

A Four-Parameter LC–MS Strategy for Reliable Ligand Screening of Cytochrome c in Herbal Medicines

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ABSTRACT

Online affinity-based ligand screening utilizing liquid chromatography (LC) integrated with various detection systems is a prevalent strategy for isolating bioactive constituents from herbal medicines (HMs). Nonetheless, the occurrence of false-positive results remains a widespread challenge due to inadequate data documentation regarding the formation and stability of the ligand-protein complex. To address this limitation and enhance the reliability of identified hits, this study developed a post-column infusion liquid chromatography–energy-resolved-affinity mass spectrometry (PCI-LC–ER-AMS) platform. This configuration enables a comprehensive "four-in-one" analytical monitoring approach, tracking: 1) the reduction in response for potential ligands; 2) the reduction in protein response; 3) ions associated with the formed ligand-protein complexes; and 4) the dissociation strength of the ligand-protein binding. As a proof-of-concept application, ligand fishing targeting Cytochrome C (Cyt C) within complex herbal matrices was performed. To validate the utility of the method, a simulated sample composed of twelve predefined ligands alongside two negative controls was subjected to LC separation and mixed with Cyt C prior to quadrupole time-of-flight mass spectrometry (Qtof-MS) analysis. In comparison to control assays lacking either Cyt C or the ligands, all twelve target ligands—and notably none of the negative controls—demonstrated reduced response intensities. These decreases were perfectly aligned with twelve negative peaks detected in the Cyt C ion current chromatogram at the corresponding retention times. Furthermore, sequential ions representing each distinct ligand-Cyt C complex were successfully recorded. Through the generation of breakdown curves, the optimal collision energy (OCE) required to disrupt the non-covalent interactions was found to be positively correlated with binding affinity. The optimized platform was subsequently applied to evaluate two herbal medicines, namely *Scutellariae Radix* (SR) and *Aconiti Lateralis Radix Preparata*. The screening successfully isolated 24 compounds exclusively from SR; among these, flavonoid glycosides demonstrated elevated OCE values and superior binding strengths relative to their aglycone counterparts. Subsequent affinity measurements and cell-based assays confirmed the pronounced interactions between Cyt C and each of the discovered components. In summary, PCI-LC–ER-AMS provides an effective, high-confidence online ligand screening methodology for Cyt C in herbal remedies via multi-dimensional "four-in-one" analytical verification.

Keywords: Energy-resolved affinity mass spectrometry, Cytochrome C, Baicalin, Post-column infusion, Ligand-protein complex

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Introduction

For millennia, herbal medicines (HMs) have played a crucial role in preserving public health and fostering civilization in China and other East Asian nations, a testament to their pronounced therapeutic efficacy [1-3]. Unquestionably, the constituent active compounds are what drive the treatment of various pathologies. Over the past several decades, HMs have served as premier repositories for lead compounds and even clinically deployed

agents, as exemplified by artemisinin derived from *Artemisia annua* [4, 5] and paclitaxel obtained from *Taxus chinensis* [6]. To date, extensive scientific endeavors worldwide have been dedicated to identifying promising chemical entities from HMs, which are widely recognized as highly intricate chemical pools [7–10]. Because of its exceptional high-throughput capabilities in isolating prospective components from complex matrices, substantial attention has been directed toward the advancement of online ligand screening methodologies facilitated by liquid chromatography (LC).

However, the occurrence of false-positive results remains widespread because the majority of screening assays determine the bioactivity of a given substance solely by monitoring a decrease in the signal response of either the compound itself [11] or a designated probe substrate [11, 12]. By integrating the chromatographic separation capabilities of LC with the qualitative and quantitative performance of tandem mass spectrometry (MS/MS), LC–MS/MS—and liquid chromatography-quadrupole time-of-flight mass spectrometry (LC–Qtof-MS) in particular—stands as the most adaptable tool for the chemical characterization of HMs [13]. To fulfill online screening requirements, multiple approaches have been pursued to enhance LC–MS/MS platforms by incorporating features that facilitate ligand-protein binding. Prevalent methodologies [14] encompass:

1. affinity chromatography, which involves the immobilization of target proteins onto stationary phase particles (such as cell membrane chromatography [15]);
2. ultrafiltration-based separation to exclude molecules retained by target proteins [16];
3. the isolation and subsequent dissociation of ligand-protein complexes to liberate the bound ligands [17]; and
4. the assessment of competitive displacement between candidate compounds and probe substrates for protein binding sites [11, 12].

Regrettably, none of these strategies are capable of directly detecting the intact ligand-protein complexes. Compounding this issue, due to the vast concentration ranges of different constituents within HMs, neither the signal reduction of a captured compound nor the signal enhancement of a probe substrate can accurately mirror the actual binding affinity within the complex. Consequently, there is an immediate demand for an analytical approach capable of directly identifying the complex and evaluating its stability.

Driven by rapid advancements in MS/MS technologies—specifically the introduction of matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) techniques—it is now feasible to analyze intact proteins and even ligand-protein complexes [18–20]. Utilizing these advanced ionization methods, native MS has emerged as the preferred analytical tool [21]. Given that the solvent environment of an LC eluate can be properly adapted via post-column infusion, and because ligand-protein binding typically occurs within a matter of seconds, LC–ESI-MS should be capable of tracking the complex by introducing aqueous protein solutions through post-column infusion. The subsequent difficulty lies in quantifying the binding strength between the ligand and the target protein. We previously established that bond strength, which corresponds to bond dissociation energy, correlates positively with the optimal collision energy (OCE) required for the precise cleavage yielding the product ion [22]. Due to the fundamental similarities between non-covalent binding in ligand-protein complexes and covalent bonds within a molecule [22], an energy-resolved MS (ER-MS) protocol should be viable for determining binding strength.

Cytochrome c (Cyt C), a monohemic globular protein situated in the mitochondria of all aerobic organisms, is essential for sustaining and terminating life via respiratory and apoptotic mechanisms [23]. As a redox-active heme protein, Cyt C transports electrons from respiratory complex III to the terminal oxygen reductase (complex IV or Cyt C oxidase), thereby playing a critical role in preserving cellular viability through a sophisticated electron-proton energy transduction pathway that culminates in ATP synthesis [24]. Disruption of Cyt C redox homeostasis causes mitochondrial dysfunction, alters intracellular ATP and reactive oxygen species (ROS) concentrations, and ultimately triggers apoptosis [25, 26]. Multiple investigations have revealed that small molecules derived from HMs possess strong binding affinities for Cyt C [27–29].

Accordingly, a post-column infusion-liquid chromatography–energy-resolved-affinity mass spectrometry (PCI-LC–ER-AMS) platform, constructed by integrating LC–ESI-Qtof-MS, ER-MS, and post-column protein infusion, should offer a unique avenue for screening high-affinity ligands for Cyt C. The instrumental configuration is illustrated in **Figure 1a**. The LC unit is responsible for resolving the intricate chemical pool into individual purified components. Each separated compound then interacts with Cyt C distributed within a relatively large volume of an aqueous medium, while the Qtof-MS system equipped with an ESI interface performs "four-in-one" monitoring of the HM constituents, Cyt C, the ligand-Cyt C complex, and the associated ligand-Cyt C binding

strength. Two prominent HMs, *Scutellariae Radix* (SR, Chinese name: Huangqin) [30] and *Aconiti Lateralis Radix Preparata* (ALRP, Chinese name: Fuzi) [31], were utilized as representative cases for Cyt C ligand screening to validate the practical utility of this approach. The resulting data are anticipated to demonstrate that PCI-LC–ER-AMS represents a promising method for high-confidence online ligand fishing while successfully eliminating false-positive results to identify authentic ligands for Cyt C.

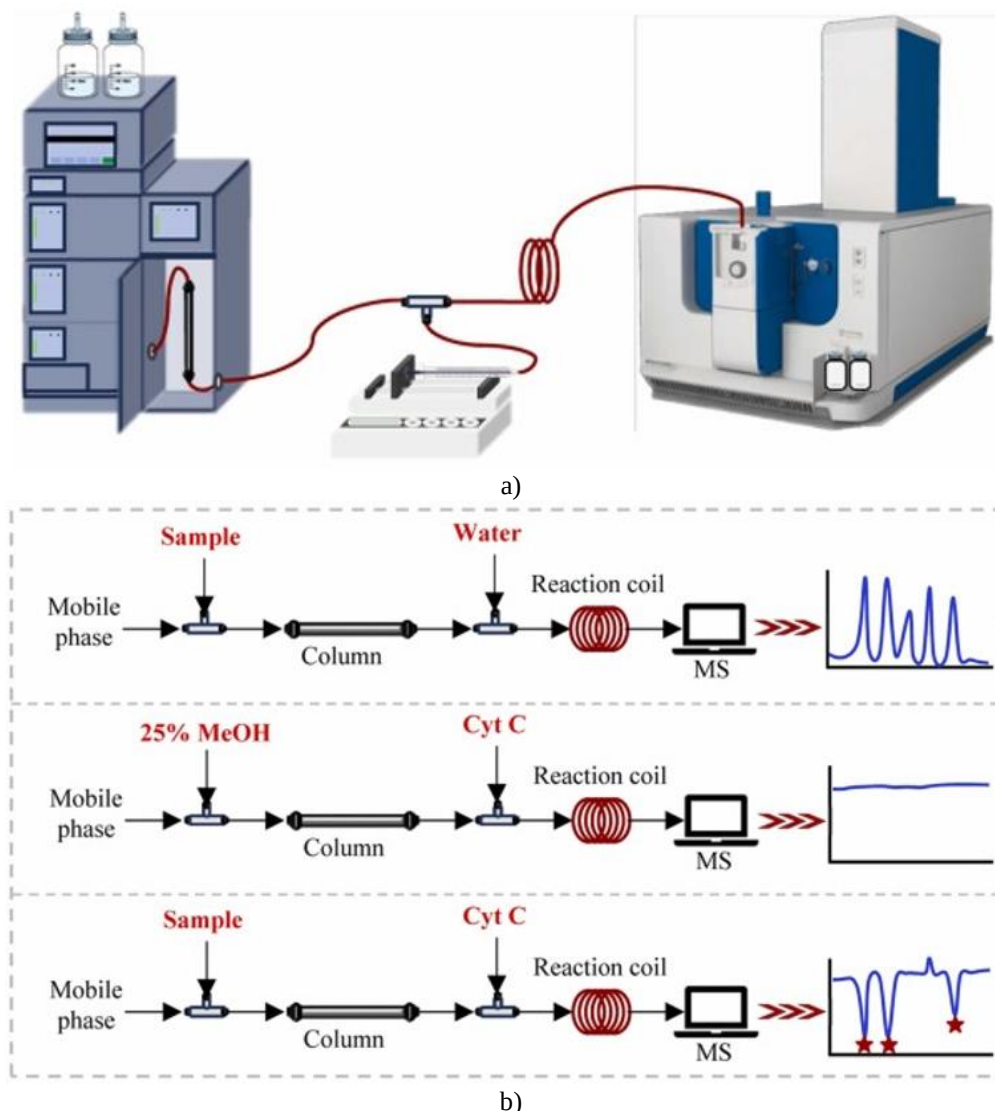


Figure 1. (a) Methodological blueprint of the PCI-LC–ER-AMS system incorporating liquid chromatography, post-column infusion, and Qtof-MS instruments; and (b) Analytical protocols for online screening of Cyt C ligands, defining parallel injections of either the herbal extract (or mixed STDs) or 25% aqueous methanol via the autosampler, combined with post-column infusion of either Cyt C or water to concurrently execute Cyt C-free, extract (or mixed STDs)-free, and extract (or mixed STDs)-Cyt C assays.

Materials and Methods

Reagents and materials

Crude specimens of SR and ALRP were obtained from Beijing Tongrentang Co., Ltd. (Beijing, China), with botanical authentication performed by co-author Prof. Pengfei Tu. Chemical reference standards, comprising apigenin, apigenin 7-O-glucuronide, baicalein, baicalin, wogonin, wogonoside, oroxylin A, oroxylin A 7-O-glucuronide, ginsenoside eR (Re), ginsenoside Rb2 (Rb2), and ginsenoside Rb3 (Rb3), were purchased from Standard Biotech Co., Ltd. (Shanghai, China). Additional reference molecules, including catechin, epicatechin, epigallocatechin gallate, gallic acid, hesperidin, tectoridin, and daidzein, were sourced from Yuanye Bio-Technology (Shanghai, China); saikosaponin A (SSA), saikosaponin C, benzoylmesaconine (BMA), and

benzoylhypaconitine (BHA) were supplied by Chengdu Desite Bio-Technology Co., Ltd. (Chengdu, China). Liquid chromatography-mass spectrometry grade solvents, including methanol, acetonitrile (ACN), dimethyl sulfoxide (DMSO), and formic acid (FA), were acquired from Thermo-Fisher (Pittsburgh, PA). High-purity deionized water (resistivity > 18.0 MΩ•cm) was generated using an in-house Milli-Q integral water purification system (Millipore, MA). Supplementary chemical agents were of analytical grade and procured from Beijing Chemical Works (Beijing, China).

Equine heart Cyt C (PDB ID: 1HRC, 12.36 kDa) was obtained from Sigma-Aldrich (Ontario, Canada). Cellular assay kits, including the AnnexinV Alexa Fluor488/PI apoptosis detection kit, were provided by Beijing Solarbio (Beijing, China). Evaluation kits for ATP, BCA, reactive oxygen species (ROS), and mitochondrial membrane potential (via JC-1 dye) were manufactured by Beyotime (Shanghai, China). Culture media components, including Dulbecco's modified Eagle's medium (DMEM), penicillin (10,000 U/mL)/streptomycin (10 mg/mL) solution, and 0.25% trypsin/2.2 mM EDTA solution, were purchased from Thermo-Fisher (Waltham, MA), while fetal bovine serum (FBS) was sourced from Corning Life Sciences (Steuben, NY).

Preparation of samples

Prior to extraction, the dried raw herbal items were pulverized into fine powders. Individual 100 mg samples of SR or ALRP powder were extracted with 10 mL of 70% aqueous methanol via an ultrasonic method for 30 min. To clarify the extracts, the mixtures were centrifuged at 12,000 rpm for 10 min, and the resulting supernatants were passed through a 0.22 µm Nylon filtration membrane.

A 25 µM working solution of Cyt C was prepared by dissolving the protein in pure water. Concentrated individual stock solutions (50 mM) for all reference standards were prepared using DMSO. To generate the simulated validation matrix (mixed STDs), appropriate volumes of fourteen distinct stock solutions—specifically catechin, epicatechin, epigallocatechin gallate, gallic acid, gallocatechin gallate, tectoridin, hesperidin, daidzein, Re, Rb2, Rb3, SSA, saikosaponin C, BMA, and BHA—were combined and diluted with 25% aqueous methanol to achieve a uniform final concentration of 0.1 µM per analyte.

Online LC–MS/MS analysis via post-column protein infusion

The instrumental design of the PCI-LC–ER-AMS architecture is detailed in **Figure 1a**. Multi-dimensional "four-in-one" monitoring was conducted using a Shimadzu LC-40ADXR UPLC instrument (Kyoto, Japan) coupled to a SCIEX 7600+ Qtof-MS system (Foster City, CA). Separation was carried out on a Waters ACQUITY HSS T3 stationary phase column (2.1×100 mm, 1.8 µm, Milford, CA) utilizing a binary mobile phase of 0.1% aqueous FA (A) and acetonitrile containing 0.1% FA (B). The column oven was held at 40°C, and the sample injection volume was 2 µL.

To achieve online protein-ligand coordination, the 25 µM Cyt C solution was introduced into the post-column eluate at a flow rate of 10 µL/min using a syringe pump (KDS100, KD Scientific, Holliston, MA) joined to a T-piece. The effluent from the T-piece was directed to the electrospray ionization source via an 80-cm segment of PEEK tubing (0.13 mm i.d.). Parallel control analyses using injections of 25% aqueous methanol or post-column infusion of pure water were executed to establish baseline profiles in the absence of ligands or proteins, respectively.

Qtof-MS parameter settings

All mass spectrometric data collection was performed in positive ionization mode. A data-dependent acquisition (DDA) method using optimized factory configurations was applied to simultaneously gather MS1 and MS2 spectra. The specific operational values were set as follows: curtain gas (CUR), 35; CAD gas, 9; GS1, 55 psi; GS2, 55 psi; source temperature (TEM), 550°C; ion spray voltage, 5500 V; declustering potential (DP), 50 V; and collision energy (CE) with a collision energy spread (CES) of 35 ± 15 eV. The mass windows for scanning MS1 and MS2 data were fixed at m/z 100–2000 and m/z 50–2000, respectively. Data logging and processing were performed via SCIEX OS software (Version 3.0).

Energy-resolved mass spectrometry via MRMHR

Following the systematic characterization of the MS1-MS2 profiles for each putative ligand-protein adduct, an online high-resolution multiple reaction monitoring MRM^{HR} method was used to gauge non-covalent binding strength. This mode was selected for its comprehensive mass range, rapid scanning rates, and sensitivity

comparable to traditional MRM. For these experiments, precursor-to-product ion transitions were built directly from the verified MS1-MS2 values of each individual complex, and stepwise increasing CE increments were applied to each ion pair. Following data extraction using SCIEX OS software, the integrated peak areas for each targeted ion transition were consolidated and exported into GraphPad Prism 9.5 software (San Diego, CA) to generate fragment-specific non-covalent breakdown curves.

Virtual screening and docking simulations

The three-dimensional atomic coordinates of Cyt C were acquired from the Protein Data Bank as a .pdb file. Structures for twelve reference ligands—specifically daidzein, hesperidin, tectoridin, epicatechin, catechin, saikosaponin C, SSA, Rb3, Rb2, and Re—alongside six primary analytes derived from SR (oroxylin A 7-O-glucuronide, wogonoside, apigenin 7-O-glucuronide, apigenin, baicalin, and baicalein) and two negative reference standards (BHA and BMA) were built using ChemBio3D Ultra 14.0 and saved as .mol2 files. AutoDockTools (Version 1.5.7, Scripps Research Institute, La Jolla, CA) was used to remove water molecules and introduce polar hydrogens to the Cyt C target before exporting it as a .pdbqt file.

The ligand configurations were likewise converted into .pdbqt format after hydrogen atom addition via AutoDockTools. To evaluate the binding affinities between the protein and the selected molecules, automated docking calculations were executed using AutoDock Vina (Version 1.2.3). Structural configurations of the resulting complexes were evaluated using PyMOL (Version 2.2.0, Schrödinger LLC, Texas) and the PLIP web server (<https://plip-tool.biotech.tu-dresden.de/plip-web/plip/index>). The strength of the intermolecular interactions was determined by the calculated free energy of binding, with an empirical threshold of -5.0 kcal/mol chosen to denote meaningful binding affinity.

Affinity characterization via surface plasmon resonance

Kinetic rates and equilibrium dissociation constants were measured at 25°C using a Biacore 8 K instrument (Cytiva, Marlborough, MA) equipped with a Series S Sensor Chip CM5. The analytical mobile phase consisted of a PBS-P solution containing 20 mM phosphate, 2.7 mM KCl, 137 mM NaCl, and 0.05% Surfactant P20 (pH 7.4). Amine coupling chemistry via sulfo-NHS/EDC was applied to activate the carboxymethylated chip matrix, which was subsequently functionalized with 100 µg/mL of Cyt C dissolved in a sodium acetate vehicle (pH 4.5). Unreacted active sites were deactivated using a 1.0 M ethanolamine solution (pH 8.0). Stock concentrations of each target molecule were prepared in DMSO at 10 mM. These stocks were diluted 50-fold into the running buffer and subsequently diluted serially by halves to yield an experimental concentration gradient spanning from 50 down to 0.39 µM. Physical binding events were detected as variations in the optical path difference (measured in nm). Data processing was conducted via the Biacore 8 K Evaluation software, and the affinity constants K_D were calculated using a standard 1:1 Langmuir interaction model.

Spectrophotometric characterization in the UV-Vis range

The compounds baicalein and baicalin were chosen as model candidates for subsequent spectroscopic and in vitro cell culture evaluations. Electronic absorption spectra for isolated Cyt C and its corresponding complexes with baicalein and baicalin were collected via a photodiode array (PDA) spectrophotometer (Shimadzu, Tokyo, Japan) according to established protocols [32].

Operationally, a mixture containing 10 µL of 0.5 mM Cyt C, 10 µL of 0.5 mM of either polyphenol, and 180 µL of PBS (pH 7.4) was prepared. For the negative control sample, the small-molecule solution was substituted with an equal volume of PBS supplemented with 1% DMSO. After allowing the components to interact for 5 min at ambient room temperature, the mixtures were analyzed in the PDA system to record absorption profiles across a spectral window of 200–800 nm.

In vitro cell maintenance and functional assays

Human breast adenocarcinoma MCF-7 cells were supplied by the BeNa Culture Collection (Shangcheng, China). The cell population was maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cultures were kept at 37°C in a humid atmosphere containing 5% CO₂. Routine passaging was performed at a 1:5 split ratio concurrently with regular replenishment of the growth media.

Quantification of cell viability

Cellular metabolic activity was measured via the CCK-8 colorimetric method [33]. Cell suspensions (5×10^3 cells in 100 μ L) were seeded into individual wells of 96-well culture plates. Following exposure to various doses of baicalin or baicalein (12.5, 25, 50, and 100 μ M in a final volume of 70 μ L of medium) for incubation periods of 24, 48, or 72 h, 30 μ L of medium supplemented with 6 μ L of CCK-8 solution was added to each well. The plates were incubated for an additional 30 min, after which the absorbance (optical density, OD) was recorded at 450 nm using a multi-mode microplate reader (PerkinElmer, MA). The survival index was calculated according to the Eq. 1:

$$(OD_{test} - OD_{blank}) / (OD_{control} - OD_{blank}) \% \quad (1)$$

Independent experiments were executed with six parallel replicates.

Intracellular ATP quantification

Endogenous ATP concentrations were evaluated using a commercial bioluminescent ATP determination kit according to the developer's instructions. In brief, MCF-7 cells were seeded into 6-well culture dishes at a density of 1×10^5 cells per well and treated with specified concentrations of baicalin or baicalein (12.5, 25, 50, and 100 μ M) for 72 h. After washing twice with cold PBS, the monolayers were disrupted on ice using 200 μ L of specialized lysis buffer. The lysates were clarified by centrifugation at 12,000 g for 5 min at 4°C. The supernatant fractions were isolated and diluted 10-fold. A linear standard curve ranging from 0.1 to 10 μ M was constructed by diluting a stock solution of ATP (5 mM) with the extraction buffer. Luminescent signals were quantified via a microplate reader. Total protein content was determined by Lowry's colorimetric method [34], and final intracellular ATP levels were reported as μ mol/mg protein ($n = 3$).

Evaluation of mitochondrial membrane potential

MCF-7 cells were distributed into 12-well plates at an initial density of 4×10^4 cells per well and allowed to adhere for 24 h before receiving treatments of baicalin or baicalein (12.5, 25, 50, and 100 μ M) for another 24 h. Shifts in the mitochondrial membrane potential ($\Delta\psi_m$) were detected using a JC-1 assay kit. The cells were rinsed twice with PBS, transitioned into serum-free media, and stained with the JC-1 fluorophore for 20 min at 37°C. Red and green fluorescence emissions were visualized using an inverted fluorescence microscope. The JC-1 dye transitions from green monomers at lower potentials to red aggregates at higher potentials; the relative shift in the red-to-green fluorescence ratio was utilized to monitor variations in $\Delta\psi_m$.

Intracellular reactive oxygen species assay

The induction of intracellular reactive oxygen species (ROS) in treated MCF-7 cells was monitored via a commercial fluorometric kit following the manufacturer's protocol. Cells were seeded at 4×10^4 cells per well in 12-well plates, left to attach for 24 h, and subsequently incubated with baicalein or baicalin for 24 h. Following three consecutive washes with PBS, the cells were stained in the dark for 20 min using 10 μ M of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Unbound probe was removed by washing three times with serum-free culture medium, and fluorescence imaging of ROS-positive cells was conducted via inverted fluorescence microscopy.

Statistical evaluation

Data gathered from at least three independent experimental replicates are expressed as mean $\pm$ SEM. Statistical differences were evaluated via one-way analysis of variance (ANOVA) within the GraphPad Prism 9.5 software suite (San Diego, CA). Differences were categorized as statistically significant at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) relative to the untreated control group.

Results and Discussion

Platform configuration and justification using the known ligands

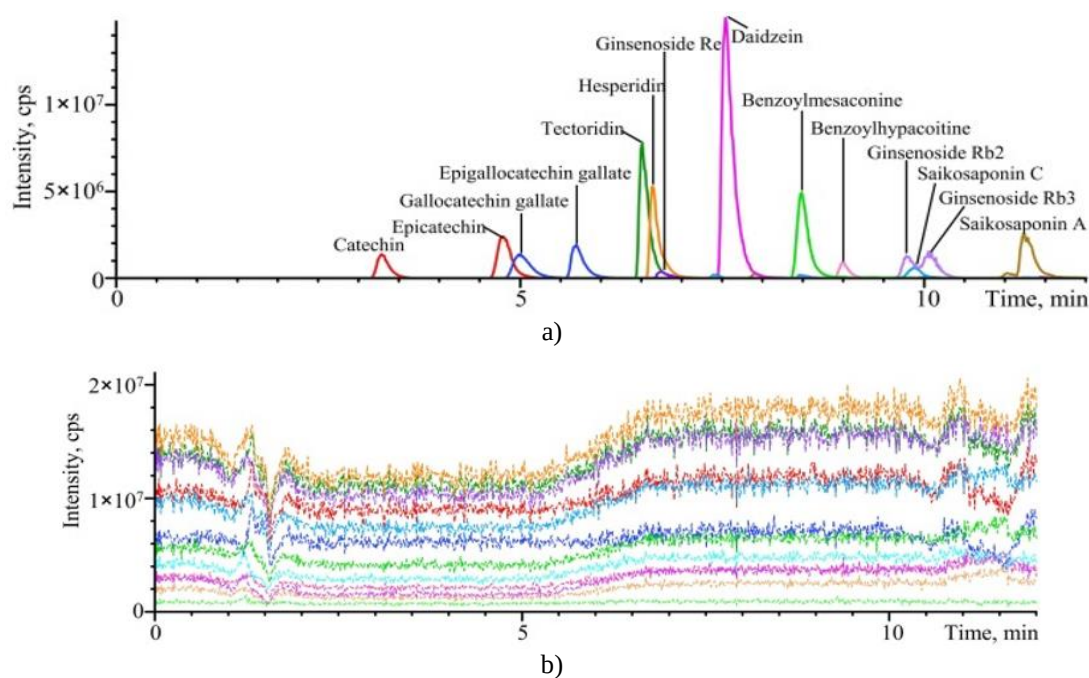
By integrating the required modules, the complete PCI-LC–ER-AMS system, illustrated in **Figure 1a**, comprises three functional components: LC separation, post-column infusion, and Qtof-MS analysis. Parallel control runs were performed using an injection of 25 % aqueous methanol (ligand-free control) and a post-column infusion of water (Cyt C-free control). Analysis of the combined STDs, SR, and ALRP followed the lower workflow shown

in **Figure 1b**, while the Cyt C-free and ligand-free assessments were executed according to the upper and middle workflows, respectively.

Significant research has focused on identifying Cyt C ligands, with numerous natural and synthetic substances—including flavonoids [35], saponins [36, 37], oligosaccharides [37], and polychlorinated biphenyls [38]—firmly established as binding agents. To validate the capability of this novel platform for the online screening of prospective ligands, a model solution was prepared utilizing twelve established ligands: catechin [27], epicatechin [29], epigallocatechin gallate [27], gallocatechin gallate [29], tectoridin [35], hesperidin [27, 35], Re [39], daidzein [27, 35], saikosaponin C [37], Rb2 [39], Rb3 [36], and SSA [37]. BMA and BHA were included as two negative control compounds.

Subsequently, considering the protonated molecular ions of all small molecules alongside the multi-charged quasi-molecular ions (i.e., $[M+nH]^{n+}$, where $n = 8, 9, 10$, etc.) of Cyt C, the superimposed extracted ion chromatogram (EIC) profiles obtained from analyzing Cyt C-free, ligand-free, and ligand-Cyt C mixtures via the protocols in **Figure 1b** are displayed in **Figures 2a–2c**, respectively. Because the eluent entering the ion source lacked Cyt C, the profile for the Cyt C-free control served as the chemical fingerprint of the combined STDs. As presented in **Figure 2a**, all reference standards are visible in this chromatogram, while signals corresponding to Cyt C are completely missing. In the absence of small molecules, a steady supply of Cyt C was introduced into the ion source, producing a stable chromatographic baseline for each quasi-molecular ion species (i.e., $[M+nH]^{n+}$, where $n = 8, 9, 10$, etc.) of Cyt C (**Figure 2b**), with a concurrent absence of protonated small molecule signals. Notably, continuous variations in the mobile phase composition caused baseline fluctuations, with the most pronounced variation occurring at 1.30 min, which represents the system void volume.

Upon introducing Cyt C into the LC effluent, the generated chromatogram (**Figure 2c**) displayed all targeted protonated molecular ions. A comparison of these small-molecule protonated ions between **Figures 2c and 2a** showed a pronounced decrease in intensity for each established ligand. Conversely, no such reduction was detected at the elution times of BMA and BHA. Remarkably, contrasting the Cyt C ion currents in **Figure 2c** with those in **Figure 2b** revealed several downward peaks exclusive to **Figure 2c**, denoting a marked drop in the abundance of these quasi-molecular ions. These negative signals precisely matched the retention times of the respective ligands. Collectively, these findings imply that complexation between the established ligands and Cyt C drives the formation of these negative peaks, which aligns with the capacity of all tested small molecules, excluding BMA and BHA, to bind with Cyt C.



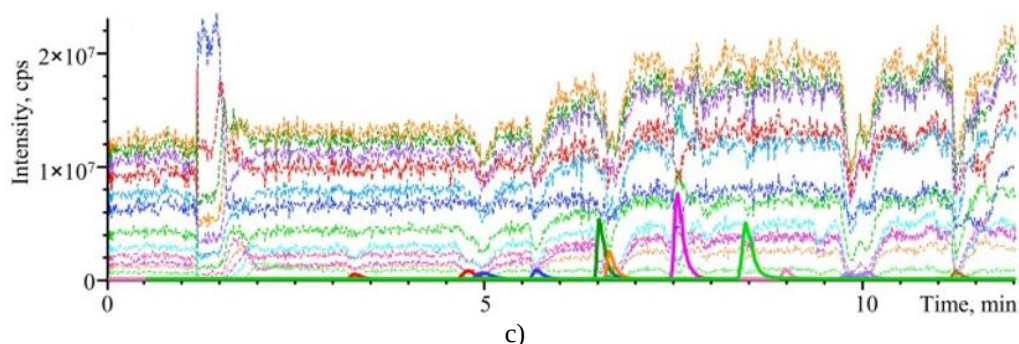
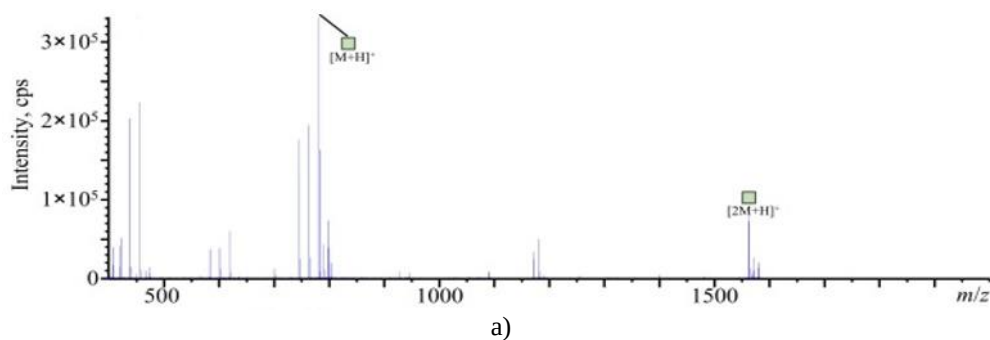


Figure 2. Typical superimposed extracted ion chromatograms for the Cyt C-free control (a), mixed reference standards-free control (b), and the combined STDs-Cyt C sample (c), acquired by monitoring the protonated molecular ions of the fourteen standard compounds and the sequential $[M+nH]^{n+}$ ($n = 8, 9, 10$, etc.) ions of unbound Cyt C.

Beyond this indirect proof, subsequent efforts focused on identifying direct evidence of ligand-protein complexation by contrasting MS spectra at matching retention times across the three datasets. Within the MS¹ spectra of the small molecules, protonated molecular signals typically predominated, while a series of protonated molecular ions across multiple charge states ($n = 8, 9, 10$, etc.) appeared throughout the MS¹ spectra derived from **Figure 2b**. Remarkably, alongside the signals present in both **Figures 2a and 2b**, supplementary ion species with higher masses than the corresponding multi-charged protonated Cyt C ions were detected, save for the negative controls.

Utilizing the MS¹ spectra at the elution time of SSA (11.23 min) as an example, the MS¹ spectrum for SSA (**Figure 3a**) displayed a dominant peak at m/z 781.4666 ($[M+H]^+$). The MS¹ spectrum for unbound Cyt C (**Figure 3b**) presented multiple ion clusters composed of monoisotopic peaks and their corresponding ¹³C -isotope variations. Since the spacing between the monoisotopic peak and its adjacent ¹³C isotope peak indicates the charge state, ions spanning charge states from 7+ to 21+ were identified. For example, the highest intensity monoisotopic signal at m/z 727.9752 was identified as the $[M+17H]^{17+}$ ion, while the peak at m/z 728.0329 was assigned to the $[^{13}\text{C-M}+17\text{H}]^{17+}$ species. Based on these $[M+nH]^{n+}$ ions, the molecular mass of unbound Cyt C was determined to be roughly 12,358.41 Da.

At the precise same retention time, the negative peak observed in **Figure 2c** yielded an MS¹ spectrum (**Figure 3c**) displaying a higher number of ion species compared to both controls. Following the subtraction of peaks associated with SSA and Cyt C, residual signals were identified as adducts formed by multi-charged protonated Cyt C bound to multiple neutral SSA molecules, verified by elemental composition analysis. Specifically, peaks at m/z 773.89, 819.80, and 865.75 (**Figure 3c**), located to the right of the m/z 727.9752 signal, were assigned as $[\text{Cyt C} + \text{SSA} + 17\text{H}]^{17+}$, $[\text{Cyt C} + 2 \times \text{SSA} + 17\text{H}]^{17+}$, and $[\text{Cyt C} + 3 \times \text{SSA} + 17\text{H}]^{17+}$, respectively. Consequently, twelve positive chromatographic peaks exhibiting pronounced response variations were resolved in the EICs corresponding to the m/z values of the ligand-protein adducts (taking $z = 15$ as an example), co-eluting exactly with their respective small-molecule ligands (**Figure 3d**).



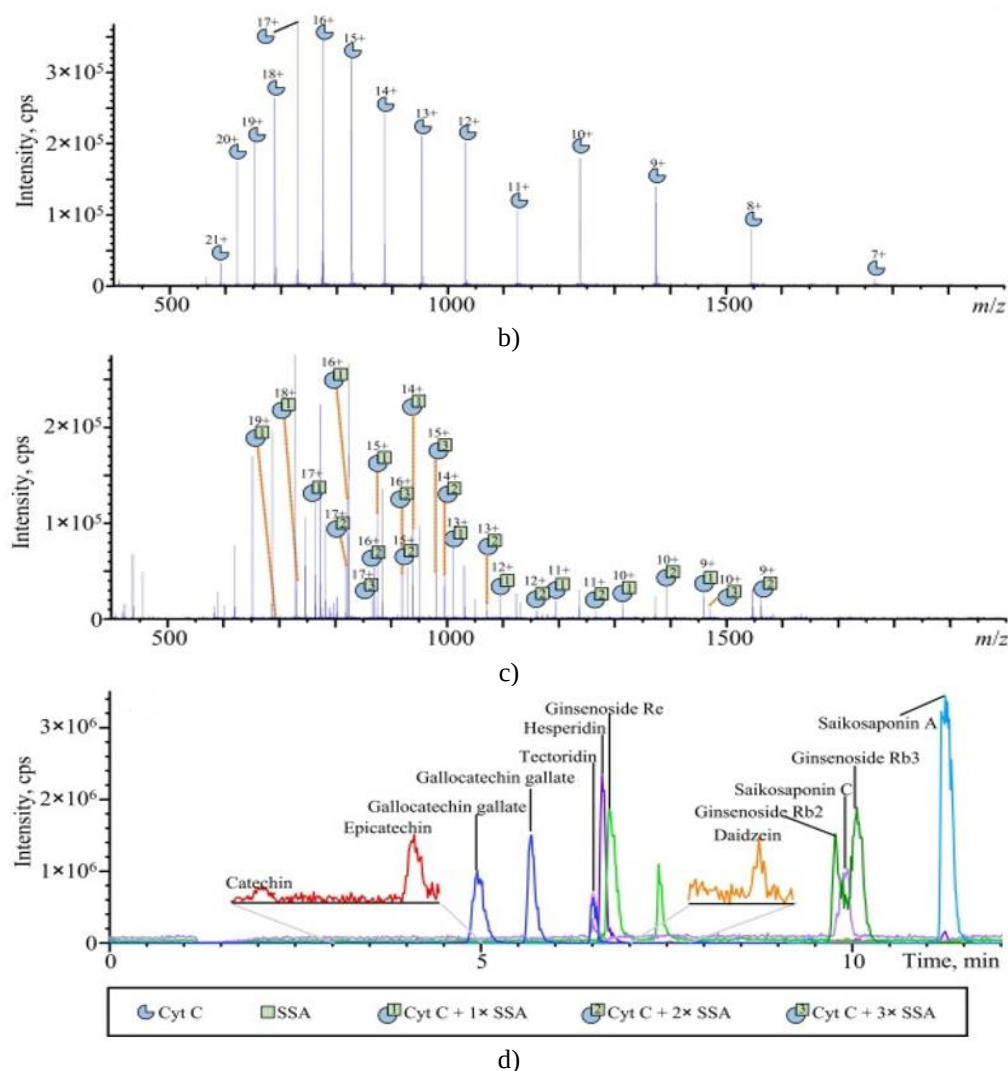


Figure 3. MS^1 spectra obtained at 11.23 min for SSA (a), unbound Cyt C (b), and the SSA-Cyt C complex (c), alongside typical extracted ion chromatograms for ligand-Cyt C complexes at a 15+ charge state generated from the mixed STDs-Cyt C analysis (d).

In conclusion, the PCI-LC–ER-AMS platform demonstrates high capability for the robust, online screening and identification of Cyt C ligands.

Binding strength comparison amongst different ligands by online ER-MS

Collision-induced dissociation (CID) facilitates the cleavage of either covalent [40] or non-covalent linkages [22], wherein the binding affinity is positively correlated with the optimal collision energy (OCE) determined via the online ER-MS workflow. Furthermore, gas-phase complexes formed between ligands and proteins closely mimic physiological states [21, 41]. Consequently, online ER-MS was configured to assess the binding affinity between Cyt C and each ligand, utilizing MRMHR to monitor the respective fragment ion abundances across incrementally increasing CE levels to construct the breakdown graph.

Regardless of the charge state, all MS^1 spectral peaks corresponding to the complexes composed of Cyt C bound to a single ligand molecule—especially the highly abundant species—were initially subjected to a fixed CE to generate MS^2 spectra. As anticipated, CID of each complex yielded fragment signals corresponding to both the free ligand and free Cyt C. For example, using m/z 876.94 ($[Cyt\ C + SSA + 15H]^{15+}$) as a representative precursor, a series of fragment ions were resolved in the MS^2 spectrum (**Figure 4a**); these included m/z 883.76 ($[Cyt\ C + 14H]^{14+}$) and 824.90 ($[Cyt\ C + 15H]^{15+}$) assigned to Cyt C, alongside m/z 781.47, 763.47, 745.46, 455.36, 437.35, and 419.33 attributed to SSA. Notably, the signals associated with SSA encompassed the majority of its characteristic fragment ion species extracted from **Figure 2a**, thereby offering robust confirmation for the structural identification of the ligands.

Subsequently, the ion transition m/z 876.94 $\rightarrow$ 824.90 was monitored via the MRMHR program to generate a Gaussian-shaped breakdown graph modeling ligand dissociation. As illustrated in **Figure 4b**, the OCE required for the dissociation of the Cyt C-SSA complex is determined to be 24.00 eV from the regression equation $Y = 52,6621 \times \exp(-0.5 \times ((X-24.00)/7.617)^2)$. Examination of all protonated molecular signals (**Figure 3c**) of the SSA-Cyt C complexes revealed distinct OCE variations across different charge states when conducting online ER-MS on the $[\text{Cyt C} + \text{SSA} + n\text{H}]^{n+} \rightarrow [\text{Cyt C} + n\text{H}]^{n+}$ ($n = 13 \sim 19$) ion transitions. This precise phenomenon was consistently observed for the remaining ligands. Furthermore, evaluating all protonated molecular signals for every ligand-protein complex yielded extensive OCE data, presenting a significant challenge for directly comparing the Cyt C binding affinities among the different ligands.

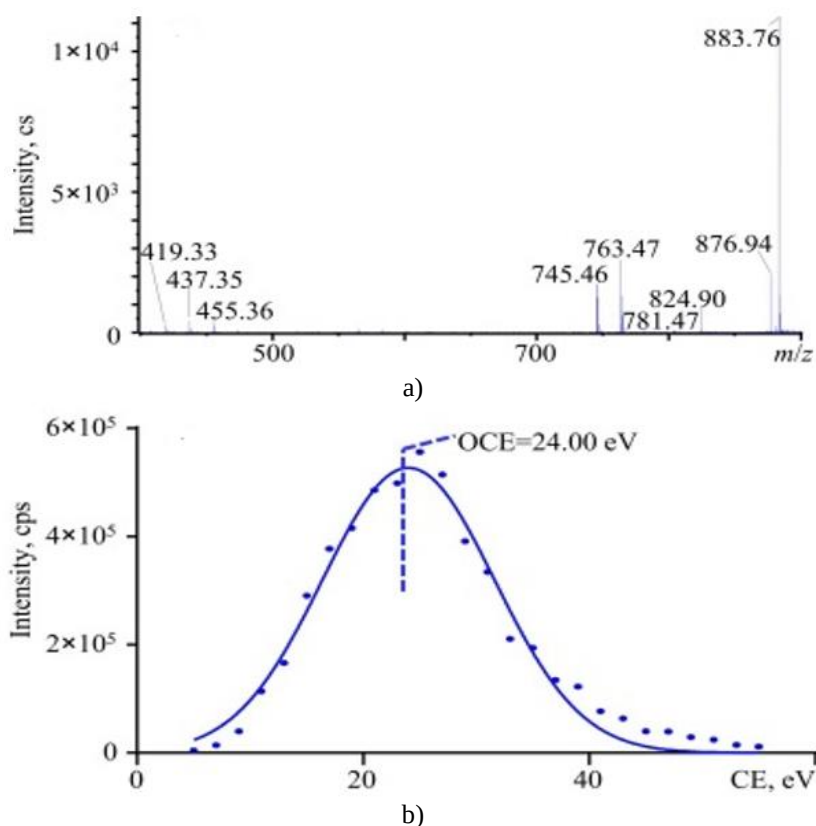


Figure 4. MS² spectrum (A) of m/z 876.94 ($[\text{Cyt C} + \text{SSA} + 15\text{H}]^{15+}$) and the corresponding MRM^{HR} response profile (B), representing the Gaussian-shaped breakdown graph ($Y = 526,621 \times \exp(-0.5 \times ((X-24.00)/7.617)^2)$, OCE = 24.00 eV), for m/z 876.94 $\rightarrow$ 824.90 ($[\text{Cyt C} + \text{SSA} + 15\text{H}]^{15+} \rightarrow [\text{Cyt C} + 15\text{H}]^{15+}$) plotted against CE.

Following a detailed evaluation of the OCEs across varying charge states, it was observed that the OCE values exhibited a rough positive linear correlation with the precursor ion masses, whereas the product ion masses exerted a minor influence [42]. Consequently, it is possible to express the binding strength of a ligand as a normalized value, designated as NOCE, by scaling the experimental OCE (EOCE) relative to the mass of the precursor ion. The conversion of EOCE to NOCE was performed using Eq. 2:

$$NOCE = E_{OCE} \times 1000 / M_{Prec} \quad (2)$$

where NOCE, EOCE, and M_{prec} denote the normalized OCE, the experimental OCE, and the precursor ion mass, respectively.

The compiled NOCE values for the twelve established ligands are presented in **Figure 5**. Based on these NOCE metrics, the twelve analytes were categorized into three distinct classes (Grades I–III). Grade I comprised Rb2, Rb3, Re, SSA, and saikosaponin C, which demonstrated the highest binding strengths. Grade III included

daidzein, catechin, and epicatechin, reflecting weak binding affinity toward Cyt C. The remaining analytes, consisting of hesperidin, tectoridin, epigallocatechin gallate, and galliccatechin gallate, were assigned to Grade II, demonstrating intermediate binding capacities.

Notably, all constituents in Grade I were saponins; their pronounced binding potentials are ascribed to the formation of extensive hydrogen bonding networks between the glycone residues of the saponins and the functional moieties (such as unbound carboxyl and amino groups) of Cyt C. Within the Grade II category, hesperidin and tectoridin are flavonoid glycosides, whereas epigallocatechin gallate and galliccatechin gallate structurally comprise flavonoid and polyphenolic subunits. Their capacity to establish hydrogen bonds with Cyt C was lower than that of the saponins due to a lower density of hydroxyl groups. However, because of the multiple aromatic rings and π - π conjugated systems present within their frameworks, they can interact with aromatic amino acid residues in Cyt C (such as phenylalanine, tryptophan, and tyrosine) via π - π stacking interactions, thereby enhancing their overall binding strength. Due to a restricted number of hydroxyl groups or aromatic rings available to engage in hydrogen bonding or π - π associations, daidzein, catechin, and epicatechin demonstrated the lowest binding capacities for Cyt C.

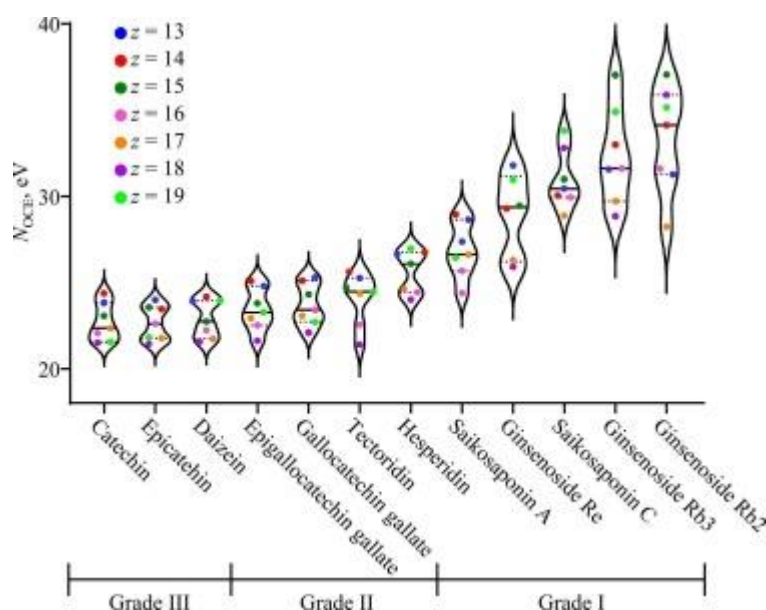


Figure 5. Violin plots illustrating the distribution patterns of NOCE values for the transitions $[\text{Cyt C} + \text{ligand} + n\text{H}]^{n+} \rightarrow [\text{Cyt C} + n\text{H}]^{n+}$ ($n = 13 \sim 19$) across all identified ligand-Cyt C complexes, along with the classification scheme (I, II, and III) representing the affinity potentials of twelve established ligands, including apigenin, apigenin 7-O-glucuronide, baicalein, baicalin, wogonin, wogonoside, oroxylin A, oroxylin A 7-O-glucuronide, ginsenoside Re, ginsenoside Rb2, ginsenoside Rb3, catechin, epicatechin, epigallocatechin gallate, galliccatechin gallate, hesperidin, tectoridin, and daidzein.

The twelve established ligands were subsequently evaluated via molecular docking simulations with Cyt C. The calculated binding energies were consistently below -5.0 kcal/mol, which represents the established threshold for meaningful ligand-protein binding [43], indicating substantial binding affinities for most compounds toward Cyt C. Promisingly, the sequence of these calculated affinities aligned reasonably well with the experimental grading tiers. The affinity values of the Grade I saponins exceeded those of the majority of Grade II flavonoids, while the compounds assigned to Grade III generally yielded low affinity scores. Furthermore, this classification scheme is fully congruent with the documented pharmacological effects of these constituents regarding Cyt C interaction available in existing literature [44-49]. In conclusion, the NOCE metric is shown to be a reliable parameter for evaluating the binding potential of candidate molecules toward Cyt C.

Online ligand screening for Cyt C from SR and ALRP

Subsequently, the PCI-LC–ER-AMS framework was deployed for the online screening of Cyt C ligands within ALRP and SR extracts, with both samples analyzed according to all experimental schemes delineated in **Figure 1b**. The superimposed EIC profiles for ALRP and SR are shown in **Figure 6**, respectively.

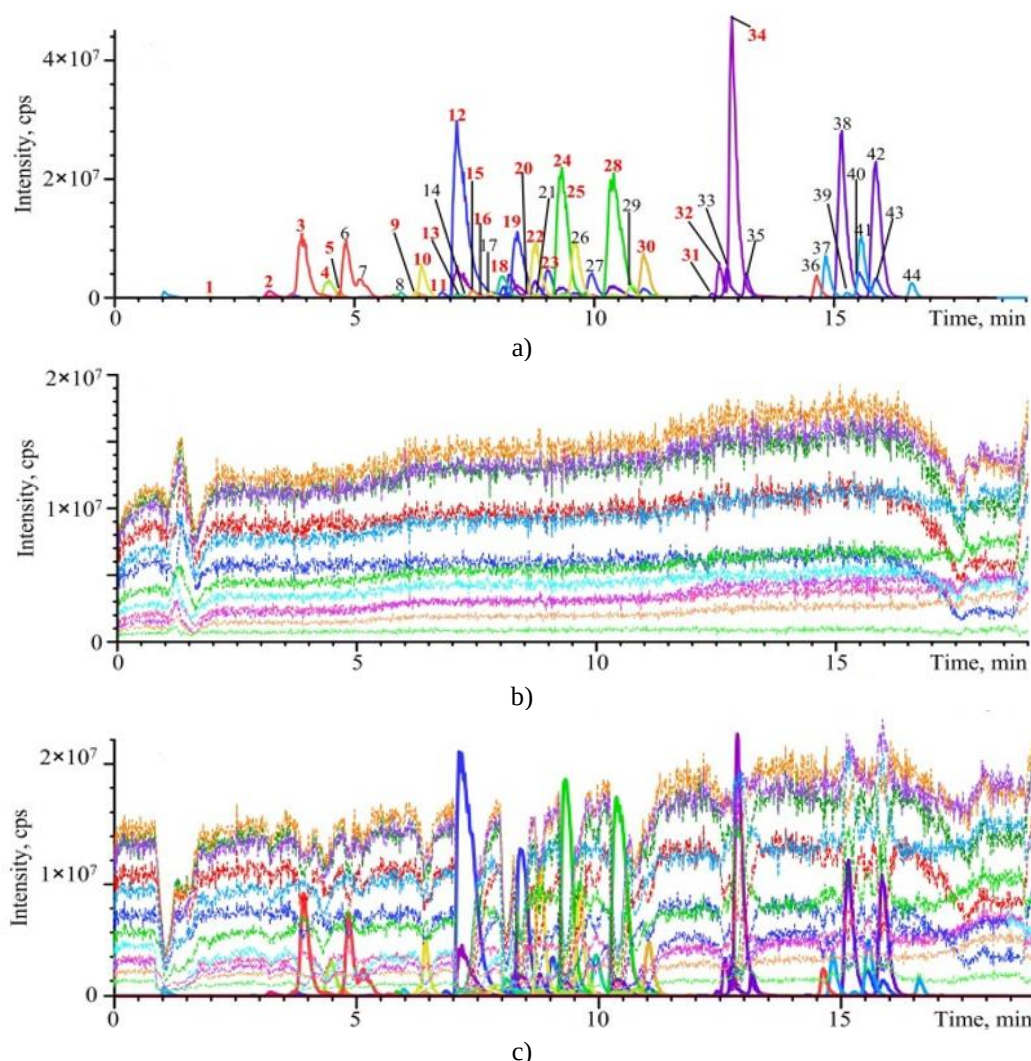


Figure 6. Typical superimposed extracted ion chromatograms for the Cyt C-free control (a), SR-free control (b), and the combined SR-Cyt C sample (c), recorded by tracking the protonated molecular ions of the primary constituents in SR (44 compounds in total, numbered 1–44) alongside the sequential $[M+nH]^{n+}$ ($n = 8, 9, 10$, etc.) ions of unbound Cyt C.

The phytochemical composition of ALRP was effectively monitored via LC–MS/MS. Through structural characterization and comparison of the acquired MS/MS spectra with literature data [50], prominent constituents were identified, including mesaconine (3.47 min), aconine (4.13 min), songorine (4.31 min), fuziline (4.75 min), neoline (5.08 min), fuzitine (5.22 min), BMA (7.99 min), benzoylaconine (8.63 min), BHA (9.01 min), mesaconitine (10.04 min), aconitine (10.82 min), hypacoitine (10.88 min), indaconitine (11.56 min), and chasmaconitine (12.07 min).

The chemical characterization of SR was performed by detailed interpretation of MS/MS spectra, leading to the tentative identification of 44 compounds (**Figure 6a**) cross-referenced against a previously established data library [51].

By comparing the profiles in **Figures 6b** and **6c**, a total of 24 compounds matching the negative peaks in **Figure 6c** were identified as prospective Cyt C ligands from SR.

Efforts were also directed toward identifying ion species representing the ligand-Cyt C complexes. Both protonated molecular ions and specific fragment ions belonging to the free ligands, unbound Cyt C, and their non-covalent adducts were successfully detected within these negative peaks (**Figure 6c**). For example, the MS¹ spectrum recorded at 10.40 min exhibited the protonated molecular ion of wogonoside at m/z 461.1077 ($C_{22}H_{21}O_{11}^+$). In addition to the signals from unbound Cyt C (e.g., $[Cyt\ C + nH]^{n+}$, where $n = 9 \sim 21$), it revealed distinctive peaks corresponding to wogonoside-Cyt C complexes, such as m/z 916.69 $[Cyt\ C + 1 \times wogonoside]$.

+ 14H]¹⁴⁺, 1022.36 [Cyt C + 2 × wogonoside + 13H]¹³⁺, 1057.75 [Cyt C + 3 × wogonoside + 13H]¹³⁺, 1578.66 [Cyt C + 4 × wogonoside + 9H]⁹⁺, and 1630.34 [Cyt C + 5 × wogonoside + 9H]⁹⁺.

Furthermore, collision-induced dissociation of the [Cyt C + wogonoside + 13 H]¹³⁺ population (*m/z* 987.05) produced an MS² spectrum featuring principal fragment ions assigned to *m/z* 1030.7622 ([Cyt C + 12H]¹²⁺), 951.7058 ([Cyt C + 13H]¹³⁺), 461.0955 ([wogonoside + H]⁺), 285.0668 ([wogonoside – C₆H₁₀O₅ + H]⁺), and 270.0429 ([wogonoside – C₆H₁₀O₅ – CH₃• + H]⁺). These combined MS¹ and MS² spectral features definitively confirm the formation of wogonoside-Cyt C complexes. The EIC traces of the ligand-Cyt C complexes (using *z* = 15 as an example) for the 24 ligands in SR produced 24 positive peaks at corresponding retention times, validating the reliability of the screening assay.

Subsequent investigations turned to assessing the binding strengths of these isolated compounds toward Cyt C. Following ER-MS analysis, Gaussian-shaped breakdown graphs for all prospective ligand-Cyt C complexes—corresponding to the ion transitions [Cyt C + ligand + *n*H]^{*n*+} → [Cyt C + *n*H]^{*n*+},—were generated. OCE values were derived using the corresponding regression equations. The OCEs calculated for each specific charge state *n*⁺ were then converted to NOCE parameters using Eq. 2.

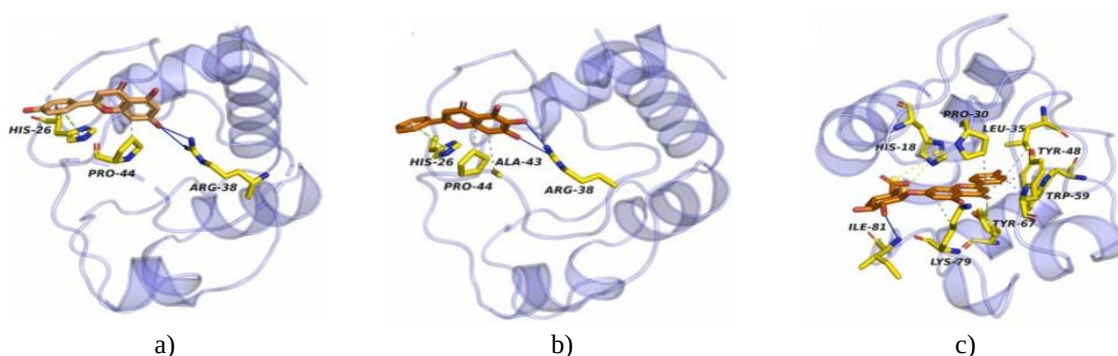
On the whole, the NOCE parameters for the flavonoid aglycone-Cyt C complexes were considerably lower than those of their glycoside counterparts. For instance, the NOCE values for baicalin and baicalein were determined to be 26.35 and 24.64 eV, respectively, at a charge state of 13⁺. In general, flavonoid glycosides carry extra hydroxyl groups from their glycone substitutions, which facilitates the generation of a greater number of hydrogen bonds with Cyt C, thereby yielding higher binding affinities. Moreover, several glycosides within SR possessed a structural advantage for establishing salt bridges due to the carboxyl moiety on their glucuronyl subunits (e.g., baicalin, apigenin-7-O-glucuronide, wogonoside, and oroxylin A-7-O-glucuronide). Owing to the presence of free hydroxyl functionalities in their frameworks, baicalein and apigenin demonstrated intermediate binding potentials toward Cyt C. For alternative aglycones, such as oroxylin A, wogonin, skullcapflavone II, and tenaxin I, one or more hydroxyl functionalities are replaced by methoxyl groups, thereby compromising their capacity to engage in hydrogen bonding with Cyt C.

Validation of binding affinity

To preliminarily corroborate the experimental data acquired from PCI-LC–ER-AMS, computational molecular docking simulations were conducted.

Regarding the two primary alkaloids derived from ALRP, both BMA and BHA exhibited binding energies exceeding –5 kcal/mol. Consequently, neither compound could function as an effective ligand for Cyt C, which strongly aligns with the observed absence of ligand-Cyt C complexation when ALRP and Cyt C co-existed. Concurrently, the six candidate ligands isolated from SR predictably demonstrated binding energies falling below –5.0 kcal/mol. Importantly, the hierarchical order of their calculated binding affinities closely matched the trend observed for their NOCE values.

The flavonoid glycosides—specifically baicalin, wogonoside, apigenin 7-O-glucuronide, and oroxylin A 7-O-glucuronide—displayed superior docking affinities compared to their aglycon counterparts, baicalein and apigenin. The docking simulations revealed that apigenin, baicalein, apigenin 7-O-glucuronide, baicalin, oroxylin A 7-O-glucuronide, and wogonoside established three, three, two, one, two, and eight hydrogen bonds, respectively, with residues within the catalytic pocket [35, 36] (**Figure 7**). Furthermore, compared to baicalein and apigenin, the glycosides (baicalin, apigenin-7-O-glucuronide, oroxylin A-7-O-glucuronide, and wogonoside) participated in a higher number of hydrophobic interactions, π -stacking arrangements, and salt bridges.



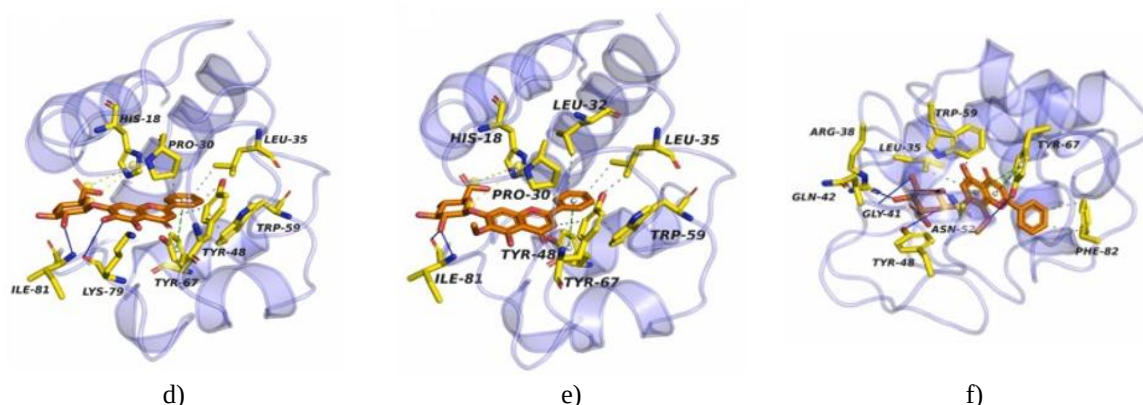
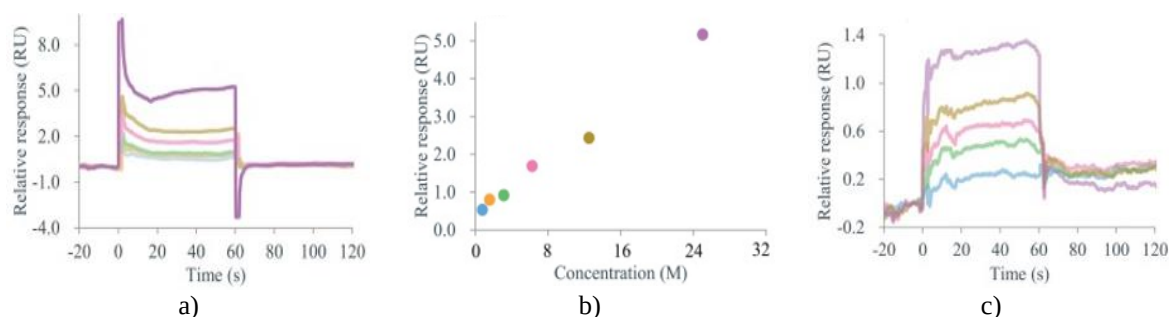


Figure 7. Computational docking profiles of Cyt C complexed with apigenin (a), baicalein (b), apigenin 7-O-glucuronide (c), baicalin (d), oroxylin A 7-O-glucuronide (e), and wogonoside (f). Intermolecular interactions with specific amino acid residues are depicted by blue, gray-dashed, yellow-dashed, and green-dashed lines representing hydrogen bonds, hydrophobic interactions, salt bridges, and π -stacking, respectively.

To rigorously validate the identification of these screened molecules as genuine Cyt C ligands, surface plasmon resonance (SPR) spectroscopy assays were performed on six representative compounds from SR: baicalein, baicalin, apigenin, apigenin 7-O-glucuronide, wogonoside, and oroxylin A 7-O-glucuronide. Furthermore, the SPR analysis included multiple positive controls (daizein, epicatechin, epigallocatechin gallate, tectoridin, Rb3, and SSA) alongside negative controls (BMA and BHA).

The empirically determined K_D values for both BMA and BHA substantially exceeded 60 μM , confirming that these two molecules do not interact as ligands with Cyt C. In contrast, the positive controls—daizein, epicatechin, epigallocatechin gallate, tectoridin, Rb3, and SSA—yielded K_D values of 36.50, 20.30, 15.70, 12.50, 3.23, and 1.63 μM , respectively, which were all notably below 50 μM . Consistent with the outcomes from the ER-MS and molecular docking analyses, the two saponins, Rb3 and SSA, demonstrated the highest degree of binding affinity. The relative order of the K_D values for daizein, epicatechin, epigallocatechin gallate, and tectoridin generally correlated with the sequence of their NOCE values.

Attention was subsequently directed toward the candidate ligands identified from SR. The four representative flavonoid glycosides exhibited markedly lower K_D values than their corresponding aglycones. The two flavonoid aglycones, apigenin and baicalein, demonstrated distinct binding modalities with Cyt C. For apigenin, the relative response versus concentration exhibited a near-linear correlation, indicating the formation of non-specific apigenin-Cyt C complexes [52] (**Figures 8a and 8b**). Conversely, baicalein demonstrated a moderate affinity for Cyt C, as indicated by a K_D value of 24.0 μM (**Figures 8c, 8d**). The glycosides, comprising apigenin 7-O-glucuronide (**Figures 8e and 8f**), baicalin (**Figures 8g and 8h**), oroxylin A 7-O-glucuronide (**Figures 8i and 8j**), and wogonoside (**Figures 8k and 8l**), displayed enhanced binding potencies, characterized by K_D values of 2.65, 1.87, 2.43, and 2.50 μM , respectively.



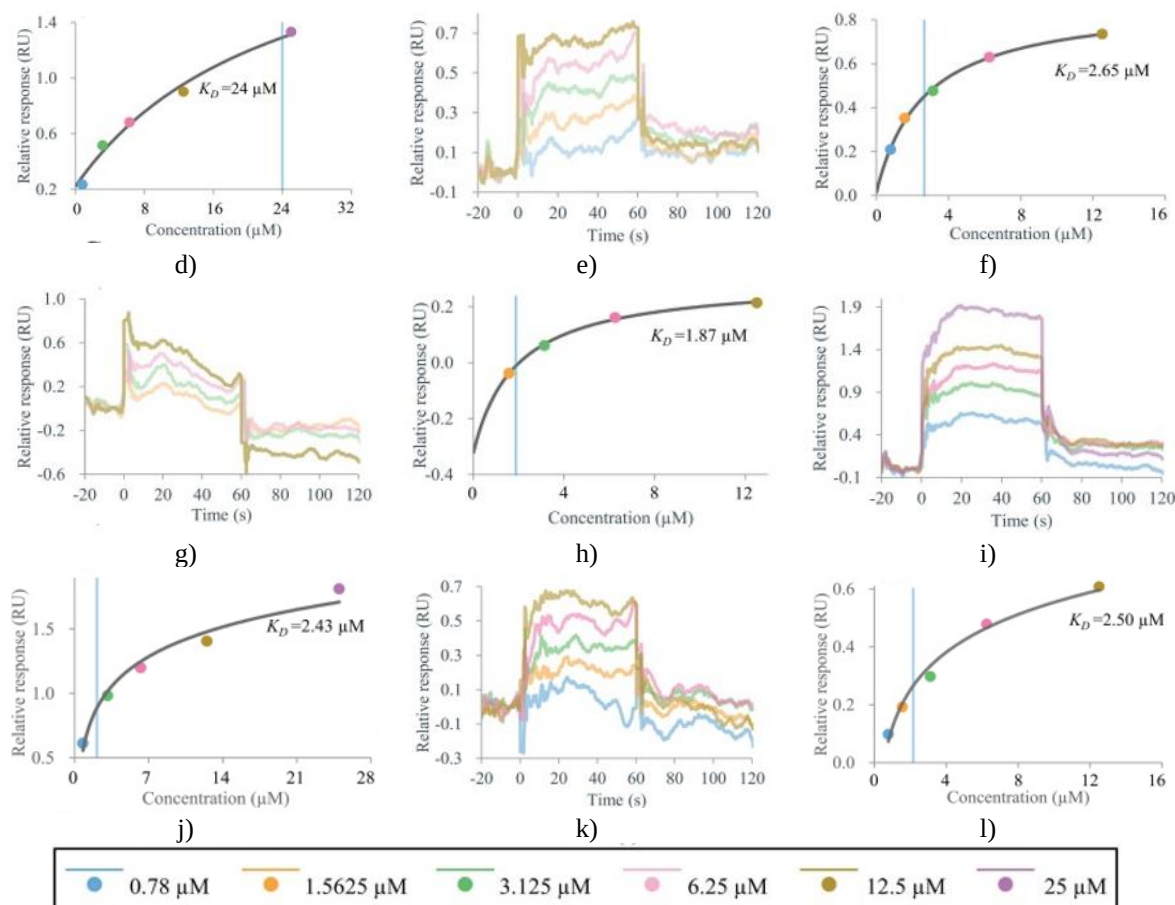


Figure 8. Interaction profiles and affinity assessments of Cyt C across various concentrations (0.78, 1.5625, 3.125, 6.25, 12.5, and 25.0 μM) of apigenin (a, b, $K_D > 25.0 \mu\text{M}$), baicalein (c, d, $K_D = 24 \mu\text{M}$), apigenin 7-O-glucuronide (e, f, $K_D = 2.65 \mu\text{M}$), baicalin (g, h, $K_D = 1.87 \mu\text{M}$), oroxylin A 7-O-glucuronide (i, j, $K_D = 2.43 \mu\text{M}$), and wogonoside (k, l, $K_D = 2.50 \mu\text{M}$) determined via SPR measurements.

In conclusion, both the computational docking simulations and the SPR spectroscopic analyses validated the experimental observations obtained from evaluating standard mixtures, SR, and ALRP via PCI-LC–ER-AMS. Crucially, the glycosides derived from SR—specifically apigenin 7-O-glucuronide, baicalin, oroxylin A 7-O-glucuronide, and wogonoside—emerge as highly promising ligands for Cyt C.

Functional regulatory evaluations of baicalein and baicalin toward Cyt C

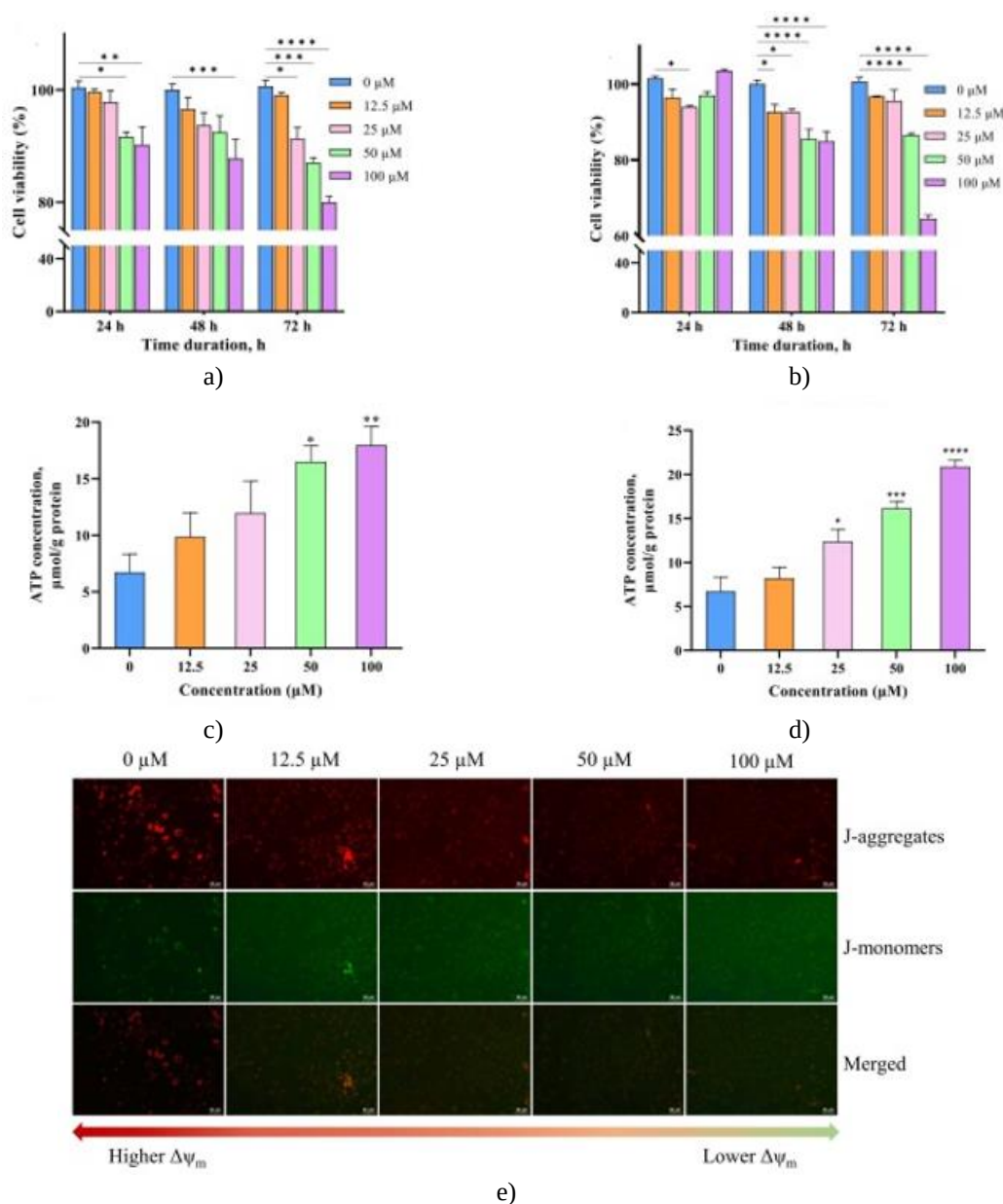
To corroborate the data obtained via PCI-LC–ER-AMS, baicalein and baicalin were chosen as representative test agents for subsequent UV spectrophotometric and in vitro cellular analyses, having been classified as weak and strong Cyt C ligands, respectively.

Because the oxidized and reduced states of Cyt C possess distinct UV-Vis profiles, their structural variations can be monitored optically. Due to its internal porphyrin chromophores, native Cyt C displays a characteristic Soret band at 409 nm and a minor absorption peak at 528 nm; these features typically shift upon complexation with a ligand. Introducing either baicalin or baicalein into the Cyt C matrix induced noticeable spectral variations. Specifically, the Soret maximum shifted to 412 nm in the presence of baicalin and to 414 nm with baicalein. Moreover, the original 528 nm peak of Cyt C resolved into two distinct peaks located at 520 and 549 nm for both the baicalin-Cyt C and baicalein-Cyt C combinations. Notably, the baicalin-Cyt C complex yielded a higher absorbance magnitude at both coordinates.

To determine the cytotoxic profiles of baicalin and baicalein, a CCK-8 screening was performed utilizing MCF-7 cell lines. **Figures 9a and 9b** track the survival rates of these cells across exposure intervals of 24, 48, and 72 h. On the whole, the two molecules demonstrated equivalent cytotoxic potencies. Each substance produced pronounced toxic responses following a 24-h exposure window at concentrations exceeding 50 μM , with the cytotoxic impact exhibiting a clear dependence on incubation duration. Because ATP serves as a primary metric

for cellular respiration, internal ATP concentrations within the MCF-7 cells were quantified after a 72-h incubation with either compound. As illustrated in **Figures 9c and 9d**, both agents increased ATP levels in a dose-dependent manner, with statistically significant disparities emerging between the treated groups and the control baseline once the dosage reached 50 μM .

Following evaluation of the $\Delta\psi_m$ in MCF-7 cells using a JC-1 fluorescent probe, analogous trends were noted for both baicalin and baicalein across all administered concentrations. As the dosage escalated, $\Delta\psi_m$ values dropped progressively, which correlated with a loss of red fluorescence and a simultaneous intensification of green fluorescence. Regarding intracellular ROS generation within MCF-7 cultures under varying treatment concentrations, both baicalin and baicalein produced matching dose-dependent increases, marked by a steady rise in green fluorescence intensity (**Figure 9g**). Synthetically, these findings indicate that by complexing with intracellular Cyt C, both baicalin and baicalein impair the protein's electron-transfer/redox activities and trigger severe mitochondrial disruption. This homeostatic failure is characterized by a rise in intracellular ATP along with a reduction in mitochondrial membrane potential. Ultimately, the downstream accumulation of cellular ROS appears to provoke lethal cellular dysfunction, resulting in cell death.



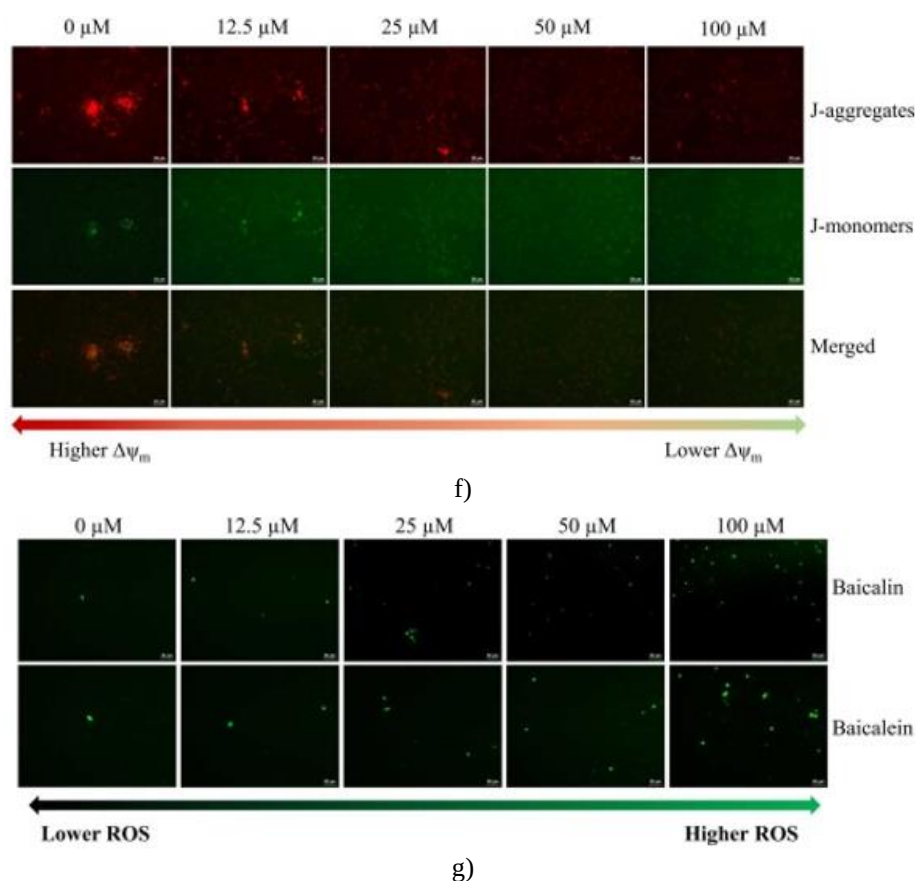


Figure 9. Survival rates of MCF-7 cells following exposure to baicalin (a) and baicalein (b) at doses of 0, 12.5, 25, 50, and 100 μM over 24, 48, and 72 h ($n = 6$); internal ATP levels after 72-h treatments with baicalin (c) and baicalein (d) at 0, 12.5, 25, 50, and 100 μM ($n = 3$); mitochondrial membrane potential $\Delta\psi_m$ profiles of MCF-7 lines subjected to 0, 12.5, 25, 50, and 100 μM of baicalin (e) and baicalein (f) for 24 h; and reactive oxygen species (ROS) induction in MCF-7 cells following a 24-h incubation with 0, 12.5, 25, 50, and 100 μM of baicalin and baicalein (g). Note: *, $P < 0.05$; , $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Following comprehensive validation of their binding parameters and biological activities, baicalin and baicalein—acting as index ligands isolated from SR using PCI-LC–ER-AMS—demonstrated definitive conjugation to Cyt C, driving both the observed UV-Vis spectral modifications and the subsequent cytotoxic events.

For several decades, the identification of bioactive natural compounds from herbal medicines (HMs) has remained a major focus of scientific research. Native mass spectrometry (MS) has gained widespread recognition as a flexible and effective biophysical technique for analyzing protein-small molecule interactions, facilitating the characterization of ligand-protein complexes within the gas phase. Ultra-performance liquid chromatography (UPLC) has been successfully integrated into numerous online ligand screening frameworks designed to identify molecules exhibiting anti-acetylcholinesterase, anti-cyclooxygenase, antiglycation, and anti-skin aging properties [10–12].

Nevertheless, these traditional methodologies are severely constrained by laborious, time-intensive sample preparation procedures and cumbersome data processing requirements. These limitations considerably hinder the rapid isolation of candidate ligands from intricate matrices such as HMs. Notably, false-positive results frequently arise because the majority of existing assays classify a given molecule as active based solely on a reduction in the response of the compound itself [11] or its probe substrate [11, 12].

In the present study, liquid chromatography (LC) was coupled with native MS via post-column protein infusion to achieve the rapid screening of potential ligands within HMs. An online energy-resolved MS (ER-MS) protocol, executed via high-resolution multiple reaction monitoring MRM^{HR} mode, was subsequently utilized to assess the binding affinities of these ligands toward the target protein. As a proof-of-concept, the inclusion of cytochrome C (Cyt C) along with twelve established ligands and two negative controls validated the efficacy of this post-

column infusion liquid chromatography energy-resolved affinity mass spectrometry (PCI-LC–ER–AMS) approach. The system reliably identified target ligands by utilizing a "four-in-one" monitoring strategy:

1. a reduction in the signal response of candidate ligands;
2. a reduction in the signal response of the target protein;
3. an increase in the signal response of the corresponding ligand-protein complex; and
4. the quantification of ligand-protein binding strength.

Non-covalent interactions, such as hydrogen bonding, van der Waals forces, and electrostatic interactions, are primary drivers of ligand-protein complex formation. Enhanced interaction forces directly correspond to a more robust binding capacity between ligands and proteins, which consequently necessitates a higher onset collision energy (OCE) to dissociate the complex into its constituent protein and ligand ions. Online ER-MS monitors the trajectories of ion abundance for either precursor or product ions against incrementally increasing collision energies (CEs), deriving the OCE by plotting breakdown curves of specific fragment ions.

Theoretically, comparing OCE values across distinct complexes allows for an assessment of their respective binding potentials toward a target protein. However, because OCE is positively correlated with precursor ion mass and negatively correlated with product ion mass, the current study configured ion transitions using identical product ions and the same charge state. Following this, the OCE values were normalized via an empirical formula to enable direct comparisons across varied complexes. Rigorous validation via surface plasmon resonance (SPR) spectroscopy, molecular docking, UV-Vis spectrometry, and cellular assays confirmed that higher normalized OCE (NOCE) values for the transition $[\text{Cyt C} + \text{ligand} + n\text{H}]^{n+} \rightarrow [\text{Cyt C} + n\text{H}]^{n+}$ ($n = 13 \sim 19$) consistently indicate superior ligand binding affinities.

Conclusion

By combining three distinct functional units—namely LC–ESI–Qtof–MS, ER–MS, and post-column protein infusion—a PCI-LC–ER–AMS framework was established to perform high-fidelity online ligand fishing for Cyt C. Twelve established Cyt C ligands served as positive controls to confirm the reliability of this novel methodology, while two alkaloids, benzylmorphine (BMA) and berberine (BHA), were applied as negative controls. For the positive controls, signal reductions were observed for both the free ligands and Cyt C, while corresponding ligand-protein complex signals appeared as inverted peaks within the extracted ion chromatograms (EICs) of the ligand-Cyt C complexes at matching retention times. Ions associated with ligand-protein complexes were completely absent in both negative controls.

Furthermore, the NOCE values tracking the dissociative binding between the ligands and Cyt C successfully categorized the binding strengths among the twelve positive controls. Both SPR and molecular docking analyses confirmed the formation of these complexes and corroborated the established affinity rankings.

Subsequent application of the PCI-LC–ER–AMS platform to isolate Cyt C ligands from two prominent HMs, *Scutellariae Radix* (SR) and *Arisaematis Rhizoma Preparatum* (ALRP), led to the identification of 24 candidate ligands from SR, whereas none were detected in ALRP. Based on the calculated NOCE values, flavonoid glucosides demonstrated a superior binding capacity toward Cyt C compared to their corresponding aglycones. Molecular docking simulations validated both the binding capabilities and the relative affinity rankings of all candidate ligands.

Following verification through SPR spectroscopy, UV-Vis spectrometry, cell viability assays, intracellular ATP content measurements, mitochondrial membrane potential analysis, and reactive oxygen species (ROS) evaluation, two representative ligands—baicalin and baicalein—were definitively shown to bind to Cyt C, thereby inducing protein dysfunction. In comparison to its aglycone counterpart baicalein, baicalin demonstrated a higher propensity for forming a baicalin-Cyt C complex via non-covalent interactions. Crucially, the PCI-LC–ER–AMS platform proves highly suitable for the online screening of ligands for Cyt C and other proteins from complex HMs by leveraging a "four-in-one" analytical methodology to effectively eliminate false-positive outcomes.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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