt, Australia. Milli-Q water was obtained in the laboratory and filtered through a 0.2 μm filter. Semi-preparative Luna CN and SphereClone ODS (250 mm × 10.0 mm, 5 μm Pd) columns were purchased from Phenomenex (Pennant Hills, Australia).

2.2. Plant material

Plant material was collected from Mount Annan Botanic Garden, Mt Annan, Sydney, Australia. Plant material prior to solvent extraction was air-dried at room temperature and crushed using a laboratory cutting mill (Fritsch, Idar-Oberstein, Germany). Plant samples were solvent extracted at room temperature in ethanol–water (80:20) for 3–5 days. The extract was then filtered and the filtrate concentrated to dryness in vacuo, using a Büchi RE 111 rotavapor.

2.3. Equipment

Chromatographic experiments were performed on a Waters LC system incorporating two 600 controllers, a 717plus autosampler, two 2487 dual wave length UV detectors and Millennium[32] Version 4.00 software running on a Compaq EVO D500 Pentium 4 1.6 GHz personal computer with 256 Mb RAM. The operating system (OS) was Windows 2000 Professional (Service Pack 2). Column switching was achieved using two 6-port, 2-position switching valves fitted with micro-electric two position valve actuators (Valco Instruments, Houston, TX, USA). Valve switching was controlled via the onboard Millennium[32] software.

2.4. Chromatographic separations

Plant extracts were dissolved in methanol and injected directly into the LC system. Regardless of whether multidimensional or gradient elution methods were employed a saturated solution of sample was prepared in methanol (∼16%, w/v). Injection volumes were 200 μl unless specified otherwise. While injection volumes greater than 200 μl may yield higher productivity, this was not investigated due to the limitations in our injection system that restricted continuous operation at higher injection loading. Increased sample volume in our current system resulted in a substantial loss of sample because of the requirements to overfill the injection loop. The exact operation of the multidimensional separations is described in the appropriate section of the text.

3. Results and discussion

3.1. Sample description

The components that were extracted from *C. floribundum* following partitioning of the dried plant material in an ethanol–water (80:20) solvent exhibited significant biological activity against the enzyme, xanthine oxidase (XO) [16]. Subsequent analysis of the crude extract using one-dimensional gradient elution HPLC under linear analytical conditions yielded the separation shown in Fig. 1. Detection was recorded using a photodiode array detector in order to gain a greater understanding of the sample complexity. While several components within this sample exhibit biological activity, the peak labelled as '*component A*' exhibited the highest specific activity [16] and is the target component for the current isolation work. Note the complexity of the chromatogram. The wide range in retention of the many different constituents is emphasised by the gradient running from 5% methanol to 100% methanol. The rise in the baseline in the region that surrounds component *A* is possibly due to a vast array of minor components eluting at this solvent composition. A substantial reduction in the peak capacity was observed for gradient elution methods employed on a nitrile column and hence further isolation studies on this column were not investigated.

3.2. Heart-cutting multidimensional separations—non-overload conditions

3.2.1. Instrumentation

The heart-cutting multidimensional HPLC system that was used in this study is illustrated in Fig. 2. Optimised mobile phase conditions were scouted at an analytical scale [17] and fine-tuned in the semi-prep and overload modes of separation reported in this communication. An acetonitrile–water mobile phase was used to elute the crude sample of *C. floribundum* on *C1* (nitrile column), which allows crude separation (Figs. 2a and 3a). The sample region containing the component of interest was heart-cut to a sample loop prior to loading onto the second column *C2* (C_{18}) (Figs. 2b and 3b,c). The sample loop served to isolate *C1* from *C2* and ensured each dimension of the system could be operated independently. Hence, separations in both dimensions could occur concurrently—an aspect that serves to maximise the production rate. The target region in the first dimension to be heart cut to the second dimension was determined by performing approximately six test separations. The area of the target compound was plotted as a function of the cut time so that the optimal and maximised recovery from the first

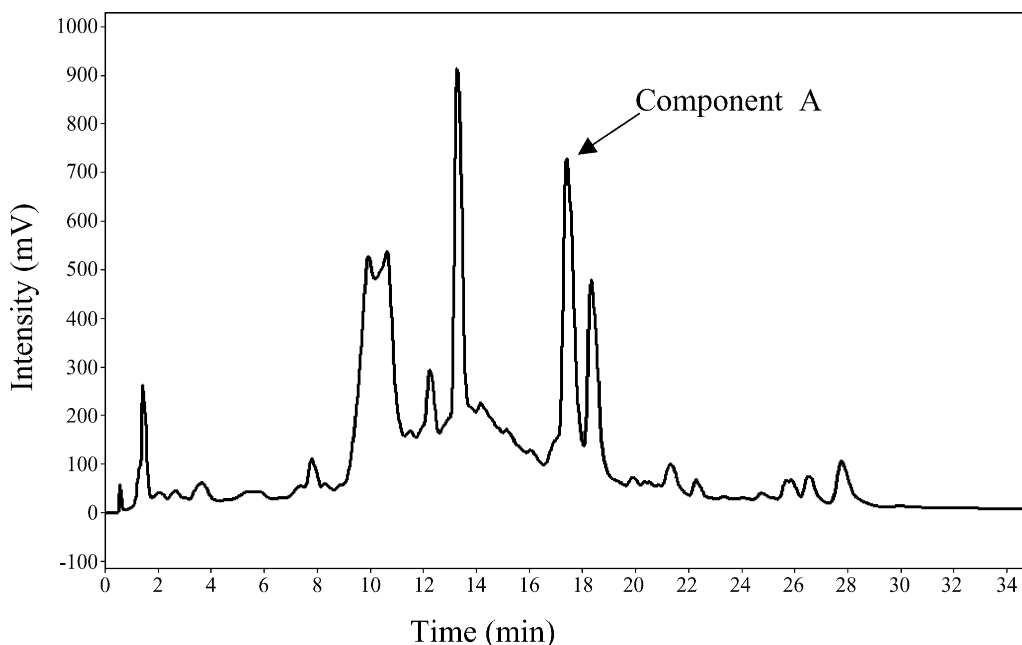


Fig. 1. Chromatogram of an ethanol–water (80:20) crude extract of *Clerodendrum floribundum*. Separation conditions involved an initial mobile phase composition (φ_i) of water–methanol (95:5) running to a final composition (φ_f) of 100% methanol in 18 min. Flow rate was 1.0 ml min^{-1}, with an injection volume of 20 µl. Detection at 270 nm.

dimension could be determined. Further, more detailed explanation of the multidimensional system may be found in previously published communications [13,14,17].

Fig. 3a illustrates the one-dimensional isocratic chromatographic separation of a crude plant sample on the nitrile column following a 200 µl injection of a saturated crude extract. The mobile phase composition (φ) used was water–ACN (30:70). Phosphoric acid was added to the mobile phase to eliminate irreversible adsorption and improve peak shape. The total run-time on the nitrile column was approximately 11 min. The region of interest that contained component *A* is indicated. For visual clarity the chromatograms are reported at a wavelength of 390 nm. We should note that we did not specifically determine the extent of retention independence between the CN and C_{18} systems. However, comparison of the separations of the crude plant extract under the various conditions employed in this study reveal that there are some very strongly retained species on the C_{18} column that

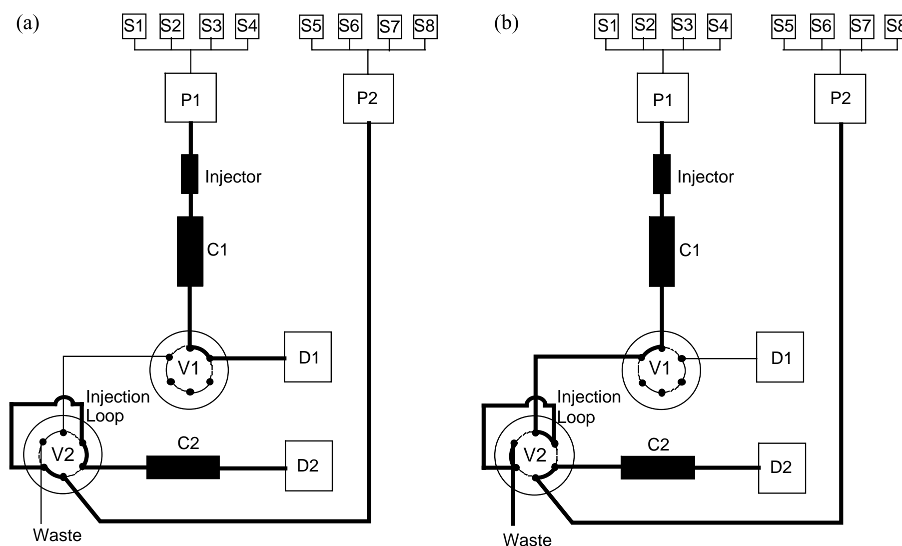


Fig. 2. Schematic diagram of the multidimensional HPLC system. (*P1–P2*): Low-pressure quaternary solvent delivery systems; (*V1-V2*): 6-port 2-position switching valves; (*C1*): column in first separation dimension; (*C2*): column in second separation dimension. (a) System configuration for elution on *C1* and *C2* (back-flush of sample loop) and (b) system configuration for the elution of a band from *C1* onto sample loop.

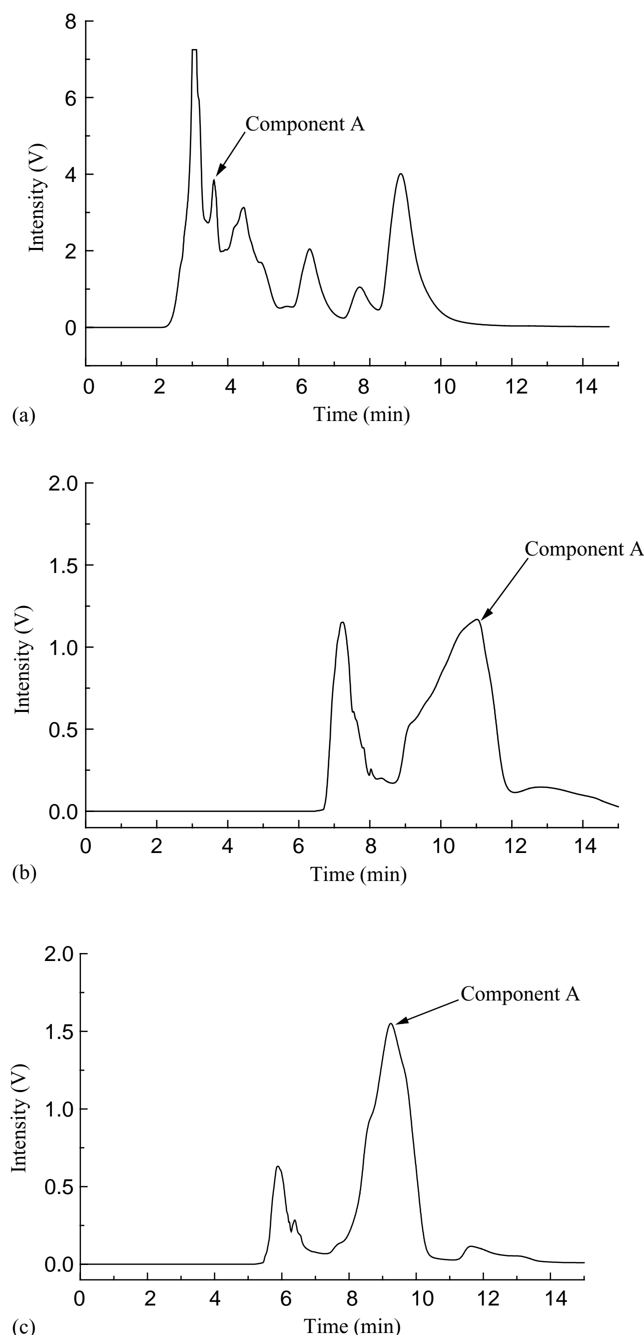


Fig. 3. Chromatograms illustrating the separation of the components in a crude extract (ethanol–water, 80:20) of *Clerodendrum floribundum*. All flow rates were 4.0 ml min^{-1}, injection volumes 200 µl. Detection at 390 nm. (a) Chromatogram illustrating the separation of the entire sample components on a nitrile column (250 mm × 10.0 mm, 5 µm Pd) in the first dimension. Mobile phase composition (φ): water–ACN (30:70); (b) chromatogram illustrating the separation of target component *A* in the second dimension (C_{18} column, 250 mm × 10.0 mm, 5 µm Pd). Mobile phase composition (φ): water–ACN (40:60). Heart-cut volume 2.0 ml and (c) chromatogram illustrating the separation of target component *A* in the second dimension (C_{18} column, 250 mm × 10.0 mm, 5 µm Pd). Mobile phase composition (φ): water–ACN (40:60). Heart-cut volume 1.2 ml.

elute essentially within the bulk sample on the CN phase. This allows isocratic separations to be undertaken on the CN phase, whereas gradients would be required on the C_{18} phase. Hence, a major advantage of the CN phase is the saving associated with time for column regeneration.

An important aspect in maximising production rate is the recovery yield. In multidimensional separations the recovery yield is essentially determined by the band broadening in the first dimension and the subsequent volume of sample that can be transported to the second dimension without adversely affecting sample distribution. In the first dimension of this separation the sample component *A* eluted in the region between approximately 3.35 and 3.85 min a total volume of approximately 2 ml. However, heart-cutting this volume and assuring 100% recovery from the first dimension to the second proved to be detrimental to the separation and hence purity in the second dimension. Effectively, recoveries in the first dimension were limited to around 75%, while the total recovery (including fraction collection from the second dimension) was approximately 70%. Fig. 3b and c, for example, illustrate the resulting second-dimension chromatograms that were obtained when the heart-cut volume was 2.0 ml (100% recovery) and 1.2 ml (75% recovery), respectively. The 2.0 ml band cut yielded an unacceptably low purity of the recovered target compound, while the 1.2 ml cut yielded a purity in excess of 99% with a total recovery of 70%.

A second factor that is important in the process of optimising the production rate is the cycle time, which is defined as the period between subsequent sample loadings. This includes any period of time associated with column regeneration or even the process of injection itself. Our initial considerations into obtaining the lowest cycle time possible and hence the highest production rate using a multidimensional approach suggest that the first dimension should be completed before the second dimension. When a multidimensional system is operated such that each dimension of the system has independent flow control, the second dimension essentially remains idle while a separation process takes place in the first dimension. The second dimension does not become active until the region of interest is heart-cut to the second dimension. Consequently, from a productivity point of view this empty separation space is counter-productive and should be utilised to improve the production rate. This simply means that sample loading onto the first-dimension column should be organised in a manner that fully utilises all separation space in the second dimension. This process of operation is in contrast to many methods of multidimensional analysis such as comprehensive two-dimensional GC (GC × GC) [18,19] where the second dimension should be rapid in comparison to the first—production rate is of little importance, rather resolution and sample profiling dominate the process.

In order to minimise the cycle time column regeneration should also be minimised or avoided altogether if possible. Hence, isocratic methods are favoured to gradient

methods. Clearly in this isolation process discussed, isocratic single-dimensional separations could not be employed as a means of purification since the sample complexity is too high with a wide variety of relative migration rates of the components in the sample (resolution would be poor and the system would require flushing to remove strongly retained species). Using a multidimensional approach, however, allows isocratic conditions to be employed. The solvent strength in the first dimension is established such that elution of the most strongly retained species is not greater than that of the most strongly retained species in the second dimension. The solvent strength in the second dimension is in turn tuned towards acceptable retention and resolution of the few components that are transported to this dimension. When this system was operated in a manner that utilised fully the separation space in the second dimension, the cycle time was 7.3 min, even though the first-dimensional separation required 11 min to be completed and the second dimension almost 14 min. This fast cycle time was achieved by taking into account four factors: (1) the void time in the first dimension being slightly longer than 2 min; (2) the time required for the injection process of 200 μl of sample was almost 3 min; (3) the effective void time in the second dimension was almost 6 min and (4) all the components in the second dimension eluted within an 8 min period. When all these factors were taken into consideration injection of a second sample onto the first column could be made before all sample components were eluted from the first column. The resulting separations are illustrated in Fig. 4a and b, which show the first and second dimensions, respectively. Note, the full utilisation of the separation space in the second dimension. With a cycle time of 7.3 min and recovery yield of 70% the production rate for a product purity of greater than 99% was equal to 0.090.

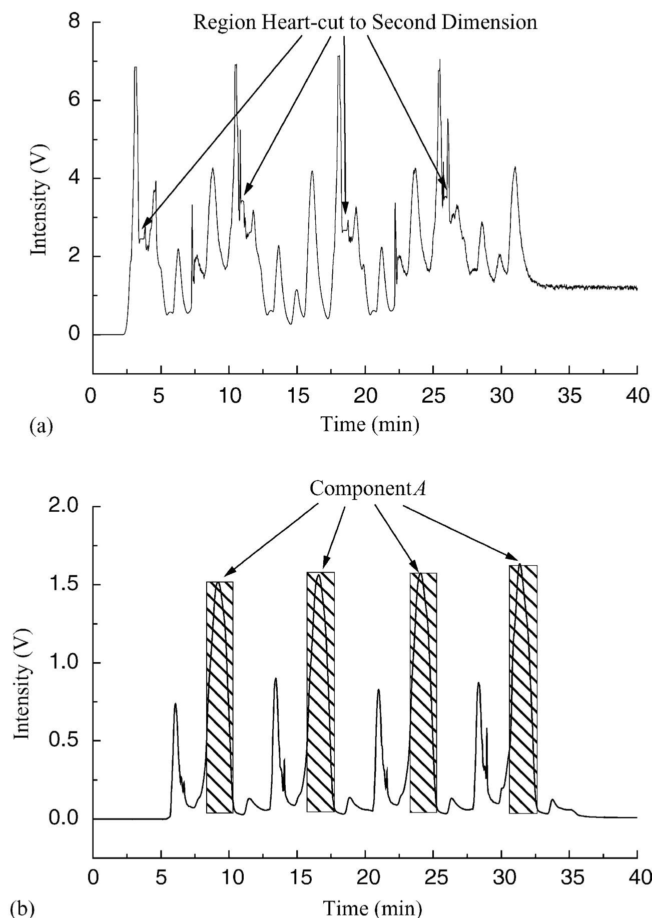


Fig. 4. Multidimensional separation of the target component from a crude extract (ethanol–water, 80:20) of *Clerodendrum floribundum*. Illustration of the cycle time and maximisation of separation space. (a) Separation in first dimension. Conditions as in Fig. 3a and (b) separation in second dimension. Conditions as in Fig. 3c.

3.3. Gradient elution one-dimensional separations—non-overload conditions

In order to compare the efficiency of the multidimensional isolation process we performed a one-dimensional gradient elution study on a C_{18} column, the same column employed in the second dimension of the multidimensional system. The loading factor and required product purity remained constant. The chromatographic conditions employed an initial mobile phase composition (φ_i) of water–ACN (60:40) followed by a steep gradient at 20% min^{-1} to a final mobile phase composition (φ_f) of 100% acetonitrile. The resulting separation is illustrated in Fig. 5. While production rate in a gradient system is dependent on the gradient steepness [9] in this particular separation higher gradient rates resulted in little further improvements in decreasing the separation time as the sample contained a number of strongly adsorbed species even in 100% acetonitrile. Hence, the cycle time was dependent on the elution of these species and then on the regeneration of the column to the initial conditions. The cycle time could, perhaps be reduced, by employing a ternary solvent system, but this was not investigated. The cycle time was 22 min, which included re-equilibration using six column volumes of the initial mobile phase during which the chromatographic system underwent the injection process. Recovery yield in this separation was high (95%) and the resulting production rate was 0.036. This rate was 0.4 times the multidimensional approach. Details of each process are presented in Table 1.

3.4. Comparison between the multidimensional and the gradient elution one-dimensional separation modes—non-overload conditions

The production rate in isocratic preparative chromatography, assuming recovery yield and product purity remain fixed, is largely determined by the sample loading and plate number [1,9]. In gradient elution, the gradient steepness parameter is also important [9]. Effectively, increasing the plate number leads to a higher peak capacity and hence higher resolving power. Higher resolving power can be obtained in three ways: (1) either gradient elution; (2) increased plate

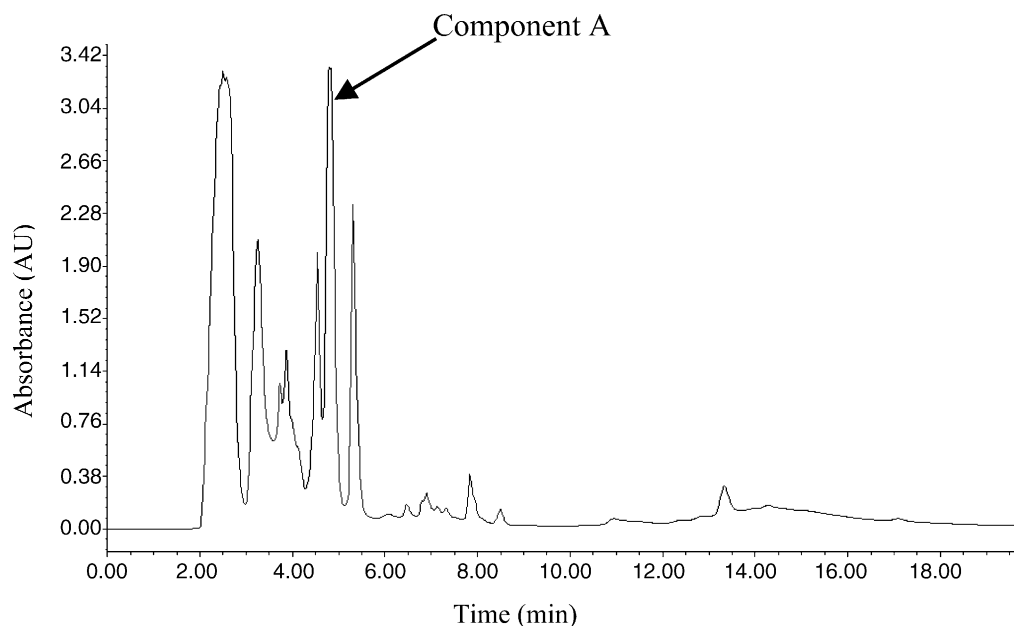


Fig. 5. Chromatogram illustrating the separation of the components in a crude extract (ethanol–water, 80:20) of *Clerodendrum floribundum* using a one-dimensional gradient system. Conditions: φ_i (water–acetonitrile 40:60), φ_f 100% methanol, gradient rate 20% min^{-1}. Conditions held at 100% acetonitrile for 17 min. Flow rate 4.0 ml min^{-1}, injection volume of 200 μl, detection at 370 nm.

number (by virtue of a reduction in particle size or increase in column length) or (3) through a multidimensional approach. If we apply a multidimensional approach, the peak capacity of the entire separation is the product of the peak capacities in each dimension (provided the two systems are orthogonal). However, correlation between each dimension reduces the overall peak capacity [10,11] to some fraction of that of the theoretical value. When correlation is high, the technique is more akin to a column-switching process. The gain in performance may often be achieved without a corresponding increase in retention time. In comparison, if the gain in separation performance is achieved through an increase in plate number in a one-dimensional system, the separation time (for constant pressure drop) must increase, hence cycle time increases. While a gradient elution approach increases the separation performance, the required time for column regeneration is usually detrimental to the overall production rate at least for species with retention factors less than around 5–7 [9]. In this particular exercise this gain in efficiency associated with a multidimensional approach has outweighed the gain in efficiency associated with the application of a gradient one-dimensional system and consequently the production rate is higher.

While the production rate of the multidimensional method was 2.5 times higher than the gradient one-dimensional mode the specific production rate, which takes into consideration the solvent consumption, was only 27% higher. This results because the multidimensional separation process employed two columns each of which were operated at the same mobile phase velocity as the gradient system. Hence, twice as much solvent was consumed in the multidimensional process. However, the multidimensional mode of operation has several factors that could be further optimised to greatly improve the recovery yield. For example, the diameter of the column in the second dimension should be greater than that in the first in order to allow a larger heart-cut volume to be transported. Had the recovery yield been 95% the production rate in the multidimensional system would have increased by a further 45%. We have found that the volume that can be heart-cut is highly dependent

Table 1
Characteristics of multidimensional and gradient elution separations of component *A* from *Clerodendrum floribundum*

System	Purity (%)	V_i (ml)	Δt_c (min)	Y_i (%)	P_r	SP_r
Multidimensional non-overload	>99	0.2	7.3	70	0.090	0.011
Multidimensional overload	>99	0.2	6.8	95	0.580	0.12
1D gradient non-overload	>99	0.2	22	95	0.036	0.009
1D gradient overload	>99	0.05	23	95	0.043	0.021
1D gradient overload	>98	0.1	23	95	0.085	0.043
1D gradient overload	<95	0.2	23	70	0.126	0.063

V_i, injection volume (dissolved in methanol); 1D, one dimension; Δt_c, cycle time; Y_i, recovery yield; P_r, production rate (g min^{-1} cm^{-2}) and SP_r, specific production (g min^{-1} cm^{-2} ml^{-1}).

on the sample and the mechanism through which retention occurs. We have, for example, been able to heart-cut 0.8 ml volumes to 4.6 mm i.d. columns 3–5 cm in length [13–15]. Quite clearly given the limitation of the heart-cutting efficiency into the second dimension for this particular sample, scale-up to overload conditions would be unfeasible under the present operating conditions. Consequently, the following discussion illustrates the operating performance of a system in which the column in the second dimension had an internal diameter 2.17 times the diameter in the first dimension.

3.5. Heart-cutting multidimensional separations—overload conditions

In order to improve the production rate by virtue of increasing the sample recovery and then expand the system to overload conditions we replaced the semi-preparative column (i.d. 10 mm) in the first dimension with an analytical column (i.d. 4.6 mm). In this manner we did not use inordinate quantities of our quite rare sample in order to obtain overload conditions. Maintaining a constant loading factor a 200 μl injection volume was applied and the resulting isocratic separation on the nitrile column in the first dimension is illustrated in Fig. 6. The distribution of component *A* is shown by the dotted line. Under these conditions, more than 95% of component *A* eluted within a 1.3 ml volume. Heart-cutting this volume onto the 10 mm i.d. column in the second dimension was entirely reasonable and the resolution of component *A* from the limiting impurity was excellent and subsequently the total recovery was determined to be 95%. An important aspect of this separation is that the concentration of component *A* in the second dimension is greater under overload conditions, than in the linear conditions previously shown in Figs. 3 and 4. Subsequently the peak shape in the second dimension was improved and it would be very likely that even larger cutting volumes could be incorporated if necessary.

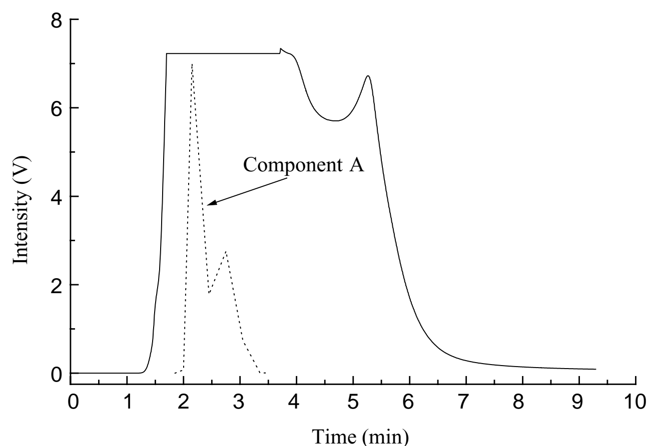
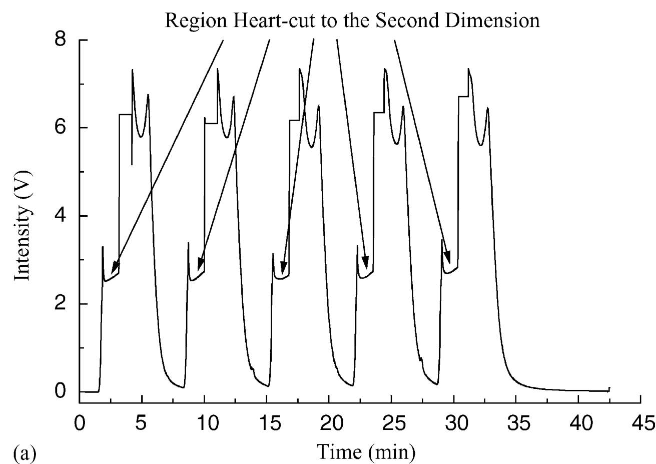


Fig. 6. Chromatogram illustrating the elution of the components in a crude extract (ethanol–water (80:20) of *Clerodendrum floribundum* under *overload* conditions. Column: CN (150 mm × 4.6 mm, 5 μm Pd). Flow rate in 1.0 ml min^{-1}. Injection volume 200 μl. Detection at 390 nm. Mobile phase composition (φ): water–ACN (30:70). Dotted line indicates the distribution of component A.



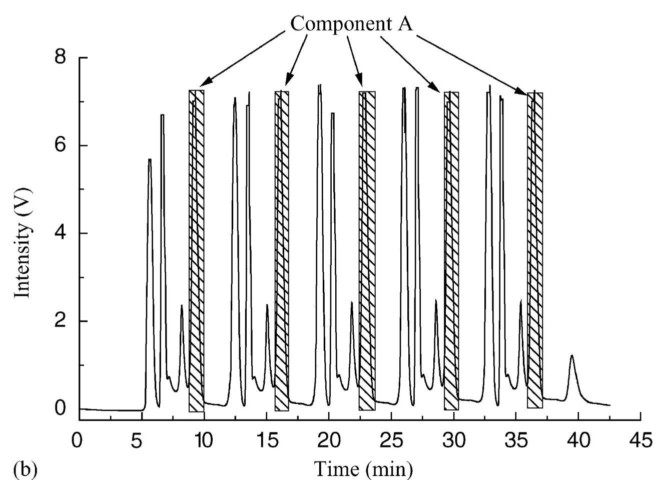


Fig. 7. Multidimensional separation of the target component from a crude extract (ethanol–water, 80:20) of *Clerodendrum floribundum* under *overload* conditions. Illustration of the cycle time and maximisation of separation space. (a) Separation in first dimension. Conditions as per Fig. 6 and (b) separation in second dimension conditions as per Fig. 3c. Heart cut volume 1.3 ml.

The chromatograms illustrated in Fig. 7 show the cycle time of this system. Five repetitions are recorded and the subsequent cycle time was 6.8 min. The purity of the collected component *A* was > 99% and with a recovery yield of 95% the production rate was 0.58 g min^{-1} cm^{-2}. This represents a vast improvement (6.4 times) over the non-overload conditions. The specific production rate was 0.12 g min^{-1} cm^{-2} ml^{-1}, a ten-fold improvement.

3.6. One-dimensional gradient separations—overload conditions

For comparison, overload conditions were performed in single-dimension gradient elution. Due to the complexity of the sample matrix the separation was difficult to fully optimise. The initial gradient conditions required higher

concentrations of water and this increased the cycle time. Furthermore, under constant loading factor conditions (200 μl injection) the recovery yield of sample was low (<70%) and even at that recovery the purity was less than 95%. The series of chromatograms shown in Fig. 8 illustrate a number of gradient processes where the loading factor is varied (injection volume 50–200 μl). The results in Table 1 compare the production rates for each of the systems discussed above. Quite clearly the multidimensional systems out performed those in a single dimension, particularly in overload conditions. For example, the overload multidimensional system was at least 1.84 times more productive than the one-dimensional gradient system and the purity of the product in the multidimensional system was higher. In order to improve the purity and yield of the gradient system the sample load was reduced (injection volume 100 μl) after which the specific productivity of the multidimensional system was 2.7 times greater. It is interesting to note that even if the recovery yield in the overload one-dimensional gradient system (injection volume 200 μl) was 100% the specific production rate would still be 28% lower than that of the overload multidimensional system.

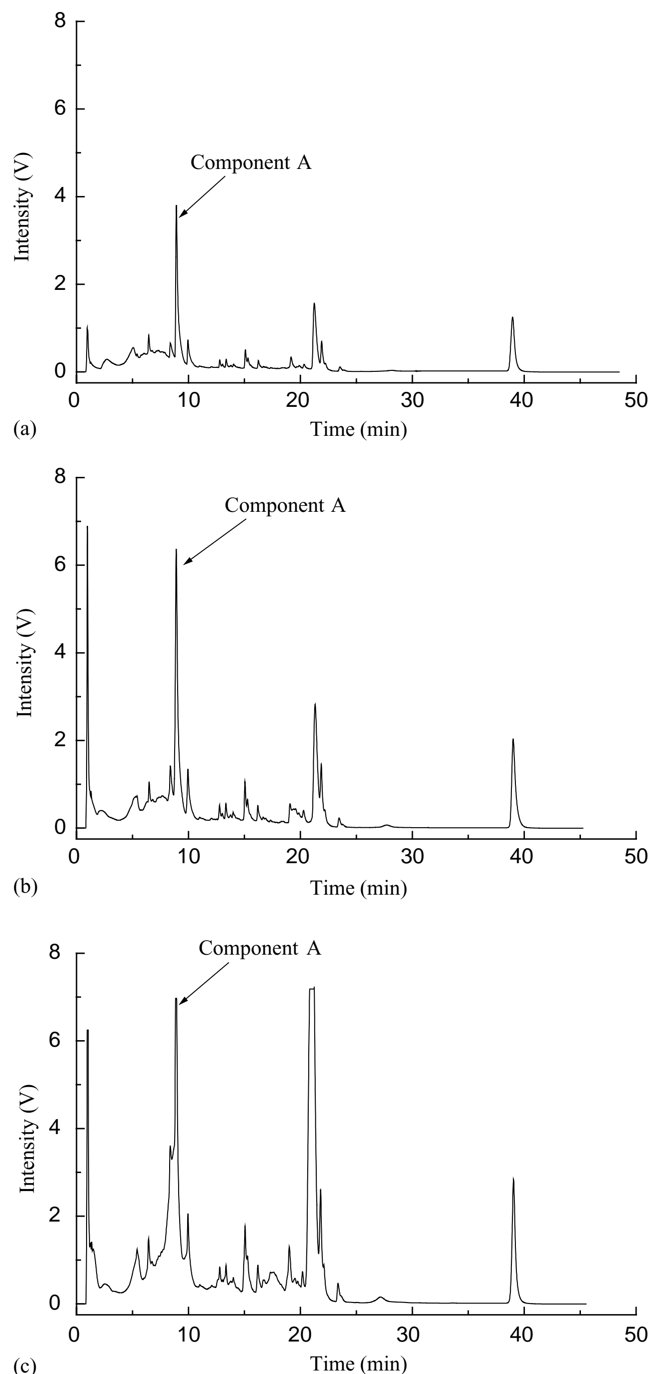


Fig. 8. Chromatograms illustrating the separation of the components in a crude extract (ethanol–water, 80:20) of *Clerodendrum floribundum* using a one-dimensional gradient system under *overload* conditions. Conditions: φ_i (water–acetonitrile, 90:10), φ_f 100% acetonitrile, gradient rate 5% min^{-1}. Conditions held at 100% acetonitrile for 17 min. Flow rate 2.0 ml min^{-1}. Injection volume of (a) 50 μl; (b) 100 μl and (c) 200 μl.

4. Conclusions

A multidimensional heart-cutting reversed-phase HPLC approach was employed in the separation and purification of an active constituent found in *C. floribundum*. When operated under overload conditions the specific productivity of the multidimensional separation approach was almost twice that of conventional gradient elution methods. The success of the multidimensional approached relied on two important aspects: (1) recovery and transportation of the component of interest into the second dimension and (2) independent operation of each dimension, which enabled the cycle time to be short with full utilisation of the separation space. The key to achieving the first aspect was to employ a column with a larger diameter in the second dimension compared to the first-dimension column. This ensured the appropriate volume of solute could be heart-cut to the second dimension. The key to minimising the cycle time was to ensure the first-dimension separation was shorter than the second dimension and consequently sample could be re-injected into the system prior to completion of the separation in the second dimension. This ensured that all separation space was fully utilised.

The final analysis of the results revealed that the specific production rate of the multidimensional system was almost twice that of the conventional gradient elution process. The final product maintained a purity of greater than 99% with a 95% recovery from the multidimensional system. Under the same loading factor the gradient elution one-dimensional method resulted in a final product of purity less than 95% with a recovery less than 70%.

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