**Isolation and Characterization of Host-selective Toxin from Helminthosporium sacchari**

(Received for publication, June 26, 1980, and in revised form, October 31, 1980)

Robert S. Livingston and Robert F. Scheffer

From the Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824

Helminthosporium sacchari infects certain clones of sugar cane and produces a toxin with the same plant selectivity as the fungus itself. The toxin was purified by use of activated charcoal plus thin layer, gel, and ion exchange chromatography. Gas chromatography (GC) of a trimethylsilyl derivative of toxin gave a single peak. Toxin was characterized by GC, mass spectrometry (MS), and NMR spectroscopy. The spectra of hydrolytic products showed that toxin contains galactose plus a C\(_{15}\)H\(_{13}\) moiety which appears to be a sesquiterpene. Spectral data and methylation procedures showed that toxin contains an oligosaccharide composed of \(\beta, 1 \rightarrow 5\) galactofuranose units (probably 5 units). Several interconvertible forms of the C\(_{15}\)H\(_{13}\) moiety were evident after acid hydrolysis. Toxin was separated from 3 closely related, nontoxic compounds ("noxins"), which contained galactose plus the C\(_{15}\)H\(_{13}\) moiety. Comparative data show that the toxin examined in this study is the same as the toxin described by Steiner and Strobel (Steiner, G. W., and Strobel, G. A. (1971) J. Biol. Chem. 246, 4350-4357). The data also show that the previously proposed structure is incorrect.

At least 15 plant-infecting fungi are now known to produce substances with selective toxicity against susceptible hosts. Such toxins are not active against non-host species, and against host genotypes that are resistant to the fungus. Several of these "host-selective toxins" have been isolated and partially characterized (6). However, only the toxin from *Alternaria mali* affecting certain apple cultivars has been characterized completely (4), the structure confirmed (8), and the molecule synthesized (2). A. mali toxin is a cyclic depsipeptide with a C-15 moiety.

*Helminthosporium sacchari* (Van Breda de Haan) Butler selectively parasitizes some cultivars (clones) of sugar cane, causing a disease known as "eyespot." Several years ago, the fungus was shown to produce a toxin with selective effects which matched those of the fungus. Steiner and Strobel (7) isolated the toxic compound and characterized it as 2-hydroxycyclopropyl-a-galactopyranoside (trivial name, helminthosporoside). The proposed structure has not been confirmed. Nevertheless, this and other work on *H. sacchari* toxin is often cited in discussions of the molecular basis of disease development and disease resistance in plants (1).

We have re-examined the toxin from *H. sacchari*. Characterization is not yet complete, but we feel that the data should be published because of the importance of the work (7) and the controversies involved (10). An abstract describing some of our work was published (3).

**MATERIALS AND METHODS**

**RESULTS**

Water-soluble Hydrolytic Products of Toxin—The aqueous phase of acid-hydrolyzed toxin was chromatographed on thin layer plates, using several different solvent systems, with diphenylamine:aniline:phosphoric acid as the indicator reagent. The R\(_F\) values and color reactions of the resulting spots matched those of galactose standards. The water-soluble fraction was then derivatized with Tri-Sil-Z and subjected to GC-MS\(^2\), using columns containing several different liquid phases. Retention times for peaks from gas chromatography matched those of derivatized galactose and galactose (\(\alpha, \beta, \gamma\) forms); mass spectra confirmed the presence of galactose but there was no indication of other sugars.

Chloroform-soluble Hydrolytic Products of Toxin—The chloroform phase of hydrolyzed toxin was subjected to gas chromatography, using a column (1.8 m) packed with OV-1 (3\%) and a temperature of 170°C. Four major peaks and several minor peaks were observed (Fig. 1). Each major peak was later characterized by MS as a 15-carbon compound; for convenience, they are identified as C-15a, C-15b, C-15c, and C-15d (Fig. 1). Enriched preparations of the major C-15 products were made by TLC followed by chromatography with an LH-20 column (see "Materials and Methods").

Possible interconversion of the C-15 products was considered. Aliquots of each of the 4 major C-15 products in aqueous trifluoroacetic acid (0.1, 0.05, and 0.01 M) were held at 95°C for 2.5 h. The solutions were then extracted with 3 equal volumes of chloroform and the combined extracts were subjected to GC. Results showed that each of the major C-15 products gave at least trace quantities of the others. For example, when C-15c was exposed to acid at 0.05 M, GC

---

1 Portions of this paper (including "Materials and Methods," some of the "Results," Fig. 5, and Tables II and III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M-1305, cite author(s), and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: GC, gas chromatography; MS, mass spectrometry; MeSO\(_2\), dimethyl sulfoxide; MeSi, trimethylsilyl; TLC, thin-layer chromatography, EI/D, electron ionization subsequent to thermal desorption from field emitters.
Isolation and Characterization of Toxin from H. sacchari

The four major products had retention times of 2.53, 2.85, 5.95, and 6.47 min. Several other products were present in smaller amounts. All products had an apparent molecular ion in the mass spectrum at m/e 218; high resolution peak matching indicated an empirical formula of C_{15}H_{21}OH.

The spectrum (Fig. 2) showed the presence of C-15d, plus two products with retention times very similar to those of C-15a and -b, plus two other products (retention times, 4.22 and 4.58 min). These data indicate that the C-15 products are unstable and are interconvertible. Available data do not establish which form of the C-15 moiety is in the toxin molecule; indeed, toxin might exist as isomers based on different forms of the C-15 moiety.

Mass Spectroscopy—The following conditions were used for intact toxin, using the Varian CH-5 spectrometer with the direct probe electron impact method: source temperature was 200°C, probe was heated from ambient to 280°C, ionization voltage was 35 eV, and ions were monitored from m/e 35 to 500. The spectrum (Fig. 2) showed the highest visible molecular ion at m/e 380. The Varian CH-5 mass spectrometer was used for high resolution peak matching; ionization was by electron impact with accelerating voltage at 70 eV. Peak matching with m/e 380 indicated that the most probable empirical formula was C_{15}H_{21}O_{10}. The low resolution spectrum had a peak at m/e 201, (C_{15}H_{21}) which appears to be C_{15}H_{21}O_{9} minus galactose. High resolution peak matching on the m/e 201 confirmed this empirical formula. There was a third peak at m/e 217; peak matching indicated that this was C_{15}H_{21}O, a carbon-hydrogen compound plus oxygen from the galactoside linkage. A peak at m/e 259 was predicted; this should be C_{15}H_{21}O_{2} (C_{15}H_{21} + C_{2}H_{2}O_{2} = C_{17}H_{23}O_{2}), or a carbon-hydrogen unit plus a portion of galactose, a known break for the galactoside linkage). The low resolution spectrum had the expected peak at m/e 259, and peak matching confirmed the predicted formula.

Intact toxin was dissolved in D_{2}O (99.7%) to exchange deuterium for the hydroxyl protons. The mass spectrum of deuterium-labeled toxin should show an increase of one atomic mass unit/exchangeable proton. Related m/e values were shifted comparably. Again, these data are consistent with a structure containing galactose, plus a unit containing 15 carbons with hydrogen (C_{17}H_{32}O_{6} + C_{17}H_{31}O_{6} = C_{34}H_{63}O_{12}), with the 15-carbon unit attached to the galactose at a single position. This would leave four free hydroxyl groups which would exchange protons for deuterium. The mild conditions required for hydrolysis (with release of galactose and a 15-carbon unit from intact toxin), plus the MS data, suggest a galactosidic linkage.

Mass spectra for methylated toxin were obtained with the Varian CH-5 spectrometer, using the EI/D method. Acceleration voltage was 1.0 kV, which gives increased sensitivity and lower accuracy (±1.0 m/e) with masses >1000. Filament current was 18 mA, and ions were monitored up to m/e 1150, which is maximum for the spectrometer. The spectrum showed ions at m/e 1060 and 1093, indicating that toxin contains at least 5 galactose units plus a C_{15}H_{21} moiety.

The four chloroform-soluble, 15-carbon hydrolytic products of toxin (C-15, a-d) were characterized by GC-low resolution MS and by high resolution peak matching. Peak matching of the m/e 201 fragment indicated that the empirical formula was the same as that determined for the ion at m/e 201 (C_{15}H_{21}) in the spectrum of intact toxin. Spectra were collected in both electron impact and chemical ionization (methane) modes; these data indicated that 218 was the probable molecular mass for all 4 products. Peak matching of the m/e 218 produced the empirical formula C_{15}H_{21}O_{9}. The C-15 products may be sesquiterpene-type compounds, with a single hydroxyl formed during hydrolysis, and with 5 points of unsaturation (double bonds or ring structures). All four C-15 hydrolytic products produced a similar fragmentation pattern (see Fig. 5 in miniprint), with variation in the relative abundance of the several fragments. This indicates that the C-15 compounds are very similar in structure, possibly differing only in the position of double bonds. The conclusion is supported by proton NMR data on the C-15 breakdown products (given below).

Most ions in the mass spectrum of toxin were also present in the C-15 moiety. Only a few ions were from galactose, which is not surprising because sugar moieties are known to give weak mass spectra. Peaks for intact toxin at m/e 43, 73, and 91 can be attributed to both the galactose and the C-15 fragment (overlapping ions were confirmed by high resolution mass spectroscopy). The ions at m/e 60 and 61 are typical of galactose and were not found in the spectra of the C-15 moiety. These sets of ions were emitted from the probe at the same high temperature, indicating that the toxin preparation is a single, large, relatively nonvolatile compound.

Proton NMR Studies—The NMR spectrum of toxin in D_{2}O contains 14 major peaks or distinct regions (Fig. 3, Table 1).
Isolation and Characterization of Toxin from *H. sacchari*

**Fig. 3. Proton NMR spectrum of toxin at 180 MHz.** Host-selective toxin (10 mg) from *Helminthosporium sacchari* was dried under reduced pressure and rinsed several times with D$_2$O to remove H$_2$O. The sample was dissolved in 0.5 ml of D$_2$O (99.7% deuterium) in a 5-mm NMR tube. The Fourier transformed spectrum was obtained from 25 transients on a Bruker WH-180 instrument. The chemical shifts in ppm for the major peaks are given in Table I.

**TABLE I**

<table>
<thead>
<tr>
<th>Peak <em>a</em></th>
<th>Shift position <em>b</em></th>
<th>Number of protons <em>c</em></th>
<th>Peak</th>
<th>Shift position <em>d</em></th>
<th>Number of protons</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.41 ppm</td>
<td>1</td>
<td>H</td>
<td>3.74 ppm</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>5.77 ppm</td>
<td>1</td>
<td>I</td>
<td>3.66 ppm</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>5.17 ppm</td>
<td>3</td>
<td>J</td>
<td>2.57 ppm</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>5.08 ppm</td>
<td>1</td>
<td>K</td>
<td>2.0-1.7 ppm</td>
<td>8</td>
</tr>
<tr>
<td>E</td>
<td>4.96 ppm</td>
<td>1</td>
<td>L</td>
<td>1.64 ppm</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>4.10 ppm</td>
<td>1</td>
<td>M</td>
<td>1.5-1.2 ppm</td>
<td>7</td>
</tr>
<tr>
<td>G</td>
<td>4.03 ppm</td>
<td>3</td>
<td>N</td>
<td>0.85 ppm</td>
<td>3</td>
</tr>
</tbody>
</table>

*a* See Fig. 5.  
*b* Calculated from the HDO peak at 4.74.  
*c* Determined from the area under each peak.  
*d* Peaks F, G, H, and I represent a total of 38 protons.

The spread of the seven peaks in the 4.91 to 5.41 ppm region is too great to result from splitting of a single group of protons. Therefore, these peaks represent five groups of protons. One group at 5.17 ppm contains three identical protons; the other peaks in this region represent one proton each. These peaks probably are from olefinic protons. Strong absorbance at 3.6 to 4.2 ppm represents approximately 35 to 40 protons on carbons with a hydroxyl group; most of this absorbance is from protons in galactose. This number of protons suggests as many as 5 or 6 galactose units/molecule of toxin. β anomic protons also absorb in the low field end of the 3.6 to 4.2 ppm region. The peaks at 4.03 ppm may be from galactose protons, but protons on the C-15 moiety that are involved in the galactose linkage could also absorb in this region. A single C-15 unit/molecule of toxin probably has no more than two protons absorbing in the 4.03 region, this is not enough to account for the large peak at 4.03 ppm. Another possibility could be more than one C-15 moiety/toxin molecule.

Proton NMR studies of the isolated C-15 moieties showed that they varied in the number of olefinic protons (2 to 4). There was a sharp singlet upfield, which represents an isolated methyl group. The sharp singlet at 4 ppm was assigned to the two protons on the carbon with the hydroxyl, which probably is adjacent to a carbon with a double bond.

The NMR spectrum of the Me$_3$Si derivative of toxin, in deuterated chloroform, was dominated by absorption at 0.25 to 0.05 ppm. The area of this absorption was proportional to the number of protons on those Me$_3$Si groups that had replaced each hydroxyl group on the original toxin. The two peaks at 1.64 and 0.85 ppm, each representing 3 protons, were used to determine the units of area/proton. To determine the number of Me$_3$Si groups/toxin molecule, the number of protons in the 0.25 to 0.05 region (125 to 150 protons) was divided by the number of protons/Me$_3$Si group (9 protons). There were 14 to 17 Me$_3$Si groups/molecule of toxin, indicating 5 galactose units.

$^{13}$C NMR Studies—The dominant characteristic of the $^{13}$C NMR spectrum of intact toxin was the strong intensity of peaks from 63.9 to 85.5 ppm (Fig. 4). These peaks are from carbons in galactose; they are much more intense than the peaks given by the C-15 moiety. The difference in intensity of the galactose and the C-15 peaks is great enough to suggest that there are several galactose units per C-15 moiety in the toxin molecule. The six to eight peaks from 116.7 to 151.2 ppm indicate six to eight olefinic carbons. These data indicate the presence of three double bonds and two ring structures per C-15 fragment, suggesting a sesquiterpene. This is consistent with the empirical formula predicted by mass spectral peak matching data. The region of the spectrum in which aliphatic carbons absorb (18 to 48.6 ppm) contains 9 to 13 peaks. The carbon atom of the C-15 moiety which shares an oxygen with the oligosaccharide will absorb in the same region as does galactose (63.9 to 85.5 ppm).

The peak at 63.9 ppm is very close to the assigned shift position for the C-6 of a galactofuranoside (63.6 ppm). The shift position of a C-1 is characteristic for α and β anomeric forms of pyranosides and furanosides. The lack of peaks at 101-104 ppm rules out α- and β-galactopyranosides and α-galactofuranoside (9). The peak at 109.8 ppm indicates a β-linked galactofuranoside (5).

**Mass Spectroscopy of Helminthosporoside**—MS data on helminthosporoside from G. Strobel (Montana State University) were taken for comparison with our preparation of toxin.

![Fig. 4. $^{13}$C NMR spectrum of toxin at 45.2 MHz.](image-url)
from *H. sacchari*. First, the Hewlett-Packard 5985-A spectrometer was used with a series of ion source temperatures and ionization voltages in the electron ionization mode. With the ion source temperature at 250°C and the ionization voltage at 70 eV, the mass spectra for the two preparations were essentially the same, except for minor peaks; these spectra were similar to the published spectrum for helminthosporoside (7). Next, the fragmentation conditions were altered to favor survival of higher mass ions: this was accomplished by using a lower ion source temperature (200°C) and a lower ionization voltage (35 eV). Under these conditions, the two preparations gave similar spectra, including the m/e 380 ion. However, Strobel’s preparation had an ion at m/e 236 which was missing from all spectra of our preparations. This difference is important because m/e 236 was considered to be the molecular ion of the toxin (7).

Further comparisons of Strobel’s preparation with ours were by high resolution peak matching, using the Varian CH-5 mass spectrometer. Ions resulting from fragments of the aglycone moiety of toxin were selected for examination, because that unit (the C-15 compound) was not included in the previously proposed structure (7). Thus, the empirical formulae were determined for the ions at m/e 145, 157, 201, 217, and 380, which were evident in the spectra of both preparations. The predicted empirical formulae for these ions were identical for both preparations. The data indicate that our toxic molecule is the same as the one reported elsewhere, and that the proposed structure (7) must be revised.

**DISCUSSION**

Our highly purified toxin contained no detectable contaminants, as shown by thin layer chromatography and by gas chromatography of derivatives. Toxin purified by gas chromatography was hydrolyzed and the hydrolytic products were characterized by mass spectrometry. These compounds appear to be convertible from one to the other. NMR data indicated that the compounds differ from each other in the positions of their double bonds and rings. The compounds may be sesquiterpenes, as indicated by the 15-carbon skeletons with double bonds. Furthermore, the molecules of some sesquiterpenes are known to be rearranged in unbranched chain. 13C NMR spectra indicate that the oligosaccharide contains β, 1 → 5 linked galactose units in the furanose form (5, 9).

An attempt was made to hydrolyze toxin with α and β-galactosidases (Sigma Chemical Co.). There was no liberation of galactose or C-15 compounds by these enzymes, used singly or in combinations (data not given).

Four different 15-carbon compounds were isolated after hydrolysis of the toxin by dilute acid. All the 15-carbon compounds had a molecular weight of 218, as determined by mass spectrometry. These compounds appear to be convertible from one to the other. NMR data indicated that the compounds differ from each other in the positions of their double bonds and rings. The compounds may be sesquiterpenes, as indicated by the 15-carbon skeletons with double bonds. Furthermore, the molecules of some sesquiterpenes are known to be rearranged in unbranched chain. The MS and hydrolysis data indicated that the 15-carbon unit is attached to the galactose chain by a galactosidic linkage. Acid hydrolysis of this linkage should give a 15-carbon unit bearing a hydroxyl group on the carbon that was involved in the galactosidic linkage. Further characterization of the 15-carbon moiety is underway.

In summary, the data discussed above show that toxin contains galactofuranose units linked by β, 1 → 5 bonds, plus a C₆H₁₂ moiety attached to the reducing end of the oligosaccharide. Several lines of evidence indicate five galactose units. The empirical formula of the aglycone unit indicates 5 points of unsaturation; spectral data indicate 3 double bonds and 2 rings (a sesquiterpene). Molecular weight of the toxin was calculated, tentatively, to be 1028.

**Acknowledgments—** We are grateful to Professors Charles Sweeney and W. H. Reusch for suggestions and help in interpretation of data, and to Dr. H. Nunez for interpretation of 13C NMR data. We also thank Professors N. E. Good, C. J. Pollard, and K. Kohmoto for helpful comments and discussion.

**Note Added in Proof—** We recently became aware of work on *H. sacchari* toxin by R. C. Beier (1980) Ph.D. thesis, Department of Chemistry, Montana State University. Beier suggests that the toxin may contain 2 galactose units and an aglycone (C₁₅H₂₀O₅).

**REFERENCES**


**Abstr. 449**
Supplementary Material

Isolation and Characterization of Non-Selective Toxin from Mucor miehei

Material and Methods

Materials

Cheesecloth and Whatman No. 1 paper.

Methods

The plates in front of a fluorescence lamp were removed from the solvent tank. Differential evaporation of the plates resulted in a sharpening of the chromatographed bands. The area was outlined quickly because it was too large for this technique. To ensure the accuracy of the procedure a check on the TLC plate was removed and sprayed with indicator. The stained bands were then sprayed with a heat-resistant silver nitrate spray. All experiments were done with a high-pressure liquid chromatograph (HPLC) (model 5010, Varian). The HPLC was equipped with an ultraviolet (UV) detector, a column oven, and a solvent delivery system. The column was a stainless steel column (3.9 mm × 30 cm) packed with 5% octadecylsilyl silica gel. The solvent was a mixture of methanol-water (95:5). The flow rate was 1.0 ml/min. The column was maintained at 30°C. The detection limit was 0.01 mg of active principle. The photos were taken with a digital camera. The pictures were processed with a computer program.

Additional references are found on p. 1710.
Isolation and Characterization of Toxin from H. sacchari

Methylation analysis of toxins. Toxin was methylated by Hofmeister's method (1), as described by Easmon et al. (2). Methyl iodide (90%) (0.3 ml) was added to 100 mg of toxin (about 0.75 mg of toxin/ml in methanol) and the mixture was added to 500 ml of 0.05 M HCl. A solution of methyl iodide was added to the mixture and the mixture was incubated at room temperature for 3 hours. The reaction was stopped by boiling, and the mixture was cooled and filtered. The filtrate was then evaporated to dryness, and the residue was dissolved in methanol. The product was then standardized and used for further studies.

Gas chromatography-mass spectrometry was used to identify partially methylated alditol acetates and to determine the identity of the products. The products were then identified by comparing their retention times and gas chromatographic-mass spectrometric properties with those of authentic samples.

RESULTS

Table 1. Solvents used in thin-layer chromatography of toxins from Helminthosporium sacchari

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Proportions</th>
<th>Rf of Toluene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>9:1</td>
<td>0.55</td>
</tr>
<tr>
<td>Aqueous acetone</td>
<td>10:1</td>
<td>0.60</td>
</tr>
<tr>
<td>Isooctane</td>
<td>20:1</td>
<td>0.55</td>
</tr>
<tr>
<td>Methanol-water</td>
<td>1:1</td>
<td>0.63</td>
</tr>
<tr>
<td>Chloroform-water</td>
<td>2:1</td>
<td>0.60</td>
</tr>
</tbody>
</table>

*Chromatograms on Silica Gel 60, 0.25 mm high performance plates from C. Merck.*

Gas chromatography of the methyl-derivatives of toxins gave a single peak. The peaks from several samples were collected from the plates and submitted for further analysis.

A sample of the methylated toxin purified by gas chromatography was combined with an equal volume of butanol. The solution was treated with 5% NaOH and the butanol was removed by distillation. The residue was dissolved in 0.05 M HCl and the solution was then evaporated to dryness. The residue was then dissolved in methanol and the solution was then standardized and used for further studies.

Table 2. Chromatographic behavior of Helminthosporium sacchari toxins and related non-toxic substances

<table>
<thead>
<tr>
<th>Chromatography system</th>
<th>Elution volume of 10% Toluene</th>
<th>Elution volume of 20% Toluene</th>
<th>Elution volume of 30% Toluene</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPC column, 1 fraction</td>
<td>65.00</td>
<td>65.00</td>
<td>65.00</td>
</tr>
<tr>
<td>CPC column, 2 fractions</td>
<td>65.00</td>
<td>65.00</td>
<td>65.00</td>
</tr>
<tr>
<td>TLC, n-butanol:water (10:1)</td>
<td>65.00</td>
<td>65.00</td>
<td>65.00</td>
</tr>
<tr>
<td>TLC, n-butanol:water (10:1)</td>
<td>65.00</td>
<td>65.00</td>
<td>65.00</td>
</tr>
<tr>
<td>TLC, methanol:water (10:1)</td>
<td>65.00</td>
<td>65.00</td>
<td>65.00</td>
</tr>
<tr>
<td>TLC, methanol:water (10:1)</td>
<td>65.00</td>
<td>65.00</td>
<td>65.00</td>
</tr>
<tr>
<td>TLC, methanol:water (10:1)</td>
<td>65.00</td>
<td>65.00</td>
<td>65.00</td>
</tr>
<tr>
<td>TLC, methanol:water (10:1)</td>
<td>65.00</td>
<td>65.00</td>
<td>65.00</td>
</tr>
<tr>
<td>TLC, methanol:water (10:1)</td>
<td>65.00</td>
<td>65.00</td>
<td>65.00</td>
</tr>
<tr>
<td>TLC, methanol:water (10:1)</td>
<td>65.00</td>
<td>65.00</td>
<td>65.00</td>
</tr>
</tbody>
</table>

*Column chromatography was used to identify peaks in each elution fraction.*

The Rf values with this solvent were determined in triplicate for each of the samples used in this study.

TLC, n-butanol:water (10:1); TLC, methanol:water (10:1).

The Rf values with this solvent were determined in triplicate for each of the samples used in this study.

References