

Optimization of sample preparation protocols for secretome metabolomics

Lena Ludwig-Radtke

Marburg University

Xenia Nagel

Marburg University

Merle Horrelt

Marburg University

Annika Block

Marburg University

Jelena Pesek

Marburg University

Maximilian Graf

Fresenius University of Applied Sciences

R. Verena Taudte

`verena.taudte@uni-marburg.de`

Marburg University

Research Article

Keywords: metabolomics, secretome, secreted metabolites, sample preparation, tumor microenvironment

Posted Date: October 26th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11295119/v1>

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Additional Declarations: No competing interests reported.

Abstract

Introduction

Metabolomics is widely used to characterize physiological or pathophysiological states, with oncology representing one area of particular interest. A growing research focus within this field is metabolic crosstalk in the tumor microenvironment (TME). Different cell populations can be present in the TME and influence one another. This multidirectional cell-to-cell crosstalk is mediated by secreted factors, including proteins, cytokine, chemokines or metabolites.

Objective

To analyze which metabolites are secreted from cells, a protocol for metabolomics-based secretome analysis is needed that also allows for a harmonized secretome analysis and thus lab-to-lab comparisons.

Methods

We systematically compared 10 previously reported solvent systems, four solvent-to-sample ratios and four extract preparation techniques to identify the optimal conditions for secretome analysis by mass spectrometry (MS)-based metabolomics. Evaluation was based on number of annotated metabolites and reproducibility.

Results

The optimized protocol consists of metabolite extraction using MeOH with 1% formic acid at a 4:1 solvent-to-sample ratio, followed by drying the extracts in a SpeedVac at 4°C. Dried extracts are then reconstituted in starting eluent and analyzed by liquid chromatography-MS.

Conclusion

We developed a standard operating procedure for holistic secretome analysis. In addition, we provide recommendations for optimal detection of individual compound classes. Together, this SOP establishes a harmonized basis for sample preparation and analysis of secreted metabolites from biological samples.

Introduction

Omics technologies have transformed biological research by providing detailed insight into the molecular architecture of living systems (Dai and Shen, 2022). Metabolomics, the most recent of these disciplines, identifies and quantifies small molecules (typically < 1,500 Da) that collectively constitute the metabolome. Because metabolite levels integrate genetic, transcriptional, and environmental influences, metabolomics provides a direct readout of physiological and pathophysiological states (Gonzalez-Covarrubias et al., 2022; Hajnajafi and Iqbal, 2025), with oncology among its most active fields of application (Gonzalez-Covarrubias et al., 2022; Rowles and Patti, 2026).

Within oncological research, the tumor microenvironment (TME) is now recognized as a critical determinant of tumor progression and treatment response (Madden et al., 2020) (Madden et al., 2020; Martínez-Reyes and Chandel, 2021). It comprises malignant cells, heterogeneous stromal and immune cell populations, secreted factors, and extracellular matrix (ECM) (de Visser and Joyce, 2023; Kim et al., 2026). Cancer cells shape this niche by reprogramming neighboring stromal and immune cells and by remodeling the ECM through proteolytic degradation or increased deposition (Mir et al., 2025; Zhao et al., 2023). These interactions and intercellular communication in the TME is mediated in part by secreted metabolites, which can act as signaling molecules that promote tumor growth and suppress antitumor immunity (Elia et al., 2022; Elia and Haigis, 2021; Mao et al., 2026). Among the oncometabolites, i.e., metabolites that accumulate aberrantly owing to dysregulated metabolic pathways, lactate and kynurenine are the best characterized (Sun and Wesolowski, 2021). Aerobic glycolysis (Warburg effect) leads to extracellular lactate accumulation and acidification, which promote tumor progression and impair immune effector function (Gu et al., 2025; van Weverwijk and de Visser, 2023). Kynurenine, generated from tryptophan via indoleamine 2,3-dioxygenase (IDO), is elevated in multiple cancer types and suppresses T-cell activity while promoting regulatory T-cell differentiation (Basson et al., 2023; Stone and Williams, 2023).

Beyond these examples, secretome composition and the immunoregulatory functions of individual secreted metabolites remain however incompletely understood. To address this gap, a robust, standardised workflow for secretome analysis via metabolomics is required. Within metabolomics, liquid chromatography–mass spectrometry (LC-MS) has become the method of choice. This is due to the fact, that high sensitivity and identification via accurate mass and spectral library matching is combined with chromatographic separation that reduces ion suppression from co-eluting compounds (Sitnikov et al., 2016). However, sample preparation is a critical determinant of the outcome, as extraction efficiency governs both metabolite coverage and extract concentration (Roca et al., 2021).

Previous comparative studies of extraction methods have predominantly addressed blood-derived matrices (Lepoittevin et al., 2023), whereas optimizations for secretome samples remain scarce. The two sample types differ considerably: plasma and serum contain 60–80 mg/mL protein, whereas conditioned media contain substantially less, particularly under serum-free conditions. Moreover, secreted metabolites are released by a limited number of cells into a comparatively large medium volume and are therefore present at lower concentrations. Extraction protocols for secretome analysis consequently need to be optimized for low analyte concentrations and a potentially narrower metabolite range. Published secretome protocols employ polar organic solvents such as methanol (MeOH) or acetonitrile (ACN), non-polar solvents such as chloroform (CHCl₃), and aqueous mixtures thereof (de Bekker et al., 2013; dos Santos et al., 2016; Lemke et al., 2017; Palevich et al., 2022; Palmer et al., 2012; Pelosi et al., 2022; Poplawski et al., 2023; Rathmann et al., 2015; Sadok et al., 2019). Solvent composition determines which metabolite classes are recovered and how efficiently. While polar solvents favor organic acids and other hydrophilic compounds, non-polar solvents improve lipid recovery. Nevertheless, systematic data comparing coverage, extraction efficiency, and reproducibility across these systems for secretome samples are lacking (Lepoittevin et al., 2023), and further parameters, such as acidification

with formic acid or extract drying, have received little attention so far. Here, we systematically compared published solvent systems, solvent-to-sample ratios, and extract preparation techniques with respect to metabolite coverage, extraction efficiency, and reproducibility. Beyond defining optimal conditions for broad metabolome coverage, we provide recommendations for solvent systems suited to specific compound classes and open the possibility for harmonization of secretome analysis via metabolomics.

Methods

Chemicals and Materials

All solvents and chemicals used were of high-performance liquid chromatography (HPLC) or LC-MS grade to ensure analytical purity. LC-MS grade water was obtained from Fisher Chemical (Geel, Belgium), and HPLC-grade water from VWR Chemicals (Rosny-sous-Bois Cedex, France). Formic acid (FA; Optima LC-MS grade) and ACN (CHROMASOLV LC-MS, $\geq 99.9\%$) were purchased from Fisher Chemical (Geel, Belgium) and Honeywell (Seelze, Germany), respectively. Isopropanol (CHROMASOLV LC-MS, $\geq 99.9\%$) was obtained from Merck (Darmstadt, Germany), while MeOH (ROTISOLV Ultra LC-MS, $\geq 99.98\%$) was sourced from ROTH (Karlsruhe, Germany). Methyl tert-butyl ether (MTBE; LiChrosolv, $> 99.8\%$) and CHCl_3 (for HPLC, $\geq 99.8\%$, amylene stabilized) were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Steinheim, Germany), respectively. Ammonium formate (HiPerSolv CHROMANORM for LC-MS, $\geq 99\%$) was obtained from VWR Chemicals (Leuven, Belgium).

Cell Culture

Secretome samples were collected from Panc1 and HEK293-F cell lines. Briefly, Panc1 and HEK293-F cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS; Fetal Constance, Anprotec, Bruckberg, Germany). At approximately 80% confluence, aliquots of the conditioned medium (secretome) were collected and stored at -80°C until extraction.

Pre-analytics/ Sample extraction

1. Comparative evaluation of solvent systems

Table 1

List of previously used extraction solvent systems for cell secretome metabolomics analysis.

No.	extraction solvent system	solvent / sample ratio (v/v)	ratio of extraction solvents (v/v)	literature
1	acetonitrile	1:1	-	De Bekker et al. (2013)
2	methanol	1:1	-	Sadok et al. (2019)
3	methanol + 0.1% formic acid	1:1	-	
4	isopropanol	0.25:1	-	Pelosi and Fernandes et al. (2022)
5	methanol : water	2:1	80:20	Poplawski et al. (2023)
6	acetonitrile : water	2:1	50:50	Palmer et al. (2012)
7	isopropanol : MTBE	2.5:1	30:70	Rathmann et al. (2015)
8	water : methanol : MTBE	3.9:1	2:3:6	Lemke et al. (2017)
9	water : chloroform	20:1	1:2	dos Santos et al. (2016)
10	water : methanol : chloroform	6:1	1:2:2	Palevich et al. (2022)

Ten different solvent systems were compared. Secretome sample were mixed with solvent systems listed in Table 1 in a solvent-to-sample ratio (v/v) of 2:1. The mixture was vortexed (REAX 2000 Vortex Mixer, Heidolph, Schwabach, Germany) and centrifuged at 4°C for 10 min at 12700 rpm in a Centrifuge 5430R (Eppendorf, Hamburg, Germany). Supernatant aliquots were transferred into two HPLC vials, dried in a SpeedVac (Refrigerated CentriVap Concentrator, Labconco Corporation, Kansas City, MO, USA) at 4°C, stored at -80°C, and reconstituted in eluent immediately prior to analysis.

2. Comparative evaluation of solvent-to-sample ratio

To determine optimal solvent-to-sample ratio, samples were extracted using MeOH with 1% FA at four different ratios i.e., 1:1, 2:1, 3:1 and 4:1 (v/v), covering ratios above and below the 2:1 ratio used above. Samples were prepared as stated above (1. Comparative Evaluation of Solvent Systems). Peak areas were multiplied by the respective dilution factor to exclude dilution effects from the comparison.

3. Comparative evaluation of extract preparations/ drying techniques

Afterwards, four different extract preparations were compared. Samples were extracted with MeOH + 1% FA at a solvent-to-sample ratio of 4:1. After vortex mixing and centrifugation at 12,700 rpm and 4°C for 10 min, the supernatant was collected. Subsequently, four different extract preparation protocols were evaluated. The first three include a drying step and were 1) drying the samples under a stream of

nitrogen (N₂) at room temperature (RT), 2) drying the samples in a SpeedVac concentrator at 4°C (SV4°C) or 3) at RT (SVRT). The dried secretome samples were stored at – 80°C until data acquisition. Immediately prior to measurement, the dried samples were reconstituted in 200 µL of eluent. The fourth protocol did not include a drying step and consisted of diluting the samples and injecting the diluted sample extracts directly (dilute-and-shoot, DS). For this, 200 µL supernatant were transferred into a HPLC vial and the diluted secretome sample injected without further processing.

4. Final protocol

100 µL secretome (cell supernatant) were mixed with 400 µL MeOH containing 1% FA and vortex mixed. After centrifugation at 12,700 rpm for 10 min at 4°C, 2 x 200 µL of the supernatant were transferred to HPLC vials, dried using a SV4°C and stored at -80°C upon sample measurement.

LC-MS Metabolomics Analysis

Untargeted analysis was performed on an Agilent 1290 Infinity HPLC system (autosampler, binary pump, thermostated column compartment; Waldbronn, Germany) coupled to an AB Sciex TripleTOF 5600 + mass spectrometer (Framingham, MA, USA). Each sample was analyzed by hydrophilic interaction liquid chromatography (HILIC) and reversed-phase (RP) chromatography, each in positive and negative ionization mode. HILIC separation used an ACQUITY UPLC BEH Amide column and RP separation an ACQUITY UPLC BEH C18 column (both 1.7 µm, 2.1 × 100 mm, with guard column; Waters, Eschborn, Germany). HILIC eluents were H₂O (A) and ACN:H₂O (95:5, v/v; B), both containing 10 mM ammonium formate and 0.1% FA. RP eluents were H₂O (A) and MeOH (B), both containing 0.1% FA. Column temperature was 40°C, flow rate 0.35 mL/min, and injection volume 10 µL. The HILIC gradient was 100% B (0–2 min), 30% B at 14 min (held to 16.5 min), 100% B at 17.5 min, and re-equilibration until 30 min. The RP gradient increased from 0.5% to 98% B over 11 min, held until 15 min, returned to 0.5% B at 15.5 min, and re-equilibrated until 30 min. The needle was washed with H₂O:MeOH (40:60, v/v) after each injection. Blank samples were used in order to allow for blank subtraction later on.

The mass spectrometer was controlled using Analyst TF 1.7.1 and equipped with a DuoSpray source with ESI probe and calibrated every five samples using APCI calibration solution. Eluate was directed to the mass spectrometer for the first 18 min and to waste thereafter. Ion spray voltage was + 5,500 V/–4,500 V, gas 1 and gas 2 50 psi, curtain gas 35 psi, source temperature 500°C, and declustering potential + 60 V/–100 V. TOF MS scans (m/z 50–900) were acquired with 0.1 s accumulation time and ± 10 V collision energy. Information-dependent acquisition (IDA) triggered product ion scans (m/z 50–900; collision energy ± 25 V, spread 15 V) for up to 10 candidate ions per cycle (intensity threshold 100 cps, isotope exclusion 4 Da, mass tolerance 50 mDa, dynamic exclusion 5 s after one occurrence).

Data processing

Raw data (.wiff) were processed in MS-DIAL (versions 5.1.230912 and 5.5.260323) using the settings listed in Supplementary Table 1. Features were filtered using the MS-DIAL filters *Blank Filter* and the *Reference Matched*, *Suggested*, and *MS2 Acquired*, and features with a retention time < 0.7 min or a spectral matching score ≤ 0.8 were excluded. Remaining features were curated manually with respect to EIC peak shape, intensity and signal-to-noise ratio (S/N), plausibility of the annotation and ontology, and correct peak assignment for multiple peaks, following a predefined decision tree (Supplementary Fig. 1). Filtered feature lists from RP+, RP-, HILIC+, and HILIC- were combined using a custom R script, and statistical analysis and visualization were performed in R/RStudio (version 2026.06.0).

Results and Discussion

Secretome samples of the pancreatic ductal adenocarcinoma (PDAC) cell line Panc1, reflecting the intended application in TME metabolomics, and of the non-tumorigenic cell line HEK293-F, included to assess generalizability, were extracted with the solvent systems listed in Table 1. To isolate the effect of solvent composition, all systems were initially applied at a solvent-to-sample ratio 2:1 (v/v), which was approximately the average of the reported ratios.

Number of Features

Across all samples and solvent systems between 3,566 (HILIC-) and 17,291 (RP+) features were detected, consistent with the typically higher feature count observed in RPPOS(Hemmer et al., 2024). Sequential filtering reduced these numbers to between 129 (HILIC-) and 212 (RP+) features, corresponding to retention rates of approximately 1.2%, 3.5%, 2.0%, and 3.6% for RP+, RP-, HILIC+, and HILIC-, respectively. Notably, the rank order of feature counts across modes was preserved throughout the filtering process (Fig. 1a).

The void volume filter (RT < 0.7 min) had the smallest effect (at most 18 features, HILIC+), whereas the *Reference Matched*, *Suggested*, and *MS2 Acquired* filters caused the largest reduction (32.7–35.1%). Overall, 545 unique features were annotated. This stringent reduction is consistent with untargeted workflows, in which typically only 10–30% of detected features carry reliable annotations (de Jonge et al., 2022; Gauglitz et al., 2022), and ensures that only confidently detected and annotated features enter biological interpretation.

1. Comparative evaluation of solvent systems

Solvent chemistry was the main source of variance in the metabolic profiles (Fig. 1b). In the PCA plot, three clusters could be observed i.e., a cluster for biphasic solvent systems based on either CHCl₃ or MTBE (light red eclipse), a cluster with ACN-based solvents (light green eclipse), and a cluster for alcoholic solvents (light blue eclipse). ACN-based solvents separated from the others along PC2, likely because ACN extracts are more polar subset of the metabolome (Höring et al., 2022), whereas biphasic and alcohol-based solvents separated along PC1. Within most solvent clusters, the two tested cell lines

formed subgroups indicating underlying cell-line-specific metabolome differences. PC1 and PC2 together explained about ~ 50% of total variance, indicating that solvent chemistry and cell line were dominant, but not the only source of variation in the data set.

Extraction Efficiency

Extraction efficiency was assessed by the number of MS2 annotated metabolites detected and their summed peak areas. Acidified MeOH (1% FA) recovered the most metabolites (144), followed by MeOH:H₂O (130), pure MeOH (129), ACN:H₂O (125), isopropanol (123), whereas pure ACN recovered the fewest (79; Fig. 1c). Biphasic systems yielded intermediate to low numbers (H₂O:MeOH:MTBE 107, H₂O:MeOH:CHCl₃ 101, isopropanol:MTBE 89, and H₂O:CHCl₃ 82). Summed peak areas largely paralleled these results (Fig. 1d). Pure MeOH and ACN:H₂O (50:50 v/v) yielded the highest cumulative signal (101.4% each, relative to MeOH + 1% FA), suggesting that acidification broadened detection mainly among low-abundant species rather than increasing overall extraction yield. MeOH:H₂O (83.8%) and isopropanol (70.9%) were intermediate whereas biphasic systems and pure ACN showed the lowest areas (37.7–44.9%). Thus, extraction yield and coverage largely coincided, with monophasic alcohol-based solvents and ACN:H₂O outperforming pure ACN and biphasic systems on both measures.

Across all solvents, 292 unique metabolites were distributed over 128 distinct patterns of solvent membership (Fig. 1e). A core set of 30 metabolites was detected in all 10 solvent systems, representing the fraction of the metabolome recovered independently of extraction chemistry. Solvent-exclusive recovery, defined as metabolites annotated in only one solvent systems, was highest for MeOH + 1% FA (15 metabolites) followed by isopropanol (12 metabolites), ACN:H₂O and MeOH:H₂O (10 each), and H₂O:MeOH:CHCl₃, H₂O:MeOH:MTBE, and pure ACN (9 each). The remaining solvents, isopropanol:MTBE and H₂O:CHCl₃, contributed fewer. Most combinations represented metabolites shared by small subsets of two to four solvents. The solvents therefore differed not only in how many but also in which metabolites they recovered.

Ontology classification (MS-DIAL) further distinguished the solvents (Fig. 2a). Free α -amino acids, medium-chain fatty acids, and acyl carnitines were the most abundant classes overall, together comprising 14–21% of annotated metabolites in nine of the 10 solvent systems. They were most prominent in the biphasic systems (19–21%). ACN:H₂O showed the highest proportions of dipeptides and long-chain fatty acids, while pure ACN showed a flatter distribution, where fatty acids, alpha-amino acids, and glycinated bile acids showed similar percentages. No class exceeded ~ 8% of any solvent's metabolites, and each solvent spanned 54–83 classes, reflecting a chemically diverse metabolome.

Reproducibility was evaluated using per-metabolite coefficients of variations (CVs) of triplicate extractions (Fig. 2b). Isopropanol yielded the most reproducible measurements (median CV 14.9%), in line with the high reproducibility reported for single-step isopropanol precipitation (Iriondo et al., 2019; Sarafian et al., 2014). Efficient protein precipitation may additionally limit residual enzymatic activity in

the extract, which has been shown to alter metabolite levels after extraction (House et al., 2024). Acidified MeOH ranked second (17.0%), possibly because acidification accelerates enzyme quenching and thereby limits metabolite interconversion during extraction (Rabinowitz and Kimball, 2007). Pure MeOH was the least reproducible (46.6%), roughly three-fold higher than either isopropanol or its acidified counterpart despite comparable metabolite coverage. Biphasic systems showed intermediate CVs (24.6–32.2%), likely because isolating a defined phase volume introduces additional variance. HEK293-F extracts showed equal or higher CVs than Panc1 extracts for nine of 10 solvents, most pronounced for ACN (27.2% vs. 37.4%), ACN:H₂O (16.4% vs. 22.4%), and isopropanol:MTBE (21.4% vs. 31.7%). Whether this reflects differences in metabolite stability, matrix composition, or matrix effects cannot be resolved from the present data. However, reproducibility benchmarks derived from a single cell line should not be generalized without verification.

Overall, monophasic MeOH- and ACN:H₂O-based extractions outperformed biphasic MTBE- or CHCl₃-containing systems in both coverage and recovery. This agrees with systematic comparisons in adherent mammalian cells, in which MeOH:H₂O was the optimal single protocol (Dettmer et al., 2011), and in human plasma, in which MeOH-based precipitation provided the highest coverage and repeatability, whereas MTBE-based liquid-liquid extraction recovered fewer features despite greater class selectivity (Sitnikov et al., 2016). Biphasic extracts were proportionally enriched in fatty acid-related classes, making them suitable when lipid coverage rather than broad polar metabolome coverage is the analytical focus. Taken together, MeOH-based monophasic extraction, particularly with acidification, is the most broadly effective choice for untargeted secretome metabolomics.

2. Comparative evaluation of solvent/ sample ratios

Four solvent-to-sample ratios commonly applied in secretome (Table 1) and plasma or serum metabolomics were compared (Fig. 3), whereby 238 metabolites were detected after extraction using MeOH + 1%FA. In the uncorrected data, total peak area increased with decreasing dilution, as expected for more concentrated extracts. Correcting for the nominal dilution factors (x 1 to x 4), however, did not equalize the groups. Instead, it reversed and amplified the difference, indicating a non-linear relationship between extraction ratio and measured signal. Two mechanisms likely contribute. Firstly, a larger solvent volume may improve extraction by penetrating the sample more completely. Secondly, and the more concentrated 1:1 and 2:1 extract carry more co-extracted proteins, salts, and lipids that suppress ionization. Both effects have been described in the literature. In plasma, protein removal by MeOH was shown to increase with precipitant/sample ratio beyond 2.5:1, whereas ACN plateaued at approximately 2:1, and acidic precipitants reduced ionization effects in LC-MS/MS (Polson et al., 2003). This supports the use of higher ratios, i.e., 3:1 or 4:1, for MeOH-based extraction.

Cell lines separated along PC1 (40.6%) and solvent ratios along PC2 (31.9%), with similar clustering in both cell lines (Fig. 3a). Summed class loadings (Fig. 3b) showed that α -amino acids (n = 7) carried the strongest combined loading, associated with both Panc1 and the ratio series, whereas purine nucleosides (n = 6) were most strongly associated with HEK293-F. Medium- and long-chain fatty acids,

dipeptides, and lysophosphatidylcholines loaded predominantly on PC2 and thus tracked the solvent ratio, whereas adenine, hypoxanthine, and thymine discriminated between cell lines independently of the ratio.

Total peak area (after dilution correction) increased from 35.3% (HEK293-F) and 38.1% (Panc1) at 1:1 to 100% at 4:1 (Fig. 3c). The ratio had little influence on reproducibility, with median CVs of 8–11% at all ratios in both cell lines (Fig. 3d). Pairwise comparisons (Welch's t-test, Benjamini–Hochberg correction, $|\log_2\text{FC}| > 1$, $q < 0.05$) revealed no metabolites significantly enriched at lower ratios. The only consistent effect was a moderate decrease of dicarboxylic acids (azelaic, suberic, dodecanedioic, and undecanedioic acid) at higher ratios. As higher ratios increased yield without compromising reproducibility, a 4:1 ratio was selected.

3. Comparative evaluation of extract preparations/ drying techniques

In many untargeted metabolomics workflows, extracts are dried and stored at -80°C when immediate analysis is not possible (Dunn et al., 2011). We therefore compared SV4 $^\circ\text{C}$ (used in all preceding experiments), SVRT, N_2 evaporation, and DS, a direct injection approach common in urine analysis (Want et al., 2010).

Cell lines separated along PC2 (26.8%), whereas PC1 (46.8%) separated DS from the three evaporative techniques within each cell line (Fig. 4a). This magnitude of separation is consistent with prior reports that showed, that the extract drying technique can substantially reshape the detected metabolome. A comparison of SpeedVac evaporation and lyophilization across three pancreatic cancer cell lines, including Panc1, for instance identified similar to this study 40–50 metabolites differing significantly in abundance between drying methods, with several metabolites detected exclusively under one technique (Petrova et al., 2019). Class loadings indicated a compositional shift rather than a uniform intensity change (Fig. 4b): phosphatidylcholines were most strongly associated with DS, whereas dipeptides, purine nucleosides, α -amino acids and derivatives, acyl amino acids, and taurine-conjugated bile acids were associated with evaporative drying, suggesting that drying and reconstitution affect lipids differently from polar metabolites and peptides. Nevertheless, summed peak areas varied only modestly (98.9–109.7% of DS; Fig. 4c), and reproducibility was comparable, with most metabolites below 30% CV in all protocols (Fig. 4D). N_2 evaporation was slightly less reproducible, consistent with the wider sample dispersion in the PCA. DS, however, produced split and broadened peaks for most metabolites (Supplementary Fig. 2), suggesting that the evaporative methods may be superior from a chromatographical standpoint.

In summary, the three evaporative methods yielded similar profiles, total signal, and reproducibility, in line with reports that N_2 evaporation, SpeedVac drying, and lyophilization do not differ significantly and that complete dryness, rather than the evaporation method, determines data quality (Luo and Li, 2023). The choice among them can therefore be guided by processing time, throughput, and sample stability. DS, in contrast, is not interchangeable with evaporative drying. Evaporative drying is recommended when broad

coverage of polar and peptide-related metabolites is required, whereas DS, alone or in combination, should be considered when phospholipids such as phosphatidylcholines are of interest. Given its throughput in our laboratory and the added sample safety at low temperature, SV4°C was used for further experiments.

4. Application

Using the final method (MeOH + 1% FA, solvent/ sample ratio 4:1, SV4°C), we compared secretome samples obtained from Panc1 and HEK293-F cells. Overall, 33 metabolites were detected at different intensities between Panc1 and HEK293-F secretomes (16 up in Panc1, 17 up in HEK293-F).

Dipeptides stood out as molecule class significantly enriched, whereby eight (Ala-Ile, Ala-Ala, Val-Ile, Val-Phe, Thr-Leu, Thr-Phe, Ser-Leu, Ala-Phe) were more abundant in HEK293-F secretomes. In contrast, free amino acids and their catabolites including glutamic acid, aspartic acid, serine, citrulline, cystine, and 3-aminoisobutyric acid, were found at higher abundancies in Panc1 secretomes. This pattern is consistent with the known capacity of PDAC cells to scavenge extracellular protein and peptides by macropinocytosis and lysosomal degradation, particularly under nutrient-poor TME conditions (Kamphorst et al., 2015). Furthermore, circulating amino acids, including the branched-chain amino acids, were found to be elevated systemically early in PDAC development (Mayers et al., 2014). Our secretome data suggest, that the differential abundance of peptide-bound and free amino acid pools is at least partly cell-autonomous, as it is already apparent under standard culture conditions.

Conclusion

Ten solvent systems, four solvent-to-sample ratios and four extract preparation protocols were systematically compared for secretome metabolomics. The optimized protocol comprises extraction with MeOH + 1% formic acid at a 4:1 solvent-to-sample ratio, drying in a SpeedVac at 4°C, storage at -80°C, and reconstitution in starting eluent prior to analysis. This protocol reproducibly yielded the largest number of annotated metabolites, although performance may vary between cell lines. Applied to Panc1 and HEK293-F secretomes, it revealed cancer-associated differences in secreted metabolites and may thus support future investigations into metabolite-driven immunoregulation within the TME.

Declarations

Author Conflict of Interest Statement

The authors declare no conflict of interest.

Compliance with Ethical Standards

This article does not contain any studies with human and/or animal participants performed by any of the authors.

Funding Declaration

This work received no external funding.

Author Contribution

LLR and XN conducted the metabolomics measurements and analyzed data. MH conducted cell culture experiments. AB provided cell culture expertise. JP conducted metabolomics measurements. MG co-supervised with RVT. RVT designed the experiments and wrote the manuscript. All authors read and approved the manuscript.

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information (Supplementary Table 1-3). Raw data can be provided upon request from the corresponding author.

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Figures

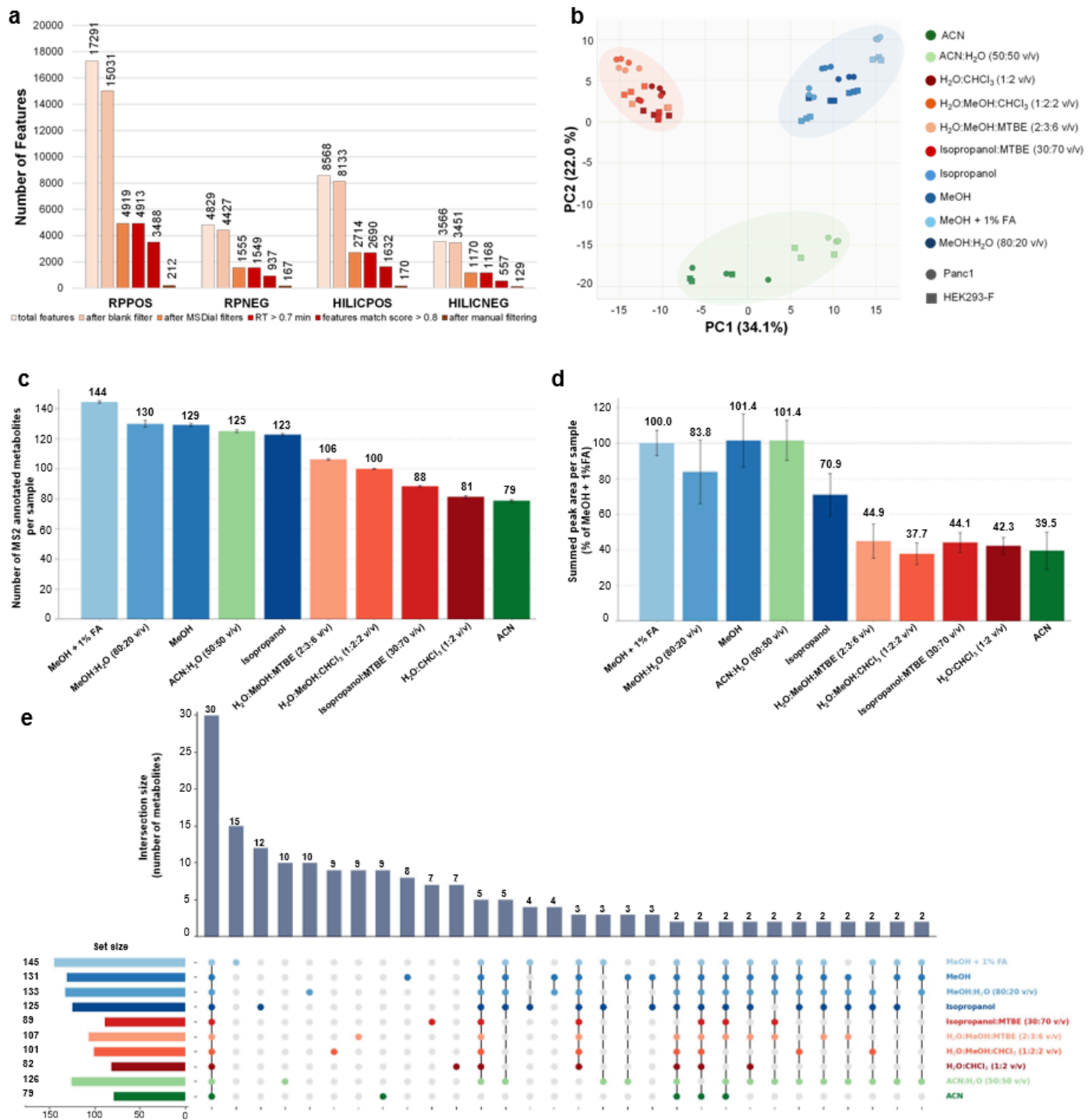


Figure 1

a - Features detected and retained after sequential filtering steps across the four LC-MS acquisition modes applied in the untargeted metabolomics workflow for all cell lines and extraction solvents tested. **b** - Principal component analysis (PCA, log₁₀ transformed, Pareto-scaled) of Panc1 and HEK 293-F secretomes extracted with 10 different solvent systems. **c** - Number of MS2-annotated metabolites detected per sample, by solvent system. Bars show the mean count across the six samples; error bars represent the standard deviation (SD) across the six individual samples (two cell lines × three replicates). **d** - Total extracted metabolite signal per solvent system. Bars show the mean summed peak area per

sample across all MS2 annotated metabolites for that solvent for individual samples (two cell lines × three replicates), expressed as a percentage of MeOH + 1% FA (set to 100%). Error bars represent the SD across the six samples (two cell lines x three replicates). **e** – Upset plot displaying the number of MS2 annotated metabolites shared between the different solvent systems. ACN: acetonitrile; CHCl₃: chloroform; MTBE: methyl tert-butyl ether; MeOH: methanol; FA: formic acid.

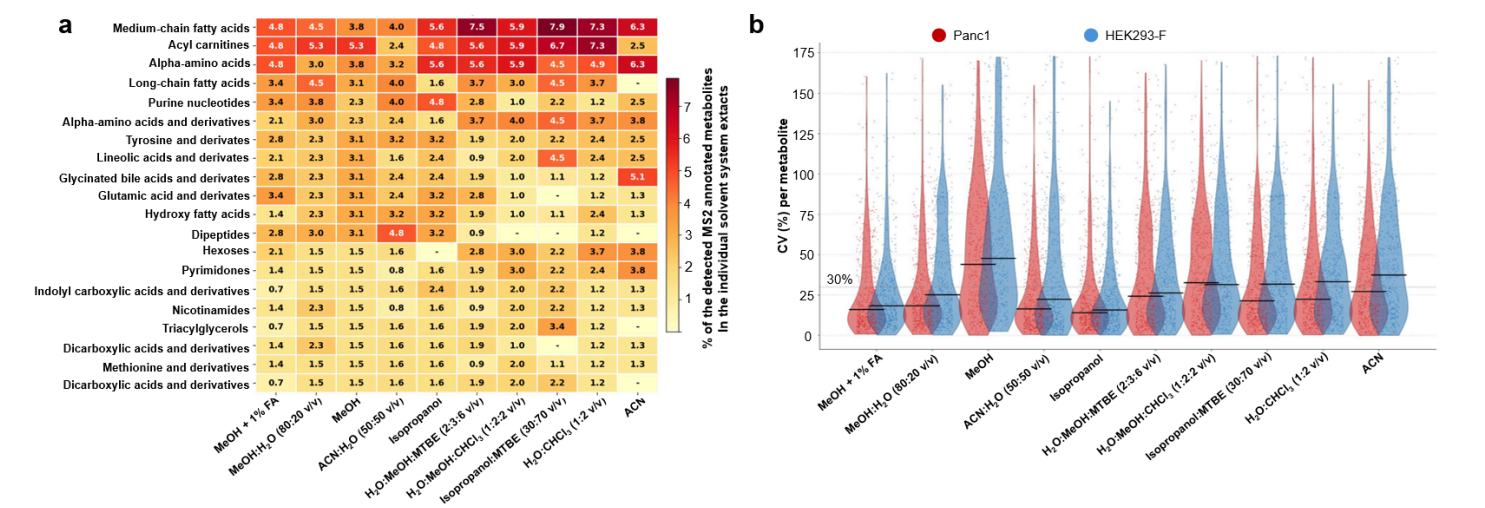


Figure 2

a – Ontology composition (top 20 classes) of MS2 annotated metabolites per solvent system (all six samples combined). **b** – Violin plots of per-metabolite CVs of triplicate measurements. Dots represent individual metabolites and horizontal lines the median. ACN: acetonitrile; CHCl₃: chloroform; CV: coefficient of variation; MTBE: methyl tert-butyl ether; MeOH: methanol; FA: formic acid.

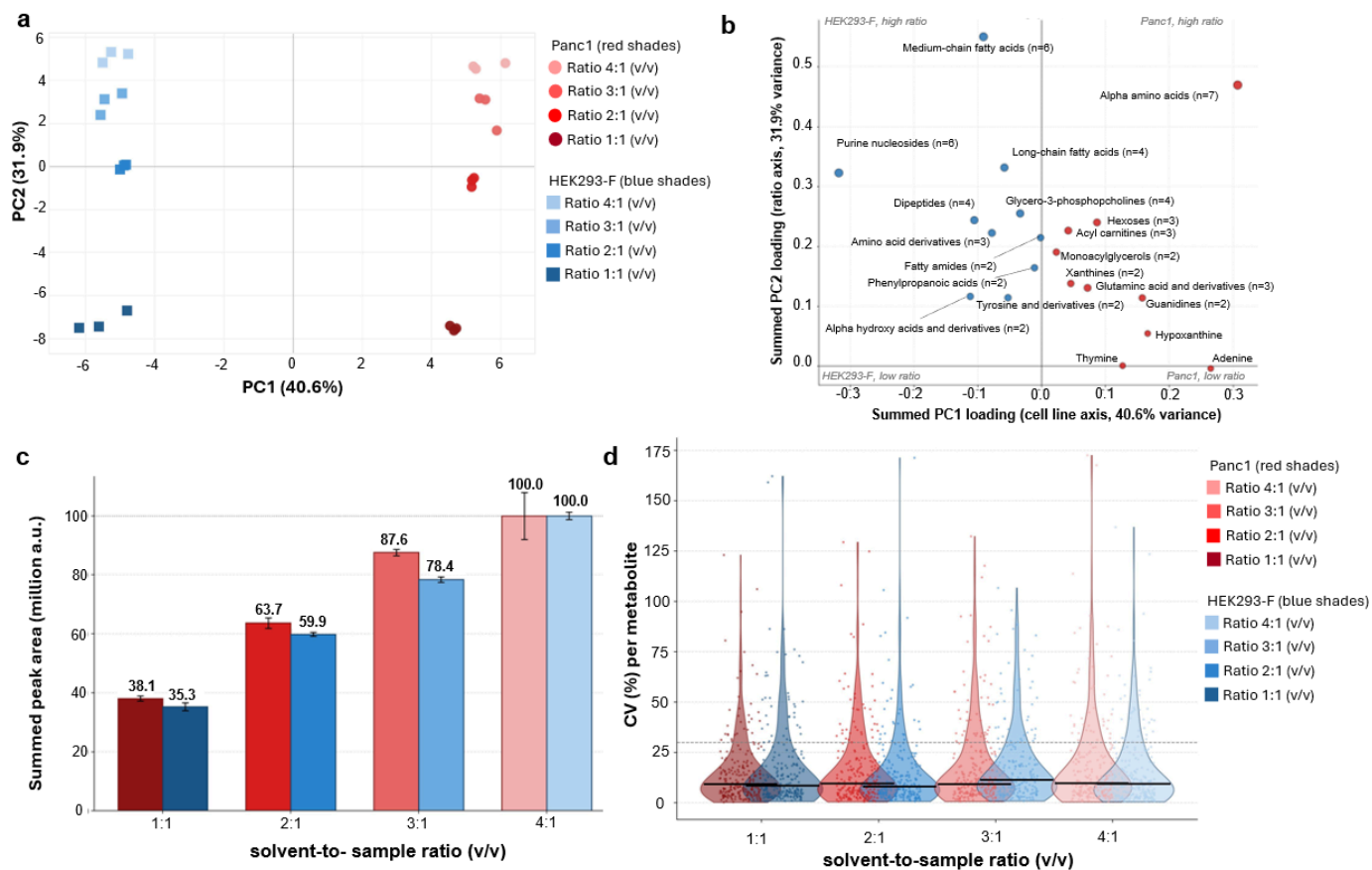


Figure 3

a –PCA (log10 transformed, Pareto-scaled) of secretomes extracted at different solvent-to-sample ratios. **b** - Ontology-level PCA loadings of the 20 highest-magnitude classes, plotted as summed PC1 and PC2 loadings. Point size scales with the number of metabolites per class (n in brackets) and color indicates the sign of the PC1 loading (red, positive; blue negative). **c**– Summed peak area per sample (MeOH + 1% FA, corrected for dilution) for ratios of 1:1 to 4:1, expressed relative to the 4:1 ratio of each cell line (100%). Bars show means $\pm$ SD (n=3). **d** Violin plots of per-metabolite CVs per ratio. SD: standard deviation; CV: coefficient of variations.

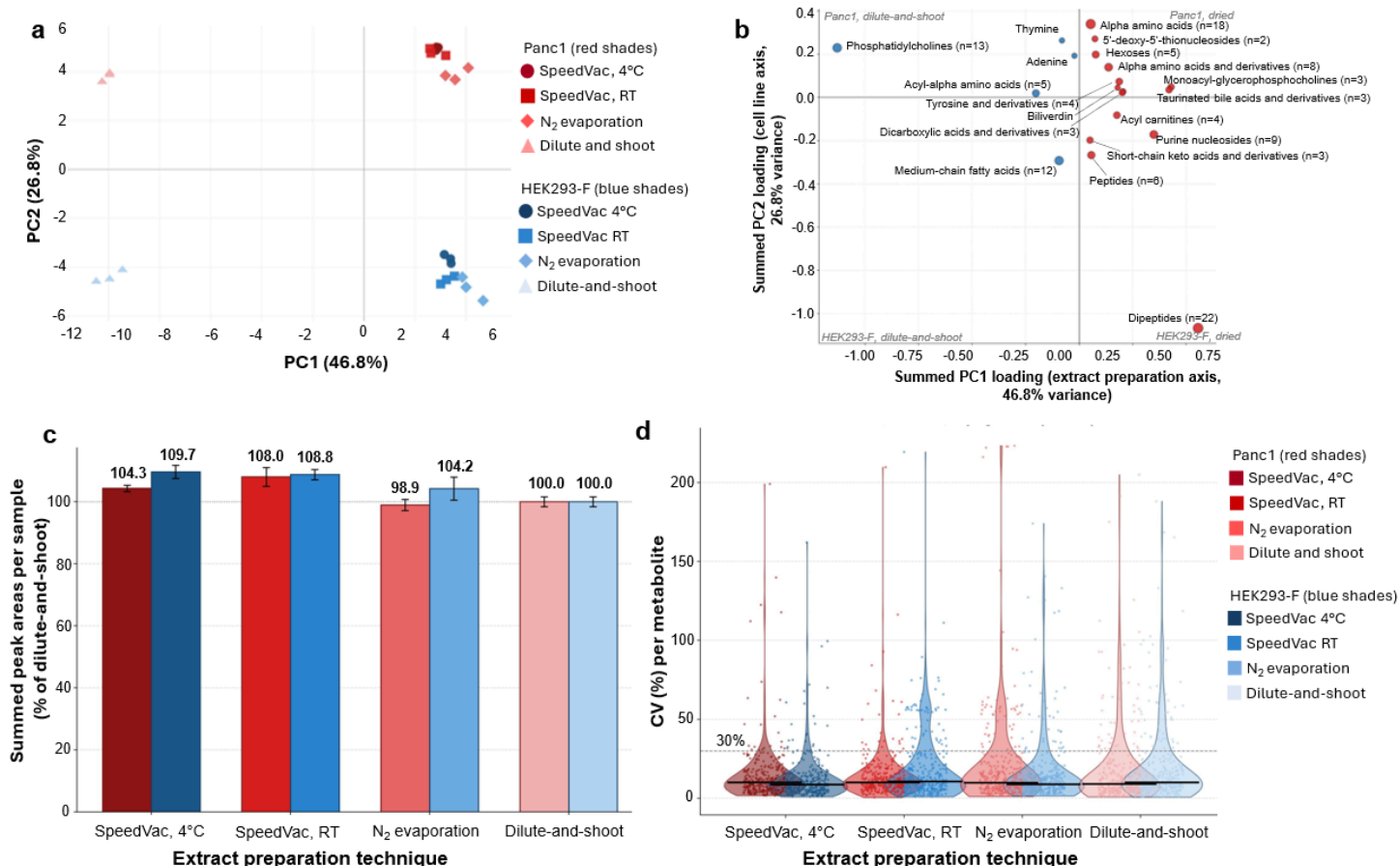


Figure 4

a - PCA (log10 transformed, Pareto-scaled) of secretomes processing with different extract preparation techniques. **b** - Ontology-level PCA loadings of the 20 highest-magnitude classes. Point size scales with the number of metabolites contributing to the class (n, given in the label). Point color reflects the sign of the PC1 loading (red, positive; blue, negative). **c** – Summed peak area per sample (normalized to dilute-and-shoot). Bars show the mean peak area per sample. Error bars represent SD (n=3). **d** - Violin plots of per-metabolite CVs per extract preparation technique. SD: standard deviation; CV: coefficient of variations.

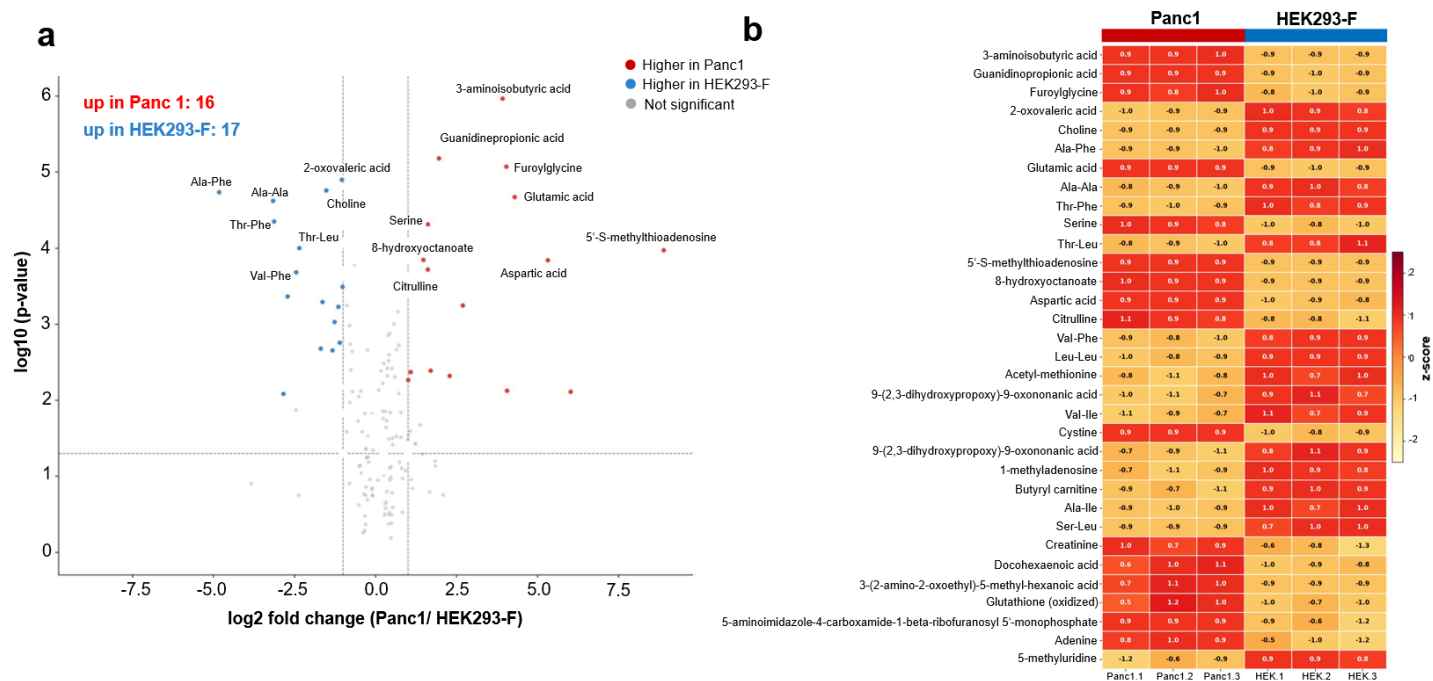


Figure 5

a – Volcano plot of metabolites in Panc1 versus HEK293-F secretomes (Welch's t-test, Benjamini–Hochberg correction); metabolites significantly higher in Panc1 ($n = 16$) are shown in red, those higher in HEK293-F ($n = 17$) in blue, and non-significant metabolites in gray. Selected metabolites are labeled. **b** – Heatmap of the 33 significant metabolites across biological replicates. Color indicates the row-wise z-score of log10-transformed peak area.

Supplementary Files

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