BIOCHEMISTRY
including biophysical chemistry & molecular biology
Biochemistry, 1996, 35(16), 5264-5271, DOI:10.1021/bi952520q

Supplementary Material for Microfilm Edition

Functional and Sequence Characterization of Coagulation Factor IX/Factor X-Binding Protein from the venom of *Echis carinatus leucogaster*

Yuh-Ling Chen and Inn-Ho Tsai

EXPERIMENTAL PROCEDURES

Materials. ECLV IX/X-bp was purified from the venom of *Echis carinatus leucogaster* by cation exchanger chromatography followed by RP-HPLC (Cosmosil C_8 column, 10×150 mm, Nakara Chemicals, Ltd., Kyoto). Trifluoroacetic acid (TFA), acetonitrile, dithiothreitol and 4-vinylpyridine were purchased from Merck, Germany. Sequencing grade Arg-C endoproteinase, trypsin, chymotrypsin, Glu-C endoproteinase and Asp-N endoproteinase were from Boehringer Mannheim (Germany).

Reduction and pyridylethylation. ECLV IX/X-bp (20 nmol) was denatured in 500 µl of 0.25 M Tris-HCl buffer (pH 8.5), containing 6M guanidine hydrochloride, 1 mM EDTA, and dithiothreitol (14 µmol) under N_2 gas at 50°C for 2.5 h. The protein was alkylated by adding 4.5 µl of 4-vinylpyridine (42 µmol) and incubated at room temperature for 2 h (Atoda *et al.*, 1991). S-pyridylethylated subunits were purified by RP-HPLC using a Cosmosil C8 column (1×15 cm). Elution was performed with a gradient of 0-30% acetonitrile (containing 0.07% TFA) over 10 min at a flow rate of 1 ml/min, followed by a gradient of 30-45% acetonitrile over 40 min. The effluent was monitored at 230 nm.

Amino acid analysis and sequencing. Amino acid analysis was performed by vapour-phase hydrolysis at 158°C for 30 min using 7M HCl/10% TFA/0.1% phenol (Chang & Liu,

1988), and the hydrolysates were derivatized to dimethyl-aminoazobenzenesulfonyl amino acids before separation by RP-HPLC (Kencht & Chang, 1986). An automated gas-phase sequencer (Model 477A, Applied Biosystems, USA) was used, and the phenylthiohydantoin derivatives of amino acids were determined by an on-line HPLC system (Hunkapiller *et al.*, 1983).

Proteolytic digestion and peptide separation. The alkylated α subunit (100 μg) was hydrolyzed with 2 μg of trypsin or Asp-N endoproteinase in 0.1 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 2 M urea at 37°C for 20 h. Digestion of the alkylated subunits (100 μg) with Glu-C endoproteinase (2 μg) was performed in 25 mM ammonium carbonate (pH 7.8) containing 1.0 M guanidine-HCl at 25°C for 20 h. Alkylated β subunit (100 μg) was hydrolyzed with 2 μg of Arg-C endopeptidase in 0.1 ml of 50 mM Tris-HCl containing 1 mM $CaCl_2$, 5 mM dithiothreitol (pH 7.5) at 37°C for 20 h. Alkylated β subunit (100 μg) was hydrolyzed with 2 μg of chymotrypsin in 0.1 ml of 50 mM Tris-HCl containing 2 M urea (pH 7.5) at 37°C for 20 h. Peptides after digestion were separated by RP-HPLC on a Cosmosil C8 column and eluted with a linear gradient of acetonitrile in 0.07% trifluoroacetic acid at a flow rate of 1.0 ml/min. The effluent was monitored at 205 nm. After HPLC the purified peptides were subjected to amino acid analyses and sequencing.

Determination of disulfide bridges. The intact protein (0.3 mg) was digested with trypsin or Glu-C endoproteinase in 25 mM ammonium carbonate buffer, pH 7.8, containing 1.0 M guanidine-HCl at 37°C or 25°C for 24h with a molar ratio of enzyme to substrate of 1 to 30. Peptides were separated by RP-HPLC chromatography as described above, and 1/5 aliquots were subjected to amino acid analyses and sequencing. The peptide E15 was further digested with 1 μg of α-chymotrypsin in 50 mM Tris-HCl containing 2 M urea (pH 7.5) at 37°C for 20 h and separated by RP-HPLC.

K-5271-M3

Table S1
Amino acid compositions of the α- and β-subunits of ECLV IX/X-bp.

Amino acid	α subunit	β subunit
	Residues / molecule	
Asp+Asn	8.0 (8)	9.5 (10)
Glu+Gln	20.9 (21)	13.8 (14)
Ser	12.7 (13)	14.4 (14)
Thr	4.5 (5)	7.2 (7)
Gly	9.2 (10)	8.3 (8)
Ala	2.7 (2)	10.2 (10)
Arg	4.6 (4)	3.6 (4)
Pro	3.3 (3)	3.1 (3)
Val	5.8 (6)	5.9 (7)
Met	0.2 (0)	1.2 (1)
Ile	3.5 (4)	1.8 (2)
Leu	7.4 (8)	5.2 (5)
Phe	5.4 (6)	6.6 (7)
Lys	17.3 (18)	9.9 (11)
His	3.8 (4)	3.1 (3)
Tyr	4.5 (5)	3.6 (4)
Cys[a]	6.5 (7)	6.7 (7)
Trp	n.d.[b] (7)	n.d.[b] (10)
Total	133	125

[a] S-pyridylethylcysteine
[b] Not determined

FIGURE LEGENDS

FIGURE S1: Purification of ECLV IX/X-bp by RP-HPLC. The protein obtained from ion-exchange chromatography was applied to a Cosmosil C8 column (1 × 15 cm) and eluted with a linear gradient of acetonitrile from 20 to 37% containing 0.07% TFA at a flow rate of 1 ml per min.

FIGURE S2: Separation of the α and β subunits. S-pyridylethylated ECLV IX/X-bp was applied to a Cosmosil C8 column (1 × 15 cm) and eluted with a linear gradient of acetonitrile from 30 to 45% containing 0.07% TFA at a flow rate of 1 ml per min.

FIGURE S3: Separation of the tryptic peptides of the alkylated α subunit by RP-HPLC. The tryptic digest was applied to a Cosmosil C8 column (1 × 15 cm) and eluted with a linear gradient of acetonitrile from 10 to 50% containing 0.07% TFA at a flow rate of 1 ml per min.

FIGURE S4: Separation of the Glu-C endoproteinase-digested peptides of the alkylated α subunit by RP-HPLC. The digest was applied to a Cosmosil C8 column (1 × 15 cm) and eluted with a linear gradient of acetonitrile from 10 to 50% containing 0.07% TFA at a flow rate of 1 ml per min.

FIGURE S5: Separation of the Asp-N endoproteinase-digested peptides of the alkylated α subunit by RP-HPLC. The digest was applied to a Cosmosil C8 column (1 × 15 cm) and eluted with a linear gradient of acetonitrile from 10 to 55% containing 0.07% TFA at a flow rate of 1 ml per min.

FIGURE S6: Separation of the Arg-C endoproteinase-digested peptides of the alkylated β subunit by RP-HPLC. The digest was applied to a Cosmosil C8 column (0.46 × 5 cm) and eluted with a linear gradient of acetonitrile from 20 to 45% containing 0.07% TFA at a flow rate of 1 ml per min.

FIGURE S7: Separation of the chymotrypsin-digested peptides of the alkylated β subunit by RP-HPLC. The digest was applied to a Cosmosil C8 column (1 × 15 cm) and eluted with a

linear gradient of acetonitrile from 5 to 50% containing 0.07% TFA at a flow rate of 1 ml per min.

FIGURE S8: Separation of the Glu-C endoproteinase-digested peptides of the alkylated β subunit by RP-HPLC. The digest was applied to a Cosmosil C8 column (1 × 15 cm) and eluted with a linear gradient of acetonitrile from 10 to 50% containing 0.07% TFA at a flow rate of 1 ml per min.

FIGURE S9: Separation of the tryptic peptides of intact ECLV IX/X-bp by RP-HPLC. The digest by trypsin was applied to a Cosmosil C8 column (0.46 × 5 cm) and eluted with a linear gradient of acetonitrile from 10 to 40% containing 0.07% TFA at a flow rate of 1 ml per min.

FIGURE S10: Separation of the Glu-C endoproteinase-digested peptides of intact ECLV IX/X-bp by RP-HPLC. The digest was applied to a Cosmosil C8 column (1 × 15 cm) and eluted with a gradient of acetonitrile from 5 to 50% containing 0.07% TFA at a flow rate of 1 ml per min.

FIGURE S11: Separation of the chymotrypsin-digested fragments of the peptide E15 (see Figure S10) by RP-HPLC. The digest was applied to a Cosmosil C8 column (0.46 × 5 cm) and eluted with a linear gradient of acetonitrile from 10 to 30% containing 0.07% TFA at a flow rate of 1 ml per min.

Fig S1
K-5271-M6

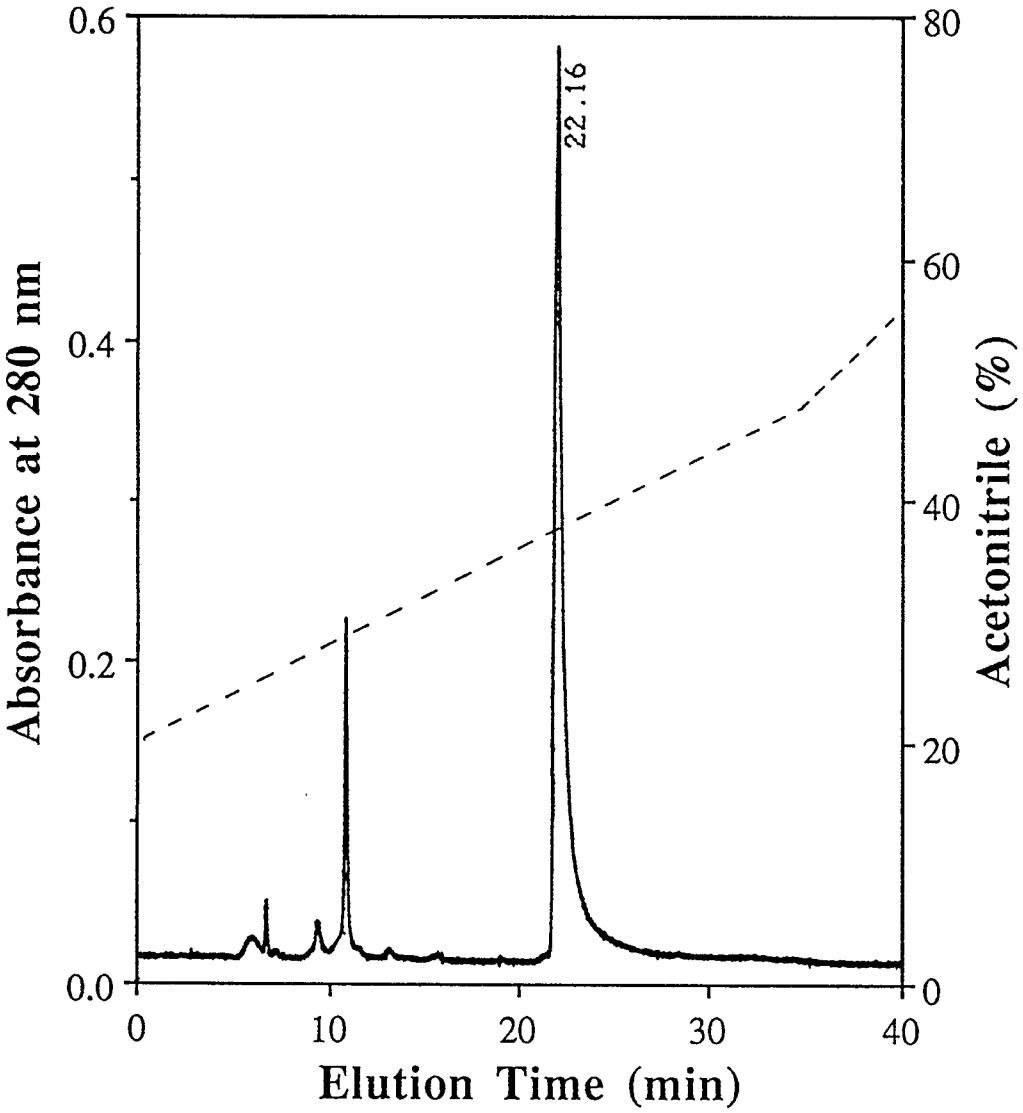
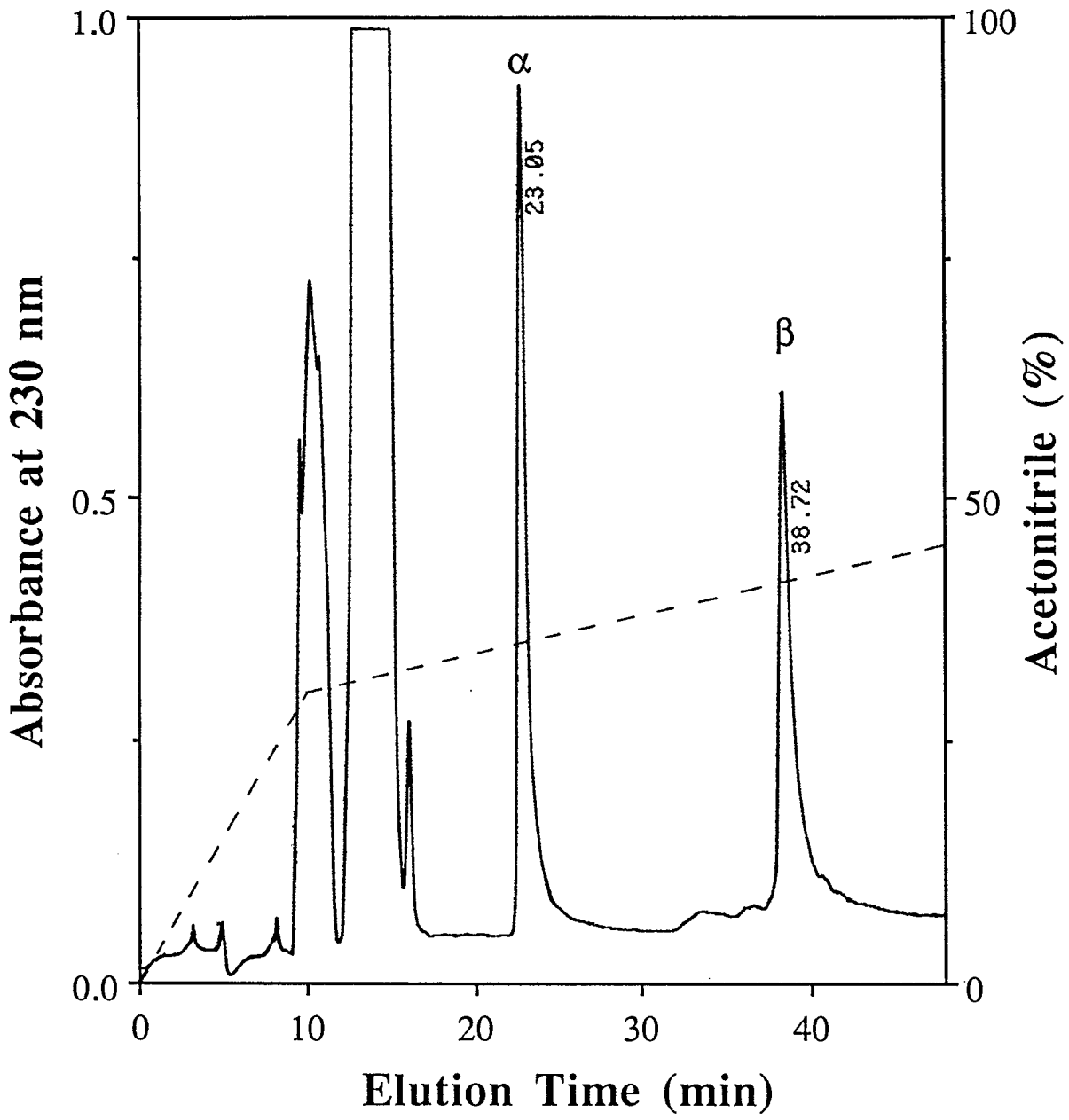
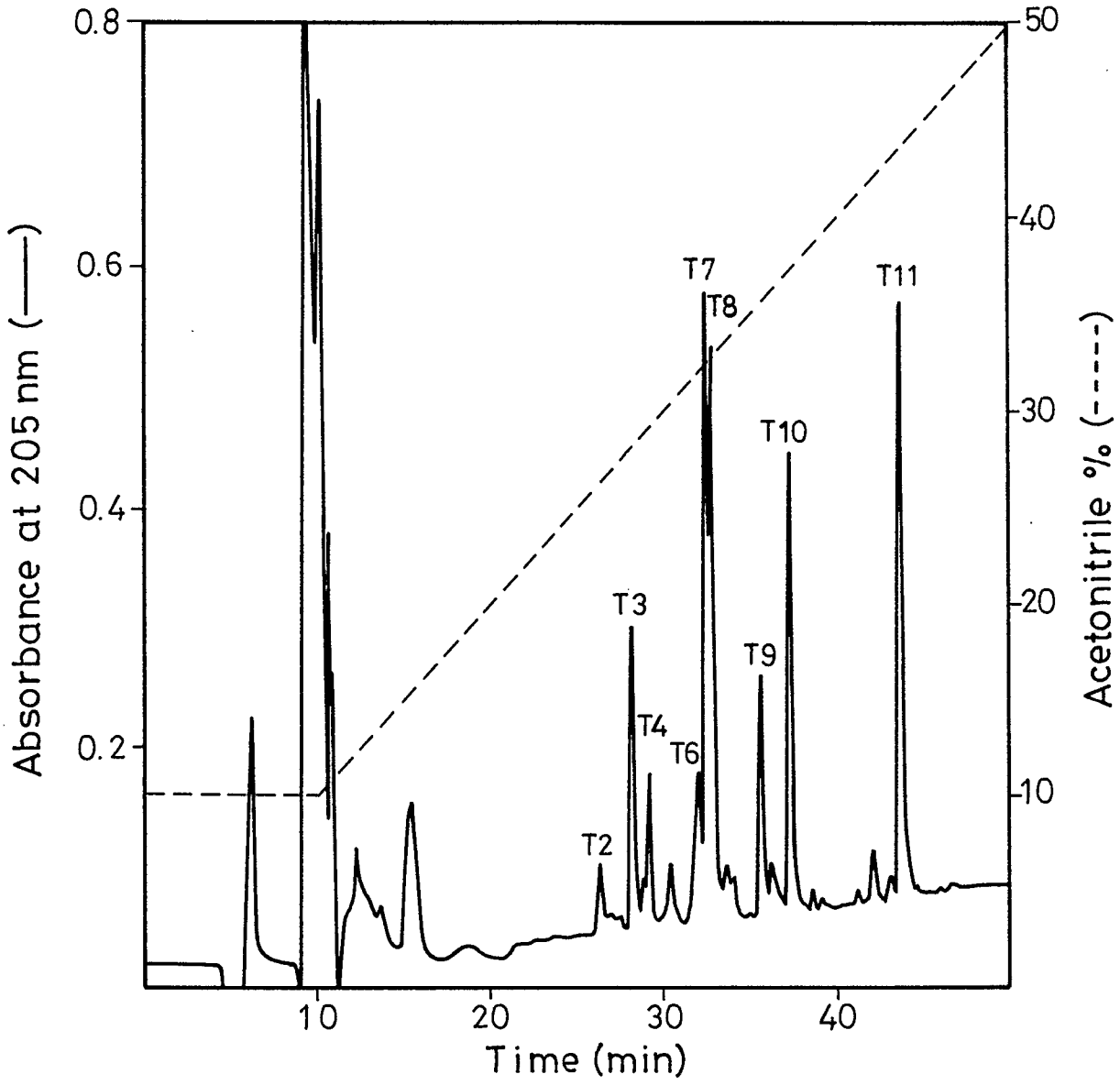


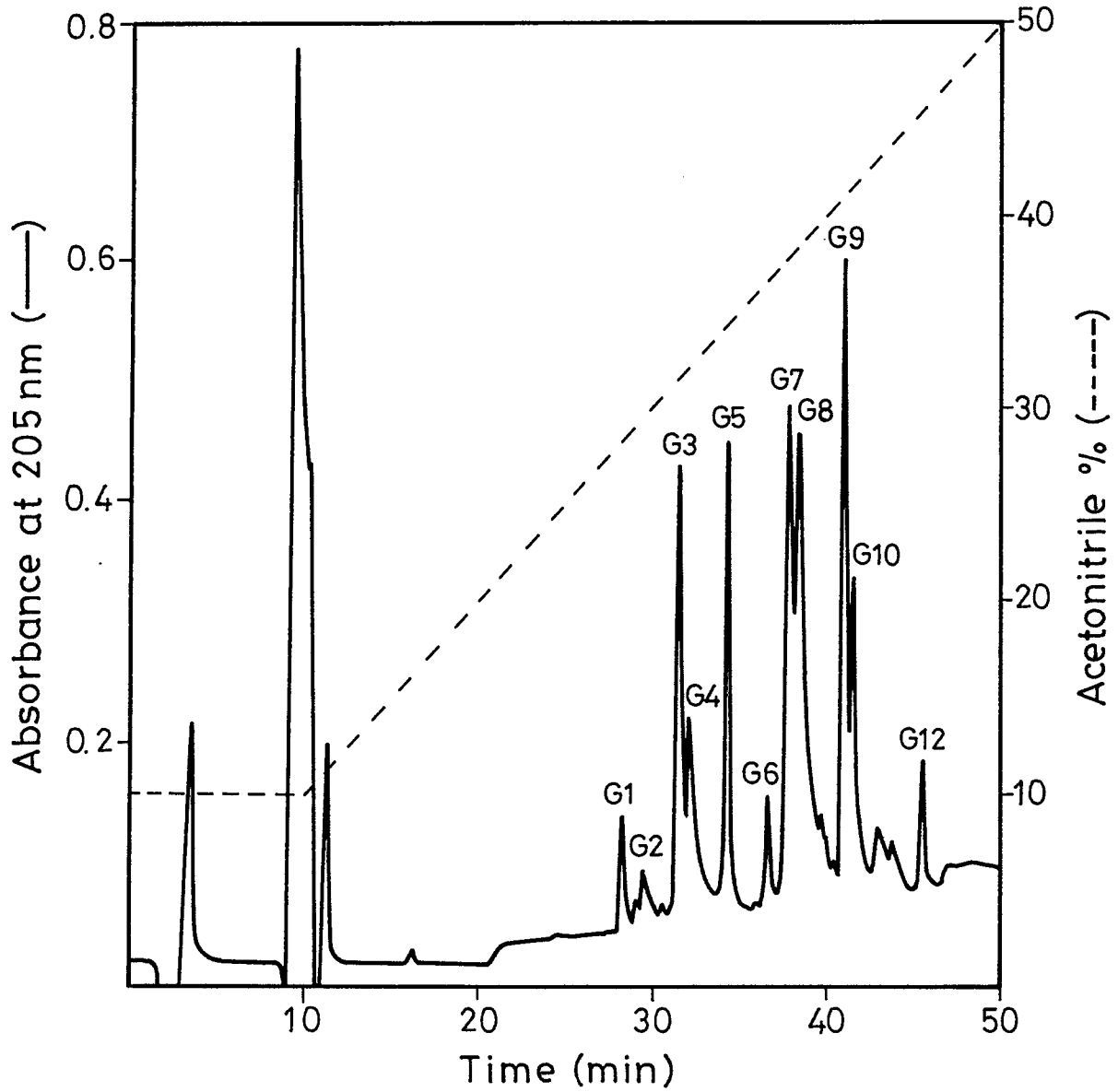
Fig. S2
K-5271-7M7



K-5271-M8
Fig. S3

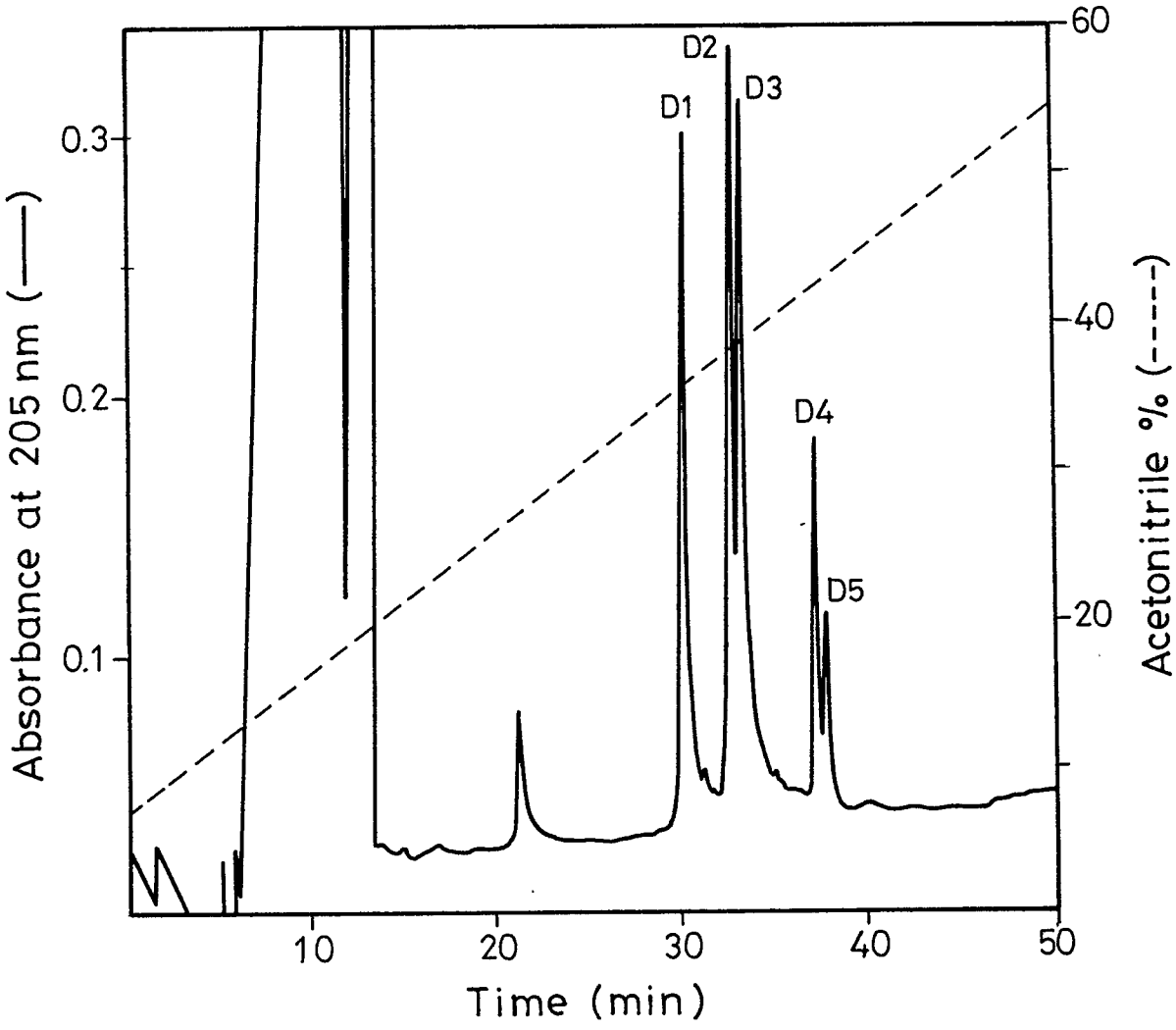


K-5271-M9
Fig. S4 1



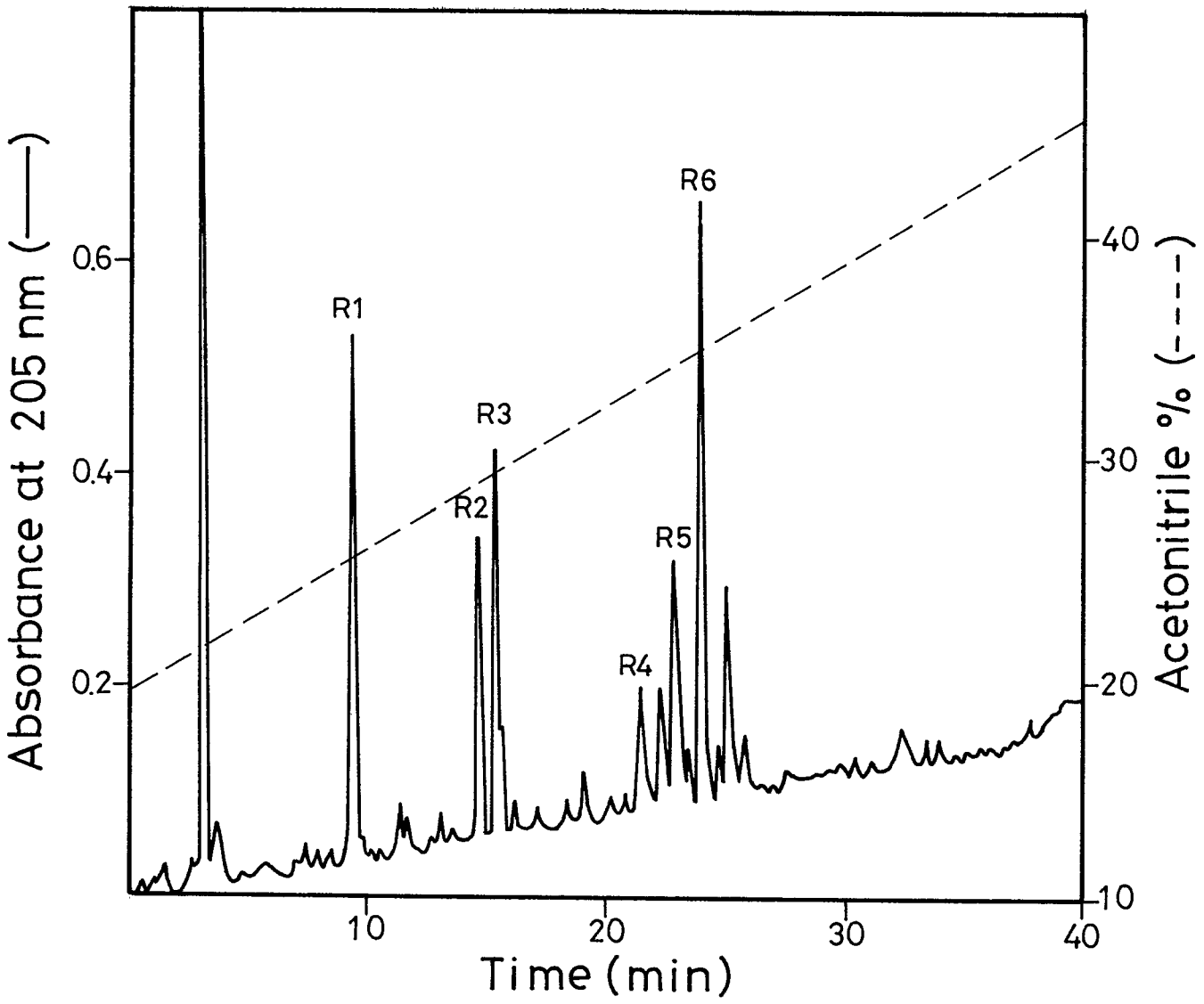
K-5271-M10

Fig. S5

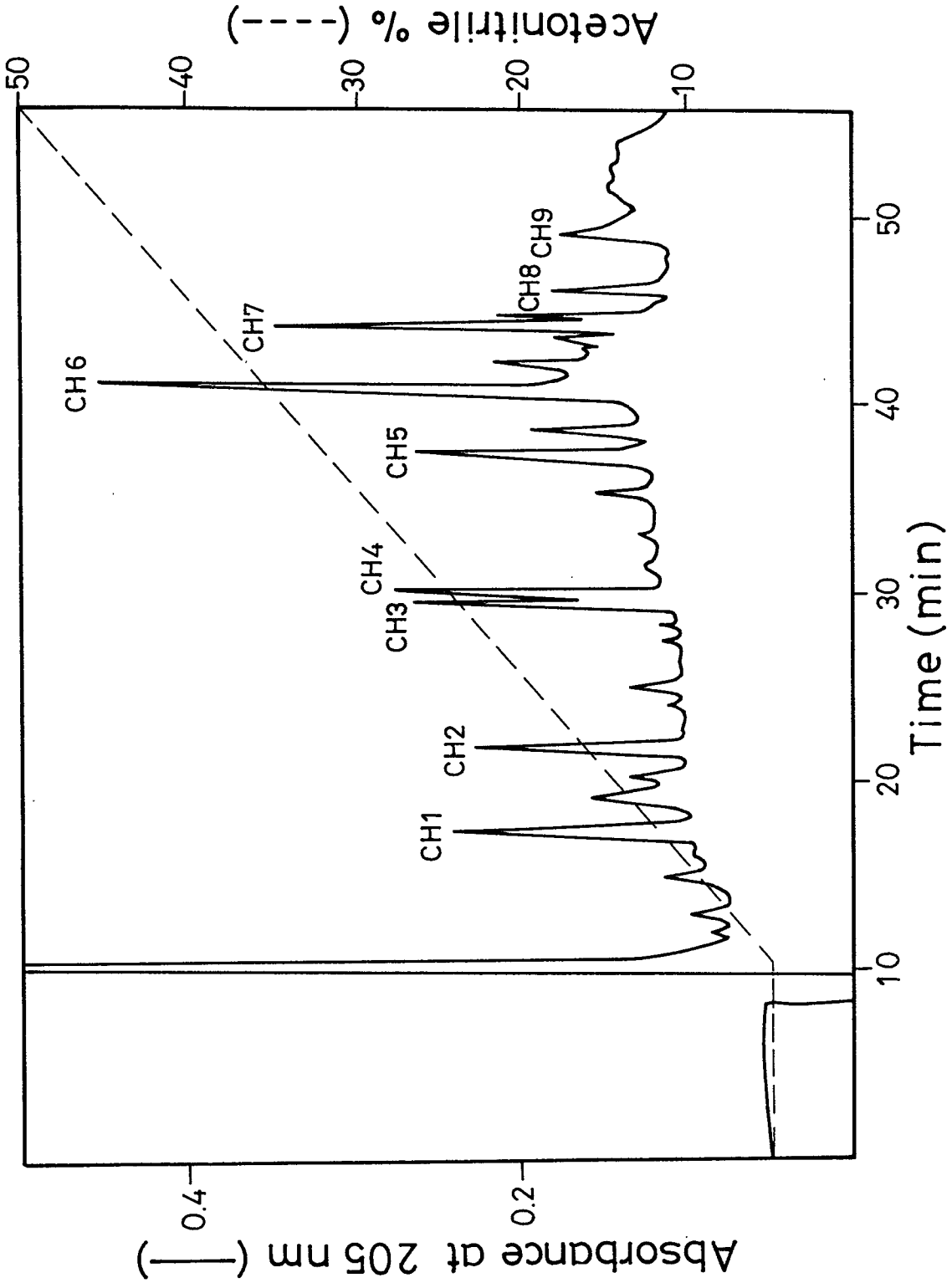


k-5271-M11

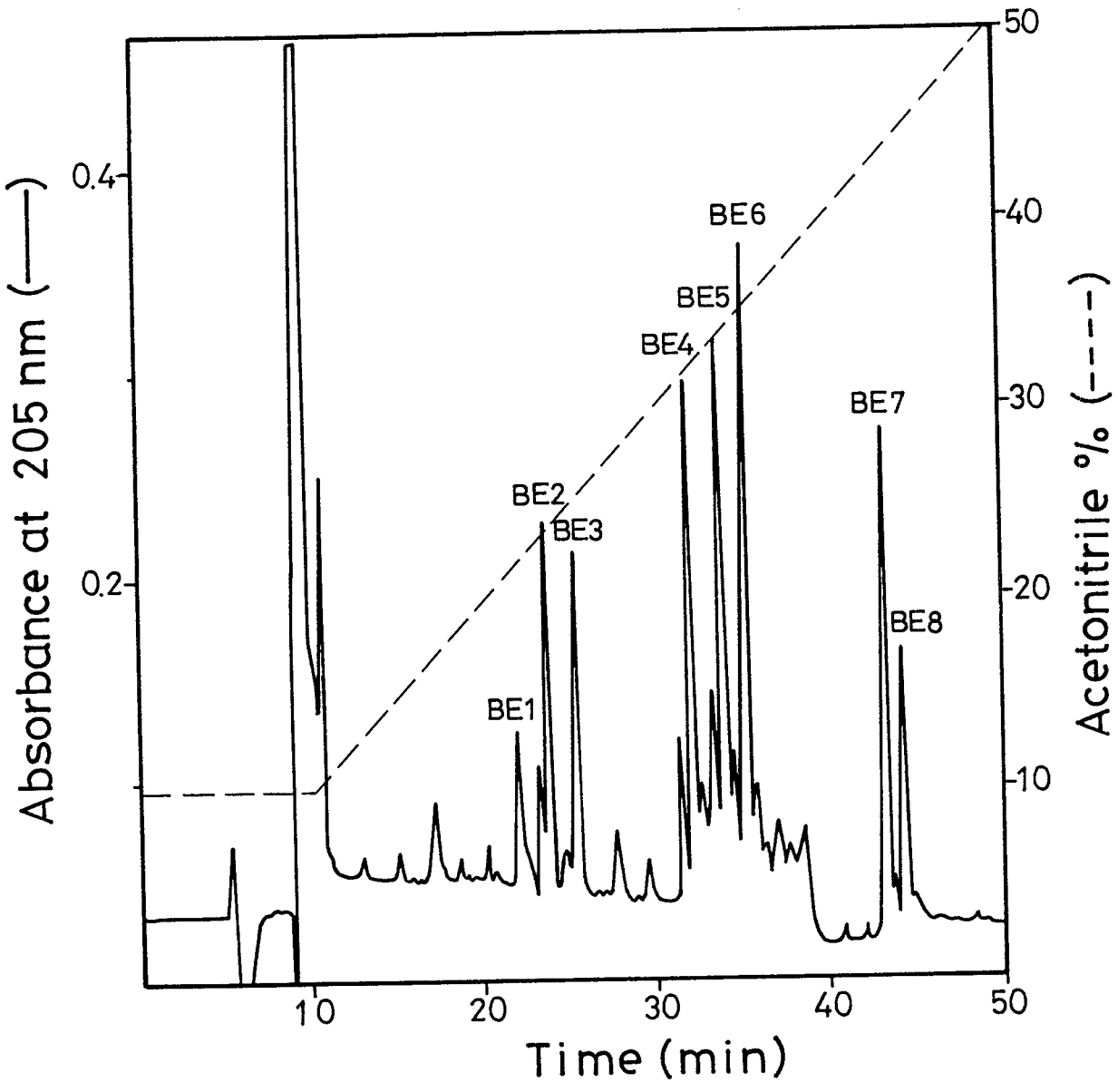
Fig. S6



K-5271-M12



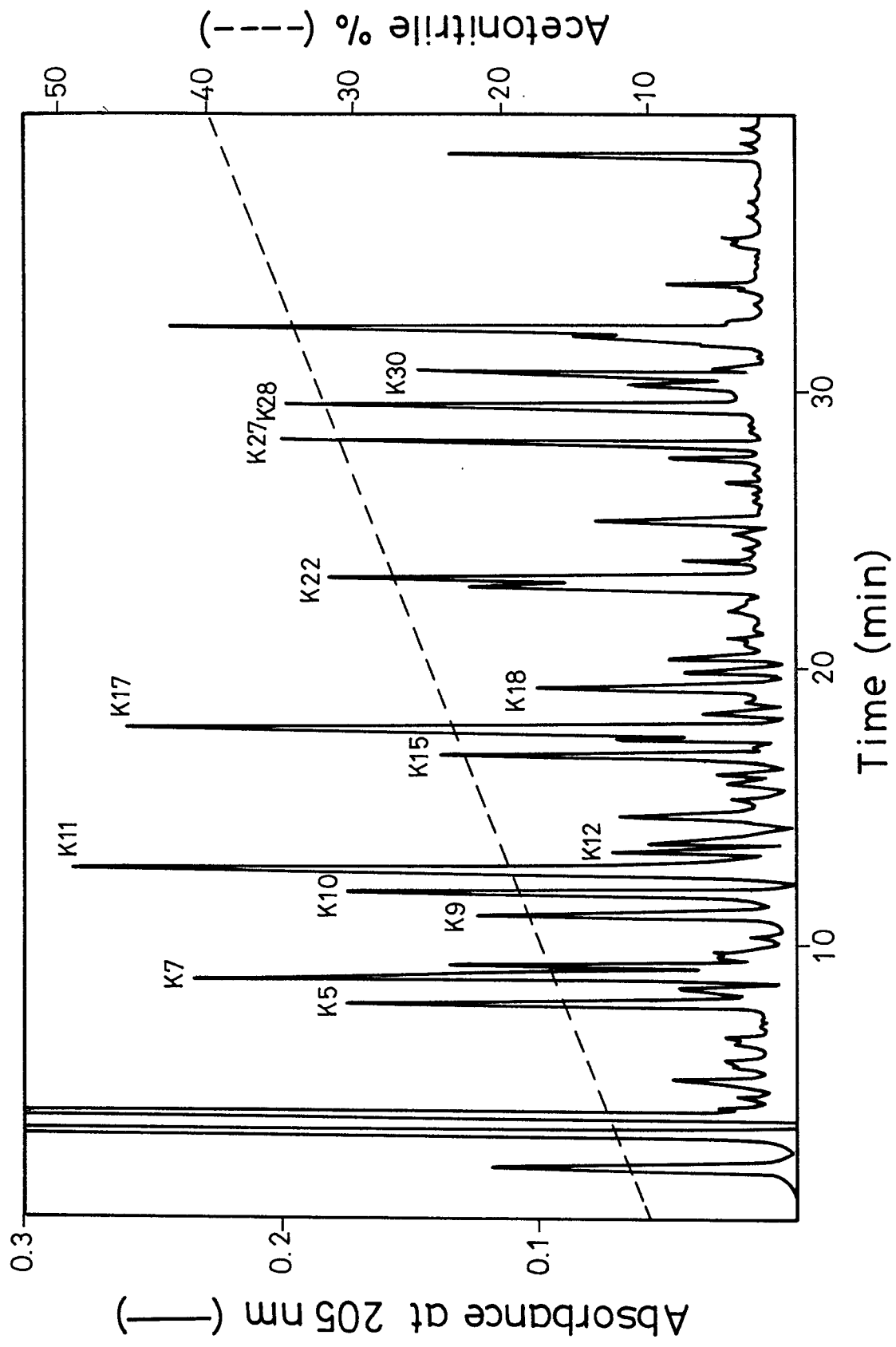
K-5271-M13
Fig. s8



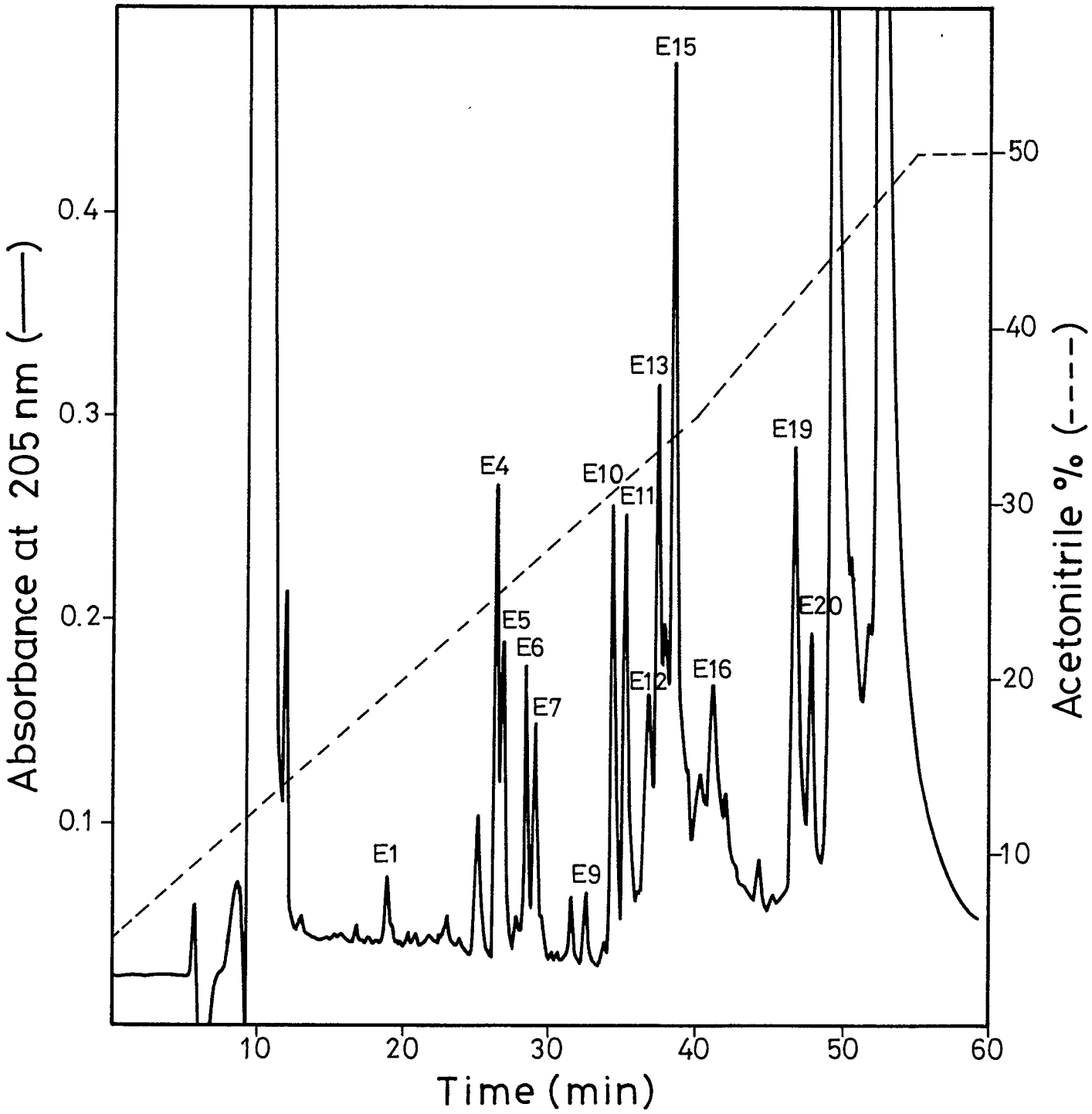
K-5271-M14

Fig. 5A

14



K-5271-MIS
Fig. 510



K-5271-M16
11

Fig. S11