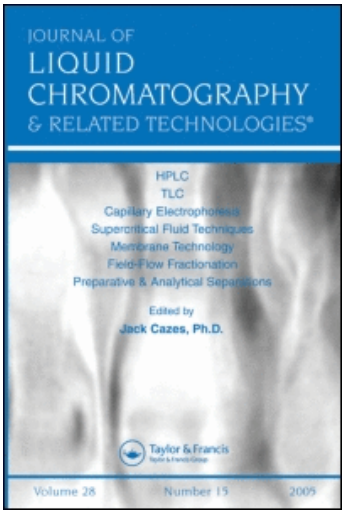

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A FACILE SEPARATION OF NONACTIN AND ITS HOMOLOGUES

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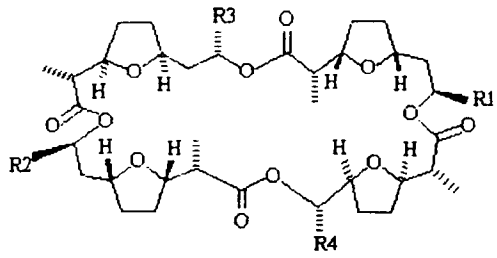
ABSTRACT

An analytical reverse phase HPLC separation of Nonactin and its homologues: Monactin, Dinactin, Trinactin and Tetranactin using Evaporative Light Scattering Detection is described.

INTRODUCTION

As part of the ongoing screening of natural products for biological activity, we needed to establish the purity of Nonactin and its homologues which had been isolated by conventional chromatography of an extract from a *Streptomyces* species. The structure of Nonactin and its homologues (collectively known as Actins) are shown in Figure 1. As one ascends the homologous series, four of the methyl groups in Nonactin are sequentially replaced by ethyl groups. The compounds are potent ionophores for alkali metals and might be expected to be chromotographically challenging. A search of the literature revealed only one preparative HPLC method.[1] Attempts to adapt this as analytical method failed, as did attempts to derive an HPLC method based on TLC conditions.[2]



NONACTIN:	R1 = R2 = R3 = R4 = **Me**
MONACTIN:	R1 = **Et**, R2 = R3 = R4 = **Me**
DINACTIN:	R1 = R2 = **Et**, R3 = R4 = **Me**
TRINACTIN:	R1 = R2 = R3 = **Me**, R4 = **Me**
TETRANACTIN:	R1 = R2 = R3 = R4 = **Et**

Figure 1. The structure of Nonactin and its homologues.

Described below is an HPLC method which separates Nonactin, Monactin, Dinactin, Trinactin and Tetranactin to baseline with good peak shape. There is also no need for mobile phase additives, as long as the HPLC system is kept as free of metal ions as possible.

EXPERIMENTAL

THF (stabilised with 0.04% butylated hydroxytoluene) and disodium EDTA were obtained from Ajax Chemicals, 9 Short Street, Auburn, NSW 2144, Australia. All other solvents were obtained from the same source and were distilled and 0.45μ filtered before use. Water was obtained from a Milli-Q purification system. Nonactin and tropolone were obtained from Sigma-Aldrich Pty Ltd, Unit 2, 14 Anella Avenue, Castle Hill, NSW 2154, Australia. Tetranactin as a 6:3:1 mixture of Tetranactin, Trinactin and Dinactin, was obtained from Fuji Gotemba Research Laboratories, Chugai Pharmaceutical Co Ltd, 135 Komakodo 1-chome, Gotemoa-shi, Shizuoka 412, Japan. Monactin, Dinactin and Trinactin were obtained by column chromatography[2] of an ethyl

acetate extract of a culture produced from *Streptomyces* A515, grown by the RSC mycology unit. Tetranactin was also purified by the same method. The identities of the purified Actins were established by FAB-MS and ^{1}H NMR spectroscopy.

The HPLC system consisted of a Waters 510 pump, Rheodyne 7125 injector with 20μL sample loop and a Varex Mark III Evaporative Light Scattering (ELS) detector kindly loaned by Alltech Associates Pty Ltd, Baulkham Hills Business Centre, NSW 2153, Australia. The drift tube temperature was set to 96°C and the nitrogen flow to 2.9 SLPM. The other detectors used were a Waters 481 variable wavelength uv detector, and a Waters 410 RI detector. The instruments were interfaced with Waters Maxima software.

The HPLC columns were Merck Lichrosorb Si 60 7μ 250mm x 4mm obtained from Merck Pty Ltd, 207 Colchester road, Kilsyth, Victoria 3137, Australia; a Waters μ-Porasil 10μ 300mm x 3.9mm and a Waters Resolve 5μ 150mm x 3.9mm obtained from Waters Australia Pty Ltd, PO Box 84 Rydalmere, NSW 2116, Australia; a YMC ODS-AQ 3μ 150mm x 4.6mm obtained from YMC Inc, 3233 Burnt Mill Drive, Wilmington, NC 28403, USA; and an Alltech Alltima C18 5μ, 250mm x 4.6mm obtained from the Alltech address given above. Mobile phase compositions are described later in the text. The Actins were dissolved in the mobile phase and 5μL containing approximately 40μg injected.

TLC was run on Merck silica gel 60 glass backed plates, gel thickness 0.25mm and size 10cm x 5cm, obtained from the above Merck address, with 2:1 ethylacetate:chloroform. After development the plates were dipped in an ethanol solution of 6% vanillin and 1% concentrated sulfuric acid, and then heated to 150°C for 5 minutes. The Actins appear as dark blue zones on a pale grey background.

RESULTS AND DISCUSSION

The HPLC method in the literature[1] used a Lichrosorb Si 60 5μ column, a mobile phase of 80:20 hexane:isopropanol (containing 4% water) and detection at 215nm. Attempts to reproduce this separation with an analytical, 7μ version of this column and a mobile phase flow of 0.8mL/min were unsuccessful, with the Actins eluting very close to the void volume and with very poor resolution. Increasing the hexane content did increase retention but at the expense of excessive peak broadening.

The TLC method described in the literature used silica gel 60 and 2:1 ethyl acetate:chloroform. Attempts to develop an HPLC method based on this used a Waters μ-Porasil column, 1:2 ethyl acetate:chloroform at 0.8mL/min and RI detection. The Actins again all eluted very close to the void volume with very poor resolution. Increasing the chloroform content led to excessive peak broadening. A similar result was obtained with the Waters Resolve column. It should be noted that on silica TLC the Actins elute as diffuse streaks rather than as well defined spots, so the poor elution behaviour in normal phase HPLC, in hindsight, is not unexpected.

The next strategy to adopt seemed to be reverse phase HPLC, although solubility tests were not encouraging, as the Actins were insoluble in both acetonitrile and methanol. They were, however, readily soluble in THF, and the first reverse phase method used a YMC ODS-AQ column, 60:40 THF(unstabilised):water at 0.8mL/min and detection at 215nm. The five Actins did separate within 20 minutes but peak shape for the last two, (Trinactin and Tetranactin) was poor. At this low wavelength the absorbance of the mobile phase was very high and, and the photomultiplier close to cutting out. Adding 1mM disodium EDTA to the mobile phase improved peak shape dramatically.

It appears that the disodium EDTA works by providing sodium ions for the Actins to complex with, as well as by chelating with trace metals, because a similar improvement in peak shape was obtained if the mobile phase was made 1mM in sodium acetate. However, the difficulties with working at this low wavelength with THF, and low sensitivity due to the low uv absorbance of the Actins, instigated a further search for a better method.

The Actins are high molecular weight, thermally stable, involatile compounds and as such, should be potentially detectable by ELS. However ELS cannot be used with involatile mobile phase additives which rules out adding sodium salts to improve peak shape. The Alltech Alltima C18 column has very low metal ion content in its silica matrix, and indeed with 60:40 THF(stabilised):water at 1mL/min and ELS detection, separated all five Actins to baseline with excellent peak shape (Figure 2). The elution order is that expected, with the retention time increasing with the number of ethyl groups in the Actin.

When trying to separate potential metal chelators it is important to not only have a metal ion-free column but also to eradicate as many metal ions as possible from the entire HPLC system. On one occasion, switching to a different pump produced a deterioration in peak shape and resolution. This

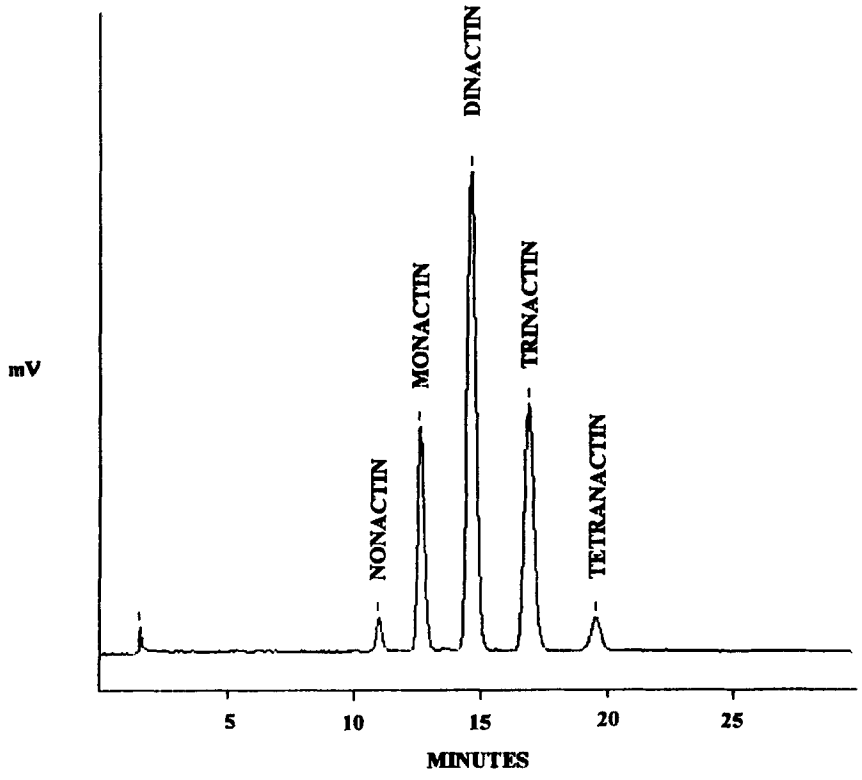


Figure 2. Separation of all five Actins from an ethyl acetate extract of *Streptomyces* A515. Retention times in minutes: Nonactin 11.1, Monactin 12.9, Dinactin 14.9, Trinactin 17.1, Tetranactin 19.8. Column: Alltech Alltima C18 5μ 250mm x 4.6mm. Mobile phase: 60:40 THF:Water at 1mL/min. Detection: ELS, Drift tube temperature 96°C, Nitrogen flow 2.9 SLPM.

pump had previously given problems with metal ions leaching into the mobile phase. Making the solution 1mM in tropolone restored separation quality. Tropolone is a metal scavenger but unlike disodium EDTA is volatile and can be used in ELS detection.

CONCLUSION

A reverse phase HPLC separation of Nonactin and its homologues at the microgram level has been achieved. The method could be adapted to semi-

preparative HPLC using either ELS detection with a post-column splitter, or the non-destructive, RI detection. It is hoped this separation will prove useful to all who work with these chromatographically challenging compounds.

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