Step by Step Guide

To

SPE Method Development

Biotage

STEP BY STEP GUIDE TO SPE METHOD DEVELOPMENT

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AFTER REVIEWING THE CHARTS, PHOTOCOPY AND FILL OUT THE FORM BELOW

Sample name: _____

Analyte of Interest: _____

Sample size: _____

Sorbent Bed Mass _____ Cartridge volume _____

Sorbent phases to try 1) _____ 2) _____ 3) _____ 4) _____

Sample pre-treatment _____

1. Condition column with _____ mL of _____at_____mL/min.
2. Equilibrate column with _____ mL of _____at_____mL/min.
3. Load sample at _____ mL per minute.
4. Remove interferences with _____mL of _____at_____mL/min.
5. Drying step _____ minutes.
6. Elute analyte with _____mL of _____at_____mL/min.

   Remember to include a soak step if possible!

THE STARTING POINT

The first decision that needs to be made is what the final analysis will be for the analyte. This will have an impact on the sample and cartridge size, as well as the final elution solvent. Gas chromatography offers higher sensitivity than HPLC, while HPLC is better suited for ionisable species and very high molecular weights. If LC-MS is available, minimal sample clean-up may be required.

Sample Size Determination

First determine the sensitivity of your analytical method. If the analytical range of your instrument is different from the one in this example, simply multiply the sample size by the same factor. The following chart assumes an analytical range for GC of 0.01 to 10 ppm of sample injected, and 2 to 20 ppm for HPLC. If your instrumental range differs from that given here, multiply the required sample volume by the appropriate factor. If greater sensitivity is required, the final elution volume can be concentrated.

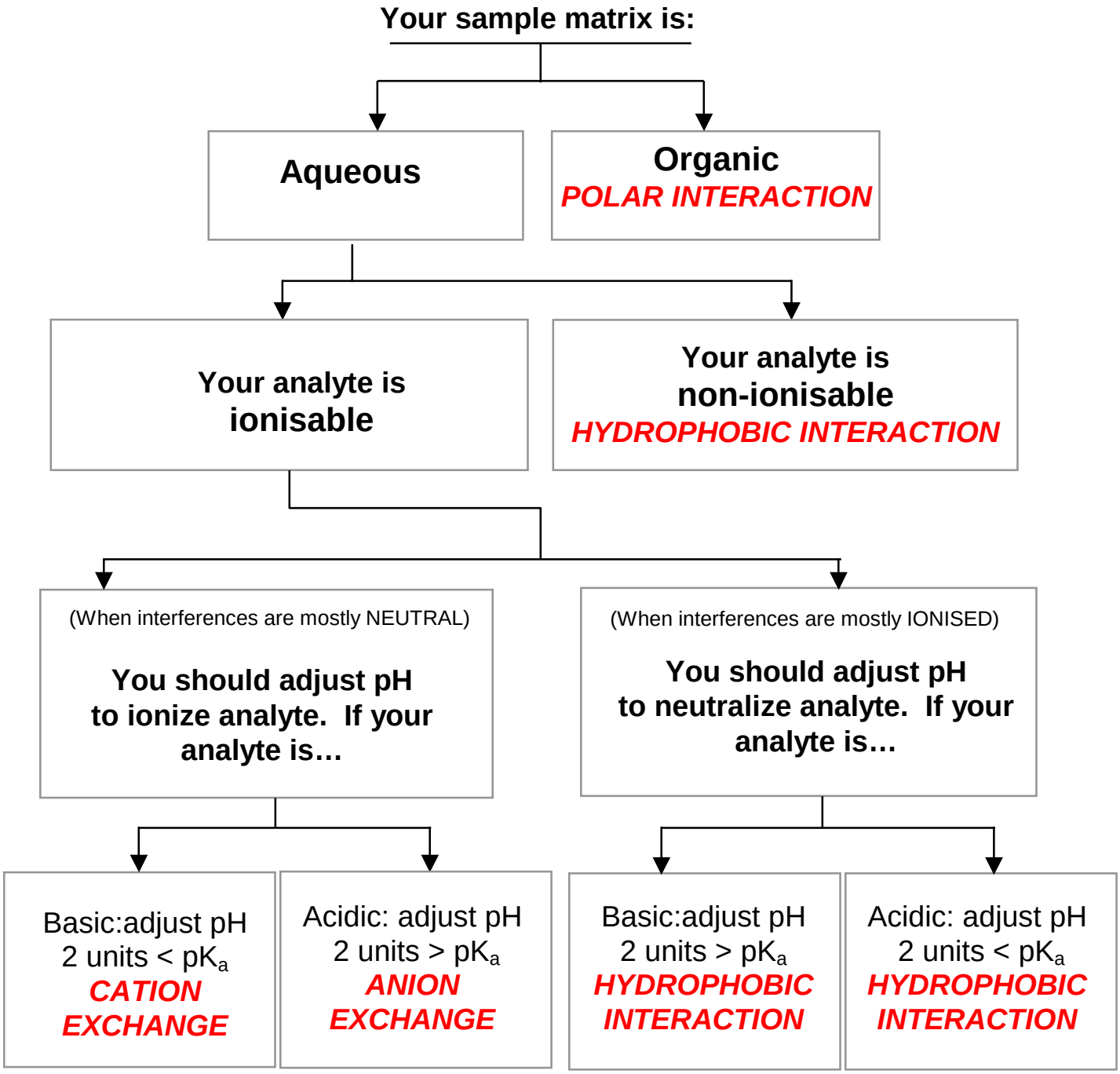
IF FINAL EXTRACT IS TO BE CONCENTRATED, THE SAMPLE VOLUME CAN BE REDUCED BY THE SAME FACTOR

Instrument	Approximate Analyte conc.	Sample Volume (mL)	Final Extract Volume (mL)	Cartridge Size
GC	1-5 ppb	500-1000	3-6	500 mg or 1 g / 6 mL
	5-50 ppb	50-500	2-4	500 mg / 6 mL
	50-500 ppb	5-50	0.5-2	100 or 200 mg / 1 or 3 mL
	0.5-50 ppm	0.25 -5	0.5-2	100 or 200 mg / 1 or 3 mL
HPLC	50-250 ppb	500-1000	3-6	500 mg or 1 g / 6 mL
	0.25-2 ppm	50-100	3-6	500 mg or 1 g / 6 mL
	2-10 ppm	2-10	0.5-2	100 or 200 mg / 1 or 3 mL
Analytical Balance	2-20 ppm	1000	3-6	500 mg or 1 g / 6 mL
	20-200 ppm	500	3-6	500 mg or 1 g / 6 mL

The ion exchanger SPE sorbents can retain approximately 0.3 to 0.4 milli-equivalents per gram (i.e. 0.3-0.4 mM/g for a singly charged species). Do the following calculation to determine the minimum bed size for an ion exchanger:

Minimum Bed Mass (mg)	=	*Sample Volume (mL)*	×	*Approx. Analyte Conc (mg/L or µg/mL)*	÷	*Molecular Wt of Analyte*	×	*Analyte Charge (abs. value)*	×	*3*

SELECT A MECHANISM *(given in italics)*



NOTE: When sample clean-up is required, but trace enrichment is not, an aqueous sample containing analytes that are water-soluble can have interferences removed by ***HYDROPHOBIC INTERACTIONS.***

SELECT A SORBENT

Once the retention mechanism is selected, the sorbent can be chosen. There will often be more than one sorbent "candidate" that can do the job. To minimize the time required for method development, and to obtain a robust method, several different phases should be tried in parallel. For example, if the primary mechanism for retention is hydrophobic, perform the extraction using C8, C18 and ENV+ to determine which gives the best results)[‡].

MECHANISM	CONSIDERATIONS	SORBENTS[‡]
Hydrophobic *ISOLUTE®* *Nonpolar Method* *Development Kit* *Recommended*	Analyte(s): Low molecular weight or polar --- Analyte(s): High M.W --- Broad range of neutral analytes of varying polarity	C6, C8, C18, ENV+® --- C2, C4, C6, PH, C8 --- C4, C6, C8, CH, Layered (e.g., C2/C18)
Polar *ISOLUTE Polar* *Method* *Development Kit* *recommended*		SI, NH2, CN, Diol
Cation Exchange *ISOLUTE Ion* *Exchange Method* *Development Kit* *recommended*	Analyte has a permanent positive charge (e.g., quaternary amine) --- Analyte(s) with pK_as between 5-10. --- 1. Analyte(s) with pK_as between 5-10. 2. Both basic and neutral analytes are present. 3. Sample matrix is dirty, and a very clean extract is required.	CBA, SI, C2* --- SCX, SCX-2[#], CBA, C2* --- HCX HCX-3 HCX-5
Anion Exchange *ISOLUTE Ion* *Exchange Method* *Development Kit* *recommended*	Analyte has a permanent negative charge. --- Analyte(s) with pK_as between 2-6. --- 1. Analyte(s) with pK_as between 2-6. 2. Basic, acidic and neutral analytes are present. 3. Sample matrix is dirty, and a very clean extract is required.	NH2 --- PEAX, SAX, NH2 --- HAX

[‡] Many of these phases are available in both endcapped and non-endcapped forms. It is recommended that non-endcapped be evaluated first.

[#] SCX-2 can be used instead of SCX to minimize hydrophobic interaction, which can occur as a secondary interaction to ion exchange. SCX-2 may allow elution with just buffer and no organic solvent. This should only be used if ionic interferences are not problematic.

* C2 is used here as a cation exchanger due to the number of easily accessible silanol sites.

PRE-TREAT THE SAMPLE

Sample pre-treatment enhances analyte retention. Use the following chart to help determine what pre-treatment may be required:

AQUEOUS MATRIX

Type of Analyte	Type of Sorbent	Pre-treatment
Neutral or Neutralized	Hydrophobic (e.g., C2, C8, C18, ENV+)	Add 0.5 to 1 % organic solvent such as methanol to samples having volumes >100 mL. If analyte is ionisable, adjust sample pH to 2 units above pK_a for bases and 2 units below pK_a for acids.
Cationic	Cation exchanger (CBA, SCX, C2, SCX-2)	Adjust pH with buffer to ensure charge on analyte (2 pH units below analyte pK_a). *IMPORTANT NOTES:* 1. If CBA phase is used, pH must not be below 7. For C2 phase, pH must not be below 5. 2. The ionic strength of the sample must not exceed 50mM for singly charged cation, or 100 mM for doubly charged cation. Samples with a high ionic strength (e.g. urine) must be diluted. 3. An appropriate buffer should be selected that will not compete with the analyte of interest. The following series lists ions on the left that will displace ions on their right: $Ba^{+}>Ag^{+}>Ca^{2+}>Zn^{2+}>K^{+}>NH_4^{+}>H^{+}>Li^{+}$
Anionic	Anion exchanger (NH2, SAX, PSA, PE-AX)	Adjust pH with buffer to ensure charge on analyte (2 pH units above analyte pK_a). *IMPORTANT NOTES:* 1. If NH2 phase is used, sample pH must not be above 7.8. 2. The ionic strength of the sample must not exceed 50mM for singly charged anion, or 100 mM for doubly charged anion. Samples with a high ionic strength (e.g. urine) must be diluted. 3. An appropriate buffer should be selected that will not compete with the analyte of interest. The following series lists ions on the right that will displace ions on their left: OH^{-} > acetate> formate> $HPO_4^{-}>HCO_3^{-}>Cl^{-}>HSO_3^{-}$> Citrate

HOW TO CONDITION AND EQUILIBRATE THE COLUMN

The appropriate solvents and/or buffers should be used to ensure that the sorbent is active. Use Chart A to help in choosing column conditioning and column equilibration solvents and Chart B for determining volumes and flow rates.

CHART A

Type of Analyte	Type of Sorbent	Column conditioning/Column equilibration
Neutral	Hydrophobic (C2, C8, C18)	Condition column with methanol. Displace excess methanol by rinsing with reagent water buffered to pH of sample. *IMPORTANT NOTE:* If ionisable species are being neutralized for a hydrophobic extraction, reagent water rinse should be followed with a buffer rinse at the pH used for sample pre-treatment.
Neutral	Hydrophobic (ENV+)	Not required *unless*: ionisable species are being neutralized for a hydrophobic extraction. Then use a buffer rinse at the pH used for sample pre-treatment. The buffer concentration should be between 10-50 mM to control ionic strength.
Cation or Anion	Cation exchanger or Anion exchanger	Condition column with methanol. Rinse with reagent water followed by a buffer rinse at the pH used for sample pre-treatment. The buffer concentration should be between 10-50 mM to control ionic strength.

CHART B

Type of Analyte	Bed Mass	Volumes, mL	Flow rates, mL/min
Silica Based	25 mg	0.1-1	0.25-2
	50 mg	0.25-1	0.5-2
	100 mg	0.5-2	1-4
	130 mg	1-2	2-4
	200 mg	1-2	2-4
	500 mg	3-5	6-10
	1 g	5-10	10-20
ENV+	25 mg	0.5-4	1-8
	200 mg	4-8	8-16

IMPORTANT NOTE: The higher flow rates listed here should be used with the greater conditioning and rinse volumes given.

HOW SHOULD THE SAMPLE BE LOADED?

The appropriate loading rate will depend on the type of analyte being extracted, and the size of the sorbent bed. Use the following chart as a guide when selecting a loading rate. It is best to start at the slowest rate for a given cartridge size when developing the method to establish the chemistry. Once the chemistry is established, the rate can be increased to minimize the time requirement. In addition, once the chemistry is optimized it may be possible to scale down the extraction with respect to bed mass and solvent volumes.

Type of Analyte	Type of Sorbent	Cartridge Size	Loading Rate (mL/minute)
Neutral	Hydrophobic (e.g., C2, C8, C18, ENV+)	1 mL 3 mL 6 mL	1-15 3-30 10-120
Cation or Anion	Cation exchanger (CBA, SCX, SCX-2, C2) or Anion exchanger (NH2, SAX, PE-AX)	1 mL 3 mL 6 mL	0.5-2 1-15 3-35

PLEASE NOTE: Columns having wider diameters allow higher flow rates, while maintaining the same retention efficiency. (Retention efficiency is a function of linear velocity).

REMOVING INTERFERENCES

The goal during the interference elution step is to eliminate as many components as possible that may interfere with the analysis, without suffering analyte losses. Use the following table as a guide:

Type of Analyte	Type of Sorbent	Interference Elution
Neutral	Hydrophobic (e.g., C2, C8, C18, ENV+)	Rinse with equilibrium buffer. If analytes are strongly retained, it may be possible to add a water miscible organic solvent to this rinse to remove additional interferences. Collect this fraction to check for analyte losses. *IMPORTANT NOTE:* Maintenance of the pH is essential if ionisable species have been neutralized for a hydrophobic extraction.
Cation or Anion	Cation exchanger (CBA, SCX, C2, SCX-2) or Anion exchanger (SAX, NH2, PSA, PE-AX)	Rinse with equilibrium buffer. *IMPORTANT NOTE:* The ionic strength of the buffer should not exceed 50 mM for singly charged analytes or 100 mM for doubly charged anions.

	Bed Mass	Rinse Volume, mL	Flow Rate, mL/min
Silica Based	25 mg	0.1-1	0.1-1
	50 mg	0.25-1	0.25-1
	100 mg	0.5-2	0.5-2
	130 mg	1-2	1-2
	200 mg	1-2	1-2
	500 mg	3-5	3-5
	1 g	5-10	5-20
ENV+	25 mg	0.5 – 4	0.5 – 4
	200 mg	4-8	4-8

Efficient elution of interfering species is dependent on adequate contact time. The smaller interference elution volumes given here should be used with the lower flow rates shown.

IS A DRYING STEP REQUIRED?

Often a drying step is included after other interferences have been removed. This is particularly important when an aqueous sample is loaded, and the analyte is eluted with a water immiscible solvent. A cartridge drying step is also required if the extract is to be evaporated to dryness. Polar sorbents require longer drying times than hydrophobic phases. See the chart below to determine drying time requirements for various phases and sorbent bed masses.

NOTE: The drying step may be significantly reduced or eliminated if a water miscible solvent is selected for the elution, but remember: there will be water in the extract, and so caution should be taken when concentrating the sample. Bulk water can be removed in ½ to 1 minute.

Sorbent	Bed Mass	Approx. Pore Volume, mL	Approx. Volume, Bulk Water Removed, mL	Drying Time (min)
C18	1 g	1.2	0.4	15
	500 mg	0.6	0.2	10
	100 mg	0.12	0.04	4
	50 mg	0.06	0.02	2
	25 mg	0.03	0.01	1
C8	1 g	1.3	0.5	20
	500 mg	0.65	0.25	15
	100 mg	0.13	0.05	6
	50 mg	0.07	0.03	4
	25 mg	0.03	0.01	3
C2 and Polar Phases	1 g	1.4	0.6	30
	500 mg	0.7	0.3	20
	100 mg	0.14	0.06	9
	50 mg	0.07	0.03	6
	25 mg	0.04	0.02	4
ENV+	200 mg	0.7	0.3	10
	25 mg	0.09	0.04	3

Drying times were determined at –20 inches vacuum or 4 litres/min gas flow

SELECT AN ELUTION SOLVENT

If final analysis is by HPLC, the end of run concentrations of the mobile phase gradient may be used as the elution solvent. If final analysis is by GC, use the following chart as a guide:

Sorbent	Analyte	Possible Solvent*
C18, C8, CH, PH, ENV+	Non-polar	1. Hexane 2. Methylene chloride
	Hydrophobic, some polar character	1. Acetone/Ethyl acetate 3:1 2. THF 3. Methanol, Acetone, Acetonitrile
	Neutral, acidic ……... Neutral, basic	1. Acidified methanol (see note below) 2. Acidified ethyl acetate (see note below) …… 1. Methanol/NH_4OH, 10% 2. Methanol/Trifluoroacetic acid, 1% 3. Methanol/Tetra-ethyl amine, 2%
PEAX, SAX, HAX, NH2-aqueous sample	Acidic	1. Hexane/Ethyl Acetate 75:25 with 1% acetic acid (see note below) 2. Acidified methanol (see note below)
SCX, HCX	Basic	1. Ethyl acetate/ammonia 98:2 2. Methylene chloride/Methanol/Ammonia 75:20:5
CBA	Basic	Ethyl acetate
SCX-2	Basic	Buffer, 0.1 M for singly charged ions, 0.2 M for doubly charged ions.
Polar Phases (SI, CN, NH2-non-aqueous sample)	Polar	1. Methanol 2. Methanol/hexane 1:1 3. Methanol/buffer

NOTE: 1% acetic acid or trifluoroacetic acid may be used to acidify elution solvent. Halogenated solvents should not be used for subsequent GC-ECD detection.

* The recommended solvents represent a starting point, however the composition of the eluting solvent must be optimized.

The following chart can be used as a guide for determining appropriate elution solvent volumes and flow rates:

	Bed Mass	Elution Volume, mL	Flow Rate, mL/min
Silica Based	25 mg	0.1-0.5	0.05-0.2
	50 mg	0.25-1	0.1-0.05
	100 mg	0.5-2	0.25-1
	130 mg	1-2	0.5-1
	200 mg	1-2	0.5-1
	500 mg	2-4	1-2
	1 g	3-6	1.5-3
ENV+	25 mg	0.2-1	0.1-0.5
	200 mg	2-5	1-2.5

Efficient elution is dependent on adequate contact time. The smaller elution volumes given here should be used at the lower flow rates shown. Alternatively, the column can be loaded with half the elution volume given above, soaked for 2 minutes, and then eluted with the second half of the elution volume. Both fractions are collected into a single collection tube. When a soak step like the one described here is included, quantitative elution is much less flow dependent.

Version 1.2

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