

AMINOGLYCOSIDE ANTIBIOTICS



Aminoglycoside broad-spectrum antibiotics, which include mainly streptomycin, gentamycin, apramycin and neomycin, are used in both human and veterinary medicine.

In addition, streptomycin is used as a plant protection agent (trade name: Plantomycin) for the treatment of the bacterium *Erwinia amylovora* on pomaceous fruit. It is applied during the flowering period and can pass via the pollen into honey. Residues of aminoglycoside antibiotics in food could potentially be very detrimental to health.

For the analysis of amino glycoside antibiotics, PICKERING provides the systems PINNACLE PCX for post-column derivatization. Their configuration (number of reagent pumps, reactor volume, etc.) differs according to the respective derivatization method.

For the aminoglycoside antibiotics gentamycin, apramycin, neomycin and streptomycin Pickering also supplies a complete application kit. The kit contains, in addition to the HPLC-column, reagents, a diluent and eluents.

Description of the Method

Gentamycin, Apramycin and Neomycin react spontaneously with *o*-phthalaldehyde (OPA) and Thiofluor[®] (2-mercaptoethanol derivative) to fluorescing isoindole derivatives. Separation can be performed on either the special Pickering ion-exchange column or on a reversed-phase-column (depending on literature).

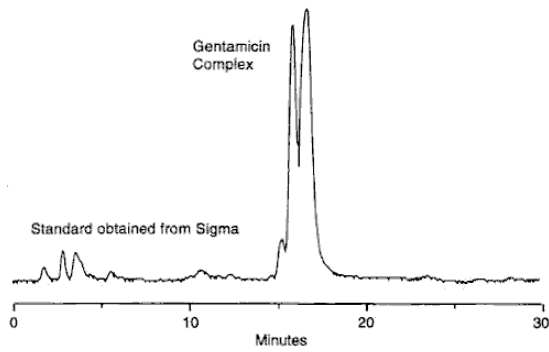
For the derivatization of Streptomycin there are several methods available! A very popular one follows exactly the method mentioned above with OPA/Thiofluor!

Extraction Procedure:

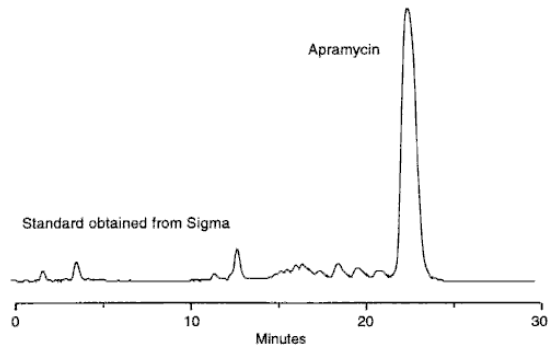
Take one part feed : 10 parts Extraction solution (w/v) Cat. No. 1700-1118 and homogenize for five minutes. Centrifuge for 10 minutes. Three layers will form: the pellet, a supernatant emulsion and a soft layer of floating fat. Carefully lift the floating fatty layer with a spatula and discard. Transfer the emulsion to a sealable vial. Coagulate the emulsion by placing the vial in a boiling water bath for 15 minutes. Centrifuge for 10 Minutes. The clear supernate is filtered (4.45 μ m Nylon) and placed in an autosampler vial.

Chromatograms

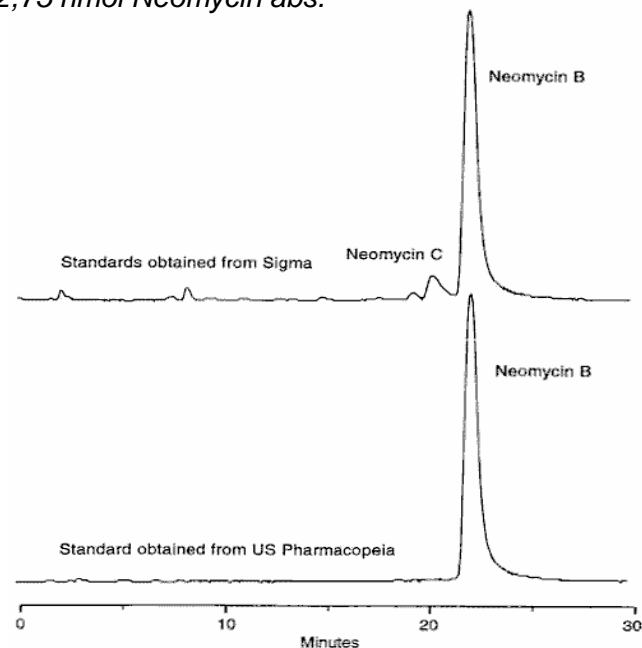
Chromatogram of Gentamycin Standard 5 µg Gentamycin-Complex abs.



Chromatogram of an Apramycin Standard 11,1 nmol Apramycin abs.

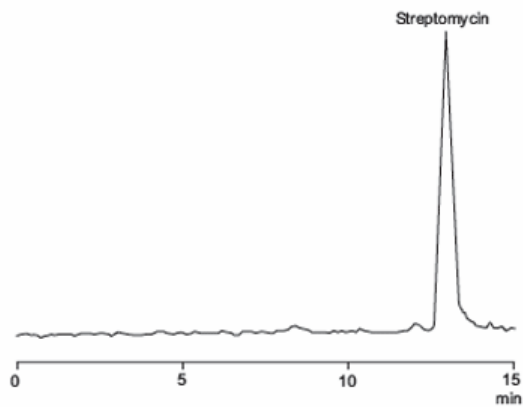


Chromatogram of a Neomycin Standard 2,75 nmol Neomycin abs.

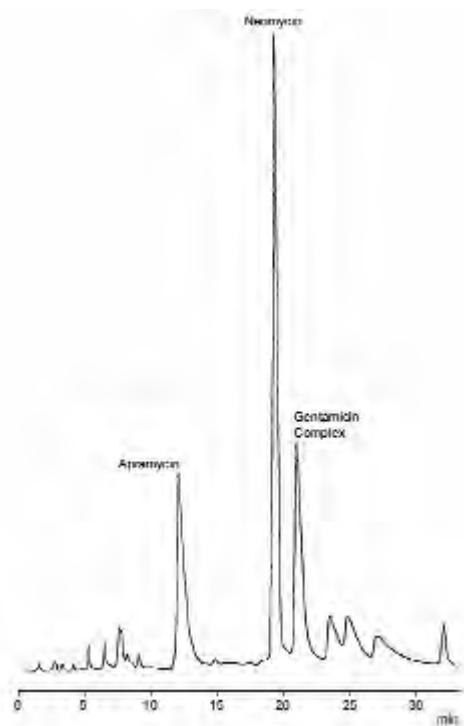


APPLICATION NOTE

Chromatogram of a Streptomycin Standard



Chromatogram of Apramycin, Gentamycin and Neomycin in one run



APPLICATION NOTE

HPLC Conditions and Derivatization Parameters

Gentamycin, Apramycin and Neomycin

HPLC	
Operation Mode	Ternary gradient
Eluent	A: 0,1 M potassium phosphate buffer, pH 6.0 (1700-1101) B: 0,1 M potassium hydroxide solution, pH 13.0 (1700-1102) C: 1,0 M sodium chloride solution (1700-1103)
Degassing	Helium- or vacuum degassed
HPLC Column	ALKION® Cation-exchange column, K ⁺ -Form
Flow Rate	0.8 mL/min
Injection volume	10 µL
Post-Column Derivatization	
Pinnacle PCX	Single-pump; 150 µL reactor
Column Oven	40 °C
Reactor Volume	150 µL
Reactor Temperature	40°C or 50°C (depending on the method)
Reagent	Solution of o-phthalaldehyde (OPA) and Thiofluor in OD104 diluent (300 mg OPA, 2 g Thiofluor and 3ml 30% Brij® in 1L diluent)
Reagent Flow	0.3 mL/min
Detektion	
Detection Type	Fluorescence detection
Excitation Wavelength	330 nm
Emission Wavelength	465 nm
Flowcell	Analytic; pressure stable up to 7 bar

APPLICATION NOTE

Caution: Extreme pH-range!

The following advice is only applicable to Pickering-Kits! As a consequence of the alkaline regenerant (pH 13), components made from Vespel may not be present in the HPLC system, instead these must be exchanged for components made from pH-inert materials (Tefzel or PEEK). For advice, please contact your LC-representative or consult your technical manual. An inert version (Titanium, PEEK) of the HPLC system is not necessary, a piston seal wash for the pump heads, however, is recommended.

To avoid corrosion of the system and contamination of the ion-exchange column with metal ions, passivating is recommended; this is especially valid for older systems. For advice, please contact your LC-representative or consult your technical manual.

Gradient Programs

Analysis of Gentamycin

TIME [min]	1700-1101 %	1700-1102 %	1700-1103%
0	43	31	26
20	9	13	78
30	9	13	78
30.1	0	22	78
31	0	22	78
31.1	43	31	26
38	43	31	26

Column temperature: 40°C

Analysis of Neomycin

TIME [min]	1700-1101 %	1700-1102 %	1700-1103%
0	60	40	0
15	13.2	8.8	78
25	11	11	78
25.1	0	22	78
26	0	22	78
26.1	60	40	0
32	60	40	0

Column temperature: 40°C

Analysis of Apramycin

TIME [min]	1700-1101 %	1700-1102 %	1700-1103%
0	67	33	0
5	67	33	0
15	14.7	7.3	78
20	14.7	7.3	78
20.1	0	22	78
21	0	22	78
21.1	67	33	0
28	67	33	0

Column temperature: 40°C

APPLICATION NOTE

Simultaneous analysis of Apramycin, Gentamycin and Neomycin

TIME [min]	1700-1101 %	1700-1102 %	1700-1103%
0	60	40	0
15	13.2	8.8	78
15.1	12	10	78
30	12	10	78
30.1	0	22	78
31	0	22	78
31.1	60	40	0
37	60	40	0

Column temperature: 50°C

APPLICATION NOTE

HPLC Conditions and Derivatization Parameters

Streptomycin – 1st possibility

HPLC	
Operation Mode	Ternary Gradient
Eluent	A: 0,1 M potassium phosphate buffer, pH 6.0 (1700-1101) B: 0,1 M potassium hydroxide solution, pH 13.0 (1700-1102) C: 1,0 M sodium chloride solution (1700-1103)
Degassing	Helium- or Vacuum degassed
HPLC Column	ALKION® Cation-exchange column, K ⁺ -Form
Flow Rate	0,8 mL/min
Injection Volume	Up to several µL
Post-Column Derivatization	
Pinnacle PCX	Dual-pump-system
Column Oven	40 °C
Reactor Volume	1. Reactor: 0.1ml (RT); 2. Reactor 0.5ml
Reactor Temperature	60 °C (second reactor)
Reagent	1. reagent: 0.75 N Sodium Hydroxide 2. reagent: 0.6 % Ninhydrin (Trione)
Reagent Flow	0,3 ml/min
Detection	
Detection Type	Fluorescence detection
Excitation Wavelength	395 nm
Emission Wavelength	500 nm
Flowcell	Analytic; pressure stable up to 7 bar

APPLICATION NOTE

Streptomycin – 2nd possibility

HPLC	
Operation Mode	Ternary Gradient
Eluent	A: 0,1 M potassium phosphate buffer, pH 6.0 (1700-1101) B: 0,1 M potassium hydroxide solution, pH 13.0 (1700-1102) C: 1,0 M sodium chloride solution (1700-1103)
Degassing	Helium- or Vacuum degassed
HPLC Column	ALKION® Cation-exchange column, K ⁺ -Form
Flow Rate	0,8 mL/min
Injection Volume	Up to several µL
Post-Column Derivatization	
Pinnacle PCX	Dual-pump-system
Column Oven	40 °C
Reactor Volume	1. Reactor: 0.5 ml; 2. Reactor: 0.1 ml (RT)
Reactor Temperature	50 °C (first reactor)
Reagent	1. reagent: 100 µl of 5% Sodium Hydrochlorite in GA116 2. reagent: Solution of o-phthalaldehyde (OPA) and Thiofluor in OD104 diluent (300 mg OPA, 2 g Thiofluor and 3ml 30% Brij® in 1L diluent)
Reagent Flow	0,3 ml/min
Detection	
Detection Type	Fluorescence detection
Excitation Wavelength	330 nm
Emission Wavelength	465 nm
Flowcell	Analytic; pressure stable up to 7 bar

APPLICATION NOTE

Post-Column Derivatization Units

Order Number	Description
1153-1012	PINNACLE PCX; single-pump, 0,15 mL reactor
1153-1052	PINNACLE PCX; dual-pump, 0.5 mL reactor

Chemicals and Columns

Application-Kits

Order Number	Description
0352-0041	Application kit for gentamycin, neomycin, apramycin and/or streptomycin analysis:
9410917	ALKION [®] , Ion-exchange column, K ⁺ -Form, 4 x 150 mm
9493020	Guard column, 3 x 20 mm, for column 9410917
O120	<i>o</i> -Phthalaldehyd (OPA), "Chromatographic Grade TM ", 5 g
OD104	OPA diluent, "Chromatographic Grade TM ", 4 x 950 mL
3700-2000	Thiofluor [®] , "Chromatographic Grade TM ", 10 g
1700-1101	Potassium phosphate/borate buffer, 0.1 M, pH 6.0, 4 x 950 mL
1700-1102	Potassium hydroxide solution, 0.1 M, 4 x 950 mL
1700-1103	Potassium chloride solution, 1.0 M, pH 6.0, 4 x 950 mL

Caution-Exchange Columns for Amino glycoside Antibiotics Analysis

Each column for the analysis of amino glycoside antibiotics is tested by applying an calibration standard and running it according to a specific gradient elution protocol. After a column passes this rigorous QC test, it receives a serial number and is packaged with its test chromatogram.

Order Number	Description
9410917	ALKION [®] , Cation-exchange column, K ⁺ -form, 4 x 150 mm
9493020	Guard column; 3 x 20 mm

APPLICATION NOTE

o-Phthalaldehyde Reagent

Primary amines such as gentamycin, apramycin and neomycin form highly fluorescent compounds when reacted with o-Phthalaldehyde (OPA) and a mercaptan under basic conditions. At a pH >9 and ambient temperature, reaction is generally complete within 1 - 30 seconds. The products of this reaction, 1-alkyl-2-alkylthio-substituted isoindoles, exhibit optimal excitation at 330 nm and maximal emission at 465 nm.

For an oxygen-sensitive reagent like OPA to remain stable for days instead of hours, it is important to start with the purest and most stable ingredients available, and to store and use the reagent under anaerobic conditions.

Using the chemicals described below, a long-lasting (up to ten days) OPA reagent for post-column derivatization of primary amines can be prepared. Each chemical is accompanied by clear instructions for formulating the reagent in your laboratory within minutes.



For the preparation of o-phthalaldehyde reagent PICKERING offers:

- 1) Ultra-pure, crystalline OPA
- 2) Borate diluent; free of heavy metals, particulates and amines
- 3) Thiofluor; crystalline substitute for 2-Mercaptoethanol

Order Number	Description
O120	o-Phthalaldehyde (OPA), "Chromatographic Grade", 5 g

o-Phthalaldehyde Diluent

For the analysis of gentamycin, apramycin and neomycin Pickering offers a sodium borate buffer with pH 10.4 capable to buffer acidic eluents.

Order Number	Description
OD104	OPA Diluent, "Chromatographic Grade™", 4 x 950 mL

APPLICATION NOTE

Thiofluor®

Pickering's Thiofluor, a solid, nearly odorless nucleophile, is a superior substitute for 2-mercaptoethanol in the preparation of OPA reagents. It forms a more stable and longer-lasting fluorophore with OPA than does 2-mercaptoethanol, while possessing the same fluorescence properties.

Unlike the volatile 2-mercaptoethanol, Thiofluor® will not migrate through the gas manifold and regulator of the OPA reagent pressurization system.

Order Number	Description
3700-2000	Thiofluor, "Chromatographic Grade™", 10 g

Potassium Phosphate Buffer

- 1) Filtered, ready to use
- 2) Free from fluorescent impurities
- 3) Cost-effective: long shelf life; can be used to the last ml
- 4) Consistent elution profiles, bottle to bottle, lot to lot

These buffers are used for the elution of aminoglycoside antibiotics according to the method developed by Pickering.

Order Number	Description
1700-1101	Potassium phosphate/borate buffer, pH 6.0, 4 x 950 mL
1700-1102	Potassium hydroxide solution, 4 x 950 mL
1700-1103	Potassium chloride solution, 4 x 950 mL

Literature

Neomycin

B. Shaikh, E. H. Allen, J. C. Gridley, *J. Assoc. Off. Anal. Chem.* **1985**, 68 (1), 29 – 36.

Gentamycin

D. Guggisberg, H. Koch, *Mitt. Gebiete Lebensm. Hyg.* **1995**, 86, 14 – 28.

Streptomycin

- 1) U. Kocher, *Lebensmittelchemie* **1996**, 50, 112 – 115.
- 2) G. C. Gerhardt, C. D. C. Salisbury, J. D. MacNeil, *J. AOAC International* **1994**, 77 (2), 334 – 336.