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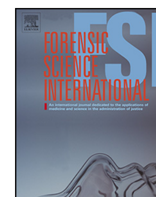

Technical Note

DryLab® optimised two-dimensional high performance liquid chromatography for differentiation of ephedrine and pseudoephedrine based methamphetamine samples

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ABSTRACT

In-silico optimised two-dimensional high performance liquid chromatographic (2D-HPLC) separations of a model methamphetamine seizure sample are described, where an excellent match between simulated and real separations was observed. Targeted separation of model compounds was completed with significantly reduced method development time. This separation was completed in the heart-cutting mode of 2D-HPLC where C_{18} columns were used in both dimensions taking advantage of the selectivity difference of methanol and acetonitrile as the mobile phases. This method development protocol is most significant when optimising the separation of chemically similar chemical compounds as it eliminates potentially hours of trial and error injections to identify the optimised experimental conditions. After only four screening injections the gradient profile for both 2D-HPLC dimensions could be optimised *via* simulations, ensuring the baseline resolution of diastereomers (ephedrine and pseudoephedrine) in 9.7 min. Depending on which diastereomer is present the potential synthetic pathway can be categorised.

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1. Introduction

The current limitations of methamphetamine impurity profiling have been recently described by Stojanovska et al. [1] who stated that '*future research is needed to address the knowledge gaps in regards to the manufacture of drugs of current interest*'. Globally, Australia has one of the highest recorded consumption rates of methamphetamine (MA) with an estimated 1/20 residents having trailed it for recreational purposes and 395,000 reported uses in 2010 [2]. Gas and liquid chromatography coupled to mass spectrometry (GC-MS and LC-MS), infrared spectroscopy (IR) and capillary electrophoresis (CE) have traditionally been the primary analytical techniques used to generate chemical information from MA seizure samples [3,4]. Other techniques have been used to study MA samples such as nuclear magnetic resonance spectroscopy (NMR) [5], ion mobility spectrometry [6], isotope ratio mass spectrometry (IRMS) [7] and high performance liquid chromatography (HPLC) [8,9]. However, having the capacity to rapidly generate chemical fingerprints of seized MA, and its impurities, is of upmost importance to Australian law enforcement agencies in order to track and identify clandestine laboratories.

Characteristic to the synthetic process, the relative concentrations of by-products in the synthetic pathway are somewhat variable. This variability, when coupled with the extensive range of cutting agents commonly used in the production of MA, forms a chemical fingerprint that can be inspected by law enforcement agencies to individualise seizure samples – at times on a batch to batch basis.

The aim of this paper is employ the superior resolving power of two-dimensional high performance liquid chromatography (2D-HPLC) to screen seizure samples and create a chemical fingerprint of production impurities and cutting agents. Two-dimensional HPLC has the capacity to separate complex mixtures *via* two independent retention mechanisms, which allows for the

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resolution of otherwise co-eluting species [10]; 2D-HPLC has not previously been used for impurity profiling of MA samples. Simulation software has been used for the first time in order to rapidly optimise a 2D-HPLC separation. This paper outlines the use of the DryLab® software package [11] in order to efficiently optimise the gradient profile of a rapid 2D-HPLC separation of key components in a model methamphetamine seizure sample. This was completed in the heart-cutting mode where only co-eluting peaks were transferred to the second dimension, as identified by simulations. This type of optimisation will ultimately lead to improved chemical fingerprinting of clandestine drug seizure samples.

2. Experimental

2.1. Standards and Samples

All standards (methamphetamine, ephedrine, pseudoephedrine, paracetamol, caffeine, benzyl alcohol, dimethyl sulfone, benzaldehyde, phenyl-2-propanone (P2P) and diphenylacetone) were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia) and the National Measurement Institute, Australian Government (Port Melbourne, Vic, Australia). High performance liquid chromatography grade acetonitrile (ACN) and methanol was obtained from Ajax Finechem (Taren point, NSW, Australia); deionized water (Continental water systems, VIC, Australia) was filtered through a 0.45 μm membrane filter (Sigma–Aldrich) prior to use. Stock solutions of all standards were prepared by dissolving in a solution of 5% aqueous ACN at a concentration of 1 mg mL^{-1} and were diluted 5-fold with 5% aqueous ACN prior to injection.

2.2. High performance liquid chromatography

An Agilent Technologies (Mulgrave, VIC, Australia) 1260 2D-chromatograph was used for all separations, including an: auto injector; capillary pump; binary pump; column thermostat; 2 position 8 port switching valve; and two diode array detectors (one in each dimension, which recorded at 200 nm). Two chromatography columns were used: the first dimension column constituted of an Agilent Poroshell 120 EC-C_{18} (4.6 mm × 100 mm, 2.7 μm particle diameter); the second dimension a Phenomenex (Lane Cove, NSW, Australia) Kinetex C_{18} (100 Å pore diameter, 4.6 mm × 100 mm, 2.6 μm particle diameter). All analysis was completed at 1 mL min^{-1} and 1 μL of all model drug solutions were injected in triplicate to ensure reproducibility. Two-dimensional HPLC was completed in the on-line heart-cutting mode, whereby a single 20 μL aliquot of the first dimension (switched at 4.31 min.) was transferred to the second dimension *via* a sample loop and an 8 port 2 position switching valve; valve timing was controlled with by the HPLC control software.

2.3. DryLab® optimisation

The DryLab® user manual requires screening data of each component from two injections with different gradient times (20 and 60 min, respectively) to optimise the separation's gradient profile [12]. Each standard was injected individually into a linear gradient over the aforementioned times with an initial mobile phase of 5% aqueous ACN that increased to 95% aqueous ACN. Retention time, heights and areas for each injection was recorded and subsequently entered into DryLab®. The gradient profile of the chromatogram generated by DryLab® was then manually adjusted in order to produce the optimum representative separation in the least amount of time and was applied to the analysis of the model seizure sample.

2.4. Model seizure samples

According to the optimised simulation the model seizure mixture was separated carried out in triplicate with 1 μL injections and a flow rate of 1 mL min^{-1} with a gradient time of 15.57 min. The initial conditions were 5% aqueous ACN to 95% aqueous ACN over 11.57 min. with a detection wavelength of 200 nm. The second dimension was completed in isocratic mode with a mobile phase of 85% aqueous methanol until all peaks were eluted; separation was recorded at 210 nm. Both columns were thermostated at 30 °C within the column heater.

3. Results and discussion

DryLab® [12] is a software tool that allows scientists to optimise several chromatographic variables *in-silico* to rapidly optimise a HPLC separation. It does this by taking the results of two gradient analyses with different gradient times, t_G, to obtain values of k_0 and S for each solute [13] When pre-elution and post-elution of the solutes can be ignored these parameters can be obtained by solving a set of simultaneous equations [13]:

$$t_{g,1} = \frac{t_0}{b_1}\log(2.3k_0b_1 + 1) + t_0 + t_D \quad (1)$$

$$t_{g,1} = \frac{t_0}{b_1}\log(2.3k_0b_1 + 1) + t_0 + t_D \quad (2)$$

where

$$b = \frac{V_0 \Delta\phi S}{t_G F} \quad (3)$$

and $t_{g,1}$ and $t_{g,2}$ are the retention times for gradient $t_{G,1}$ and $t_{G,2}$, respectively; $\Delta\Phi$ is the change of the strong mobile phase component, Φ, during the gradient; t_0 is the time required to elute an un-retained marker and t_D is the dwell time of the system, *i.e.* the time required for concentration changes in the mobile phase to leave the pump and enter the head of the column. Using the above equations it is relatively easy to simulate how subtle changes to the gradient can change the elution order and resolution of a HPLC separation; the software has the capacity to optimise 3 different experimental parameters simultaneously, however this functionality was not required for this work. This is an asset when designing a rapid 2D-HPLC separation when co-eluting peaks can be rapidly identified. DryLab® facilitates this process by generating a resolution map to identify the optimal gradient time, which can then be further manipulated by adding extra gradient steps in the gradient editor. Targeted 2D-HPLC, known as heart-cutting, is a protocol whereby only select portions of co-eluting peaks are transferred to the second dimension; this is important for rapid 2D-HPLC when a comprehensive analysis is superfluous. It is significant that the simulation software could predict co-elution and identify when this occurs so that the target analysis could be streamlined.

A model seizure sample of MA with commonly associated side products and cutting agents was prepared for *in-silico* optimisation, specifically: methamphetamine, ephedrine, pseudoephedrine, paracetamol, caffeine, benzyl alcohol, dimethyl sulfone, benzaldehyde, P2P and diphenylacetone. The predicted, optimised DryLab® simulation is illustrated in Fig. 1 and the separation in Fig. 2. Note that nicotinamide was not included in the simulated prediction as it eluted in the void volume, *i.e.* was not retained.

It is important to note that the optimal separation provided a relatively fast gradient time (*i.e.* t_G) of 11.57 min. to resolve all peaks of interest, with the exception of the ephedrine and pseudoephedrine diastereomers (peak D/E), which co-eluted, however, the time taken to elute all 10 compounds was only

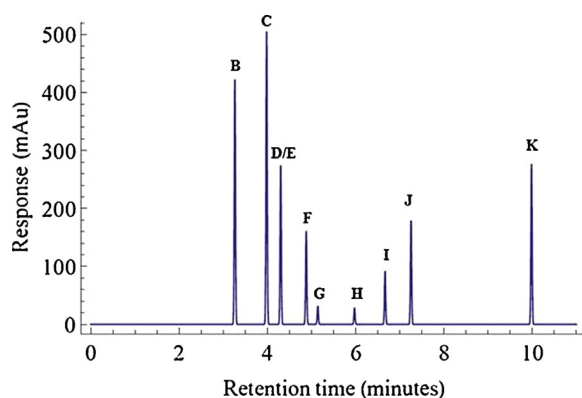

Fig. 1. Optimised DryLab® prediction B – paracetamol, C – caffeine, D/E – ephedrine/pseudoephedrine, F – methamphetamine, G – benzyl alcohol, H – dimethyl sulfone, I – benzaldehyde, J – phenyl-2-propanone, K – diphenylacetone.

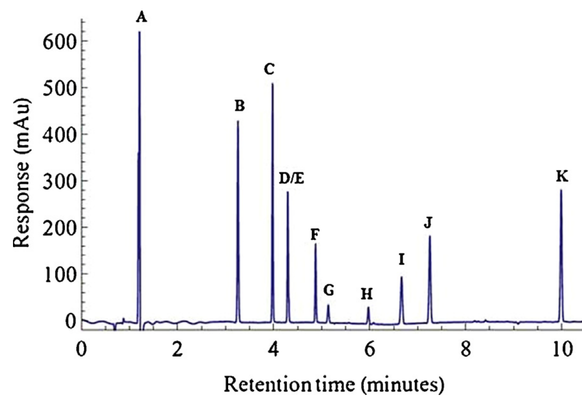

Fig. 2. Separation of the model methamphetamine sample. A – nicotinamide (B–K as for Fig. 1).

9.7 min. This separation was completed with an ACN mobile phase with a C_{18} HPLC column. The model seizure sample was analysed in triplicate with experimental parameters matching those provided by the software, Fig. 3. The direct comparison of the model methamphetamine sample and the *in-silico* prediction shows excellent correlation.

Importantly, the co-elution of the ephedrine and pseudoephedrine was observed in the real separation, as predicted by the simulation software. Co-elution of diastereomers is a common problem in chromatography, and is a great challenge for the identification of the precursor involved in the synthesis of MA. Ephedrine and pseudoephedrine were resolved from the other compounds and each other with a simple 2D-HPLC separation protocol whereby the peak (D/E) at a retention time of 4.31 min. in the first dimension was transferred to the second dimension with a 20 μL sample loop and an 8 port 2 position switching valve; the second dimension was operated with a methanol mobile phase on a C_{18} column, the different selectivities afforded by these solvents was sufficient to allow the resolution of all model compounds. The second dimension separation is illustrated in Fig. 4.

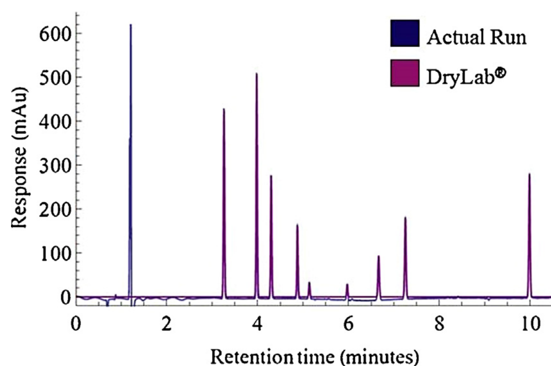

Fig. 3. Overlay of optimised DryLab® prediction and separation of the model sample.

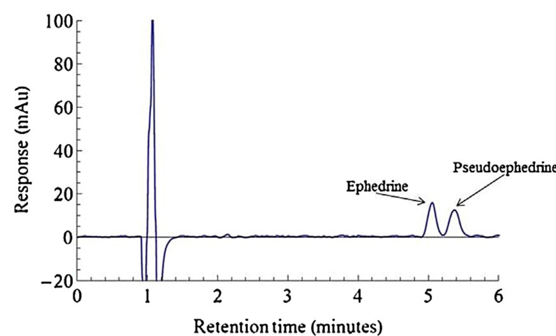

Fig. 4. Second dimension separation; separation of ephedrine and pseudoephedrine (both 1.3×10^{-3} M) from the overlapping peak (D/E).

The ephedrine and pseudoephedrine have been baseline resolved with retention times of 5.05 and 5.38 min, respectively in the second dimension. This is an important finding for three reasons:

1. The diastereomers have been separated;
2. A simple C_{18}:C_{18} two dimensional separation was sufficient; and
3. The total analysis for the separation of all model compounds, including discrimination between ephedrine and pseudoephedrine, was completed in 15.75 min.

4. Conclusions

There are many challenges in the development of optimised chromatographic separations which can be reduced by the use of *in-silico* optimisation. This study displays the effectiveness of this approach to the analysis of forensic samples highlighting an excellent match between an *in-silico* and real world separation of the same component mixture. This methodology allows the saving of time with a minimal amount of analytical runs being performed, subsequently saving resources in terms of laboratory consumables, labour costs and time. Importantly the saving in solvent waste makes this method development procedure much more environmentally friendly than contemporary methods. The methodology proved to be effective in predicting the co-elution of species. These species require a broader separation space which can be developed *in-silico* before a time consuming multidimensional separation.

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References

[1] N. Stojanovska, S. Fu, M. Tahtouh, T. Kelly, A. Beavis, K.P. Kirkbride, A review of impurity profiling and synthetic route of manufacture of methylamphetamine, 3,4-methylenedioxymethylamphetamine, amphetamine, dimethylamphetamine and p-methoxyamphetamine, Forensic Sci. Int. 224 (2013) 8–26.

[2] Australian Government Australian Institute of Health and Welfare, 2010 National Drug Strategy Household Survey Report. National Drug Strategy Household Survey Report Drug Statistics Series, 2011.
[3] T. Bassindale, A. Coxon, S. Russell, Illicit drugs and toxicology (expert evidence), in: I. Freckelton, H. Selby (Eds.), Expert Evidence, Thomson Reuters Lawbook Co., Australia, 2010.
[4] Y. Qi, I.D. Evans, A. McCluskey, Australian Federal Police seizures of illicit crystalline methamphetamine ('ice') 1998–2002: impurity analysis, Forensic Sci. Int. 164 (2006) 201–210.
[5] S. Armellin, E. Brenna, S. Frigoli, G. Fronza, C. Fuganti, D. Mussida, Determination of the synthetic origin of methamphetamine samples by 2H NMR spectroscopy, Anal. Chem. 78 (2006) 3113–3117.
[6] J.R. Verkouteren, J.L. Staymates, Reliability of ion mobility spectrometry for qualitative analysis of complex, multicomponent illicit drug samples, Forensic Sci. Int. 206 (2011) 190–196.
[7] S. Benson, C. Lennard, P. Maynard, C. Roux, Forensic applications of isotope ratio mass spectrometry – a review, Forensic Sci. Int. 157 (2006) 1–22.
[8] Y. Makino, Simple HPLC method for detection of trace ephedrine and pseudoephedrine in high-purity methamphetamine, Biomed. Chromatogr. 26 (2012) 327–330.
[9] H.X. Li, M.Y. Ding, K. Lv, J.Y. Yu, Simultaneous separation and determination of ephidrine alkaloids and tetramethyl-pyrazine in ephera sinica stapf by HPLC, J. Liq. Chromatogr. Relat. Technol. 25 (2002) 313–320.
[10] J.C. Giddings, Two-dimensional separations: concept and promise, Anal. Chem. 56 (1984) 1258–1270.
[11] Molnar-Institute for Applied Chromatography, DryLab, retrieved 19/08/2014, http://molnar-institute.com/drylab/.
[12] R.M. Krisko, K. McLaughlin, M.J. Koenigbauer, C.E. Lunte, Application of a column selection system and DryLab software for high-performance liquid chromatography method development, J. Chromatogr. A. 1122 (2006) 186–193.
[13] J.W. Dolan, L.R. Snyder, M.A. Quarry, Computer simulation as a means of developing an optimized reversed-phase gradient-elution separation, Chromatographia 24 (1987) 261–276.