•Dried blood Spot • LC-MS • Untargeted metabolomics

Evaluation of Dried Blood Spot Extraction Strategies for Untargeted Metabolomics Workflow

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Background

Dried blood spot (DBS) collection represents an extremely convenient decentralized blood micro-sampling method for its limited invasiveness and minimal storage space requirement. To merge this offline sampling method seamlessly into the liquid chromatography-mass spectrometry (LC-MS) based untargeted metabolomics workflow, it is critical that all metabolites can be recovered from the DBS with high efficiency. While metabolite extraction protocols from traditional matrices, such as plasma, have been reported, the protocol transfer to release metabolites from millimeter sized DBS punch is not straightforward due to DBS specific metrics. In this project, the optimal extraction strategy for working with milligrams of paper matrix sorbent saturated with microliters of blood will be investigated from the extraction efficiency and the workflow automation compatibility consideration.

Highlights

- An internal standard QC panel was designed to cover the entire m/z and polarity range of the human metabolome.
- The pre-analytical practice for various DBS processing stages, including the choice of recovery solvent before reconstitution, processing tube material, are systematically investigated.
- We have confirmed that the choice of reconstitution solvent for injection can have significant effect on analyte recovery. The sample processing tube also shows compound specific differential binding effect.
- We have determined the compound specific differential partitioning behavior between paper matrix and various solvent systems. We have confirmed that solvent systems that have high partitioning ratio do not necessarily have high extraction efficiency to release metabolite from the paper matrix in dehydrated state.
- We report that the extraction efficiency can be normalized with well-defined experiments.
- **Here we recommend the use of 1:1 H2O:IPA as the final reconstitution solvent for injection, and MeOH as the extraction solvent for automation and throughput considerations. We currently Eppendorf "pcr clean" tubes, but, are investigating these effects.**

Evaluation Strategy

A DBS QC panel, including CIL Metabolomics QC1 & QC2 kit, Avanti Splash mix kit, and individual metabolites to cover the m/z and polarity range of the metabolome.

A HILIC method (Figure 1(a)) and RPLC method (Figure 1(b) & 1(c)) were then optimized for the DBS QC panel and the possible adduct ion formation for each DBS QC panel species was performed on an Agilent 1290 Infinity UPLC system hyphenated with TIMSTOF Pro2 mass spectrometer equipped with VIP-HESI source.

Extraction strategy evaluation was carried out in a quantitative and backward approach: Fixed volume aliquot of the DBS QC solution at pre-determined concentration was used as the starting material at each step along the DBS pre-analytical workflow in the backward fashion. The Reconstitution solvent recovery effect, sample processing tube recovery effect, and extraction solvent & DBS partitioning effect, were investigated and the resulting recovery ratios were then used for data normalization purposes.

Results

H2O:IPA was our best solvent system for metabolite recovery and Eppendorf "PCR clean" tubes are (currently) recommended as the final dry & reconstitution processing tube from the automation perspective. While $CHCl_3$ is widely used for liquid sample based lipid extraction, its partitioning ratio was unmeasureable for polar metabolites and low for phospholipids against paper matrix, the requirement for glassware to handle $CHCl_3$ further argues against $CHCl_3$ for DBS extraction.

For DBS extraction, organic based solvent systems are favored from the throughput perspective, as they do not release dried blood debris from paper, while aqueous based solvent systems can release the dried blood from the paper, indicating more downstream sample clean-up steps are required. However, this is likely because we are extracting from a large spot of dried blood, and the ratio of extraction solvent to blood volume is too low. Future experiments will be focused on re-designing the QC panel concentration so that smaller sub-punches and less blood can be used for re-evaluation.

Additional Findings

We also found that the metabolite-on-paper stability needs further investigation. For example, alpha-ketoglutaric acid is poorly recovered using all aqueous solvent systems and MeOH in the extraction control experiments, which all have high partitioning ratio for alpha-ketoglutaric acid.

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Conflicts of Interest

There are no conflicts of interest to report.

DBS QC Elution Profile under HILIC & RPLC

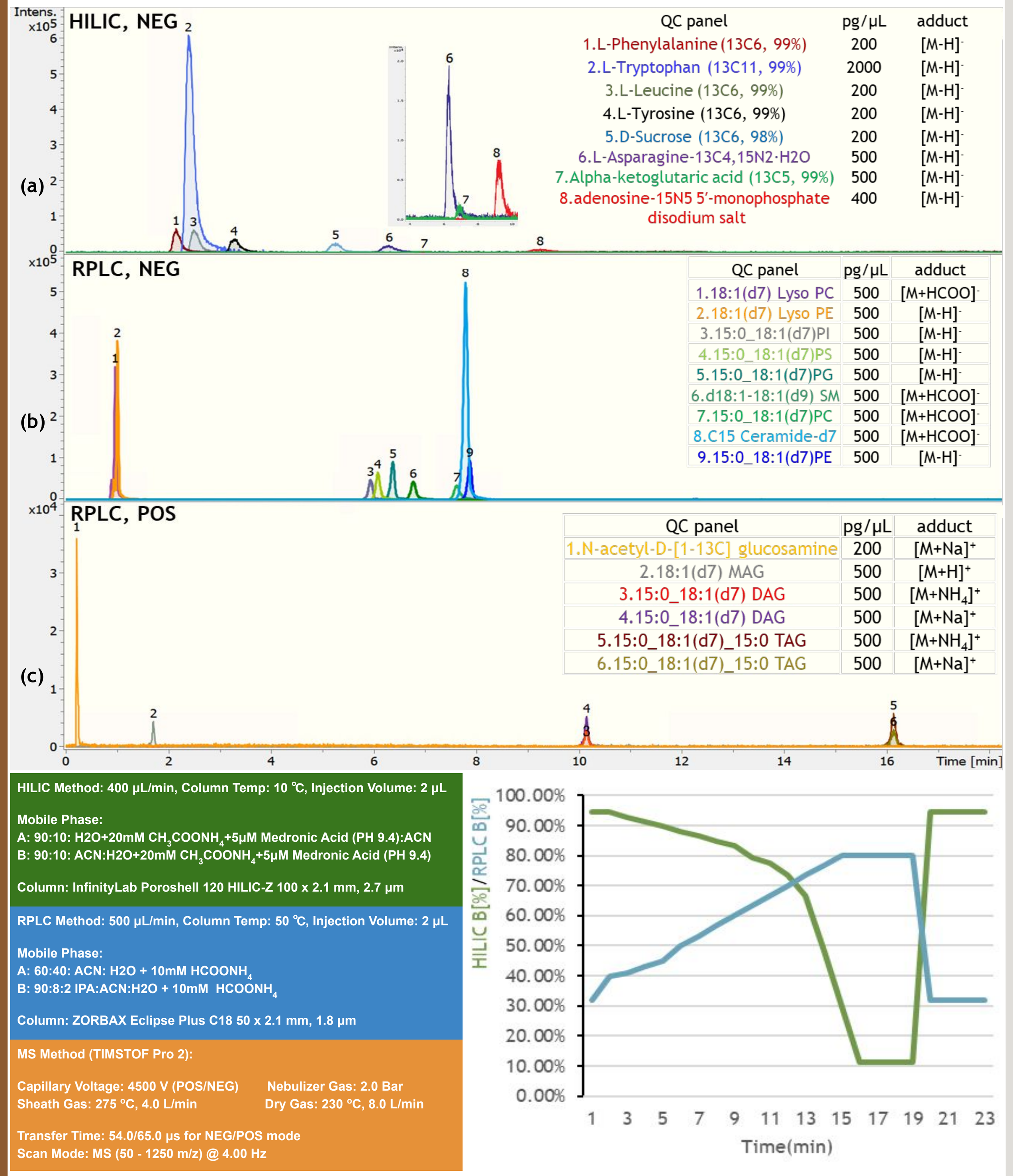
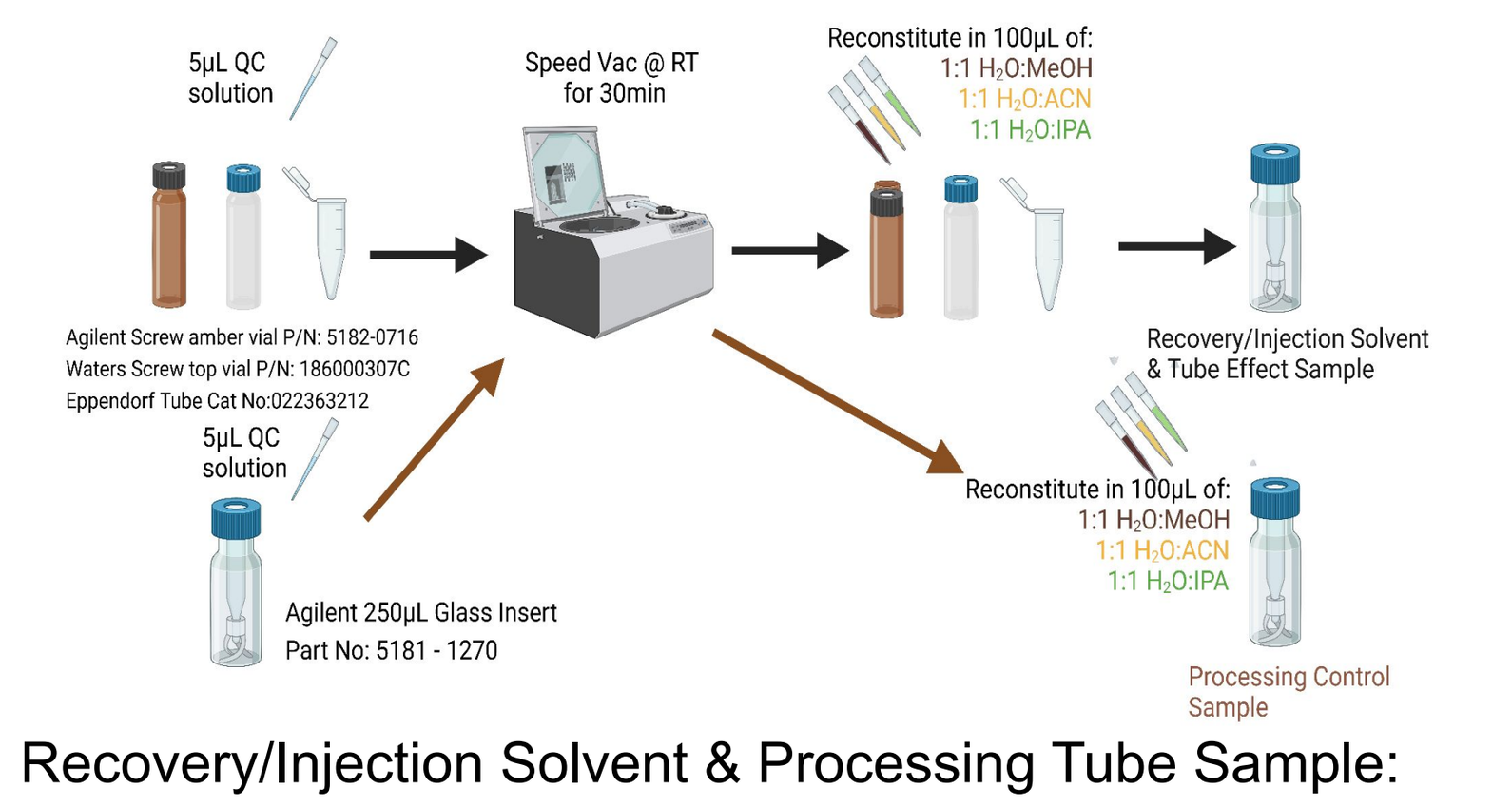


Figure 1: Overlaid Extracted Ion Chromatograms (EICs) showing QC panel elution profile under HILIC (a) and RPLC (b) and (c). The QC compound identification is achieved by either RT matching with other standards (e.g, Standard H for amino acids) or through MS/MS level validation (e.g, lipid fragmentation pattern). The DBS QC panel information, including the adduct form used for EIC extraction and stock concentration, is shown in insert. The optimized LC and MS methods are shown at the bottom.

Recovery/Injection Solvent & Processing Tube Effect

1:1 H_2O:IPA is our best recovery solvent for DBS QC panel
Waters Vial & Eppendorf tube have comparable recovery ratios



Despite the many degrees of freedom in DBS pre-analytical workflow design, the final step is usually common, which consists of reconstitution of dried extracted sample for injection. Thus, it is important that the injection solvent can recovery analytes from the processing tube with high efficiency.

Recovery/Injection Solvent & Processing Tube Sample: 5 uL of DBSQC solution pipetted into processing tube, after speed vacuum dry for 30 min at room temperature, 100 uL of 1:1 H2O:MeOH, H2O:ACN, H2O:IPA is added to the tube for recovery and transfer into glass insert.

Processing Control Sample: 5 uL of DBSQC solution directly pipetted into glass insert, after speed vacuum dry for 30 min at room temperature, 100 uL of 1:1 H2O:MeOH, H2O:ACN, H2O:IPA is added to the glass insert for reconstitution.

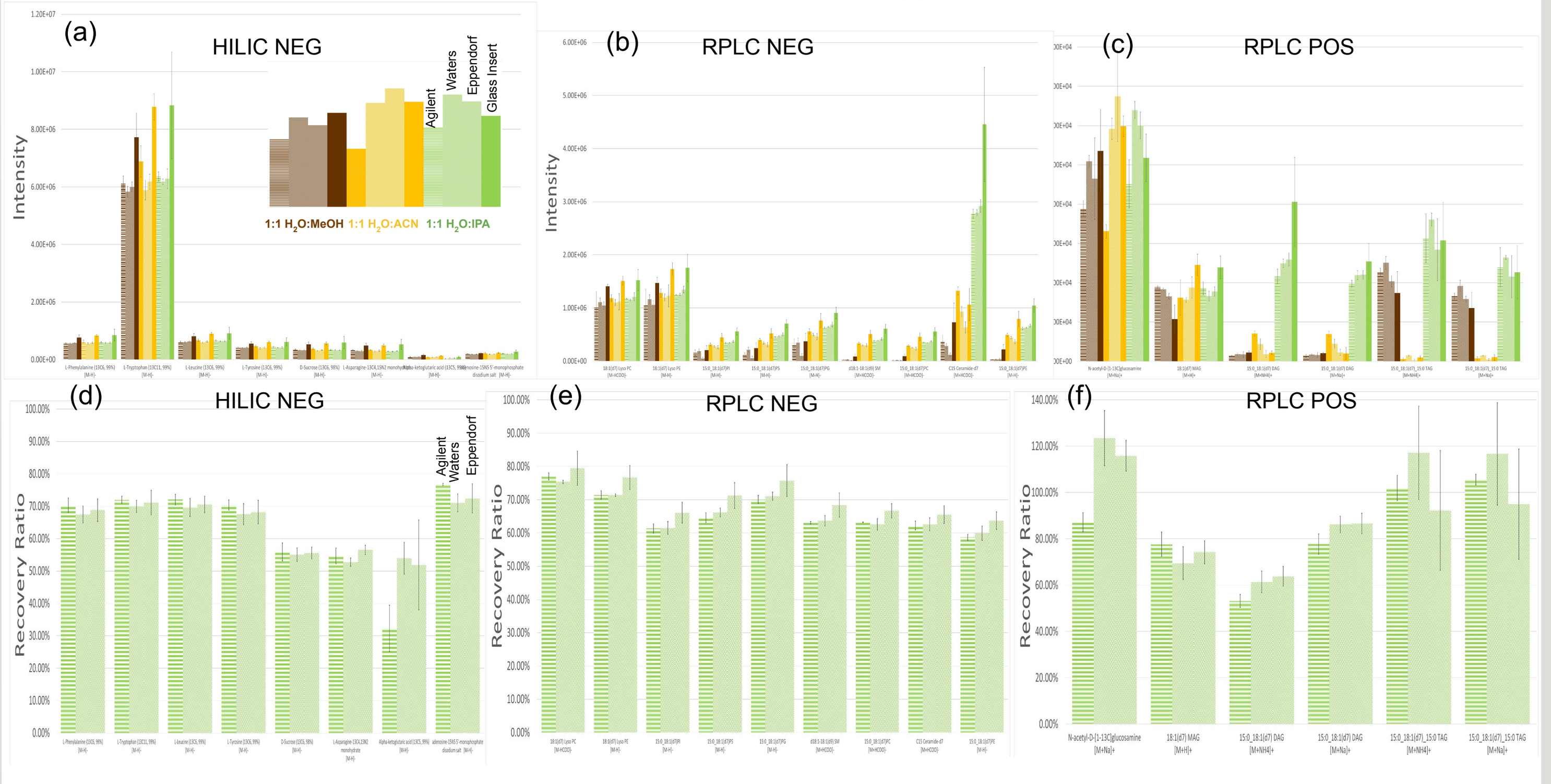
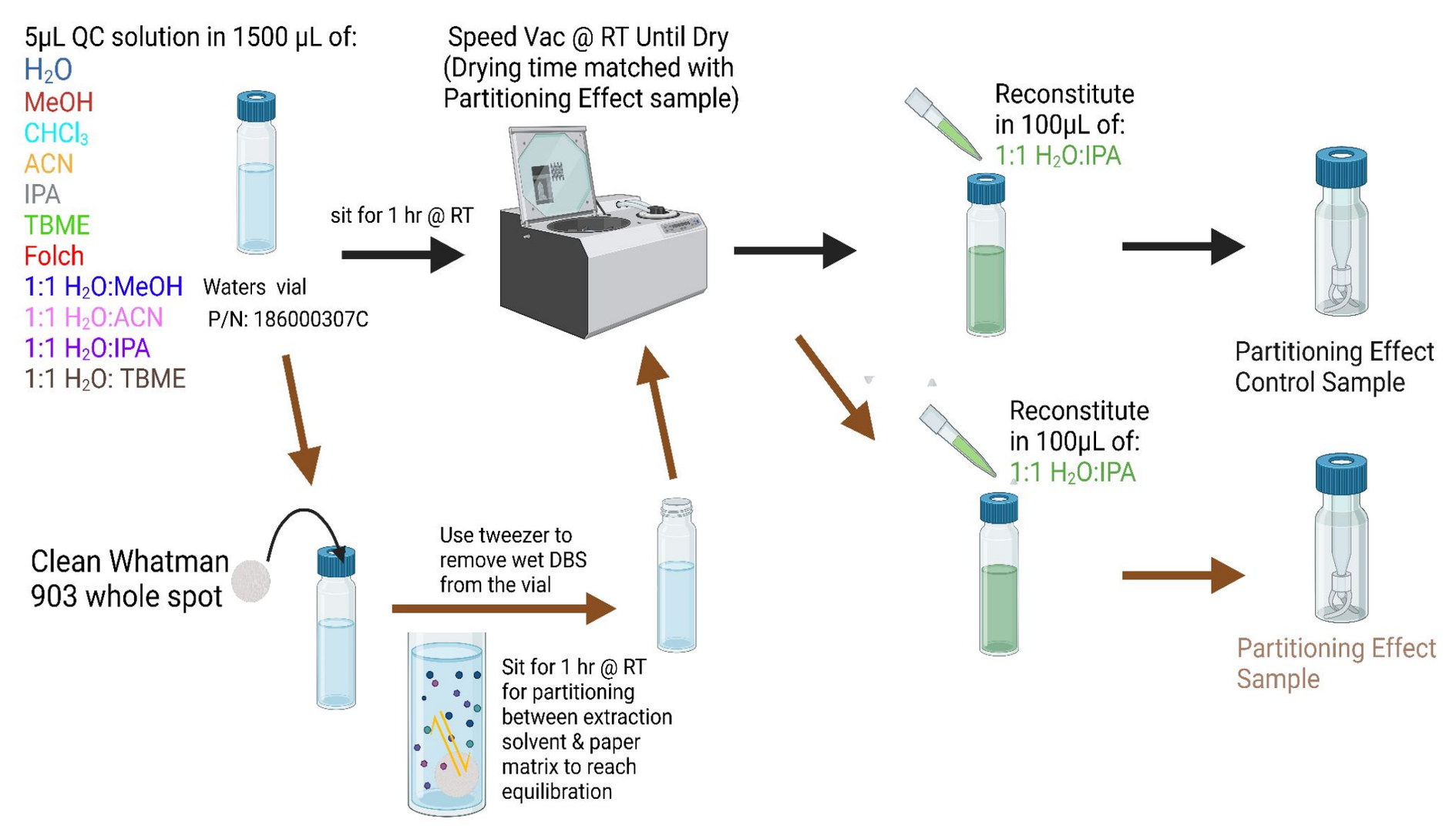


Figure 2: Recovery/Injection solvent (color coded) & processing tube (pattern coded) selection decision making based on absolute intensity and recovery ratio. (a)-(c) indicate 1:1 H_2O:IPA has comparable intensity among polar metabolites and the highest intensity across the lipids panel. (d)-(f) show the processing tube effect on recovery ratio across the QC panel, with Waters vial and Eppendorf tube being comparable in the recovery performance. Recovery ratio = QC panel EIC peak area from recovery & processing tube sample /QC panel EIC peak areafrom process control sample

Extraction Solvent & Paper Matrix Partitioning Effect

$CHCl_3$ has negligible partitioning ratio for polar metabolites and is low for phospholipids against paper matrix



A high partitioning ratio corresponds to low percentage of compound being absorbed into the paper matrix (better).

The extraction solvent lists are from literature search and they covers a wide polarity range; With $CHCl_3$ and Folch in use, Waters glass autosampler vial is selected as the processing vial.

The partitioning ratio of DBS QC panel between various solvent systems and blank DBS paper spot is an alternative way for determining the suitable extraction solvent. Compound specific partitioning preference between extraction solvent and paper matrix at equilibrium is observed.

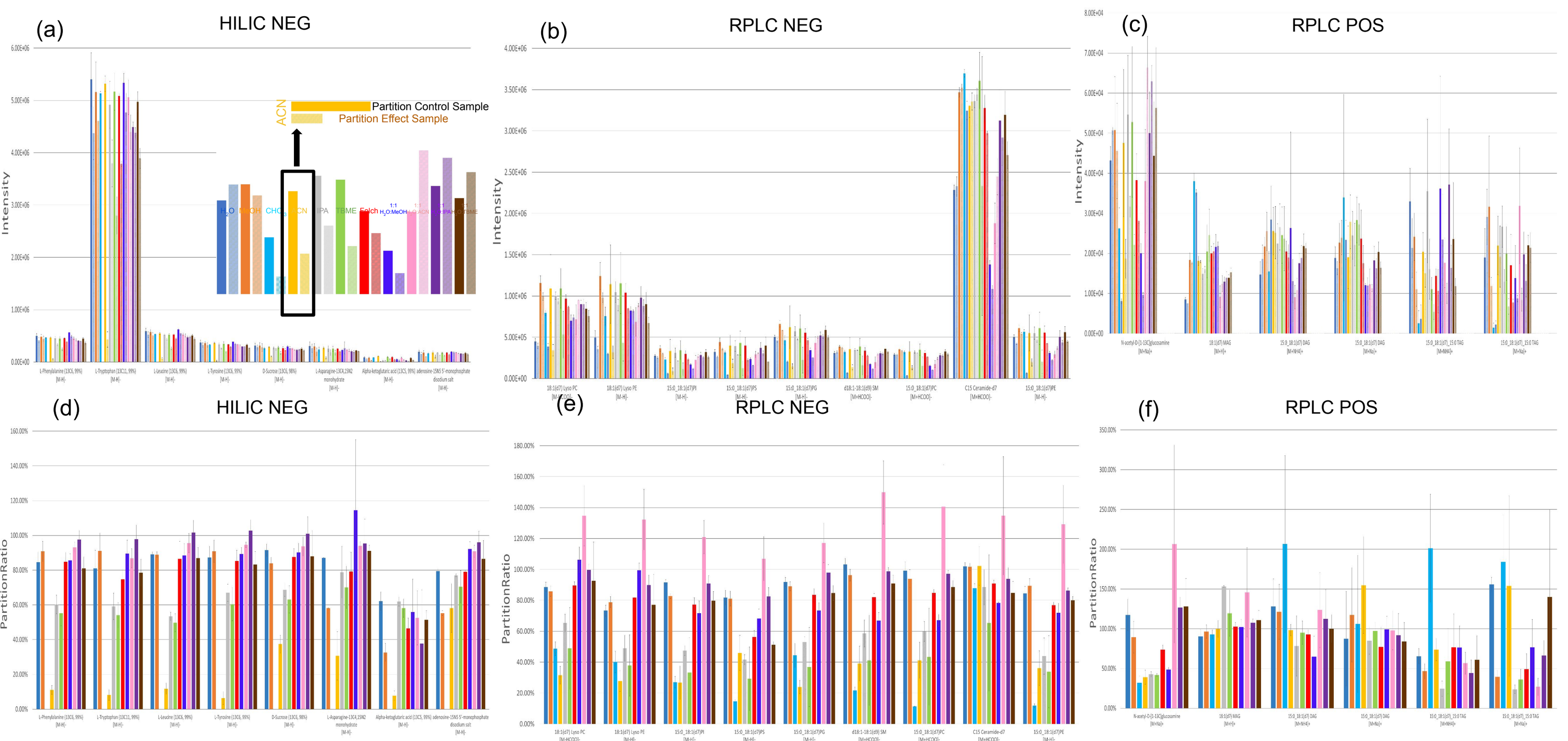
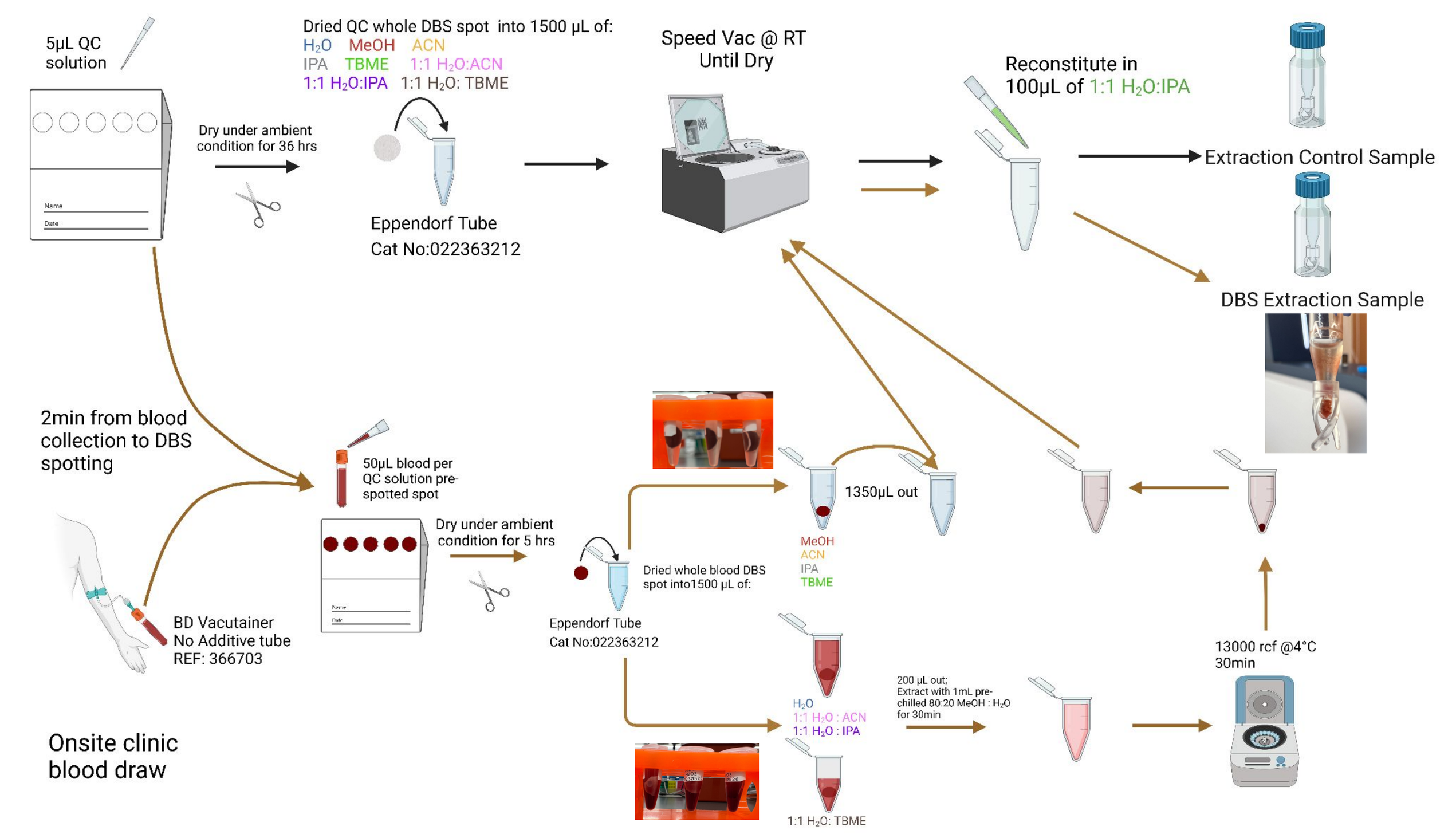


Figure 3: DBS QC panel partitioning behavior between various solvent (color coded) and paper matrix. (a)-(c) show QC panel EIC intensity variation between Partitioning Effect Control Sample and Partitioning Effect Sample(same color,pattern coded). In the $CHCl_3$ case, all polar metabolite EICs are at noise level, indicating their preference to partition into paper matrix against $CHCl_3$. (d)-(f) show the QC panel partitioning ratio.

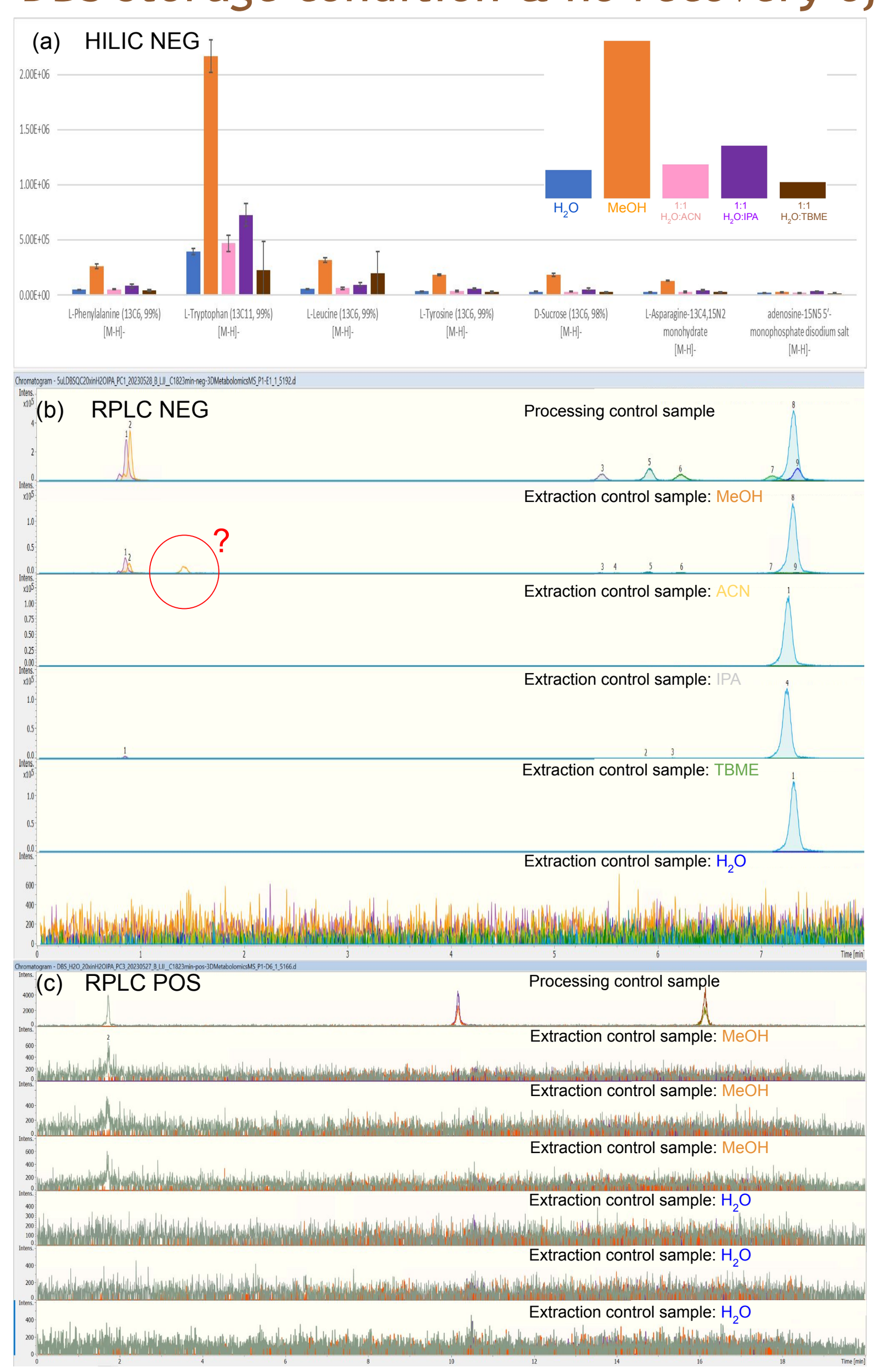
DBS Extraction Evaluation Strategy



5 uL of DBS QC solution was spotted onto Whatman 903 paper and dried under ambient condition for 36 hours prior to the blood draw. No Additive Vacutainer tube is used for onsite intravenous blood draw and quickly spotted onto QC solution pre-conditioned spots. The extraction control spots and DBS spots are processed 5 hours later together. For all extraction control spots, extraction was performed by equilibrating with the extraction solvent for 1 hour with occasional vortexing, the extracted sample was sent for downstream drying and reconstitution with the spot removed from the eppendorf tube. For all DBS extraction samples, after 1 hour of equilibration with the extraction solvent, organic extraction do not release cell debris from the paper; Whereas for all aqueous based extraction solvents, extra sample clean-up steps are required as the cell debris got released from the paper. With pre-chilled 80:20 MeOH:H2O for further protein precipitation, extraction, and dilution, the final sample was still too concentrated to not clog the column. Further experiments using more concentrated QC solution and less blood volume on smaller spots (e.g, pre-cut sub-punches) are planned.

MeOH Works Best in Breaking the Metabolite/Paper Interaction in Dehydrated State

DBS storage condition & no recovery of non-polar lipids still needs further investigation



The **Extraction Control** sample directly reflects the efficiency of the extraction solvent in breaking the intact interaction between QC component and paper matrix in the dehydrated state. While MeOH does not have the highest partitioning coefficient across the QC panel, **the extraction control experiments indicate MeOH is most efficient in extracting out polar metabolites and phospholipid QC components from the dried paper matrix.** The ideal extraction solvent thus shall be capable of breaking the paper/analyte interaction in dehydrate condition first and also have a high partitioning coefficient as in the liquid equilibration condition.

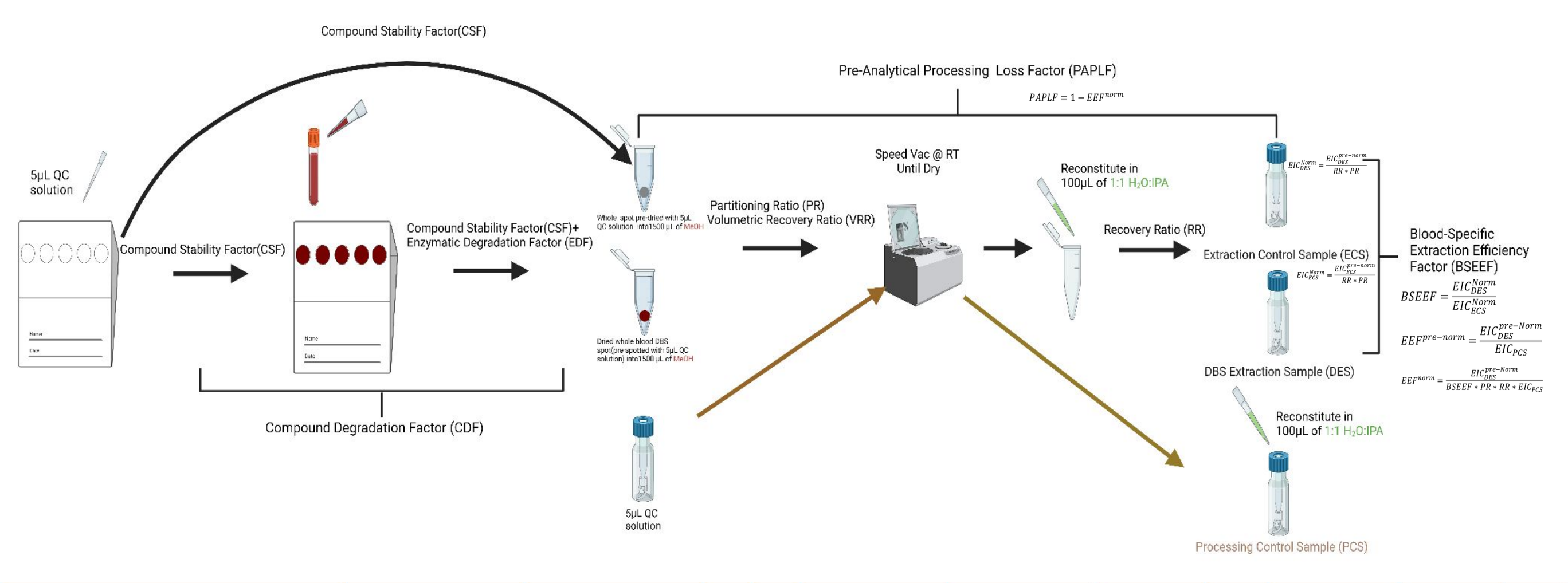
It is surprising H_2O, in our hands, is less efficient in extracting out polar metabolites and very poorly recovers lipids from the paper, its partitioning ratio is high across the DBS QC panel.

The limited recovery of monoacylglycerol species and no recovery of diacylglycerol and triacylglycerol lipid species needs further investigation (compare to Fig 1c). Storage condition (including photobleaching and/or oxidation) is an obvious direction; Paper-assisted acyl migration is another assumption for future investigation.

Figure 4: Extraction efficiency comparison for different extraction solvents using selected DBS QC panel EICs under HILIC and RPLC. In (a), under HILIC NEG mode, all polar metabolites except alpha-ketoglutaric acid (why?) can be extracted using aqueous based solvent and MeOH, which has the highest intensity, while all other organic solvents seem incapable of extracting out polar metabolites from paper (data not shown). In (b), phospholipid panel extraction efficiencies for all organic solvent and H2O are compared, MeOH can recover all phospholipids as compared to the processing control sample despite at lower intensities, other organic solvents can all extract out ceramide with comparable intensity to that of MeOH, yet very limited recovery for other phospholipid species, H_2O extraction returns only noise level signal. In (c), only limited recovery of the MAG species for MeOH extraction, as compared to the processing control sample.

Extraction Efficiency Normalization Strategy

Pre-analytical normalization parameters can be calculated from well-defined experimental results



HILIC_NEG_MeOHExtraction	EIC_Prenorm_ECS	EIC_Prenorm_DES	PR	RR	EIC_Norm_ECS	EIC_Norm_DES	EIC_PCS	BSEEF	EEF_Prenorm	EEF_Norm	PAPLF
L-Phenylalanine (13C6, 99%)	259218	83091	0.91	0.69	402798	133007	851626.5	0.33	0.10	0.47	0.53
L-Tryptophan (13C11, 99%)	2167679	1119159	0.91	0.71	3262164	1727950	8830536	0.53	0.13	0.37	0.63
L-Leucine (13C6, 99%)	317817	178919	0.89	0.71	493831	285203	910768.75	0.58	0.20	0.54	0.46
L-Tyrosine (13C6, 99%)	181156	149892	0.91	0.68	290075	241766	612992.75	0.83	0.24	0.47	0.53
D-Sucrose (13C6, 98%)	184101	150822	0.84	0.56	385399	323545	589128.25	0.84	0.26	0.65	0.35
L-Asparagine-13C4,15N2 monohydrate	127157	66992	0.58	0.57	390715	203560	529349.75	0.52	0.13	0.74	0.26
adenosine-15N5 5'-monophosphate disodium salt	25684	6063	0.55	0.72	64034	15143	272421	0.24	0.02	0.24	0.76

Table 1 : Extraction efficiency normalization for MeOH extraction using polar QC metabolite panel EICs under HILIC NEG mode. Each factor/ratio calculation formula is defined and can be found on the upper normalization strategy workflow. The lipid QC panel result is not shown here due to high concentration of endogenous lipids sharing the same m/z.

While the stability of many compounds preserved on DBS is know, thru this work, we attempt to begin the formulation of a method by which *class based* measurement corrections for the *extraction* of compounds can be determined as well.

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