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Reversed-phase high-performance liquid chromatography of tenuazonic acid and related tetramic acids

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ABSTRACT

A reversed-phase high-performance liquid chromatographic system for the determination of the fungal toxin, tenuazonic acid, (5*S*,8*S*)-3-acetyl-5-*sec*.-butyltetramic acid, is described. The system utilizes a column packed with deactivated end-capped C_{18} silica with a high carbon load to overcome the problem of poor chromatographic performance of this β-diketone on reversed-phase liquid chromatography which previously necessitated the use of anion-exchange, ligand-exchange or ion-pairing methods. The reversed-phase system allows the separation of tenuazonic acid from its (5*R*,8*S*)-diastereomer, *allo*-tenuazonic acid and was applied to the detection of tenuazonic acid in cultures of *Alternaria alternata* and *Phoma sorghina*. By means of diode-array ultraviolet detection, (5*S*)-3-acetyl-5-isopropyltetramic acid was observed in extracts of culture material. This metabolite was purified using the analytical reversed-phase system and was identified by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

INTRODUCTION

Tenuazonic acid [(5*S*,8*S*)-3-acetyl-5-*sec*.-butyltetramic acid, TA, Fig. 1) is a fungal toxin produced by various species of *Alternaria* as well as by *Phoma sorgh-*

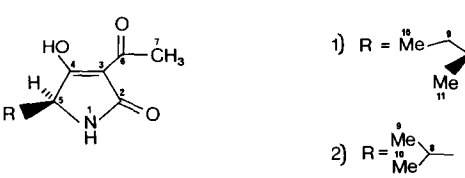

Fig. 1. Chemical structures of (1) TA and (2) 3-acetyl-5-isopropyltetramic acid.

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ina and *Pyricularia oryzae*. It is a potent inhibitor of protein biosynthesis and possesses a wide range of biological activities, including antitumour, antibiotic and antiviral properties [1].

The determination of TA by high-performance liquid chromatography (HPLC) has recently been reviewed [2]. TA is a β-diketone with metal chelating properties and also contains a secondary amine moiety. Both these properties are detrimental to efficient chromatographic performance on silica-based packing materials [3,4]. The amine moiety is susceptible to interactions with active silanol groups on the column packing material, thus causing broad, tailing chromatographic peaks. β-Diketones present a distinctly different problem in that they are strong metal chelators and tend to bind to metal ions present in packing materials that have been insufficiently demineralized. The effect of metal ion contamination of packing material can cause severe peak tailing or the total absorption of the component [3,4]. Thus the HPLC separation and determination of TA has been hampered by poor chromatographic efficiency which produces a broad, tailing peak in reversed-phase systems [2,5,6]. In order to overcome these effects and to improve chromatographic performance, previous workers have used either ion-pair, anion-exchange or ligand-exchange chromatography [2,4,7].

This study reports the use of a high-carbon-loaded octadecyl silica packing material to achieve the efficient reversed-phase HPLC of TA. This system was used to determine TA extracted from fungal culture material of *Alternaria alternata* and *Phoma sorghina*. Modifications to the mobile phase composition and flow-rate allowed the separation of TA from its diastereomer, *allo*-TA. An unidentified component detected by diode-array UV detection in culture material of *A. alternata* was isolated and shown to be the 5-isopropyl analogue of TA by nuclear magnetic resonance (NMR) spectroscopy.

EXPERIMENTAL

Reagents

Methanol, sodium dihydrogenphosphate, orthophosphoric acid, dichloromethane, sodium hydrogencarbonate, sulphuric acid and hydrochloric acid were analytical-grade reagents from Merck (Darmstadt, Germany).

Preparation of TA

The copper salt of TA was synthesized by the method of Harris *et al.* [8] at the Research Institute for Nutritional Diseases (Tygerberg, South Africa) and stored in this form, as the free acid is unstable. The copper salt was converted to the free acid by cation-exchange chromatography. A 30-mm mini-column of Bio-Rad AG 50W-X12 (200–400 mesh, hydrogen form) was prepared in a Pasteur pipette and pre-washed with 4 ml of methanol. The column was loaded with 4 mg of copper tenuazonate in 0.5 ml of methanol and TA was eluted with 15 ml of methanol. The TA was diluted to 50 ml with methanol and its concentration

calculated from UV absorbance measurements at 277 nm (ε_{277} = 12 980) [5]. This dilute methanol solution of TA was stable for at least two months at −20°C.

Preparation of iso-TA

Iso-TA, a mixture of TA and *allo*-TA [9], was prepared by refluxing 0.1 mg of TA for 16 h in 0.1 *M* sodium hydroxide. The products were extracted with dichloromethane after acidification to pH < 2 with 1 *M* hydrochloric acid. The dichloromethane was evaporated and the products dissolved in 2 ml of methanol.

Extraction of culture material

Lyophilized, single-conidial cultures of *A. alternata* and *P. sorghina* from the culture collection of the South African Medical Research Council (MRC) were used to inoculate autoclaved yellow corn kernels [10]. The corn cultures were incubated in the dark at 25°C for 21 days after which the material was dried (45°C; 24 h) and ground in a laboratory mill. Cultures of *A. alternata* MRC 5494 and MRC 5495 were grown in Czapek-Dox liquid medium (Oxoid, London, U.K.) in shake culture at 25°C in the dark for seven days and blended prior to extraction.

TA was extracted from the corn culture material by the method of Scott and Kanhere [5]. Ground culture material was blended with methanol, centrifuged (10 min at 500 *g*) and filtered. An aliquot (10 ml) of the filtrate was diluted with an equal volume of water, acidified with 0.5 ml of 5 *M* sulphuric acid and extracted twice with 15 ml of dichloromethane. The aqueous phase was discarded and the organic phase extracted with 15 ml of 5% sodium hydrogencarbonate. The organic phase was then discarded and the aqueous phase acidified with 15 ml of 1 *M* hydrochloric acid and re-extracted twice with 25 ml of dichloromethane. The combined extracts were washed with 20 ml of water, then evaporated to dryness and the residue was dissolved in methanol.

TA was similarly extracted from liquid culture material following acidification of an aliquot (10 ml) with sulphuric acid.

Chromatographic conditions

TA was chromatographed at room temperature on a reversed-phase, isocratic HPLC system consisting of a Waters Assoc. (Milford, MA, U.S.A.) Model 510 pump and U6K injector. The analytical Phenomenex column (250 mm × 4.6 mm I.D.; Rancho Palos Verdes, CA, U.S.A.) was prepacked with Ultracarb 7 ODS 30 reversed-phase material of 7 μm particle size. A Waters Guard-Pak pre-column fitted with a Waters Resolve C_{18} cartridge (10 mm × 5 mm I.D.) packed with 10-μm particles was installed to protect the analytical column. Injections of 5–10 μl were made with a Waters U6K injector. The detector was a Model HP1040A diode-array UV detector from Hewlett-Packard (Waldbronn, Germany). The spectrum of the eluate was monitored from 210 to 320 nm and quantification by peak-area measurement was achieved at 277 nm. Alternatively, the column eluate

was monitored at 277 nm with a Waters Lambda-Max variable-wavelength Model 381 UV detector and quantification was by peak-area measurement using a Waters 745 data module. The detector response was calibrated by triplicate injections (5 μl) of TA standard (40 μg ml^{-1}). Chromatographic separation was achieved using either one of two mobile phase systems. The first mobile phase system was methanol–0.1 *M* sodium dihydrogenphosphate (2:1, v/v) adjusted to pH 3.2 with orthophosphoric acid. The flow-rate was 1 ml min^{-1}. The second mobile phase system was used for the separation of TA and *allo*-TA and consisted of acetonitrile–0.1 *M* sodium dihydrogenphosphate (1:3, v/v) adjusted to pH 3.2 with orthophosphoric acid and the flow-rate was 1.5 ml min^{-1}.

Isolation and identification of 3-acetyl-5-isopropyltetramic acid in cultures of A. alternata MRC 5495

A liquid culture of *A. alternata* MRC 5495 was grown in Czapek-Dox medium, enriched with 40 g l^{-1} valine. The fungal culture was incubated with shaking in the dark at 25°C for seven days. After blending, the sample was extracted using the method of Scott and Kanhere [5]. The 3-acetyl-5-isopropyltetramic acid was isolated from this extract on a semi-preparative basis using the Ultracarb 7 ODS 30 analytical column incorporated in an automated system consisting of a Waters WISP autoinjector, Waters Model 590 programmable pump and Waters automated switching valve. The mobile phase was methanol–0.1 *M* sodium dihydrogenphosphate (3:2, v/v) adjusted to pH 3.2. The autoinjector was programmed for repeated injection of aliquots of the sample extract and the eluate fraction corresponding to the chromatographic peak of 3-acetyl-5-isopropyltetramic acid was collected using the automated switching valve. The combined fractions were acidified with hydrochloric acid and extracted with dichloromethane to give 3-acetyl-5-isopropyltetramic acid. The structure of the compound was deduced from the ^{1}H and ^{13}C NMR data recorded for a sample (20 mg) in $C^{2}HCl_3$ on a Bruker AC-300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C.

RESULTS AND DISCUSSION

Chromatography of TA

In order to overcome the poor chromatographic performance of TA on reversed-phase systems, the use of a C_{18} reversed-phase column containing a high (31%) carbon-loaded silica which had been deactivated and exhaustively end-capped, was investigated. Using a mobile phase of methanol–0.1 *M* sodium dihydrogenphosphate (2:1, v/v) at pH 3.2 (mobile phase system 1), 200 ng TA eluted as a sharp, symmetrical peak at 9.9 min retention time. The chromatographic peak shape was excellent. The chromatographic efficiency calculated on this peak was 3200 theoretical plates, while the peak symmetry calculated at 10% of peak height (A_{10}) was 1.0. This excellent peak shape was maintained for low amounts injected such that the detection limit using the Waters Lambda-Max

UV detector was 0.4 ng injected at a signal-to-noise ratio of 4:1. The response with this detector was linear within the 1–80 ng range tested. The retention time of the TA peak was reproducible (mean time 9.9 min for sixteen injections; relative standard deviation 0.3%). These data indicate the satisfactory chromatographic performance of TA on this reversed-phase system using an Ultracarb column, even down to the sub-nanogram detection limit. The efficiency obtained by this sytem is superior to that previously published in a comparison of the ion-pair, ligand-exchange and anion-exchange systems [2].

Due to its acidic nature (pK_a = 3.5) [1], the retention of TA on a reversed-phase system is dependent on its degree of ionization which in turn is pH-dependent. Fig. 2 shows the capacity factor of TA as a function of the pH of the mobile phase. The capacity factor and hence the retention time of TA increases as its degree of ionization is reduced at lower pH values. The sensitivity of TA retention time to pH can provide an important selectivity factor in its chromatography. Despite the range in retention times, the peak symmetry achieved in this reversed-phase system is maintained.

Separation of TA and allo-TA

On standing for a period of some months, TA is partially converted into the diastereomer, *allo*-TA by epimerization of the C-5 chiral centre [9]. In order to investigate the ability of this reversed-phase system to separate the diastereomers, iso-TA (a mixture of TA and *allo*-TA) was synthesized. When chromatographed using mobile phase system 1, *allo*-TA and TA were only partially separated, with the former compound eluting slightly before TA. The selectivity factor (α) was 1.06, a result which was found to be similar to that previously reported for this separation using ligand-exchange chromatography [2]. Optimization of the mobile phase for improved separation of the diastereomers resulted in almost baseline separation and a selectivity factor of 1.13 using a mobile phase of acetonitrile–0.1 *M* sodium dihydrogenphosphate (1:3, v/v) at pH 3.2 (flow-rate 1.5 ml

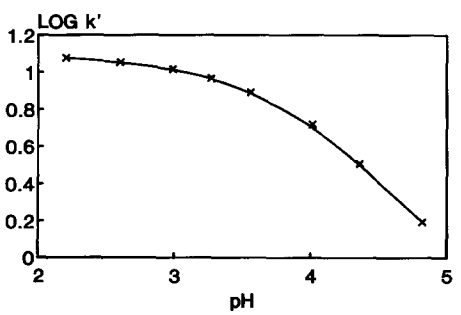


Fig. 2. Influence of mobile phase pH on the capacity factor of TA. Mobile phase was acetonitrile–methanol–0.1 *M* sodium dihydrogenphosphate (4:1:1, v/v) with a flow-rate of 1.5 ml min^{-1}. The pH was adjusted with orthophosphoric acid.

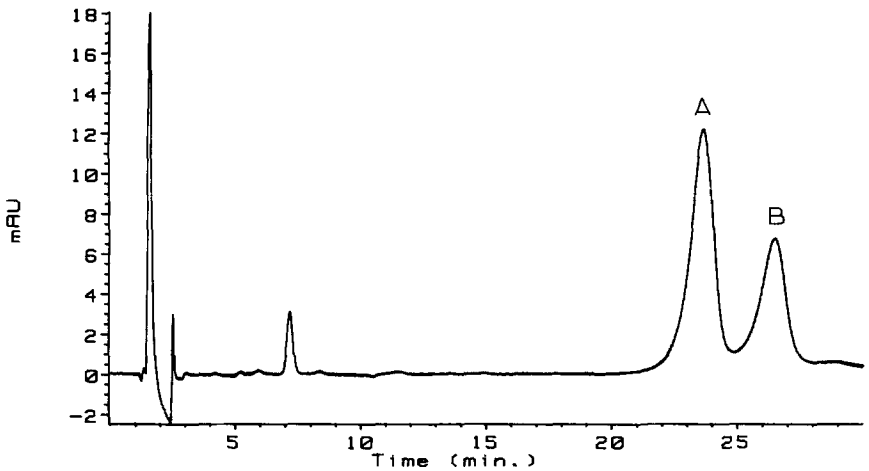


Fig. 3. Chromatogram of a mixture of *allo*-TA (peak A, 510 ng) and TA (peak B, 390 ng). Chromatographic conditions are given in the text.

min^{-1}; mobile phase system 2). This separation (Fig. 3) is better than the selectivity of 1.10 reported for anion-exchange chromatography [2].

Determination of TA in fungal cultures

This reversed-phase HPLC system for TA was applied to the chromatographic separation of extracts of various fungal cultures. Fig. 4 shows the chromatogram of an extract of *A. alternata* MRC 3968, where TA elutes at 9.6 min using mobile

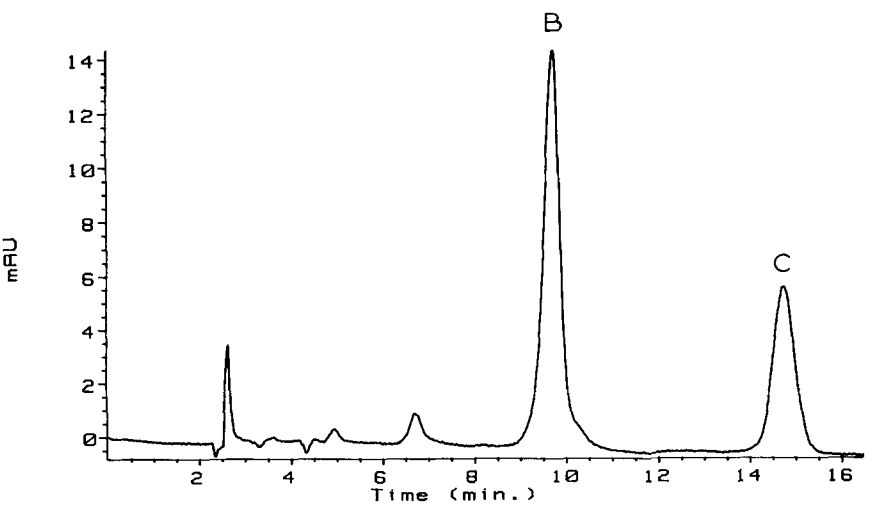


Fig. 4. Chromatogram of an extract of *A. alternata* MRC 3968. Chromatographic conditions are the same as for TA standard. Peak B corresponds to 180 ng TA. Peak C was identified as alternariol.

phase system 1. The identity of this peak was confirmed by both co-elution of added standard and by comparison of its UV spectrum with that of standard TA, using the diode-array detector. The compound eluting at 14.7 min (Fig. 4) was identified as alternariol (another *Alternaria* toxin) by comparison of its retention time and its UV spectrum with that of a standard.

Chromatograms of an extract of *P. sorghina* MRC 561 run with mobile phase system 1 showed an unresolved peak eluting prior to TA. This extract was subsequently chromatographed with mobile phase system 2 optimised for the separation of TA and *allo*-TA (Fig. 5). The previously unresolved shoulder was separated and eluted at 25.4 min. This compound was identified as *allo*-TA by spectral comparison and by spiking the sample with standard. The slightly longer retention times in Fig. 5 as compared to those in Fig. 3 result from batch variations in the mobile phase.

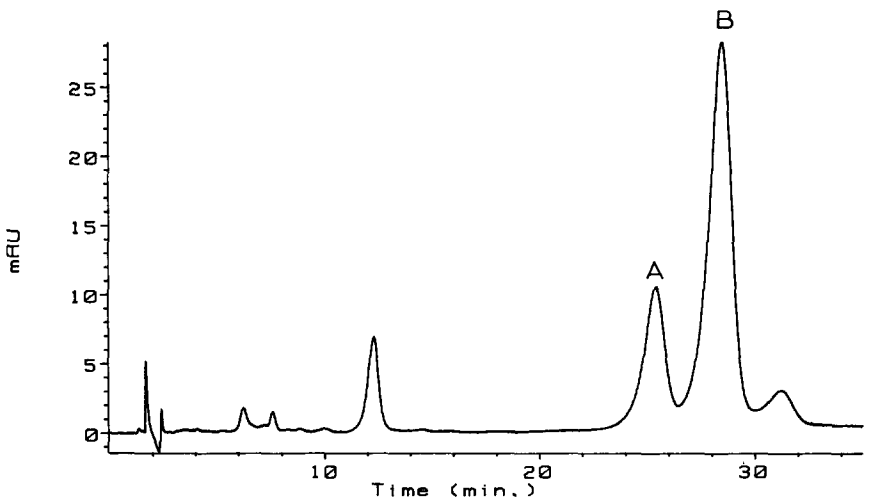


Fig. 5. Chromatogram of an extract of *P. sorghina* MRC 561. Chromatographic conditions are the same as for the separation of TA and *allo*-TA. Peaks: A = *allo*-TA (450 ng); B = TA (1600 ng).

Validation of the TA extraction method

The extraction method for the determination of TA was validated with respect to precision, accuracy and extraction recovery from samples spiked with TA standard. The precision was assessed by six replicate determinations of TA in each of two liquid cultures of *A. alternata*, one containing 3.9 $\mu g\ ml^{-1}$ TA and the other containing 41.4 $\mu g\ ml^{-1}$ TA. The reproducibility of sample extraction and analysis was 7.1% relative standard deviation at the low level and 2.0% relative standard deviation at the high level.

The accuracy of the method was assessed by analysis of blank samples of medium spiked in quadruplicate with TA standard to yield samples of known TA

concentration at two levels, namely 6 and 43 μg ml^{-1}. Determination of TA in these samples gave mean recoveries of 88 ± 7.5% relative standard deviation at the low level and 100 ± 4.5% at the high level.

Analytical recoveries from a liquid culture sample naturally containing 3.9 μg ml^{-1} TA were determined by the addition of TA standard at levels equivalent to 7 and 48 μg ml^{-1} TA. The mean recovery of added TA standard based on triplicate analyses at each level was 88 ± 0.4% relative standard deviation at the low level and 91 ± 0.9% at the high level. These recoveries are in accordance with those determined by Scott and Kanhere [5].

Identification of 3-acetyl-5-isopropyltetramic acid in fungal cultures

Extracts of liquid cultures of *A. alternata* MRC 5494 and MRC 5495 were also run on this reversed-phase system. Fig. 6 shows a chromatogram of an extract of MRC 5495. The presence of TA eluting at 9.8 min using mobile phase system 1 is clearly shown. The unidentified compound eluting at 6.8 min had a UV spectrum identical to that of TA (Fig. 6), suggesting the presence of a compound similar to TA. A similar compound eluting at 12.3 min was identified in the chromatogram

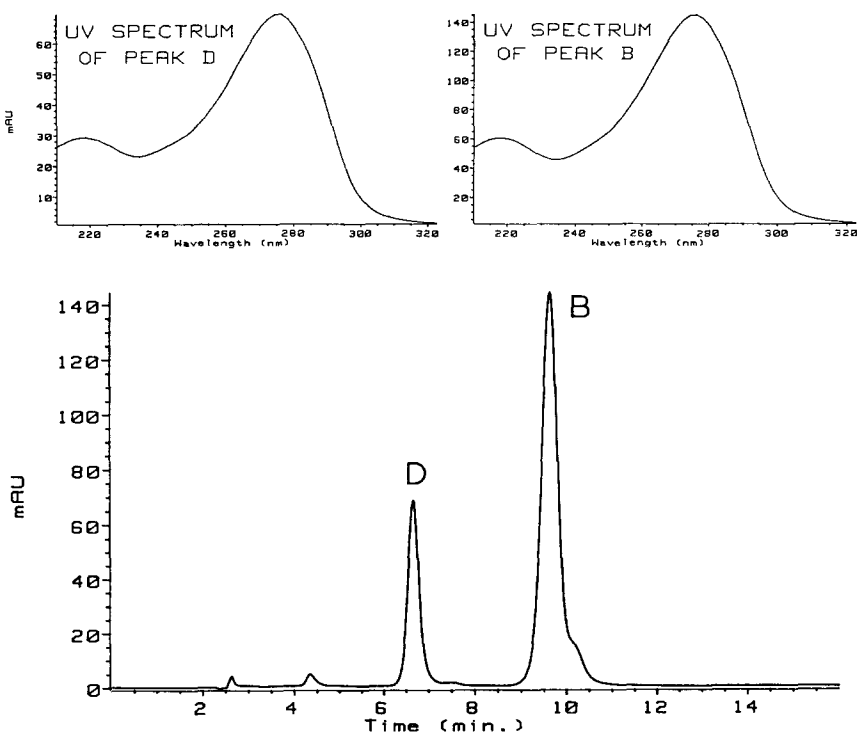
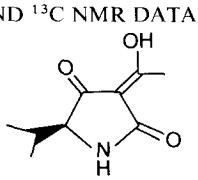


Fig. 6. Chromatogram of an extract of *A. alternata* MRC 5495 showing TA (peak B, 1800 ng) and the 5-isopropyl analogue (peak D, 500 ng) together with their UV spectra. Chromatographic conditions are the same as for TA standard.

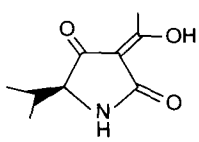
of *P. sorghina* MRC 561 (Fig. 5). Previous workers [2,7,11] have reported the natural occurrence of 3-acetyl-5-isopropyltetramic acid, an analogue of TA derived from valine. Originally identified by Joshi *et al.* [11] in cultures of *A. tenuis* grown on rice, it has also been found and confirmed in *Alternaria*-contaminated tomatoes [7]. Its presence in *Pyricularia oryzae* culture filtrates was suggested but not confirmed [2]. In order to confirm the production of 3-acetyl-5-isopropyltetramic acid by the cultures used in the present investigation, the unidentified compound was isolated from a liquid culture of *A. alternata* MRC 5495 grown in a medium enriched with valine. The metabolite was purified by semi-preparative HPLC and a sample investigated by ^{1}H and ^{13}C NMR spectroscopy. The ^{1}H and ^{13}C NMR data are collated in Table I and support the proposed 3-acetyl-5-isopropyltetramic acid structure for the metabolite. The ^{13}C NMR data show that in non-polar solvents (*e.g.* $C^{2}HCl_3$) the 3-acetyl-5-isopropyltetramic acid exists as an equilibrium mixture of two external tautomers in which the *exo*-enol form "b" predominates. This tautomer is also the form in which 3-acetyl-5-isopropyltetramic acid exists in the crystalline state [12].

TABLE I

^{1}H AND ^{13}C NMR DATA FOR 3-ACETYL-5-ISOPROPYLTETRAMIC ACID



Form a



Form b

Atom	δ_C (ppm)		δ_H (ppm)	
	Form b	Form a	Form b	Form a
1	–	–	6.972 s	6.748 s
2	175.71 S	169.80 S	–	–
3	102.30 S	105.60 S	–	–
4	195.36 S	200.76 S	–	–
5	67.45 D	64.22 D	3.711 d (3.6 J/Hz)	3.876 d (3.8 J/Hz)
6	184.56 S	189.08 S	–	–
7	19.46 Q	20.60 Q	2.422 s	2.474 s
8	30.12 D	30.21 D	2.20 m	2.20 m
9	19.35 Q	19.01 Q	0.824 d (6.8 J/Hz)	0.858 d (6.8 J/Hz)
10	15.86 Q	16.20 Q	1.017 d (7.0 J/Hz)	1.017 d (7.0 J/Hz)

Table II summarizes the levels of TA and 3-acetyl-5-isopropyltetramic acid determined in the fungal cultures used in this study and shows, as previously indicated from mass spectroscopy [11], that the 5-isopropyl analogue occurs at lower levels than TA.

TABLE II

LEVELS OF TA AND 3-ACETYL-5-ISOPROPYLTETRAMIC ACID IN SELECTED CULTURES

Culture	TA	3-Acetyl-5-isopropyltetramic acid[a]
A. alternata MRC 3968[b]	360 μg/g	–
A. alternata MRC 5494	2 μg/ml	0.5 μg/ml
A. alternata MRC 5495	85 μg/ml	30 μg/ml
P. sorghina MRC 561	810 μg/g[c]	85 μg/g

[a] Calculation based on the assumption that the molar extinction coefficient is the same as that of TA.
[b] MRC number represents the accession number of the lyophilized culture as deposited at the Medical Research Council (Tygerberg, South Africa).
[c] Includes *allo*-TA (180 μg/g).

Previous studies [13] on the antitumour, cytotoxic and antibacterial activities of TA and its synthetically produced 5-substituted analogues (including 5-isopropyl) showed that the activities of the analogues were generally much lower than those of TA. Similarly, the 5-isopropyl analogue has been shown to be only weakly phytotoxic [14]. Hence, these differences in biological activities make it essential to use a chromatographic method such as the reversed-phase system described in this report which effectively separates these two naturally occurring substituted tetramic acids.

CONCLUSION

The use of a high-carbon-loaded octadecyl silica, which has been deactivated and end-capped, as column packing provides efficient reversed-phase HPLC for TA. This sysem allows separation of TA from its diastereomer, *allo*-TA, as well as from its naturally occurring valine-derived analogue. In addition, no column deterioration, as noted previously for other chromatographic methods [2], was observed over a period of several months during which fungal cultures were analysed.

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