THE FINAL WORD

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INSIGHT OF 'MY IC'

SAMPLE PROCESSING TECHNIQUES IN BIO ANALYSIS

Basically there are three techniques, which are used in bio analytical sample preparation.

1) Precipitation (PPT)
2) Liquid liquid extraction (LLE)
3) Solid Phase extraction (SPE)

1)PRECIPITATION

"The underlying mechanism of precipitation is to alter the solvation potential of the solvent, more specifically, by lowering the solubility of the solute by addition of a reagent."

In general the use of organic vaporizable solvent is preferred which have minimum effect on the compound of interest and suitable for instrument of analysis. We generally use Methanol, Acetonitrile as universal solvents.

Precipitation of blood can be done either with the above solvents and sometimes with ZnSO4, TCA and others.

Precipitation techniques can be used when you had got a good protein binding ability of drug, to find about protein binding of particular drug it can be found on online sites as drugbank. The more percent of protein binding gives us an idea that drug can be extracted by disruption of protein, that is what is been mentioned in the start of the discussion. "Solute=drug, reagent=our solvent"

Advantages of protein precipitation: Fast method of extraction, Low solvent requirement against LLE, more cost effective.

Disadvantages of Protein precipitation: High amount of unknown interference (phospholipids), considered less clean techniques then LLE and SPE, Sample reproducibility is a challenge in many cases.

Sample Processing Care:

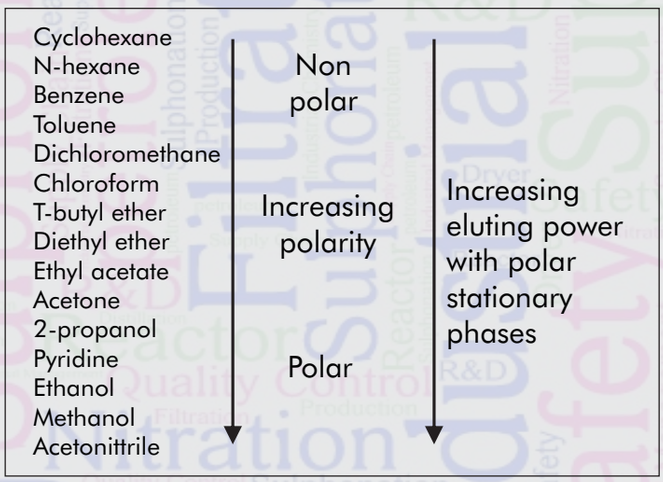
- Amount of Solvent addition (It can decide the % recovery of analyte of interest also uniformity is to be maintained through-out for even extraction)
- Vortex time/mixing time shall be monitored.
- Centrifugation time shall be optimized for better and uniform results.
- PPT evaporation shall be

2)LLE

Liquid-liquid extraction (LLE) is widely used as a simple and robust sample preparation technique in bio analytical sample preparation. When extracting ionisable compounds, most bio analysts adjust the pH of the sample to achieve fully unionized compounds. Usually, a generally accepted rule is applied to adjust the pH of the aqueous phase, known as the pKa+/-2 rule, depending on the acid/base characteristics of the analyte.

For acidic compound = Adjusting pH below Acidic side or pKa(-2) is more favorable . While for Basic compounds = Adjusting pH above Basic side or pKa(+2) is more favorable.

Also addition of buffer to adjust pH of sample is also playing role in uniformity of final extraction, recovery will always be point to be seen when selecting buffer, it is quite possible some compounds will not require any pH conditions to be extracted, but that will always open a chance of response variation.



Based upon the choice of solvent we can optimize recovery and reproducibility for extracted samples. Considering the above fact that is been shown in the table, we also shall find data if the water solubility of particular solvent is there or not, as water solubility increases the polarity increases and we here intend to extract with non-polar solvents. In case if you need to use double LLE or SPE LLE or PPT LLE, density difference will help you to separate your solvent layers, and with practical approach (1:2) (1:3) can be checked to find out whether to aspirate or discard the unwanted layer.

Sample Processing Care:

- Amount of extraction solvent taken to extract shall be optimized to check the response variability, It shall be checked that extraction response with 3 ml of solvent shall not vary more that 10% when extraction solvent is used 2 ml. This will ensure that method is robust and not prone to variability. (The above 10% shall be checked for both analyte as well as ISTD)
- Try to use Techniques like flash freezing for optimum sample clean up, rather than separating regular way.
- Keep a note on the lot numbers of solvents that are been used during the sample analysis, try to keep them uniform, which will ensure and eliminate doubt or any uncertain result due to solvent.
- For even sample extraction and low variability evaporation drying time shall be monitored and ensure complete drying before reconstitution.
- Vortex time shall be optimized for even recovery and sample reproducibility.
- During evaporation ensure that nozzles of evaporator are wiped with methanol before placing the samples, this will minimize the chance of possible contamination
- Remaining extraction solvent used during sample processing shall be discarded after a batch processing, rather than putting it back to the stock solvent.

- A continuous without pulse vortex is more suggested , rather than pulse vortex, this is not a mandatory requirement but sample results with types of solvent shall be compared.

3)SOLID PHASE EXTRACTION

Above two methods are used with bio analysis, but when after performing the above two techniques still sample is unsuitable for analysis and need for a cleaner sample is required, we can go for solid phase extraction. The reasons can vary to select SPE processes are from:

- **Too dirty** - contains other sample matrix components that interfere with the analysis.
- **Too dilute** - analyte(s) not concentrated enough for quantitative detection.
- Present **sample matrix not compatible** with or harmful to the chromatographic column/system.

General SPE approaches:
Bind & Elute Strategy

- Most common
- Bind: Analytes bind to tube, matrix comp. are washed off
- Elute: Eluent changed to remove analytes from tube
- Eluate is concentrated prior to HPLC or GC analysis

Fractionation Strategy (Form of Bind & Elute)

- Retain and sequentially elute different classes of compounds by modifying eluent pH or % organic.

Different type Retention which contains reversed phase, normal phase and ion exchange retentions.

Reversed phase retention: non-polar, hydrophobic interactions (lipophilic compounds), it will retain via vander waals or dispersion forces.

Normal Phase retentions: Polar interaction, dipole-dipole,Pi-Pi, Hydrogen bonding. Analyte with hydroxyl group, carbonyls, amines, functional group with resonance properties.

Ion Exchange retentions: Electrostatic attraction of charged functional groups of analyte to oppositely charged functional groups on the sorbent.
Eg. If sorbent is containing SO3- group it will attract and bind NH3+ group present in sample analyte. The above type of exchange is called strong cation exchange, and vice versa for –NH4+ (Quat Ammonium) Group will attract and bind to CH3COO- group present in sample analyte , this type of ion exchanges are known as Strong anion exchange.

Types of ion exchange:
Mix Mode Cation Exchange (MCX) – Useful to retain Compounds with Positive charge.
Mix Mode Anion Exchange (MAX) – Useful to retain Compounds with Negative charge.

For cation exchange samples, acid wash can be optimized after sample loading to get a cleaner sample, while during elution it will follow like dissolves like fundamentals and applying a high strength of ammonium hydroxide in Methanol(5% or as optimized) will elute the sample.

Similarly for Anion exchange samples, basic wash can be optimized after sample loading to get a cleaner sample, while during elution it will follow like dissolves like fundamentals and applying a high strength of Formic acid in Methanol(2% or as optimized) will elute the sample.

HLB : Hydrophilic lipophilic balance is a reversed phase stationary phase made up from a specific ratio of two monomers, hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene. Universal sorbent for acidic, basic, and neutral compounds.

Other modes are also available like WAX and WCX, these are for weaker acids and weaker base compounds, which will serially follow MCX and MAX respectively.

General HLB protocol
Condition :1 ml CH_3OH
Equilibrate : 1 ml H_2O
Load: 1 mL spiked sample** (generally acidic conditions/ 2 units lower than its PKa
Wash (Mild Wash): 1 mL 5% CH3OH in H2O
Elute (Strong Elution): 1 mL CH3OH
Evaporate and Reconstitute: 40 °C/under nitrogen stream
200 ML mobile phase

If the fraction from this step contains the analyte, make this adjustment for optimum sample recovery:

Load: The Oasis HLB sorbent has been found to retain ionizedanalytes more strongly than silica-based reversed-phase sorbents. However, recoveries may be enhanced whenanalyte ionization is suppressed.
For acidic analytes,adjust the sample pH to at least two pH units below thepKa of the acid.
For basic analytes, adjust the pH to atleast two pH units above the pKa of the conjugate acid.

Wash : Recoveries of very polar analytes can be increased by using only 1 mL of water (not 5% methanol in water) as the wash solution.

First Elution: If an acceptable recovery of analyte(s) is obtained in this fraction (usually > 90%), no adjustments are necessary.

Second Elution:
For very nonpolar analytes, stronger solvents such as acetonitrile, methylene chloride or ethyl acetate may be substituted, or used in sequence. In addition, for ionizable analytes, methanol may needed to be modified with the addition of 2% acid or 2% base, as appropriate. If solvents stronger than methanol or acetonitrile are used for the elution, then a preliminary conditioning step should be performed prior to the methanol conditioning step. For example, if ethyl acetate is to be used as an eluent, condition the cartridge with 1 ml of ethyl acetate, followed by 1 ml of methanol and 1 ml of water.

Generic Oasis MCX (pKa 2-10) Method for Extraction of Basic Compounds(WAX Also pKa <1 Strong Acid)

Condition: CH3OH
Equilibrate: H2O
Load: Spiked and acidified sample
Wash 1: 2% HCOOH in H2O
Elute 1 (Wash 2): CH3OH
Elute 2: 5% NH4OH in CH3OH
Evaporate and Reconstitute: 20% CH3OH in H2O

Generic Oasis MAX (pKa 2-10) Method for Extraction of Acidic Compounds(WCX Also pKa> 10 strong bases)

Condition: CH3OH
Equilibrate: H2O
Load: Spiked and acidified sample
Wash 1: 5% NH4OH in H2O
Elute 1 (Wash 2): CH3OH
Elute 2: 2% HCOOH in CH3OH
Evaporate and Reconstitute: 20% CH3OH in H2O.

Sample Processing Care:

- While using RP phases like C18 it shall be taken care that SPE tube drying shall not be done , during conditioning and before sample loading.
- Select on the polarity from non-polar C18, C8, Ph and CN polar SPE tubes as per requirements.
- Over- drying care shall be taken care in each and every step for the above mentioned phases.
- Tubes arrangements in SPE and functioning of SPE will always decide the uniformity of extracted samples.
- Only in some cases you can give wash of mid polar solvents like Acetone, or non-polar solvent DCM, can be used with C18 phases not suitable for RP phases.

- During evaporation ensure that nozzles of evaporator are wiped with methanol before placing the samples, this will minimize the chance of possible contamination.
- Applying gradual pressure for proper elution is required.
- Always check the pressure before elution or any step during processing to avoid any sample error for quantification.
- Always ensure that tube is completely eluted before next SPE step.
- Always ensure uniform volume in all tubes during SPE processing.
- By Centrifugation of samples before loading will help throughout smooth sample processing.
- For low intensity compounds elution in parts can maximize the recovery upto 30%. (500μL+500μL)
- Try to minimize use of basic buffers during sample preparation when using RP or phases like C18 this has shown phospholipids interferences or ion enhancements in the

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IC 2002-2004

SPORTS ACHIEVEMENTS OF 'MY IC'

- Winner of interclass cricket tournament
- Runners up in volleyball interclass tournament

PLACEMENT STATUS OF 'MY IC'(JANUARY-2018)

sr. no.	Name of the industry	Post	NO. of students selected
1	LAMBADA, AHMEDABAD	Research Associate-Bio Analytical	1
2	GLENMARK PHARMA, ANKLESHWAR	QA OFFICER	2
3	LUPIN LTD., BHOPAL	PRODUCTION OFFICER	3

NATIONAL SEMINAR ORGANIZED BY 'MY IC' WITH DEPARTMENT OF CHEMISTRY-SHUATS, ALLAHABAD ON 20TH JANUARY, 2018

NATIONAL SEMINAR ON ADVANCEMENT IN CHEMICAL TECHNOLOGY-A GREEN PERSPECTIVE