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ION-PAIR, ANION-EXCHANGE AND LIGAND-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TENUAZONIC ACID AND 3-ACETYL 5-SUBSTITUTED PYRROLIDINE-2,4-DIONES

M. H. LEBRUN*
Cryptogamie, CNRS UA 086, Bat. 400, Université Paris-Sud, 91405 Orsay (France)
and
F. GAUDEMER, M. BOUTAR, L. NICOLAS and A. GAUDEMER
Chimie de Coordination Bioorganique, CNRS UA 255, Bat. 420, Université Paris-Sud, 91405 Orsay (France)
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SUMMARY

The ion-pair, ligand-exchange and anion-exchange chromatography of the fungal metabolic tenuazonic acid (TA) and its related 3-acetyl 5-substituted pyrrolidine-2,4-diones were studied. Ion-pair chromatography was performed on a C_{18} column with a mobile phase composed of cetrimide, phosphate buffer in water–methanol and a metal complexant (ethylenediamine) to improve the peak sharpness. Addition of the same metal complexant to the mobile phase of the anion-exchange chromatographic system also improved its efficiency. TA and its 5-substituted analogues derived from valine and leucine were separated with the ion-pair and anion-exchange chromatographic systems. With ligand-exchange chromatography, TA could only be separated from its valine analogue. These chromatographic systems were used for the detection of TA in the culture filtrates of the fungus *Pyricularia oryzae* and in infected rice leaves. Deproteinated culture filtrates could be rapidly analysed for their TA content by anion-exchange chromatography. However, this system was not suitable for the detection of TA in the infected rice leaf as interfering compounds were coeluted with TA. Ion-pair and ligand-exchange chromatographic systems allowed the efficient quantification of TA in infected leaves.

INTRODUCTION

Tenuazonic acid (3-acetyl 5-*sec.*-butylpyrrolidine-2,4-dione; TA; Fig. 1) is a toxic metabolite derived from isoleucine[1,2] which is produced by several fungi: *Alternaria tenuis, alternata, longipes, mali, citri, oryzae and kikuchiana*[3–7], *Phoma sorghina*[8] and *Pyricularia oryzae*[9–11]. TA exhibits toxicity towards plants[5,9] and animals[6,12,13] and is weakly bactericidal[14] and viricidal[15].

Chromatographic methods have been developed for the detection of TA in *Alternaria*-contaminated food and feeds for animals. First, Harvan and Pero[16] described the gas chromatography of TA trimethylsilyl derivatives. This technique was

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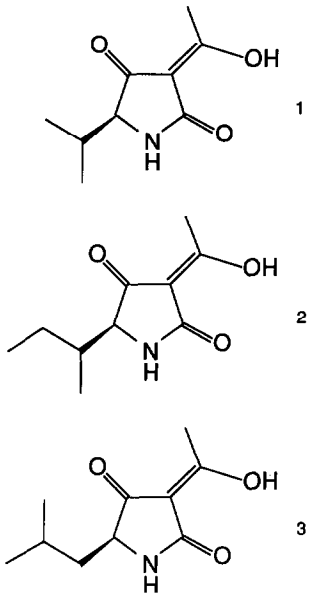

Fig. 1. Structures of 3-acetyl-5-isopropylpyrrolidine-2,4-dione (1), 3-acetyl-5-*sec.*-butylpyrrolidine-2,4-dione (2) and 3-acetyl-5-isobutylpyrrolidine-2,4-dione (3).

applied to the detection of TA in contaminated tomatoes[17]. Then, reversed-phase chromatography with UV detection at 280 nm was employed by Heisler *et al.*[18] using a 10-μm C_{18} column with methanol–water (90:10) as the mobile phase. TA was more retained with increasing proportion of methanol, suggesting that residual silanol groups were responsible for its retention. This technique has been used in the detection of TA in contaminated fruits[19,20]. As TA has metal-chelating properties[3,21], Scott and Kanhere[22] developed a ligand-exchange chromatographic system with dodecyldiethylenetriamine and zinc sulphate in water–acetonitrile (50:50) with a 10-μm C_{18} column. This method allowed the detection of TA in tomato paste[22] and was applied with slight modifications to the chromatography of cyclopiazonic acid[23,24], which has the same metal-complexing structure as TA. A simplification of this method was described by Stack *et al.*[25], who used zinc sulphate in methanol–water solutions with a C_{18} packing. Scott and Kanhere[22] also used a silica-based weak anion exchanger to detect TA. Finally, a gradient of acetonitrile buffered with trifluoroacetic acid and a C_{18} packing were described by Frisvad[26].

In addition to these chromatographic improvements, Joshi *et al.*[27] detected, together with TA, a small amount of a TA analogue derived from valine (3-acetyl 5-isopropylpyrrolidine-2,4-dione) by mass spectrometric analysis of their copper salts. This compound was also detected by Stack *et al.*[25] in *Alternaria*-contaminated tomatoes, without derivation, by capillary gas chromatography coupled with mass spectrometry. This metabolite might interfere with the determination of TA if they are coeluted. Therefore, we have compared different high-performance liquid chromatographic (HPLC) systems for their ability to separate TA from other 3-acetyl 5-substituted pyrrolidine-2,4-diones. We used isocratic systems, which allow the

analysis of a large number of samples in a short time (less than 15 min per sample). Three distinct chromatographic systems were studied. First, ion-pair chromatography with alkylammonium ion was adapted to the chromatography of TA and its 5-substituted analogues with 5-μm C_{18} columns. Then, we studied the behaviour of TA and its 5-substituted analogues during anion-exchange and ligand-exchange chromatography. We have applied these methods to the detection of TA produced by the fungus *Pyricularia oryzae* in culture media and in infected rice leaves.

EXPERIMENTAL

Extraction of metabolites from culture filtrates

Isolates of *Pyricularia oryzae* were obtained from the CIRAD-IRAT culture collection and stored as dried paper disks at −20°C. The strains were grown on rice medium agar (using paddy rice 20 g, Difco yeast extract 2 g and agar 20 g per litre). Liquid cultures (Fries medium[28]) in Roux flasks (100 ml) were inoculated with mycelial implants. The flasks were incubated for 15–30 days at 26°C in the dark. At the end of the culture, the mycelium was separated from the culture medium by filtration through a filter-paper (No. 111, Durieux). The culture filtrate was either extracted with an organic solvent or treated with acetonitrile to precipitate the proteins. In the first instance, 2 ml of the culture filtrate were acidified to pH 2 with 5 *M* hydrochloric acid and extracted with 2 ml of ethyl acetate. After agitation of the tube on a Vortex mixer and decantation for 30 min, the organic phase was taken up with a Pasteur pipette and transferred to another tube. This ethyl acetate phase was then evaporated in a Speed-Vac evaporator and treated with 2 ml of 5% sodium hydrogencarbonate solution and 2 ml of methylene chloride. The aqueous phase was removed and centrifuged at 1500 *g* for 10 min to remove particles in suspension. For direct injection of the culture filtrates, proteins were removed as described by Jehl *et al.*[29], by precipitation with acetonitrile followed by a centrifugation at 1500 *g* for 10 min.

Inoculation of rice with Pyricularia oryzae spores and extraction of infected leaves

Pyricularia oryzae spores were produced on rice agar, suspended in water (10^5 spores ml^{-1}) containing 0.8% gelatine (bovine type II, Sigma) and sprayed on the rice leaves (5 leaf stage) with a hand atomizer. Seven days after the inoculation, the infected leaves were collected and crushed in ethanol with an Ultra-Turrax (100 mg fresh weight in 1 ml). After filtration on a glass-fibre filter (Whatman GF/C), the ethanol was evaporated in a Speed-Vac apparatus. The residue was dissolved in acidified water and extracted with ethyl acetate. The organic phase was then extracted with 5% sodium hydrogencarbonate solution. The aqueous phase was centrifuged at 1500 *g* for 10 min and injected.

Tenuazonic acid and 3-acetyl 5-substituted pyrrolidine2,4-diones

Physical data for TA and its analogues synthesized from different amino acids have been presented elsewhere[30]. D-*allo*-TA was synthesized independently from D-*allo*-isoleucine (Bachem, Switzerland). Its characteristics are as follows: $[\alpha]_D^{22}$, +150° (H_2O; *c* 1); ^1H NMR (250 MHz, sodium salt, $C^2H_3O^2H$), δ 0.78 (3H, d, C-9), 1.0 (3H, t, C-8), 1.4 (2H, m, C-7), 1.9 (H, m, C-6), 2.45 (3H, s, C-11), 3.72 ppm (H, d, C-5).

Reagents

HPLC-grade acetonitrile was obtained from Carlo Erba and methanol (Rectapur) from Prolabo. Water was obtained daily from a Milli-Ro-Milli-Q system (Millipore). Tetrabutylammonium hydroxide (TBAOH, Aldrich), hexadecyltrimethylammonium bromide ($C_{16}N$, Merck), octadecyltrimethylammonium bromide ($C_{18}N$, Fluka, 4-dodecyldiethylenetriamine (C_{12}-dien, Kodak), ethylenediamine (Merck), Na_2EDTA (Merck), zinc sulphate (Merck), ammonium acetate (Merck), potassium monophosphate (Merck), phosphoric acid (Merck) and potassium chloride (Merck) were of analytical-reagent grade. For stock solutions, tetrabutylammonium hydroxide and ethylenediamine were dissolved in water (1 *M*) and the solution was adjusted to pH 6 with 80% phosphoric acid.

Chromatographic equipment

The isocratic liquid chromatograph consisted of a Model 110A pump (Beckman), a Model 210 injector (Altex) with a 20-μl loop and a Model 330 UV detector (Beckman) with a 8-μl cell and filters for detection at 254 and 280 nm. Chromatograms were recorded on an HP-3380 A calculator (Hewlett-Packard). Reversed-phase C_{18} columns were obtained from Beckman (Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml, stainless steel) and anion-exchange columns from Chrompack (Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml or 100 × 4.6 mm I.D., V_0 = 1.25 ml, stainless steel; 5 μm, 100 × 3 mm I.D., V_0 = 0.7 ml, glass). The support for the 5-μm Ionospher-A cartridge was obtained from Chrompack (Chromsep, 135 mm). Dead volumes of each column were measured by weighing the column filled with two different solvents[31].

Composition of mobile phases

Ion-pair chromatography. The ion-pairing reagents (tetrabutylammonium phosphate, hexadecyltrimethylammonium bromide or octadecyltrimethylammonium bromide) were added at a concentration of 5 m*M* to the water–methanol (45:55) mobile phase buffered with potassium phosphate (10 m*M*, pH 6). Ethylenediamine phosphate or Na_2EDTA were added to this mobile phase at a concentration of 1 m*M*, if necessary. The flow-rate was fixed at 1 ml/min.

Anion-exchange chromatography. Acetonitrile (5–20%) was mixed with water buffered with potassium phosphate (10 m*M*, pH 6). Ethylenediamine phosphate (1 m*M*) or Na_2EDTA (0.3 m*M*) were added if necessary. The flow-rate was fixed at 2 ml/min for the 10-μm Ionispher-A packing and at 1 ml/min for the 5-μm Ionospher-A packing.

Ligand-exchange chromatography. 4-Dodecyldiethylenetriamine (5 m*M*) and zinc sulphate (5 m*M*) were added to the water–methanol (25:75) mobile phase buffered with ammonium acetate (30 m*M*, pH 6). The flow-rate was fixed at 1 ml/min.

Determination of pK values in water

p*K* values were obtained from measurements of the UV absorption of solutions of defined pH. Sodium salts of 3-acetyl 5-substituted pyrrolidine-2,4-diones have two UV absorption maxima, at 240 and 280 nm. Their acidic forms have a low UV absorption at 240 nm and maxima at 220 and 275 nm. Sodium salts of TA or its analogues were dissolved in 0.01 *M* potassium chloride solution at a concentration of

0.1 m*M*. UV absorptions were recorded at 240 nm from pH 6 to 2. These values (obtained from three independent experiments) were used to construct a graph with the equation $y = pH_i = a + bx$, with $x = \log[D_i - D_{min})/(D_{max} - D_i)]$, where D_i is the absorption at 240 nm of the solution at pH_i, D_{max} the absorption of the sodium salt (pH $\geqslant$ 7) at 240 nm and D_{min} the absorption of the acid at 240 nm. p*K* values were calculated from the linear regression curve for $x = 0$ ($y = pK$).

RESULTS

Tenuazonic acid has a strong metal complexation capacity[3,21,32] and maximum UV absorbance at 280 nm[1,2]. Isocratic reversed-phase chromatography of TA on a fully capped C_{18} column with different water–methanol mixtures at neutral pH led to broad peaks or a very low retention. The addition of a metal complexing reagent (EDTA) or the acidification of the mobile phase with phosphoric acid (pH 2) did not give better results. Therefore, we chromatographed TA on a C_{18} column by ion pairing or ligand exchange and with a silica-based anion-exchange column.

Ion-pair chromatography of tenuazonic acid

Scott and Kanhere[22] used the ion-pairing reagent tetrabutylammonium phosphate (TBA, 5 m*M*) for the chromatography of TA[22] with water–methanol (40:60) as the mobile phase and a 10-μm C_{18} column (μBondapack, Waters Assoc.). Adaptation of this method to a 5-μm C_{18} column (Ultrasphere-ODS, Beckman) did not give good results. TA was rapidly eluted in water–methanol (50:50), even with a high TBA concentration ($k' = 1.8$ with 10 m*M* TBA, Fig. 2). Further, the efficiency of this chromatographic system was low ($N \leqslant 300$). Another ion-pairing reagent, cetrimide ($C_{16}N$), has been widely used for the ion-pair chromatography of organic acids[33], and also for the bitter iso-α-acids from hops[34], which have an enolic structure comparable

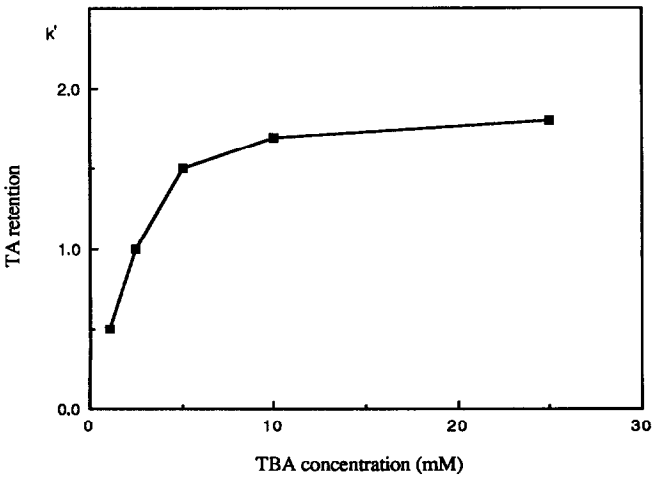

Fig. 2. Effect of the concentration of the ion-pairing reagent TBA on TA retention. TBA = tetrabutylammonium phosphate. Mobile phase, methanol–water (1:1) buffered with phosphate (10 m*M*, pH 6), column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D.; flow-rate, 1 ml/min.

TABLE I

EFFECT OF ION-PAIRING REAGENTS AND METAL COMPLEXANTS ON THE ION-PAIR CHROMATOGRAPHY OF TA

Mobile phase: 55% methanol in phosphate buffer (10 m*M*, pH 6) at 1 ml/min. Column: C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml.

Compound	*TA retention (k')*	*Selectivity (α)*		*Efficiency (N)*
		TA–VT	*LT–TA*	
$C_{18}N$ (5 m*M*)	5.4	1.63	1.23	2000
$C_{16}N$ (5 m*M*)	4.5	1.60	1.18	2000
$C_{16}N$ (5 m*M*) + EDTA (1 m*M*)	3.3	1.70	1.20	1700
$C_{16}N$ (5 m*M*) + ethylenediamine (1 m*M*)	4.8	1.60	1.20	2500

to that of TA. Addition of 5 m*M* $C_{16}N$ to a water–methanol (45:55) mobile phase buffered with phosphate (10 m*M*, pH 6) allowed a sufficient retention of TA on a 5-μm C_{18} column, (k' = 4.5, N = 2000, Table I). When an ion-pairing reagent with a longer apolar chain (5 m*M*, $C_{18}N$) is added to the water–methanol (45:55) mobile phase buffered with phosphate, TA is slightly more retained (k' = 5.4, Table I). However,

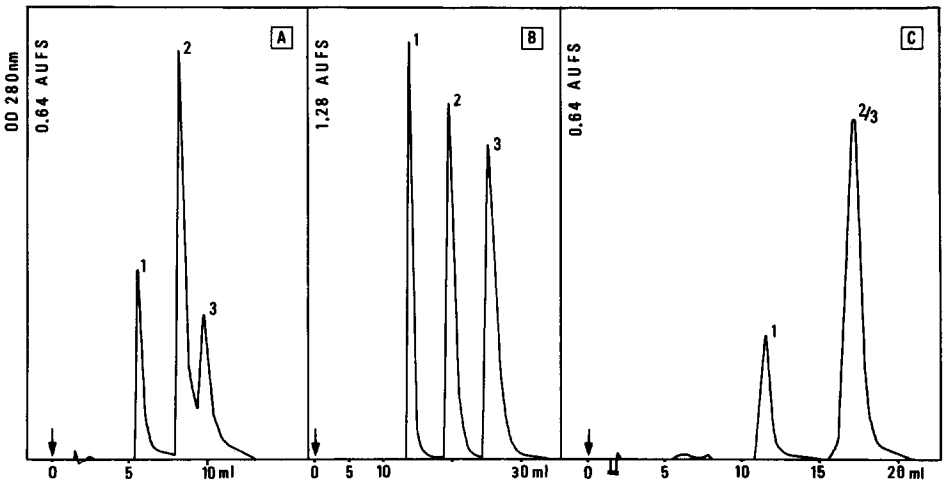

Fig. 3. Separation of 3-acetyl 5-substituted pyrrolidine-2,4-diones by ion-pair, anion-exchange and ligand-exchange HPLC. Injection of 20 μl of VT, TA and LT (0.1, 0.3 and 0.1 m*M* in A and C; 1 m*M* each in B). Peaks: 1 = VT (3-acetyl-5-isopropylpyrrolidine-2,4-dione); 2 = TA (3-acetyl-5-*sec.*-butylpyrrolidine-2,4-dione); 3 = LT (3-acetyl 5-isobutylpyrrolidine-2,4-dione). (A) Ion-pair chromatography. Mobile phase, cetrimide (5 m*M*), ethylenediamine (1 m*M*) and phosphate buffer (10 mM) (pH 6) with 55% methanol at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml; detection at 280 nm, 0.64 a.u.f.s. (B) Anion-exchange chromatography. Mobile phase, phosphate buffer (10 m*M*) (pH 7) and KCl (0.1 *M*) with 2.5% acetonitrile at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml; detection at 280 nm, 1.28 a.u.f.s. (C) Ligand-exchange chromatography. Mobile phase, ammonium acetate buffer (30 m*M*) (pH 6), C_{12}-dien (5 m*M*), $ZnSO_4$ (5 m*M*) with 70% methanol at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D.; detection at 280 nm, 0.64 a.u.f.s.

the low solubility of $C_{18}N$ in water–methanol mixtures makes it less suitable than $C_{16}N$. Changing the buffer from phosphate to acetate increased the retention time of TA but decreased the efficiency and gave broad peaks. The separation of TA from its analogues derived from valine (VT) and leucine (LT) is shown in Fig. 3a. With water–methanol (45:55) and 5 m*M* $C_{16}N$, TA is clearly separated from VT ($\alpha = 1.6$) but less satisfactorily from LT ($\alpha = 1.18$). The TA–LT separation was slightly improved with increase in the water content of the mobile phase (60% water, $\alpha = 1.3$).

The efficiency of this chromatographic system decreased with time, as a result of two major alterations to the column. First, ion-pairing reagents slowly dissolved the silica particles at the head of the column[35]. Repacking of the column with small amounts of the original solid phase restored the column efficiency[36]. This column deterioration can be prevented if a silica saturation column is placed before the injector[35]. Second, the accumulation of metal ions or complexes on the solid phase might interfere with the retention of TA. Such problems have been reported during the chromatography of iso-α-acids of hops[37], leukotrienes[38] and fusaric acid[39]. For the last two, the addition of EDTA to the mobile phase was necessary in order to achieve good peak resolution[38,39]. Therefore, we cleaned up the column with a mobile phase containing a metal-complexing reagent (10 m*M*, EDTA). This treatment restored its original efficiency. Further, the addition to the mobile phase of a metal-complexing agent (1 m*M* EDTA or ethylenediamine phosphate) prevented this column alteration. The addition of ethylenediamine phosphate to the mobile phase did not change the retention time of TA. In contrast, EDTA slightly decreased the retention time of TA (Table I). The reproducibility of the retention time of TA was satisfactory with ethylenediamine in the mobile phase ($k' = 4.8 \pm 0.2$, $n = 5$).

Anion-exchange chromatography of tenuazonic acid

Scott and Kanhere[22] used a 10-μm weak anion-exchange column and a mobile phase composed of acetonitrile (2.5%) buffered with phosphate (50 m*M*) and potassium chloride (0.1 *M*) for the chromatography of TA. We tested different anion-exchange columns (Chrompack, Ionosher-A, 5 and 10 μm) for their chromatographic behaviour towards TA. The retention of TA was dependent on the proportion of acetonitrile in the mobile phase (Table II, $k' = 4.6$ with 5% acetonitrile and 0.1 *M* potassium chloride), but also on the type and the concentration of the buffer. For example, a 30 m*M* sodium acetate buffer gave the same retention time as a 10 m*M* phosphate buffer, but an ammonium acetate buffer increased the retention time of TA (1.5-fold). The separation of TA from its 5-substituted analogues is complete with the 10-μm Ionospher-A packing and the mobile phase described by Scott and Kanhere[22] (Fig. 3b). An increase in the proportion of water in the mobile phase improved the selectivity of this separation (Table II). However, the selectivity of the separation with a 5-μm Iomospher-A packing was not improved.

The efficiency of the anion-exchange column decreased with time. This column deterioration was experimentally reproduced by the injection (ten times) of concentrated metal salt solutions which are used for the growth of the fungus. Washing the column with EDTA (10 m*M*) overnight restored its original efficiency. This column deterioration could be prevented by the addition of a metal-complexing reagent to the mobile phase (Table III). EDTA (1 m*M*) decreased the retention time of TA, particularly with the 5-μm Ionospher packing, for which a 0.3 m*M* concentration

TABLE II

EFFECT OF THE PERCENTAGE OF ACETONITRILE IN THE MOBILE PHASE ON THE SEPARATION OF TA AND ITS 5-SUBSTITUTED ANALOGUES BY ANION-EXCHANGE CHROMATOGRAPHY

Mobile phase: phosphate buffer (10 m*M*, pH 7)–KCl (0.1 *M*)–acetonitrile (2.5–10%) at 2 ml/min. Column: Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml.

Acetonitrile (%)	*TA retention (k')*	*Selectivity (α)*	
		VT–TA	*TA–LT*
2.5	5.2	1.55	1.35
5	4.6	1.50	1.30
10	3.6	1.45	1.25

should be used to allow a sufficient retention of TA. In contrast, ethylenediamine phosphate only slightly decreased the retention time of TA. Neither reagent modified the selectivity of the separation of TA from its 5-substituted analogues. Therefore, we used ethylenediamine for the ion-exchange chromatography of TA.

When crude culture filtrates samples were injected, the retention time of TA irreversibly decreased with time, even with the addition of a metal-complexing reagent to the mobile phase. Deproteinization of the culture filtrates by precipitation with acetonitrile overcame this problem. The reproducibility of the retention time of TA between experiments was then more satisfactory ($k' = 6.2 \pm 0.34$, $n = 4$) with a mobile phase consisting of 5% acetonitrile, 10 m*M* phosphate buffer (pH 6) and 1 m*M* ethylenediamine phosphate.

Ligand-exchange and metal complex chromatography

We adapted the ligand-exchange chromatographic system described by Scott and Kanhere[22] to short 5-μm C_{18} columns. We used a water–methanol (25:75) mobile

TABLE III

EFFECT OF ADDITION OF METAL-COMPLEXING REAGENTS ON THE ION-EXCHANGE CHROMATOGRAPHY OF TA

Mobile phase for Ionospher-A (5 μm) column: phosphate buffer (10 m*M*, pH 6)–acetonitrile(15%) at 1 ml/min. Mobile phase for Ionospher-A (10 μm) column: phosphate buffer (10 m*M*, pH 6)–acetonitrile (5%) at 2 ml/min. Columns: Ionospher-A (5 μm), 100 × 3 mm I.D., V_0 = 0.7 ml; Ionospher-A (10 μm), 250 × 10 min I.D., V_0 = 3.2 ml.

Eluent	*TA retention (k')*	
	Ionospher-A (5 μm)	*Ionospher-A (10 μm)*
Mobile phase	7	6.8
Mobile phase + 1 m*M* EDTA	2.7	5.1
Mobile phase + 1 m*M* ethylenediamine phosphate	5.6	6.2

phase with an ammonium acetate buffer (pH 6, 30 m*M*), zinc sulfate (5 m*M*) and C_{12}-dien (5 m*M*). This mobile phase gave a good retention of TA ($k' = 5.8$). The separation of the 5-substituted TA analogues was only partially achieved with this system as LT was coeluted with TA (Fig. 3c, Table V). Further, all the 5-substituted TA analogues with side-chains of the same size as TA (*n*-butyl) or longer (5-benzyl) were not separated from TA. Hence this chromatographic system is not selective for these TA analogues.

With this chromatographic system, the reproducibility of the retention time of TA was not satisfactory ($k' = 5.8 \pm 1.2$, $n = 5$). We studied the effect of different parameters that could be responsible for this low reproducibility of the retention time of TA. First, we compared the effect of temperature with a thermostated column. An increase in temperature from 22 to 28°C decreased the retention time of TA from 6 to 5.2 (with 75% methanol), but increased the column efficiency (from $N = 1500$ to 2000). These temperatures may occur as the lower and upper limits of room temperature. Therefore, the column must be thermostated in order to reduce the variability in retention time. Second, the addition of zinc in a slight excess (1.3 fold) increased the retention time of TA 1.05-fold but decreased the efficiency 0.75-fold. In contrast, an excess of C_{12}-dien (2-fold) decreased the retention time of TA (0.85-fold). Hence these slight modifications to the mobile phase could lead to differences in the retention time of TA from one batch to another.

Withdrawal of the C_{12}-dien from the mobile phase as described by Stack *et al.*[25] led to a poor retention of TA, even with 60% water ($k' = 1.8$), which is different from their results. This difference might be due to the C_{18} columns employed, as Stack *et al.* used a Waters C_{18} column (μBondapack), which might be more hydrophobic than the column we used (Ultrasphere-ODS). Further, this last chromatographic system did not show any selectivity for the TA analogues as they were all coeluted.

Separation of tenuazonic acid diastereoisomers

Synthetic or natural TA (L-*iso* diastereoisomer) can be contaminated by various amounts of its D-*allo* diastereoisomer[30]. To make a quantitative measurement of TA, we have to take into account this contaminating isomer. For this reason we compared different chromatographic systems (ion-pair, anion-exchange, ligand-exchange) for their ability to separate the four TA diastereoisomers. The reference compounds were obtained individually by synthesis from the corresponding amino acids[30]. Ion-pair chromatography did not allow their separation. L-*allo*-TA and D-*allo*-TA were slightly separated from L-*iso*-TA by anion-exchange and ligand-exchange chromatography ($\alpha = 1.06$–1.10, Table IV). This is in agreement with the report of Scott and Kanhere[22], who showed that ligand-exchange chromatography on a C_{18} column with zinc and C_{12}-dien was able to separate L-*iso*-TA from D-*allo*-TA. D-TA and L-TA were not separated with any of the chromatographic systems tested. Hence, ligand- and anion-exchange chromatography can be used to determine the amount of D-*allo*-TA in synthetic or natural samples of L-TA.

Separation of 3-acetyl 5-substituted pyrrolidine-2,4-diones

Synthetic analogues of TA with apolar substituents of increasing size at position 5 were submitted to ion-pair, ligand-exchange and anion-exchange chromatography. The analogues with the longer substituents were the more retained in all chromato-

TABLE IV

SEPARATION OF TA DIASTEREOISOMERS BY LIGAND-EXCHANGE AND ANION-EXCHANGE CHROMATOGRAPHY

Values given are retentions (k') with selectivity factors (k'_1/k'_2) in parentheses. Ligand-exchange chromatography: mobile phase, 70% methanol in ammonium acetate buffer (30 m*M*, pH 6), C_{12}-dien (5 m*M*) and zinc sulphate (5 m*M*), at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml. Anion-exchange chromatography: mobile phase, 5% acetonitrile in phosphate buffer (10 m*M*, pH 7) and KCl (0.1 *M*) at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml.

Mode	L-*TA*	L-*allo-TA*	D-*TA*	D-*allo-TA*
Ligand-exchange	11[a]	10.3[a] (1.07)	11 (1.00)	10.3 (1.07)
Anion-exchange	4.6	4.1 (1.10)	4.6 (1.00)	4.1 (1.10)

[a] Retention of the compound alone.

graphic systems (Table V). However, the separation of TA from its isomers (*iso*- and *n*-butyl) or from the analogue with a longer substituent at position 5 (benzyl) was achieved only with ion-pair and anion-exchange chromatography. The order of elution of these compounds cannot be related to their pK values as they are all similar (Table V). Hence the hydrophobic side-chains of these compounds might participate in their retention on the column.

Determination of tenuazonic acid produced by Pyricularia oryzae

Pyricularia oryzae is able to produce TA when grown on synthetic media[9,10] or in infected rice leaves[11] at concentrations between 0.01 and 1 m*M*. Its quantification requires a rapid and sensitive chromatographic assay with no interferences from the culture media or plants from which it is isolated. Quantification of TA was performed with external TA standard solutions with concentrations ranging from 10 μ*M* to 1 m*M* (20-μl loop) with detection at 280 nm. For all the chromatographic systems tested, a linear relationship between the logarithm of the amount of TA injected and the

TABLE V

SEPARATION OF 3-ACETYL 5-SUBSTITUTED PYRROLIDINE-2,4-DIONES BY ION-PAIR, ANION-EXCHANGE AND LIGAND-EXCHANGE CHROMATOGRAPHY

Values given are retentions (k'). Ion-pair chromatography: mobile phase, 55% methanol in phosphate buffer (10 m*M*, pH 6) and cetrimide (5 m*M*) at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml. Anion-exchange chromatography: mobile phase, 5% acetonitrile in phosphate buffer (10 m*M*, pH 7) and KCl (0.1 *M*) at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml. Ligand-exchange chromatography: mobile phase, 75% methanol in ammonium acetate buffer (10 m*M*, pH 6) and $ZnSO_4$–C_{12}-dien (5 m*M*) at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml.

Mode	*5-Methyl*	*5-Ethyl*	*5-Isopropyl (VT)*	*5-sec.-Butyl (TA)*	*5-Isobutyl (LT)*	*5-n-Butyl*	*5-Benzyl*
Ion-pair	1.65	2	2.85	4.5	5.6	5.8	7.1
Anion-exchange	2.4	2.75	3.05	4.6	6	6.5	11.5
Ligand-exchange	2.8	3.4	4.35	5.8	5.8	5.8	5.8
pK	3.00	3.00	3.05	3.10	3.05	3.00	2.95

logarithm of the peak area was obtained over this range of concentrations. This linear relationship was extended to 3 μM TA with the ion-pair and ligand-exchange chromatographic systems. We were not able to detect TA in amounts lower than 3 μM (12 ng) by anion-exchange chromatography, as it has a low efficiency at low TA concentrations and a variable baseline at low attenuation. In contrast, we were able to detect as little as 1 μM (4 ng) TA by ion-pair and ligand-exchange chromatography (Table VI).

For the analysis of culture filtrates, different preparations of the samples to be analysed were performed, depending on the chromatographic system used. For ion-pair or ligand-exchanged chromatography, removal of the apolar compounds from the culture filtrates was necessary in order to avoid contamination of the C_{18} packing of the column. For this purpose, the culture filtrates were acifified to pH 2 and extracted with ethyl acetate. The organic phase was then extracted with sodium hydrogencarbonate. With this procedure, TA was recovered in 75% yield ($\pm 6\%$, with 0.2 mM TA, $n = 3$). Anion-exchange chromatography required the removal of the proteins from the culture filtrates to avoid deterioration of the column. A rapid protein precipitation was performed with the addition of acetonitrile (50% final concentra-

TABLE VI

COMPARISON OF ION-PAIR, LIGAND-EXCHANGE AND ANION-EXCHANGE CHROMATOGRAPHY OF TA AND 3-ACETYL 5-SUBSTITUTED PYRROLIDINE-2,4-DIONES

Ion-pair chromatography: mobile phase, 55% methanol in phosphate buffer (10 mM, pH 6), ethylenediamine (1 mM), cetrimide (5 mM) at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D. Ligand-exchange chromatography: mobile phase, 75% methanol in ammonium acetate buffer (30 mM, pH 6), C_{12}-dien (5 mM) and zinc sulphate (5 mM) at 1 ml/min and 22°C; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml. Anion-exchange chromatography: mobile phase, 5% acetonitrile in phosphate buffer (10 mM, pH 6), ethylenediamine (1 mM) at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml.

Parameter	*Ion-pair chromatography*	*Ligand-exchange chromatography*	*Anion-exchange chromatography*
Retention [k'(TA)]	4.8	5.8	6.2
Efficiency (N)	2500	2000	1000
Selectivity (VT–TA)	1.60	1.30	1.45
Selectivity (TA–LT)	1.2	1	1.25
Selectivity (L-*allo*-TA–LTA)	1	1.05	1.10
Advantages	Reproducibility sensitivity (4 ng[a]), selectivity (TA–LT–VT), no interference (leaf)	Sensitivity (4 ng[a]), strong retention of TA, no interference (leaf)	Reproducibility, selectivity (TA–LT–VT), rapid sample preparation
Disadvantages	Prepurification of sample	Prepurification of sample, low selectivity (TA–LT), low reproductibility	Interference (leaf), low sensitivity (12 ng[a])

[a] Minimum amount detected, injection of 20 μl and detection at 280 nm.

tion) followed by the removal of acetonitrile by extraction with methylene chloride[29]. With this treatment, TA was recovered in 90% yield from culture filtrates (±4%, with 0.03–0.3 m*M* TA, $n = 4$). As shown in Fig. 4, TA was the major UV-absorbing metabolite of *Pyricularia oryzae* culture filtrate. The extraction and the purification by sodium hydrogencarbonate partition of the culture filtrate removed most of the UV-absorbing metabolites, but not all. We detected trace amounts of a metabolite that could correspond to the valine analogue of TA (arrow 1 in Fig. 4). The production of TA varied from 10 μ*M* to 1 m*M*, depending on the strain of *Pyricularia oryzae*, but most of the strains produced between 0.3 and 0.6 m*M* TA.

The analysis of the infected plants required purification of the ethanolic leaf extract. After evaporation of the ethanol, the residue was partitioned between acidified water and ethyl acetate and the organic phase was extracted with sodium hydrogencarbonate. Extracts from non-infected plants were analysed with the three chromatographic systems. We found compounds that were eluted almost at the same retention time as TA in anion-exchange chromatography (Fig. 5, B_1 and B_2). The presence of these compounds did not prevent the detection of TA in the infected leaves, but

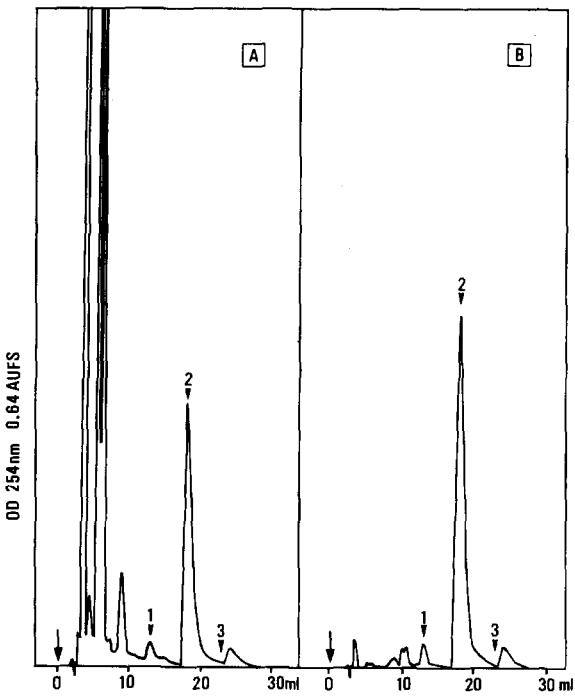

Fig. 4. Anion-exchange chromatography of *Pyricularia oryzae* culture filtrates. (A) Injection of the deproteinated culture filtrate containing 1 m*M* TA. (B) Injection of the culture filtrate extract purified by partition with sodium hydrogencarbonate (× 1.5 compared with A). Retention times of VT (1), TA (2) and LT (3) are indicated by arrows and were determined by co-injection of a standard solution with the sample. Culture filtrates were obtained from the *Pyricularia oryzae* strain MAD4 (Madagascar) after 30 days of growth on Fries medium. Mobile phase, phosphate buffer (10 m*M*) (pH 7), KCl (0.1 *M*) and 5% acetonitrile at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D.. Detection at 254 nm to detect most of the UV-absorbing metabolites, 0.64 a.u.f.s., 20 μl injected.

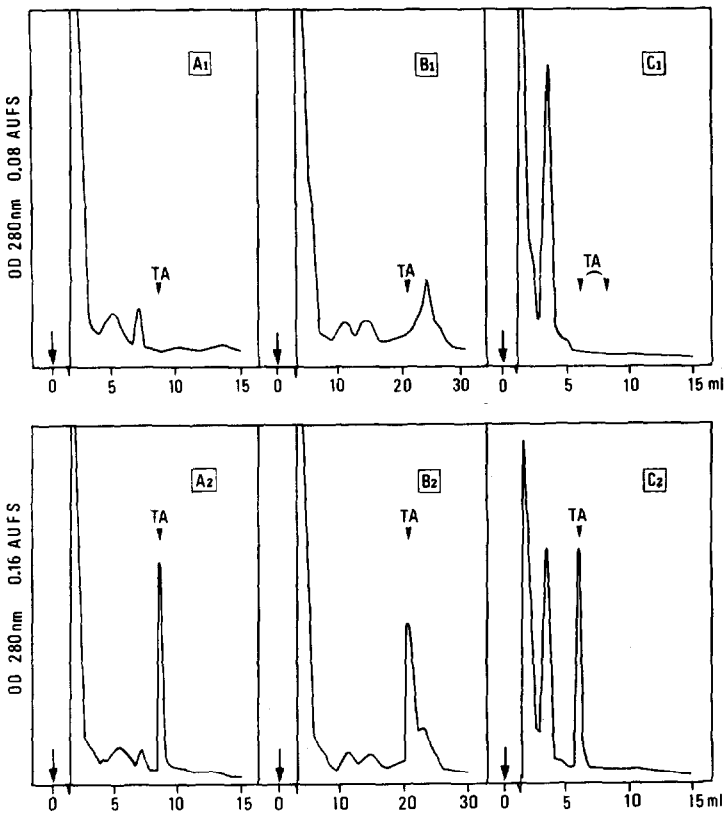

Fig. 5. Anion-exchange, ion-pair and ligand-exchange HPLC of rice leaf extracts. The TA retention time, determined by co-injection of TA and the sample, is indicated by an arrow. Samples injected (20 μl): (1) extract of non-infected rice leaves, dilution 0.25; (2) extract of leaves infected by *Pyricularia oryzae* containing 0.16 m*M* TA (7 days after inoculation), dilution 0.25. (A) Ion-pair chromatography. Mobile phase, cetrimide (5 m*M*) and phosphate buffer (10 m*M*) (pH 6), ethylenediamine (1 m*M*) with 55% methanol at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D.; detection 280 nm. (B) Anion-exchange chromatography. Mobile phase, phosphate buffer (10 m*M*) (pH 6), ethylenediamine (1 m*M*) with 5% acetonitrile at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D.; detection at 280 nm. (C) Ligand-exchange chromatography. Mobile phase, C_{12}-dien (5 m*M*) and zinc sulphate (5 m*M*) with ammonium acetate buffer (30 m*M*) (pH 6) with 80% methanol at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D.; detection at 280 nm.

interfered with its quantification. Further, we were not able to detect small amounts of TA (10 μM; 40 ng). With ion-pair and ligand-exchange chromatography, TA was eluted after the plant components (Fig. 5, A_1, A_2, C_1 and C_2). These two last chromatographic systems were used for the determination of TA in infected leaves. The TA concentration that we detected in these leaves was around 0.1 m*M* 7 days after the beginning of the infection. We did not detect any TA analogues in the infected leaf extracts.

DISCUSSION

The three isocratic liquid chromatographic systems studied were applied to the determination of TA in fungal culture filtrates and infected leaves (Table VI). Anion-exchange chromatography was the best suited for the rapid monitoring of TA production by *Pyricularia oryzae* strains. The main advantages of this system are its rapidity and its selectivity, as it allowed the use of culture filtrates without extraction and a good separation of 5-substituted TA analogues. However, we were not able to detect trace amounts of TA ($< 3\ \mu M$) because of its lower efficiency ($N = 1000$). In contrast, ion-pair or ligand-exchange chromatography could be used to detect TA in this range (1–10 μM). Ion-pair and ligand-exchange chromatography were best suited for the determination of TA in infected rice leaves. We were not able to use anion-exchange chromatography as TA was not separated from compounds present in the non-infected leaves. TA was more retained with ligand-exchange than ion-pair chromatography as it was necessary to use a higher proportion of methanol to obtain the same retention time (80% compared with 55% for $k' = 5$). This is related to the strong metal-complexation capacity of TA. Therefore, TA was more clearly eluted after plant components than with ion-pair chromatography. Hence ligand-exchange chromatography was used to determine TA in leaf extracts, despite the low reproducibility of the retention time of TA and the low selectivity of the separation of the 5-substituted analogues of TA. Because of this last characteristic, some samples of infected leaf extracts were also analysed by ion-pair chromatography to detect whether or not some TA analogues were produced. No TA analogues were detected in any of the leaf samples analysed. This result allowed us to use ligand-exchange chromatography for the determination of TA in leaf extracts.

CONCLUSIONS

We have studied the behaviour of tenuazonic acid in three different HPLC systems. Ion-pair chromatography was performed with the best results with the ion-pairing reagent cetrimide and a phosphate buffer with water–methanol mixtures. With this chromatographic system, we were able to separate TA analogues substituted at position 5 with apolar substituents of different size. Anion-exchange chromatography with silica-based packings allowed the separation of all the 5-substituted TA analogues and also some of its diastereoisomers (D-*allo*-TA and L-*allo*-TA). The selectivity factors of these separations were higher than with ion-pair chromatography. With time, the efficiency of ion-pair and anion-exchange chromatography might decrease. Washing with EDTA solution restored the initial performances of the columns. Addition of a metal-complexing reagent (ethylenediamine) to the mobile phase prevented this column deterioration. These chromatographic systems were used to detect and determine TA in *Pyricularia oryzae* culture filtrates and infected rice leaves. We chose anion-exchange chromatography for the analysis of the culture filtrates for its rapidity, simplicity and selectivity. Extracts of infected leaves could only be analysed by ion-pair and ligand-exchange chromatography as interfering compounds were coeluted with TA during anion-exchange chromatography.

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