Analytix Notes

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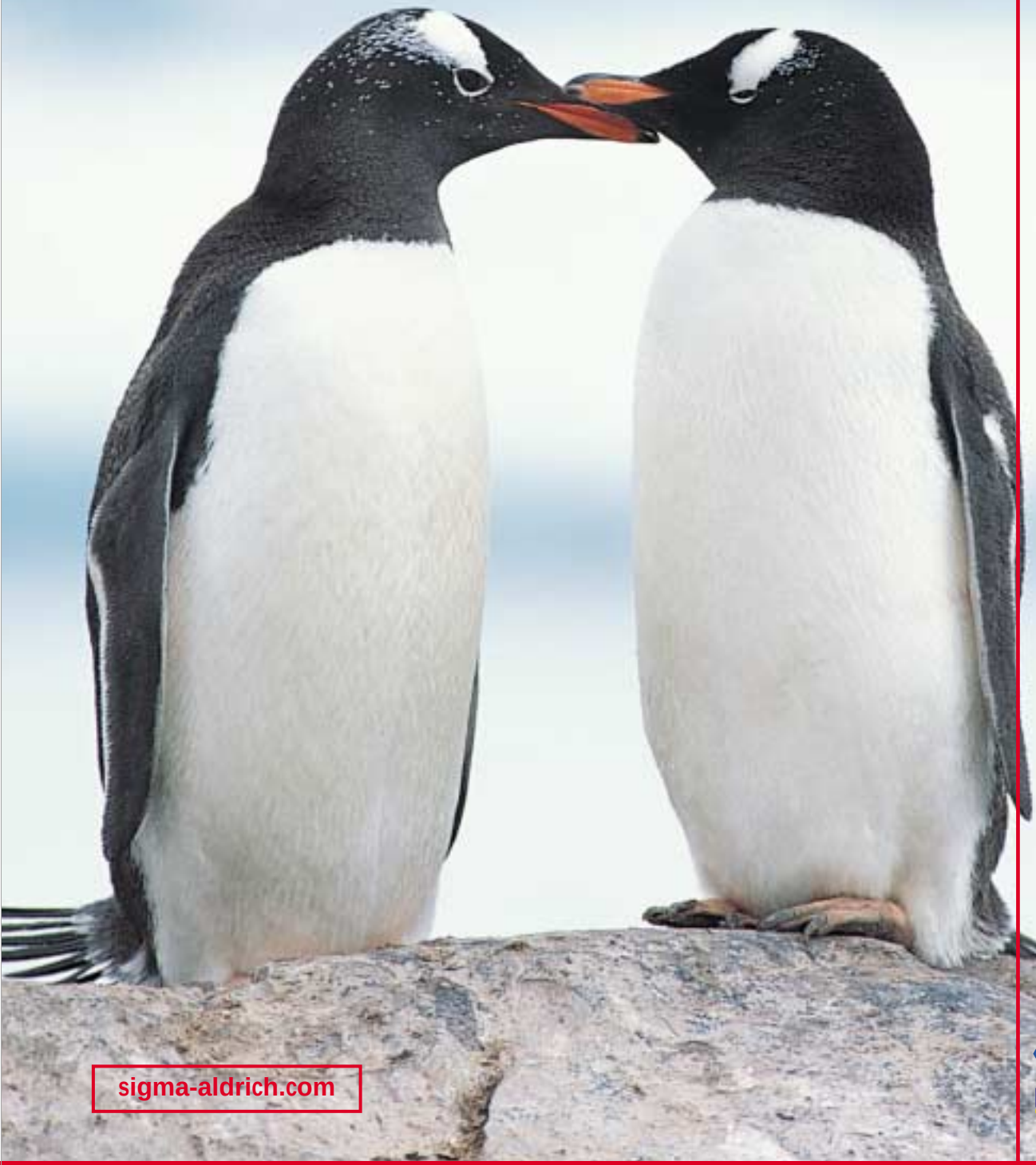
Ion Pair Chromatography



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Ion Pair Chromatography

"Color writing. Chromatography is arguably the most widely used technique in the modern analytical laboratory. Whether used for separating and identifying environmental contaminants from industrial wastewater or separating and identifying proteins from blood serum, the principles are the same, each in some way based on the differential solubility of individual chemical species."
- James F. Ryan in Today's Chemist at Work, 2001

One of the biggest challenges facing scientists working in pharmaceutical research and drug discovery is the separation and identification of biological substances. Since most of these compounds are ionic or polar, the use of reversed phase-high performance liquid chromatography (RP-HPLC) is somewhat restricted.

In the past, the approach used to separate charged analytes was ionic suppression. This technique is based on the pH adjustment of the mobile phase to result in a non-ionised analyte. However, this requires extensive method development and is only suitable for single compounds or simple mixtures where the pKa's of the analytes lie close together. Furthermore, the silica supported on bonded columns is only stable within a pH range of 2-8.

The limitations of ionic suppression led to the development of *Ion Pair Chromatography* (IPC). IPC is a more general and applicable approach that allows the separation of complex mixtures of polar and ionic molecules. The selectivity is determined by the mobile phase: the organic eluent is supplemented with a specific ion-pairing reagent. The IPC reagents are large ionic molecules having a charge opposite to the analyte of interest, as well as a hydrophobic region to interact with the stationary phase. The counter-ion combines with the ions of the eluent, becoming ion pairs in the stationary phase. This results in different retention, thus facilitating separation of analytes. IPC is now an established and reliable technique which provides:

- Reduced separation times
- Highly reproducible results
- Sharper peak shapes
- Simultaneous separation of ionized and non-ionized analytes in one run
- Wide choice of additives to improve separation.

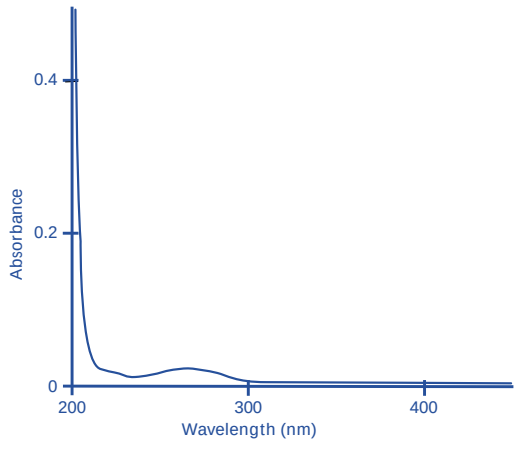

Figure 1. UV absorption performed on tetrabutylammonium bisulfate (Cat. No 86853). Concentration: 10% in water, measured against water. Cell: quartz (1cm).

The benefits of Fluka IPC reagents

In RP-HPLC, the purity of eluent additives means better reproducibility and reliability together with improved accuracy. Additives such as buffers or IPC reagents can lead to impurity peaks if the quality is not sufficient. Only products that have been carefully analysed and tested for functional performance will guarantee you an application without problems.

Over the past 14 years, Fluka has studied this well-established and reliable technique, and is now proud to offer you the widest range of accurately IPC-tested products available in the market. These include tailor-made reagents for anionic (quaternary ammonium and phosphonium salts) and cationic (alkanesulfonates) determinations. In addition to extensive analytical testing, our R&D laboratories have also developed a large number of application notes to help you to resolve your samples.

Fluka IPC reagents are of the highest purity and exhibit minimal extinction in the low UV (**Figure 1**). They have excellent transparency down to 200 nm, even at high concentrations. In addition, they are tested for the absence of insoluble matter (**Figure 2**). Non-absorbing impurities such as redox traces, which may interfere with the sample, are also checked. Finally, the suitability test is performed using a very steep gradient (**Figure 3**).

Figure 2. Filter test performed on tetrabutylammonium bisulfate (Cat. No 86853).
20 ml of the reference solution is filtered through a Millipore® 0.45 µm Membrane filter. After air drying it is compared with a blank. The filter shows no insoluble matter.

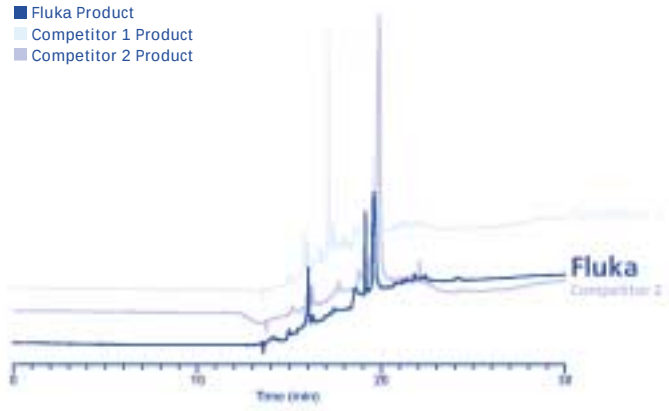

Figure 3. Gradient test for Sodium 1-heptanesulfonate monohydrate (Cat. No 51832)

Columns: Discovery C18™ 150 x 4.6mm, 5 µm (Cat. No 504955)
Mobile Phase: Mobile phase A – water (5 mM heptanesulfonic acid); Mobile phase B – acetonitrile
Flow Rate: 1.0 ml/min
Temperature: 35ºC
Detection: UV, 205 nm
Gradient: Mobile phase A – water and 5 mM heptane-sulfonic acid; Mobile phase B – acetonitrile

Time (min)	%A	%B
0	100	0
10	100	0
20	0	100
30	0	100

Two challenging mixtures resolved with Fluka IPC reagents

Separation of biogenic amines

Biogenic amines such as adrenaline, dopamine, tyramine and tryptamine play important roles as monoamine neurotransmitters. The analysis of these cations by RP-HPLC is not easy: their retention times are similar and interferences are often observed. The addition of an IPC reagent can be of help to resolve such mixtures.

The successful separation of cations by IPC is often obtained by using alkylsulfonic acid sodium salts. For these monoamines, the popular sodium-1-heptanesulfonate (Cat. No 51832) was chosen. **Figure 4** shows how the mixture containing adrenaline, dopamine, tyramine and tryptamine was efficiently resolved.

Separation of nucleotides

It can be a challenge to separate mixtures containing nucleotides by RP-HPLC, as they cover a wide range of polarities and functionalities. IPC can be the key to resolve them, as the separation of anions becomes possible using quarternary ammonium salts.

In this case, the IPC reagent is tetrabutylammonium bisulfate (Cat. No 86853). The chromatogram obtained for a sample containing nucleotides is shown in **Figure 5**. The mixture was separated with excellent resolution and highly reproducible results.

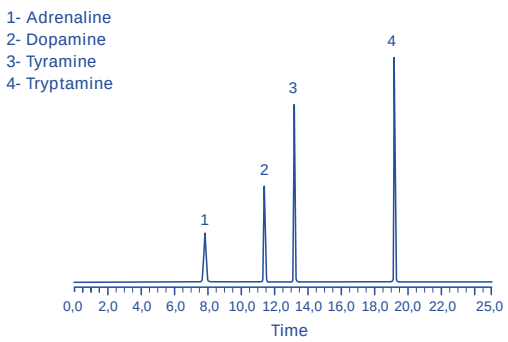

Figure 4. Mixture of biogenic amines resolved by IPC

Columns: Discovery™ C18 Column (250 x 4.0 mm) ID, 5 µm (Cat. No 04971-40)
Eluent: acetonitrile: heptanesulfonic acid buffer pH 2.4:
Buffer concentration: 0,005 M heptanesulfonic acid sodium salt (Cat. No 51832) + 0,01 M phosphoric acid (Cat. no 79606)
Weigh-in: ~ 2 mg in 10 ml acetonitrile/phosphoric acid (0.01 M) 1:9
Acetonitrile gradient: t=0 min : 6%, t=5 min : 6%, t=18 min : 25%
Flow: 1.5 ml/min
Detection: 220 nm
Injection volume: 20 µl
Temperature: ambient
Detector: UV 1000
Pump: P 4000

SUPELCO

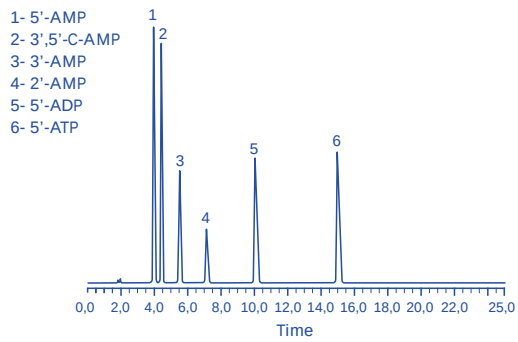

Figure 5. Mixture of nucleotides resolved by IPC

Column: Discovery™ C18 Column (250 x 4.0 mm) ID, 5 µm (Cat. No 504971-40)
Eluent: acetonitrile: tetrabutylammonium buffer pH 7.0
Gradient Buffer concentration: 0,005 M tetrabutyl-ammonium hydrogensulfate (Cat. No 86853) + 0,01 M Na_2HPO_4*$12H_2O$ (Cat. No 71649).
Weigh-in: ~ 4 mg in 10 ml acetonitrile / water 1:9
Acetonitrile gradient: t=0: 10%; t=4 min 10%; t=14 min: 25%

Flow: 1.5 ml/min
Detection: 254 nm
Injection volume: 20 µl
Temperature: ambient
Detector: UV 1000
Pump: P 4000

SUPELCO

How to select the right IPC reagent

If you have a mixture of ionic and non-ionic analytes, we recommend that you start by optimising the method for the non-ionic components. Then select the appropriate IPC reagent to provide the necessary counter ion. Alkyl sulfonates are a good first choice for basic solutes, whereas quaternary amines are useful for the acidic ones. Halogenated IPC reagents are only suitable for isocratic applications and should not be used in gradient systems. **Tables 1** and **2** give you an overview of IPC reagents for the separation of cations and **Table 3** shows IPC reagents suitable for the separation of anions.

After selecting the appropriate IPC reagent, the method can be further optimised by adjusting the pH and concentration. For short or medium chain length IPC reagents, a 0.005 M solution is suitable for most separations. The optimum concentration of long chain IPC reagents varies from 0.0005 M to 0.002 M. In **Table 4** you'll find a selection of buffers and buffer concentrates for exact pH adjustment. All buffers are tested for suitability for Chromatography

Cat. No.	Compound	Carbon length
02374	1,2-Ethanedisulfonic acid disodium salt	C2
81808	2-Propanesulfonic acid sodium salt monohydrate	C3
19022	1-Butanesulfonic acid sodium salt	C4
76952	1-Pentanesulfonic acid sodium salt monohydrate	C5
52862	1-Hexanesulfonic acid sodium salt monohydrate	C6
51832	1-Heptanesulfonic acid sodium salt monohydrate	C7
74882	1-Octanesulfonic acid sodium salt monohydrate	C8
75073	Octyl sulfate sodium salt	C8
74316	1-Nonanesulfonic acid sodium salt	C9
30631	1-Decanesulfonic acid sodium salt	C10
71443	Sodium decyl sulfate	C10
94133	1-Undecanesulfonic acid sodium salt	C11
44123	1-Dodecanesulfonic acid sodium salt	C12
71726	Sodium dodecyl sulfate	C12
52263	1-Hexadecanesulfonic acid sodium salt	C16
74734	Sodium 1-octadecanesulfonate	C18

Table 1. Selection of solid IPC reagents suitable for cation separation sorted by carbon chain length.

Cat. No	Compound	Carbon length
19029	1-Butanesulfonic acid sodium salt concentrate (~0.33 M)	C4
76954	1-Pentanesulfonic acid sodium salt concentrate (~0.33 M)	C5
52864	1-Hexanesulfonic acid sodium salt concentrate (~0.33 M)	C6
51834	1-Heptanesulfonic acid sodium salt concentrate (~0.33 M)	C7
74886	1-Octanesulfonic acid sodium salt monohydrate, concentrate (~0.33 M)	C8
71735	Sodium dodecyl sulfate concentrate (~0.33 M)	C12

Table 2. Selection of IPC reagent concentrates suitable for cation separation sorted by carbon chain length.

Concentrates available in packages with 6 ampoules. Dilute to 1-liter with HPLC grade water (Cat. No 95304) to obtain a 0.005 M eluent solution.

Cat. No.	Compound	Carbon length
02799	Tetramethylammonium sulfate	C1
87708	Tetramethylammonium bromide	C1
87724	Tetramethylammonium hydrogensulfate	C1
87727	Tetramethylammonium hydrogensulfate concentrate (~0.33 M)	C1
87728	Tetramethylammonium hydroxide concentrate	C1
86608	Tetraethylammonium bromide	C2
86626	Tetraethylammonium hydrogensulfate	C2
86635	Tetraethylammonium hydroxide concentrate	C2
88103	Tetrapropylammonium bromide	C3
88106	Tetrapropylammonium hydrogensulfate	C3
88109	Tetrapropylammonium hydroxide concentrate	C3
86832	Tetrabutylammonium hydroxide solution, 1.0 M in water	C4
86846	Tetrabutylammonium bromide concentrate	C4
86847	Tetrabutylammonium hydrogensulfate concentrate	C4
86851	Tetrabutylammonium hydroxide concentrate	C4
86852	Tetrabutylammonium chloride	C4
86853	Tetrabutylammonium hydrogensulfate	C4
86854	Tetrabutylammonium hydroxide solution, ~40% in water	C4
86857	Tetrabutylammonium bromide	C4
86862	Tetrabutylammonium chloride concentrate	C4
86899	Tetrabutylammonium dihydrogenphosphate concentrate	C4
86903	Tetrabutylammonium iodide	C4
86915	Tetrabutylphosphoniumbromide	C4
86925	Tetrabutylphosphonium hydrogensulfate	C4
87997	Tetrapentylammonium bromide	C5
87297	Tetrahexylammonium bromide	C6
87299	Tetrahexylammonium hydrogen sulfate	C6
87313	Tetrahexylammonium dihydrogenphosphate concentrate	C6
87296	Tetraheptylammonium bromide	C7
87996	Tetraoctylammonium bromide	C8
30518	Decamethonium bromide	C10
87578	Tetrakis(decyl)ammonium bromide	C10
44239	Dodecyltrimethylammonium bromide	C12
44243	Dodecyltrimethylammonium hydrogensulfate	C12
87208	Tetradecyltrimethylammonium bromide	C14
87215	Tetradecyltrimethylammonium hydrogensulfate	C14
52363	Hexadecyltrimethylammonium dihydrogenphosphate	C16
52367	Hexadecyltrimethylammonium bromide	C16
52371	Hexadecyltrimethylammonium hydrogensulfate	C16
52382	Hexadecyltrimethylammonium hydroxide concentrate	C16

Table 3. Overview of solid IPC reagents suitable for anionic separation sorted by carbon chain length (longest chain is shown).

Products in Red: recommended for initial trials.

Cat. No.	Compound
82606	CAPS buffer solution, 20 mM, pH 10.0
82607	CAPS buffer solution, 20 mM, pH 10.5
82608	CAPS buffer solution, 20 mM, pH 11.0
82581	Citric acid/Sodium hydroxide buffer solution, 20 mM, pH 2.5
82582	Citric acid/Sodium hydroxide buffer solution, 20 mM, pH 3.0
79607	ortho-Phosphoric acid 50%
79606	ortho-Phosphoric acid 85%
79626	Phosphoric acid concentrate, (~0.66 M)
79629	Phosphoric acid/di-Sodium hydrogenphosphate concentrate, (~0.33 M)
79628	Phosphoric acid/Potassium dihydrogenphosphate, concentrate, (~0.33 M)
82622	Potasium phosphate buffer solution, 150 mM, pH 3.0
60221	Potassium dihydrogenphosphate
60232	Potassium dihydrogenphosphate concentrate (~0.66 M)
82583	Sodium citrate buffer solution, 20 mM, pH 3.5
82584	Sodium citrate buffer solution, 20 mM, pH 4.0
82585	Sodium citrate buffer solution, 20 mM, pH 4.5
82586	Sodium citrate buffer solution, 20 mM, pH 5.0
82587	Sodium citrate buffer solution, 20 mM, pH 5.5
82588	Sodium citrate buffer solution, 20 mM, pH 6.0
82578	Sodium phosphate buffer solution, 100 mM, pH 2.5
82599	Sodium phosphate buffer solution, 100 mM, pH 3.0
82637	Sodium phosphate buffer solution, 100 mM, pH 7.0
82634	Sodium phosphate buffer solution, 100 mM, pH 8.0
82635	Sodium phosphate buffer solution, 50 mM, pH 2.5
82635	Sodium phosphate buffer solution, 50 mM, pH 2.5
82636	Sodium phosphate buffer solution, 50 mM, pH 7.0
82633	Sodium phosphate buffer solution, 50 mM, pH 8.0
82589	Sodium phosphate buffer solution, 20 mM, pH 6.5
82589	Sodium phosphate buffer solution, 20 mM, pH 6.5
82591	Sodium phosphate buffer solution, 20 mM, pH 7.0
82592	Sodium phosphate buffer solution, 20 mM, pH 7.5
82593	Sodium phosphate buffer solution, 20 mM, pH 8.0
82601	Sodium phosphate buffer solution, 20 mM, pH 8.5
82603	Sodium phosphate buffer solution, 20 mM, pH 9.0
82605	Sodium phosphate buffer solution, 20 mM, pH 9.5
71648	Sodium phosphate dibasic concentrate I (~0.33 M)
71651	Sodium phosphate dibasic concentrate II (~0.50 M)
71653	Sodium phosphate dibasic/Potassium phosphate monohydrate, concentrate (~0.33 M)
82594	Sodium tetraborate buffer solution, 20 mM, pH 8.0
82602	Sodium tetraborate buffer solution, 20 mM, pH 8.5
82604	Sodium tetraborate buffer solution, 20 mM, pH 9.0

Table 4. Selection of Buffers tested for Chromatography applications.

Concentrates available in packages with 6 ampoules. Dilute to 1-liter with HPLC grade water (Cat. No 95304) to obtain a 0.005 M eluent solution.

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Product Manager
Fluka/Riedel-de Haën
Tel: 0041/81/755-2839
Fax: 0041/81/755-2824
Email: rwalz@sial.com

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