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Development of an analytical platform for the affinity screening of natural extracts by SEC-MS towards PPARα and PPARγ receptors

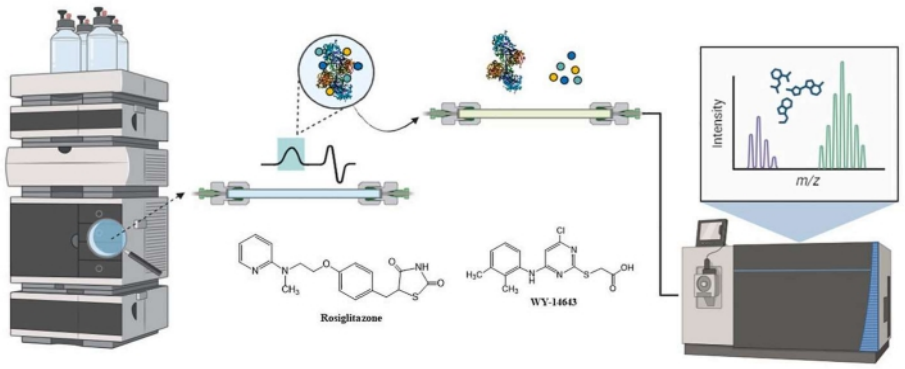
G. De Soricellis [a,b,1], F. Rinaldi [a,1], S. Tengattini [a], C. Temporini [a], S. Negri [c,b], D. Capelli [d], R. Montanari [d], H. Cena [e,f,b], S. Salerno [a], G. Massolini [a], F. Guzzo [c,b], E. Calleri [a,b,*]

[a] Department of Drug Sciences, University of Pavia, Viale Taramelli 12, Pavia, 27100, Italy
[b] National Biodiversity Future Center (NBFC), Palermo, 90133, Italy
[c] Department of Biotechnology, University of Verona, Verona, 37134, Italy
[d] Institute of Crystallography (IC), National Research Council (CNR), Via Salaria Km 29.300, Rome, 00016, Italy
[e] Laboratory of Dietetics and Clinical Nutrition, Department of Public Health, Experimental and Forensic Medicine, University of Pavia, Via Bassi 21, Pavia, 27100, Italy
[f] Clinical Nutrition and Dietetics Service, Unit of Internal Medicine and Endocrinology, ICS Maugeri IRCCS, Pavia, 27100, Italy

HIGHLIGHTS

- A new SEC-AS-MS method has been developed for the affinity screening of new potential PPARα and PPARγ ligands.
- The SEC-AS-MS method has been evaluated using known ligands.
- The system has found application on an *Allium lusitanicum* methanolic extract.
- Saponins, known PPARγ ligands, have been selectively fished by the receptors from the vegetable matrix.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear receptors and represent the targets for the therapeutical treatment of type 2 diabetes, dyslipidemia and hyperglycemia associated with metabolic syndrome. Some medicinal plants have been traditionally used to treat this kind of metabolic diseases. Today only few drugs targeting PPARs have been approved and for this reason, the rapid identification of novel ligands and/or chemical scaffolds starting from natural extracts would benefit of a selective affinity ligand fishing assay.

Results: In this paper we describe the development of a new ligand fishing assay based on size exclusion chromatography (SEC) coupled to LC-MS for the analysis of complex samples such as botanical extracts. The known PPARα and PPARγ ligands, WY-14643 and rosiglitazone respectively, were used for system development and evaluation. The system has found application on an *Allium lusitanicum* methanolic extract, containing saponins, a

* Corresponding author. Department of Drug Sciences, University of Pavia, Viale Taramelli 12, Pavia, 27100, Italy.
E-mail address: enrica.calleri@unipv.it (E. Calleri).
[1] These authors contributed equally to this work.

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class of chemical compounds which have attracted interest as PPARs ligands because of their hypolipidemic and insulin-like properties.

Significance: A new SEC-AS-MS method has been developed for the affinity screening of PPARα and PPARγ ligands. The system proved to be highly specific and will be used to improve the throughput for the identification of new selective metabolites from natural souces targeting PPARα and PPARγ.

1. Introduction

The discovery of a new drug activity is primarily related to its affinity for the biological target. Several affinity-based technologies are today available to detect and characterize a ligand-binding event and the selection of the appropriate technology is a key step as the available technologies offer different type of information. From a systematic point of view the approaches used to investigate drug-protein interactions can be divided into separative and non-separative methods. The first group involves the separation of the free ligands from the bound compounds while the second group relies on the detection of a change of a physicochemical property of either the ligand or the protein because of the binding event. In this group highly informative techniques are present such as isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) [1].

In the group of separative methods, liquid chromatography has received attention in several drug discovery projects when coupled to MS detection [2]. LC-MS methods used to assess drug–protein interactions can be divided into two groups: frontal affinity chromatography coupled to mass spectrometry (FAC-MS) where the target is immobilized on a chromatographic support and the binding equilibrium between target and analyte is established inside the affinity column [3–7], and affinity selection techniques coupled to mass spectrometry (AS-MS) where the binding equilibrium and the subsequent separation of bound ligands from unbound ligands is achieved before LC-MS analysis [8].

AS-MS techniques have been invented to address the high-throughput screening (HTS) demands of combinatorial chemistry. Several are the advantages of this approach: AS-MS is a label free technique, it is faster and consumes fewer supplies than the one compound-one well approach of HTS. The different AS-MS techniques have a common step that is the incubation of a mixture of small molecules with the pharmacological target, but they differ from the method used to separate the bound compounds from the unbound compound [9]. Three main AS-MS methods have been described: pulsed ultrafiltration (PUF) AS-MS, size exclusion chromatography (SEC) AS-MS and magnetic microbead affinity selection screening (MagMASS) AS-MS. PUF and SEC AS-MS are solution-phase screening approaches while MagMASS uses biological targets immobilized on magnetic microbeads. Despite of the fact that the most used support is magnetic beads, as the immobilization format, other solid affinity selection methods based on immobilized targets using lectin-based, phosphoinositide or diamond-based affinity materials are also employed to achieve the selection of the ligands of interest before MS analysis [10].

Among AS-MS methods, the most interesting approach in view of automation is SEC-AS-MS since the two chromatographic dimensions can be coupled in a single system. SEC-AS-MS, invented by Kaur et al. [11], begins with the incubation of potential ligands with a soluble macromolecular target until equilibrium is reached. SEC is first used to separate the ligand–receptor complexes from unbound compounds. The early eluting ligand-receptor complexes is then loaded onto an HPLC reversed phase column either off-line or on-line. The receptor–ligand complexes are therefore denatured by the presence of organic solvent in the mobile phase and bound ligands are eluted into a mass spectrometer for identification.

Although combinatorial chemistry currently receives more emphasis for lead discovery, natural products still represent a successful source of drugs and drug leads. It is estimated that less than 10 % of the world's biodiversity has been evaluated for potential biological activity. In the last years the use of AS-MS approach has been extended to ligand fishing starting from natural extracts. In this regard the AS-MS approach can facilitate the rapid isolation and identification of new ligands and scaffolds for a particular pharmacological target [12–15].

Currently, most natural products researchers use the process of bioassay-guided fractionation, which is labor-intensive and time-consuming. Typically, a natural extract is analyzed for its pharmacological activity, and ligands isolation is carried out following an iterative process that continues until an active compound is isolated for identification. In this regard AS-MS can be considered a valuable alternative for ligand fishing starting from natural product extracts.

The peroxisome proliferator activated receptors (PPARs) are members of the nuclear receptor family that play a central role in the regulation of storage and catabolism of dietary fats. The three subtypes of PPARs (designated α, β and γ) bind to fatty acids and to fatty acids metabolites and regulate the expression of genes involved in the transport, metabolism and buffering of these ligands within cells. In addition, recent studies provide evidence of the involvement of PPARs ligands in controlling inflammatory response [16].

PPARs have been used as targets in the treatment of diabetes, metabolic syndrome, and dyslipidemia. The thiazolidinedione antidiabetic agents (e.g. rosiglitazone and pioglitazone) are PPARγ agonists while the fibrate anti-atherosclerotic, hypolipidemic agents (e.g. fenofibrate and gemfibrozil) are PPARα agonists. Despite their wide prescription, PPARγ-activating drugs revealed unwanted effects that cannot be underestimated. To overcome these side effects, novel PPARs ligands have been identified. These include PPARα/γ dual agonists or PPARα/β/γ pan-agonists, which beneficially alter carbohydrate and lipid metabolism in a coordinate manner and selective PPARs modulators with robust anti-diabetic efficacy and fewer adverse effects than currently available agonists. This kind of compounds represents the new frontier for future PPAR agonists with improved therapeutic activity and less side-effects.

Natural products have been an important source for the discovery of new ligands that target PPARγ [17–19]. Several natural ligands have been identified for the PPARγ isoform, whereas only a few natural ligands of PPARα have been reported to date [20,21].

The rapid identification of novel ligands of PPARα or PPARγ from complex extracts of botanicals and dietary foods would benefit of a sensitive and selective ligand fishing assay. As far as we know only an ultrafiltration affinity selection LC-MS assay method has been described and applied to the evaluation of an *Artemisia argyi* extract, where eupatilin was identified as a selective PPARα ligand [22].

The main objective of the present research was the set-up and evaluation of an analytical platform for the affinity screening of natural extracts by SEC-MS towards PPARs receptors (α and γ) isoforms. In this paper an off-line bidimensional system based on SEC and RP chromatography was set-up. At first, the system was tested using known ligands, namely rosiglitazone and pirinixic acid (WY-14643) for the γ and α isoforms, respectively. Afterwards, the system has found application on a real sample: a methanolic *Allium lusitanicum* extract was selected owing to its composition consisting of saponins, known PPARγ ligands [18].

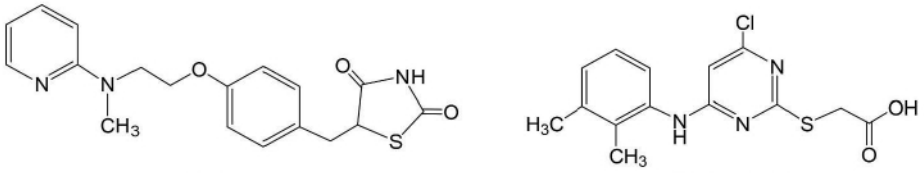

Fig. 1. Molecular structure of rosiglitazone and WY-14643.

2. Materials and methods

2.1. Reagents and chemicals

Acetonitrile (ACN) was purchased from PanReac AppliChem ITW Reagents (Cinisello Balsamo, Italy), while methanol (MeOH), ethanol (EtOH), monobasic potassium phosphate (KH_2PO_4), ammonium acetate (NH_4OAc) and sodium azide were from Sigma-Aldrich (Milan, Italy). Formic acid (FA) was from Merck KGaA (Darmstadt, Germany). Anhydrous sodium sulphate (Na_2SO_4) was purchased from Alfa Aesar GmbH (Karlsruhe, Germania). Deionized water was obtained using a Milli-Q Water purification system (Millipore Corporation, Bedford, MA). Buffer solutions were filtered through a 0.45 μm membrane filter before their use. All reagents were of analytical grade. Reference compounds rosiglitazone and WY-14643 were purchased from Sigma-Aldrich (Milan, Italy).

The human PPARγ and PPARα ligand binding domains (LBDs) were expressed as N-terminal His-tagged proteins by using a pET28 vector and purified onto a Ni^{2+}-nitriloacetic acid column (GE Healthcare, Milan, Italy) [23].

The LBD solutions were stabilized with 30 % glycerol and stored at −20 °C.

Plants of *Allium lusitanicum* Lam. were received from the "Centro Biodiversità Vegetale e Fuori Foresta" (Montecchio Precalcino, Vicenza, Italy), which is a public body dedicated to safeguarding the germplasm of local woody and herbaceous species. The plants were then cultivated and maintained in the greenhouse facility of University of Verona.

2.2. Sample preparation

2.2.1. Stock and working solutions

Stock solutions of rosiglitazone (1.0 mM) and WY-14643 (3.0 mM) were prepared dissolving the reference standards in MeOH and EtOH respectively and stored at 4 °C. The chemical structures are reported in Fig. 1.

For the linearity study, stock solutions were properly diluted with Milli-Q water to obtain concentrations in the range of 40–5000 nM (rosiglitazone) or 100–5000 nM (WY-14643).

Saturation binding experiments were performed with rosiglitazone or WY-14643 solutions in the concentration range 100–5000 nM incubated with 10 μM PPARγ or PPARα LBDs. Solutions with a total volume of 500 μL were prepared in potassium dihydrogen phosphate buffer (KH_2PO_4 0.1 M, 0.05 % NaN_3, pH = 7) and were incubated overnight at 4 °C before the analysis.

2.2.2. Allium lusitanicum sample preparation

Pools of leaves were collected on July 21st, 2022, in triplicate from plants in vegetative growth (two plants for each replicate). The samples were immediately frozen in liquid nitrogen, ground to powder using an A11 basic analytical mill (IKA-Werke, Staufen, Germany) and stored at −80 °C. About 1 g of frozen powder was extracted with 10 vol (w/v) of 100 % LC-MS grade methanol (Honeywell, Seelze, Germany). The samples were vortexed for 30 s, sonicated on ice for 10 min in a 40-kHz ultrasonic bath (SOLTEC, Milano, Italy) and centrifuged at 14,000×*g* for 10 min at 4 °C. The supernatants were collected and stored at −20 °C.

For the affinity assays, 1 ml of supernatant, corresponding to about 100 mg of fresh material, was dried with a speed-vac system (Heto-Holten; Frederiksborg, Denmark). The dry plant extract was re-dissolved in 500 μL of MeOH prior to use. 50 μL of the solution was then withdrawn and incubated overnight at 4 °C with 10 μM PPARγ or PPARα LBDs, diluting the solution to 500 μL with potassium dihydrogen phosphate buffer (KH_2PO_4 0.1 M, 0.05 % NaN_3, pH = 7). MeOH content was kept up to 10 % (v/v) to avoid protein unfolding.

2.3. Chromatographic systems and methods

2.3.1. LC-ESI-HRMS Allium lusitanicum fingerprinting

For the chemical analysis, the methanolic extract was diluted 1:5 with LC-MS grade water (Honeywell) and passed through 0.22 μm Minisart filters (Sartorius-Stedim Biotech, Göttingen, Germany). LC-ESI-HRMS analysis was carried out in negative and positive ionization modes (injection volumes, 5 and 1 μL, respectively), and by FAST-DDA negative ionization mode, with the instruments and methods previously described [24], with the following modification: the elution gradient started with 1 % B, held to 1 % B for 1 min, then increased to 40 % B at 10 min, to 70 % B at 13.5 min, to 90 % B at 15 min and to 99 % at 16.5 min. Subsequently, the method remained in 99 % B for 3.5 min and was then decreased to 1 % B at 20.1 min. The method remained in isocratic (1 % B) and ended at 25 min.

For the metabolite putative identification, the accurate mass (deduced from *m/z* ratio), retention time and fragmentation pattern (MS/MS, from the FAST-DDA analysis) were considered and compared with a proprietary library of authentic standard compounds, with an *in silico* proprietary library of plant compounds and with scientific literature and public databases such as MoNA (https://mona.fiehnlab.ucdavis.edu/) and MassBank (https://massbank.eu/MassBank/Search). The analysis in positive ionization mode was used only to confirm the molecular ions detected in negative ionization mode.

2.3.2. SEC AS-LC-MS analyses

The SEC AS-LC-MS analyses were carried out with two systems.

System 1. SEC analyses were carried out with an Agilent HPLC series 1100 system (Santa Clara, CA, USA), equipped with mobile phase online degasser, quaternary pump, manual injector, column thermostated compartment and variable wavelength detector, controlled by an Agilent ChemStation software (Rev. B.04.03-SP1 [108]). A TSK Gel Super SW2000 column (300 × 4.6 mm ID, 4 μm) from Tosoh Bioscience was used and analyses were carried out at room temperature (25 °C) setting an isocratic elution with potassium dihydrogen phosphate buffer (KH_2PO_4 0.1 M, 0.05 % NaN_3, pH = 7) as mobile phase at a flow rate of

350 μL/min, with an injection volume of 20 μL. PPARγ and PPARα LBDs and its complexes were detected at λ = 280 nm. The peak corresponding to the complexes was eluted in the window 8.5–10.5 min and this fraction (750 μl) was entrapped on an Agilent Zorbax Eclipse Plus C_{18} analytical trap column (12.5 × 4.6 mm ID, 5 μm) directly connected to the detector outlet. The trap column was then washed with water for 6 min for desalting whilst retaining the ligand-receptor complexes.

System 2. The guard column was connected to the analytical column, an XTerra MS C_{18} column (250 × 2.1 mm ID, 5 μm) from Waters and integrated in a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with mobile-phase online degasser, quaternary pump, autosampler, column thermostated compartment and variable wavelength detector controlled by Chromeleon software (6.8 version). The compounds identification was achieved through mass spectrometry (MS) using a linear ion trap mass spectrometer (LTQ) equipped with an elettrospray ion source (ESI) (Thermo Fisher Scientific, Waltham, MA, USA) and controlled by X-calibur software (2.0.7 version). Mass data processing was performed using Bioworks Browser (Thermo Fisher Scientific, revision 3.1).

Different chromatographic methods were set up on System 2 to monitor the bound fraction of the two known ligands (rosiglitazone and WY-14643) and the fished analytes from *Allium lusitanicum* extract desorbed from the trap column.

Rosiglitazone analysis. Rosiglitazone analysis was performed in isocratic conditions using NH_4OAc buffer (50 mM, pH = 5) (solvent A) and acetonitrile (solvent B), 50:50 (v/v). The flow rate was kept constant at 350 μL/min and temperature was hold at room temperature. Rosiglitazone detection was performed at λ = 242 nm and setting up the following ion source parameters for positive ion mode acquisition: source voltage 4.6 kV, capillary voltage 4 V, sheath gas flow rate 9 (arbitrary units), auxiliary gas flow rate 7 (arbitrary units), capillary temperature 250 °C, tube lens voltage 95 V. Full scan mass range was set up in the 300–400 Da range. For quantitative analysis peak areas were defined by the extraction of the $m/z = 358.43$.

WY-14643 analysis. WY-14643 was eluted in isocratic conditions using a mixture of water 0.1 % formic acid (solvent A) and acetonitrile (solvent B), 50:50 (v/v). The flow rate was kept constant at 350 μL/min and temperature was hold at room temperature.WY-14643 detection was conducted at λ = 244 nm while the mass spectrometry parameters for negative ion detection were set as follows: source voltage 4 kV, capillary voltage −21 V, sheath gas flow rate 48 (arbitrary units), auxiliary gas flow rate 18 (arbitrary units), capillary temperature 250 °C, tube lens voltage −95 V. Full scan mass range was set up in the 300–400 Da range. For quantitative analysis peak areas were defined by the extraction of the $m/z = 322.80$ g/mol.

Allium lusitanicum analysis. Chromatographic separation of the *Allium lusitanicum* fished analytes was performed through gradient elution where the mobile phase was water 0.1 % formic acid (solvent A) and acetonitrile (solvent B), with a constant flow rate of 350 μL/min. Specifically, after an isocratic step at 1 % B for 1 min, solvent B was pumped following a linear gradient from 1 % to 40 % in 9 min. Then, three sequential additions of the organic solvent B were performed from 40 % to 70 % in 3.5 min, from 70 % to 90 % in 1.30 min and from 90 % to 100 % in 2.5 min. Lastly, an isocratic step was carried out at 100 % for 10 min. *Allium lusitanicum* was analyzed operating in positive and negative ion mode at the following instrumental conditions: source voltage 4 kV, capillary voltage −44 V, sheath gas flow rate 43 (arbitrary units), auxiliary gas flow rate 18 (arbitrary units), capillary temperature 250 °C, tube lens voltage −90 V. Full scan mass range was set up in the 300–1850 Da range.

2.4. SEC AS-LC-MS method assessment

2.4.1. Linearity

As reported in paragraph 2.3.2, rosiglitazone and WY-14643 solutions with concentration ranging from 40 to 5000 nM and 100–5000 nM respectively, were prepared and injected on System 2. Linearity was assessed on seven concentration levels and three independent determinations were performed for each point. The two calibration curves were obtained by linear regression analysis.

2.4.2. Precision

Method precision for the two known ligands was evaluated as data repeatability. For the purpose, seven standard solutions of rosiglitazone and WY-14643 in the same concentration range of the calibration curve were analyzed in triplicate. Relative standard deviations (RSDs) on peak areas at each concentration level and the overall average RSD were determined. Furthermore, precision over retention times was assessed. For each sample RSD was calculated and the average RSD were obtained.

2.4.3. Specificity

Rosiglitazone (10 μM) was incubated with PPARγ (10 μM), in presence and in absence of a 10 M urea solution added as the denaturing agent. Specifically, the negative control containing 10 M urea solution was let equilibrate with the receptor for 4 h at 4 °C and vortexed prior rosiglitazone addition, diluting the media with 100 mM phosphate buffer (KH_2PO_4 0.1 M, 0.05 % NaN_3, pH = 7). The media was thus let equilibrate overnight. The sample containing the standard molecule and the intact receptor was prepared according to the protocol reported in paragraph 2.2.1. The samples were injected in System 1 and analyzed off-line by System 2 following the corresponding methods described in Paragraph 2.3.

2.4.4. Reference binding isotherms

Saturation binding experiments (for rosiglitazone and WY-14643) were performed by the overnight incubation of a fixed amount of LBD (10 μM) with 6 increasing concentrations of the reference ligands in the range 100–5000 nM. The solutions were prepared in phosphate buffer (KH_2PO_4 0.1 M, 0.05 % NaN_3, pH = 7) and incubated overnight at 4 °C, as described in Paragraph 2.2.1. The samples were injected in System 1 and analyzed off-line by System 2 following the corresponding methods described in Paragraph 2.3. Rosiglitazone and WY-14643 ion currents were extracted for each level of concentration and their content, expressed in pmol, was calculated integrating the area of the peaks, deriving the corresponding concentration from the calibration curve. The binding curves were then built correlating the pmol of bound ligand per g of receptor ratio ($pmol_{bound}/g_{receptor}$) to the ligand concentration. The dissociation constant (K_D) values were obtained by non-linear regression analysis of the data with a one-site binding (plateau followed by one phase association) by GraphPad Prism (version 9.5.1). The goodness-of-fit of the collected data was assessed through the R^2 coefficient determination.

3. Results and discussion

3.1. Method development

The focus of the present research was the set-up of an analytical platform for the affinity fishing and identification of new ligands for the γ and α isoforms of PPAR receptors starting from natural extracts.

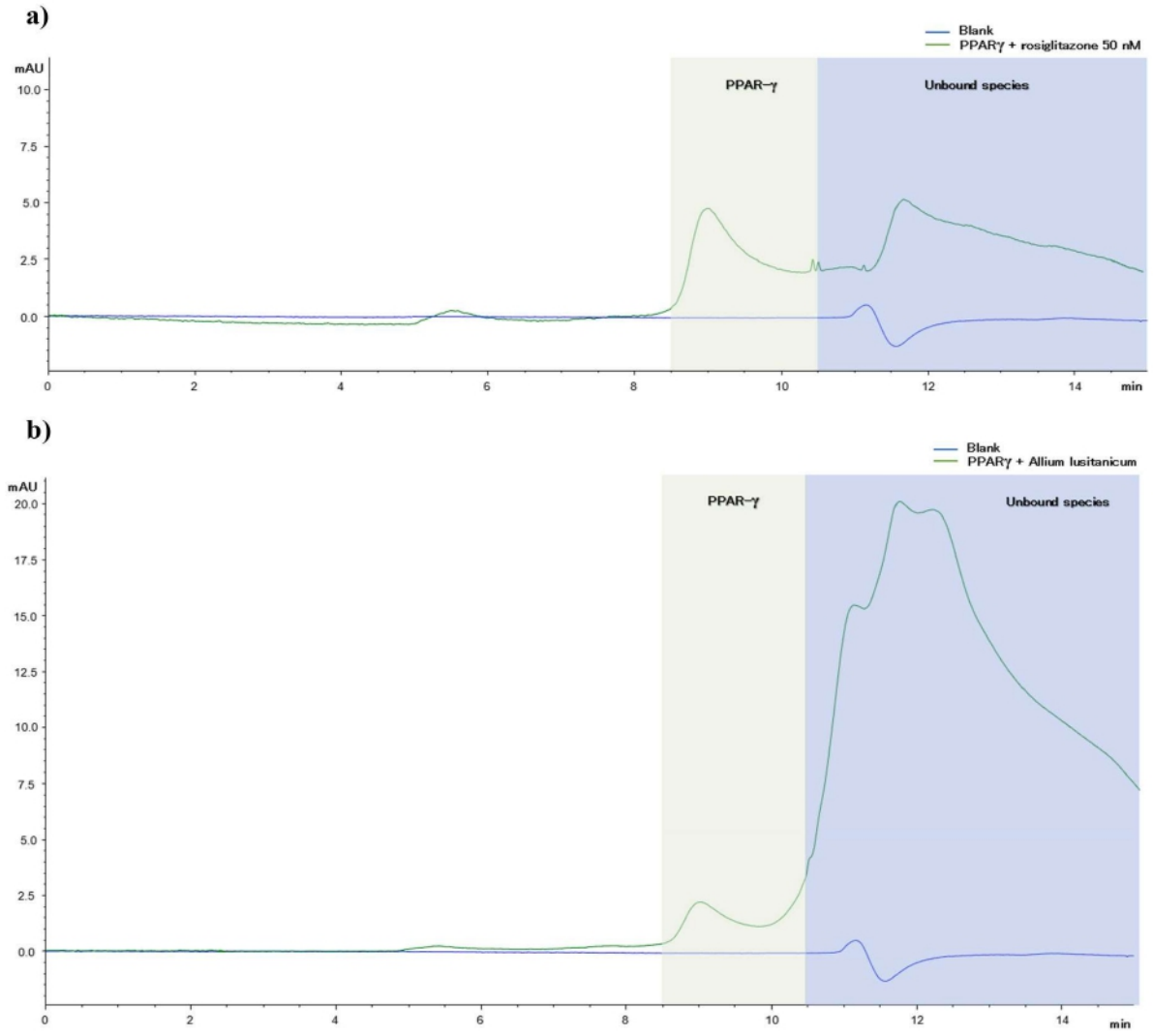

Fig. 2. SEC-UV chromatographic traces of PPARγ (10 μM) in the incubation media with a) rosiglitazone (50 nM) and b) *Allium lusitanicum*.

The development of the analytical procedure can be summarized in three main steps: 1) incubation of the PPAR-LBDs with the potential ligands; 2) separation and isolation of ligand-target complexes by SEC; 3) denaturation of the complexes and identification of the bound ligands by RPLC-UV-MS.

Size exclusion chromatography was selected for the first time as affinity selection method for PPARs receptors to isolate ligand-receptor complexes within plant matrixes due to the significant difference between the molecular weights of PPARs LBD (~33 kDa) and the unbound small molecules.

The LBD was selected instead of the full-length receptor since it was previously demonstrated that the other domains do not affect the binding to the binding site [25,26].

A SEC column with an operational mass range of 1–150 kDa was therefore selected relying on its efficiency in the separation of the target and its complexes in respect to the unbound small molecules.

In terms of chromatographic conditions, the preservation of ligand-receptor complexes throughout the elution represents a key point in this procedure. Thus, the denaturation of LBDs and the dissociation of the complexes had to be prevented. In this regard, SEC mobile phase was set-up to avoid harsh conditions such as high ionic strength and extreme pH conditions. Phosphate buffer was selected as mobile phase buffer as well as storage buffer. Moreover, the addition of a bacteriostatic agent, sodium azide, was considered to inhibit microbial growth.

The interaction between the LBDs and the potential ligands contained in the plant matrix represents another key point of the present research, thus fishing conditions were carefully defined. The same buffer used as SEC mobile phase was selected as incubation medium thanks to its nondenaturing nature. Incubation time was also taken into consideration to maximize the interaction with the PPARγ/α LBDs. Specifically, multiple trials carried out on standard samples incubated for 4 h and 6 h resulted in incomplete ligand fishing, while the best results were obtained after overnight incubation.

In plant extract fishing, the addition of a proper amount of extract is another crucial point as it must be adequately considered not only to favour the interaction with the receptor but also for sensitivity reasons. In this context, when the concentration is too high it could induce protein alterations and/or denaturation [27]. Conversely, diluted

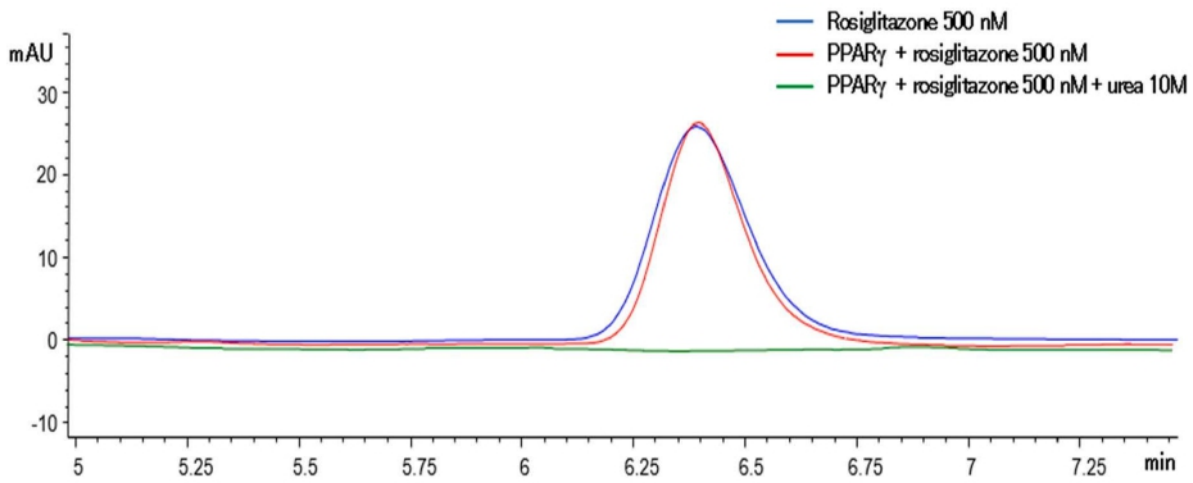

Fig. 3. Reversed phase chromatographic traces of rosiglitazone standard solution (500 nM) (blue line), the incubation media comprising rosiglitazone (500 nM) and PPARγ (10 μM) (red line) and the incubation media after denaturation (green line)

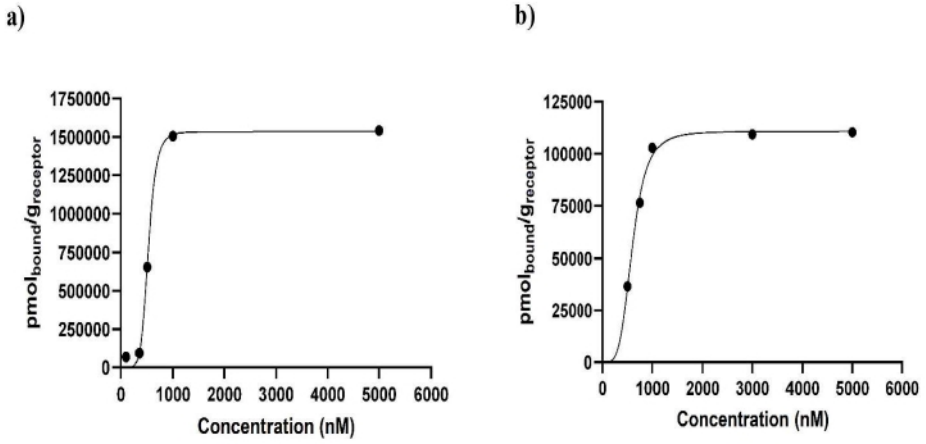

Fig. 4. Binding isotherms obtained through non-linear regression fitting for a) rosiglitazone (100–5000 nM) and b) WY-14643 (100–5000 nM).

solutions could prevent the interaction between ligands and receptor as well as their detection. Aiming to preserve PPARs biological activity, the incubation was carried out at 4 °C.

Methanol content in the incubation media was considered as well, due to the denaturing effect exerted by organic solvents over proteins. The plant methanolic extract was kept up to 10 % (v/v) to avoid protein unfolding exerted by MeOH while preserving complex stability and ensuring the detection of the fished analytes. The complete sample preparation procedure is described in Paragraph 2.2.

Relying on the theoretical basis of SEC technique, the high-molecular weight compounds such as the free target and the complexes are eluted before the solvent front. Conversely, the small molecules that do not show any affinity for the target flow through the column with higher retention times, thus allowing the separation of the two fractions. Therefore, SEC elution was optimized to the set-up a proper elution window for ligand-receptor complexes.

The SEC-UV analysis of the PPARs LBD of the two isoforms showed a main peak at approximately 9 min and a solvent front at 12 min, allowing to define an analysis time of 15 min and to set-up a proper elution window (8.5–10.5 min) corresponding to 700 μL elution volume. Fig. 2 shows the SEC chromatographic traces of 10 μM PPARγ LBD incubated with rosiglitazone (50 nM) (Fig. 2a) and *Allium lusitanicum* extract (Fig. 2b).

For complexes isolation, the use of a trap column was considered since it offers the possibility to retain and concentrate the fraction of interest, as well as to remove salts deriving from SEC mobile phase. C_{18} trap and analytical columns were selected for their ability to retain the ligand-receptor complexes and to separate the small molecule ligands, respectively. The trap column was thus connected at the SEC column outlet for the defined time interval (8.5-10-5 min). The trap column was also crucial for the following desalting step. A 6-min washing step with water using a flow rate of 350 μL/min was found to be effective for salts removal. Moreover, the trap is fundamental to avoid ligands loss due to complex instability. The subsequent elution in acidic conditions and the gradual addition of the organic solvent promotes protein denaturation, thus inducing ligands dissociation from the complexes and allowing their separation through reverse-phase chromatography. The elution conditions reported in Paragraph 2.3 were proved appropriate to obtain a LC-MS trace for the fished ligands (see Paragraph 3.2).

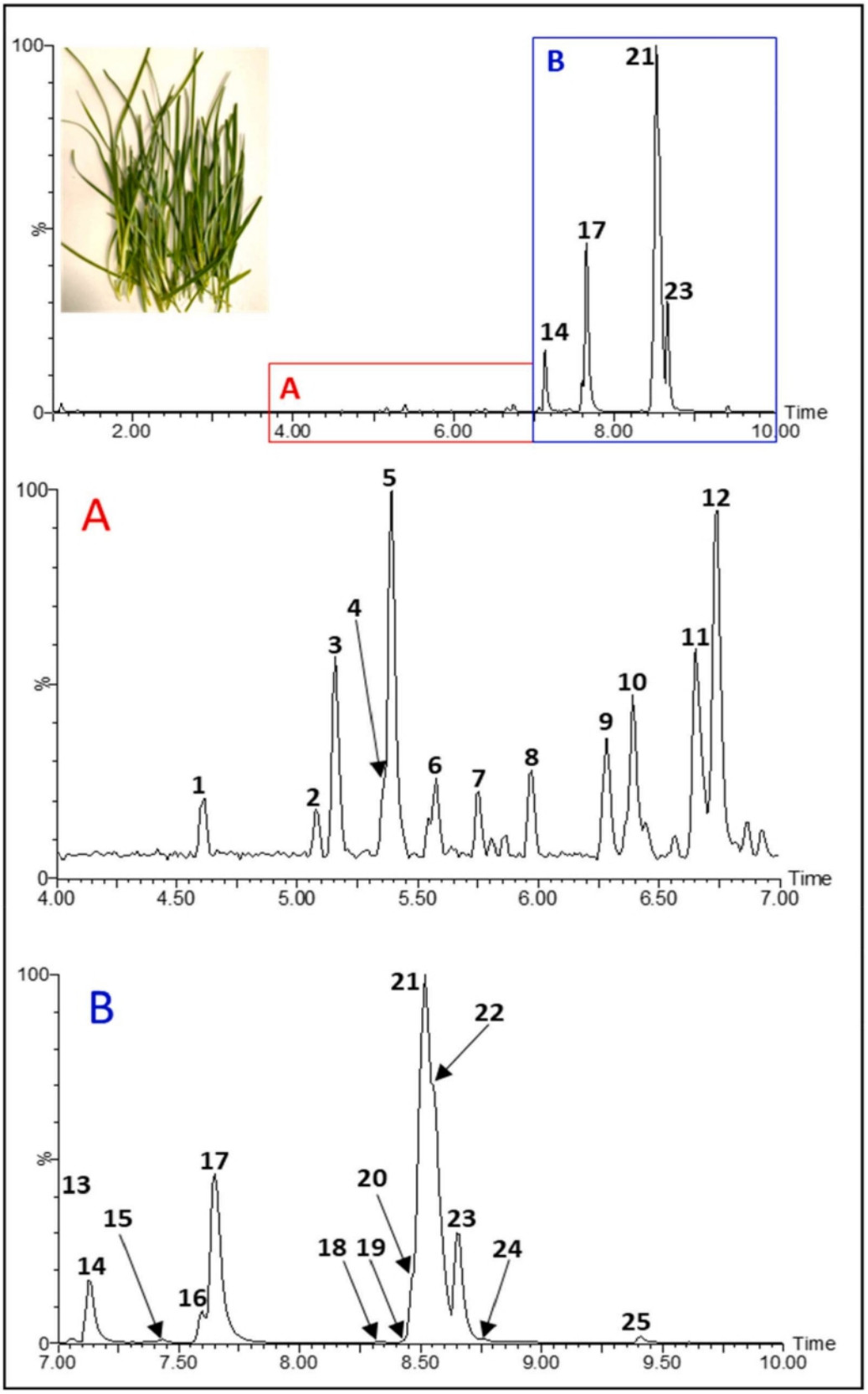

Fig. 5. Full scan chromatogram of *Allium lusitanicum* leaf methanolic extract, in negative ionization mode. A) and B) provide the zoom of the chromatogram. The characteristics of each numbered peak (including putative annotation, retention time, molecular formula, experimental *m/z* and main adduct in negative and positive acquisition modes, mass error in negative mode and the main fragments observed in ms/ms) are shown in Table 1.

Table 1
Attribution of the peaks observed in leaf methanolic extract of Allium lusitanicum, characterized through HRMS fingerprinting and shown in Fig. 5, displaying the id, retention time (in minutes), molecular formula, experimental *m/z* determined and main adduct in negative and positive acquisition modes, mass error (negative mode), in ppm, and the main fragments observed in ms/ms. UI = unidentified.

ID	Putative identification (H, hexose; dH, desoxyhexose)	Retention time (min)	Elemental formula	experimental *m/z* (−)	ESI (−) main adduct	experimental *m/z* (+)	ESI (+) main adduct	mass error (−), (ppm)	Fragments (−)
1	sinapic acid derivative	4597	$C_{19}H_{34}O_9$	451,216	$[M + HCOOH–H^+]^-$	429,207	$[M + Na^+]^+$	3882	119.034; 149.024; 164.053; 179.056; 193.014; 223.060; 405.216; 451.219
2	benzylalcohol O-(O-pentosyl-hexoside)	5075	$C_{18}H_{26}O_{10}$	447,149	$[M + HCOOH–H^+]^-$	425,14	$[M + Na^+]^+$	1208	89.024; 101.024; 113.022; 131.036; 143.039; 149.046; 161.047; 191.062; 233.067; 269.107; 293.083; 401.144
3	sinapic acid hexose	5159	$C_{17}H_{22}O_{10}$	431,192	$[M + HCOOH–H^+]^-$	409,182	$[M + Na^+]^+$	−171,647	119.036; 179.058; 190.025; 205.050; 223.060; 385.115; 431.192
4	sinapic acid hexoside	5347	$C_{17}H_{22}O_{10}$	385,112	$[M-H^+]^-$	409,107	$[M + Na^+]^+$	1465	164.048; 193.014; 208.038; 223.060
5	UI	5379	–	433,206	$[M-H^+]^-$	411,19	$[M + Na^+]^+$	–	161.052; 189.132; 209.043; 224.067; 387.203; 433.208
6	UI	5576	–	433,207	$[M + HCOOH–H^+]^-$	411,199	$[M + Na^+]^+$	–	177.056; 183.012; 197.0127; 325.183; 353.066
7	kaempferol-*O*-(O-hexosyl-hexosyl-desoxyhexoside)	5743	$C_{33}H_{40}O_{20}$	755,203	$[M-H^+]^-$	757,218	$[M + H^+]^+$	−0,581	255.033; 284.034; 285.038; 755.206
8	phenylethyl primeveroside	5961	$C_{19}H_{28}O_{10}$	461,165	$[M + HCOOH–H^+]^-$	439,154	$[M + Na^+]^+$	−0,646	101.024; 113.024; 119.034; 131.036; 149.046; 161.044; 179.058; 191.057; 221.065; 251.081; 293.090; 311.102; 415.160
9	kaempferol-*O*-desoxyhexoside derivative	6284	–	729,189	$[M-H^+]^-$	–		–	255.030; 284.034; 285.038; 431.158; 641.193; 729.185
10	UI	6388	–	551,270	$[M + HCOOH–H^+]^-$	529,263	$[M + Na^+]^+$	–	373.224; 505.265; 551.265
11	UI	6651	–	553,289	$[M + HCOOH–H^+]^-$	531,279	$[M + Na^+]^+$	–	101.024; 131.036; 161.047; 233.70; 271.024; 284.030; 295.038; 375.239; 507.282; 553.84
12	UI	6732	–	553,286	$[M + HCOOH–H^+]^-$	531,279	$[M + Na^+]^+$	–	–
13	saponin class IV4 + 2 hexoses + 1 desoxyhexose	7057	$C_{45}H_{74}O_{19}$	963,483	$[M + HCOOH–H^+]^-$	941,47	$[M + Na^+]^+$	−3114	447.312; 609.364; 771.417; 917.48
14	saponin class IV4 + 2 hexoses + 1 desoxyhexose	7128	$C_{45}H_{74}O_{19}$	963,481	$[M + HCOOH–H^+]^-$	941,47	$[M + Na^+]^+$	−1451	447.312; 609.364; 771.423; 917.480
15	saponin class V3 + 3 hexoses + 3 desoxyhexoses	7421	$C_{62}H_{102}O_{32}$	678,325	$[2M-H^+]^-$	1381,656	$[M + Na^+]^+$	−23,588	433.332; 678.331; 678.832; 757.441; 903.505; 1065.550; 1193.91
16	saponin class IV4 + 2 hexoses + 1 desoxyhexose + acetyl group	7597	$C_{47}H_{76}O_{20}$	1005,492	$[M + HCOOH–H^+]^-$	983,483	$[M + Na^+]^+$	−1989	447.312; 609.364; 771.423; 917.480
17	saponin class IV4 + 2 hexoses + 1 desoxyhexose	7650	$C_{47}H_{76}O_{20}$	1005,492	$[M + HCOOH–H^+]^-$	983,483	$[M + Na^+]^+$	−1989	447.311; 609.364; 771.423; 899.468; 917.477; 959.485
18	UI saponin	8329	–	1241,580	$[M + HCOOH–H^+]^-$	1219,561	$[M + Na^+]^+$		755.26; 901.482; 1195.579
19	saponin class IV3 + 2 hexoses + 2 desoxyhexoses + 1 pentose	8442	$C_{56}H_{92}O_{26}$	1225,592	$[M + HCOOH–H^+]^-$	1203,575	$[M + Na^+]^+$	−7343	755.420; 901.482; 1015.11; 1033.519; 1179; 583
20	saponin class IV3 + 2 hexoses + 2 desoxyhexoses	8463	$C_{51}H_{84}O_{22}$	1093,545	$[M + HCOOH–H^+]^-$	1071,536	$[M + Na^+]^+$	−1554	431.319; 593.369; 755.426; 901.482; 1047.541
21	saponin class IV3 + 2 hexoses + 3 desoxyhexoses	8515	$C_{57}H_{94}O_{26}$	1193,598	$[M-H^+]^-$	1217,597	$[M + Na^+]^+$	−2513	431.315; 575.356; 593.369; 755.420; 883.470; 901.482; 1047.541; 1193.598
22	saponin class IV3 + 2 hexoses + 1 desoxyhexose + 1 pentose	8546	$C_{50}H_{82}O_{22}$	1079,531	$[M + HCOOH–H^+]^-$	1057,522	$[M + Na^+]^+$	−3705	431.319; 593.369; 755.426; 901.482; 1033.526

(*continued on next page*)

Table 1 (*continued*)

ID	Putative identification (H, hexose; dH, desoxyhexose)	Retention time (min)	Elemental formula	experimental *m/z* (−)	ESI (−) main adduct	experimental *m/z* (+)	ESI (+) main adduct	mass error (−), (ppm)	Fragments (−)
23	saponin class IV3 + 2 hexoses + 1 desoxyhexoses	8651	$C_{45}H_{74}O_{18}$	947,487	[M + HCOOH–H$^+$]$^-$	925,479	[M + Na$^+$]$^+$	−1785	431.315; 593.374; 755.426; 901.482
24	saponin class III3 + 1 pentose + 1 hexose +3 desoxyhexoses	8756	$C_{56}H_{90}O_{25}$	1207,573	[M + HCOOH–H$^+$]$^-$	1185,564	[M + Na$^+$]$^+$	0,816	869.455; 1015.511; 1161.573;
25	saponin class IV3 + 2 hexoses + 3 desoxyhexoses + acetyl group	9412	$C_{59}H_{96}O_{27}$	1281,611	[M + HCOOH–H$^+$]$^-$	1259,59	[M + Na$^+$]$^+$	−0,039	755.420; 901.482; 1047.541; 1175.594; 1193.598; 1235.609

3.2. Method assessment

The evaluation of the integrated affinity selection method was at first carried out using rosiglitazone and WY-14643, selected as reference ligands of PPARγ and PPARα, respectively.

Before performing affinity binding experiments, the operational concentration range was defined by assessing the linearity and precision of the two RPLC-UV-MS methods.

The specificity of the binding procedure was also evaluated using freshly prepared receptor as a positive control and denatured receptor as a negative control. Saturation binding experiments were carried out to confirm the affinity fishing ability of the system.

3.2.1. Linearity

Rosiglitazone and WY-14643 solutions with concentration ranging from 40 nM to 5000 nM and 100 nM–5000 nM, respectively, were analyzed in System 2, comprising the trap column and the C_{18} analytical column. A good linear relationship was obtained in the investigated range, being the calibration curve equations $y = 1850.1x + 140669$ ($R^2 = 0.9984$) for rosiglitazone and $y = 101.76x + 8230.5$ ($R^2 = 0.9987$) for WY-14643.

3.2.2. Precision

The repeatability, calculated as relative standard deviation (RSD), was evaluated by triplicate injections of the two reference molecules in the same concentration range of the calibration curve. A good repeatability was obtained both in terms of peak areas and retention time. On peak areas, rosiglitazone provided values comprised in the range between 0.77 % and 3.95 %, with an average RSD = 1.42 %. WY-14643 gave RSD values of 0.26–1.40 %, with an average value of 0.65 %. RSDs values for rosiglitazone retention times were found to range from 0.1 % to 0.4 %, resulting in an average RSD of 0.3 %, whereas WY-14643 provided RSDs ranging from 0.23 % to 0.77 % with a mean value equal to 0.42 %.

3.2.3. Binding specificity

The purpose of the assay was to demonstrate the ability of the PPARγ/α LBDs to fish the ligands, as well as the ability of the analytical procedure to preserve complex stability for its isolation. To this aim, the interaction between PPARγ and rosiglitazone was selected as a model; the LBD was incubated with the reference molecule both in presence and in absence of a denaturing agent and the two samples were analyzed by SEC-RPLC-UV. In this context, the analysis of the sample containing the LBD and the reference molecule showed the presence of a peak at 6.4 min corresponding to rosiglitazone (Fig. 3, red trace). Conversely, the chromatogram acquired on the sample incubated in a 10 M urea solution was characterized by the complete absence of any observable signal, indication that no rosiglitazone was bound to denatured LBD (Fig. 3, green trace). This significative evidence corroborates the specificity of the binding interaction.

3.2.4. Reference binding isotherms

Saturation binding experiments were carried out overnight by incubation of a constant receptor concentration (10 μM) with increasing ligand concentrations, followed by the quantification of the bound ligand. The binding curves and the K_D values estimated by a non-linear regression analysis are reported in Fig. 4. Good correlation coefficients were obtained, and a saturation trend was observed for both the PPAR isoforms. Interestingly, K_D values were found in agreement with literature data, being $K_D = 600$ nM for rosiglitazone and K_D of 523 nM for WY-14643.

More specifically, in a work describing the use of SPR technology to study interactions with PPARγ-LBD, a K_D value of 760 nM was obtained for rosiglitazone [28]. This result is coherent with the value of 600 nM calculated by SEC AS-MS. However, it should be emphasized that rosiglitazone-PPARγ K_D values reported in literature are quite variable [28–30].

Fewer literature data on WY-14643 K_D determinations are available [31]. However, the value obtained by SEC AS-MS is comparable to the EC_{50} value declared by most suppliers and reported by Willson and colleagues [32], with values ranging from 630 nM for murine PPARα receptor to 5 μM for the human receptor.

Due to the variety of the current literature data, we decided to validate the SEC AS-MS results by performing an affinity study using the same receptor expression batch and the same reference ligands. Experiments were carried out using a well-established protocol on a Creoptix WAVE Grating-Coupled Interferometry (GCI) instrument, based on the same principles of SPR. K_D values of 407 nM and 765 nM were found for PPARγ and PPARα, respectively (see Figs. S1 and S2 in Supplementary data), consistent with the dissociation constants obtained by our method.

It must be pointed out that, as far as we know, this is the first example of K_D determination by SEC AS-MS.

3.2.5. Allium lusitanicum fingerprinting

To verify the applicability of the fishing procedure on real samples such as a complex vegetable matrix, the SEC AS-MS method was applied to an *Allium lusitanicum* extract owing to its composition, mainly consisting of saponins, well-known PPARγ ligands [18].

The methanolic extract of *Allium lusitanicum* leaves was before analyzed and characterized by HR-UPLC-ESI-MS. The obtained full scan chromatogram could be roughly divided in two parts, with more polar

Table 2
List of the compounds identified through SEC-RP-MS analysis in *Allium lusitanicum* extract incubated with PPARα and PPARγ.

	Compound	Retention time (min)	PPARα	PPARγ
1	Sinapic acid derivative	8,02	✗	⌄
2	Benzyl alcohol O-(O-pentosyl-hexoside)	8,72	✗	⌄
3	Sinapic acid hexose	8,83	✗	⌄
4	Sinapic acid hexoside	9,09	⌄	⌄
5	UI	8,95	✗	⌄
6	UI	8,95	✗	⌄
7	Kaempferol-*O*-(O-hexosyl-hexosyl-desoxyhexoside)	9,42	⌄	⌄
8	Phenylethyl primeveroside	9,68	✗	⌄
9	Kaempferol-*O*-desoxyhexoside derivative	9,68	✗	⌄
10	UI	9,94	✗	⌄
11	UI	9,94	✗	⌄
12	UI	9,94	✗	⌄
13	Saponin class IV(4) + 2 hexoses + 1 desoxyhexose	10,35	✗	⌄
14	Saponin class IV(4) + 2 hexoses + 1 desoxyhexose	10,35	✗	⌄
15	Saponin class V (3) + 3 hexoses + 3 desoxyhexoses	10,44	✗	⌄
16	Saponin class IV(4) + 2 hexoses + 1 desoxyhexose + acetyl group	10,87	✗	⌄
17	Saponin class IV(4) + 2 hexoses + 1 desoxyhexose	10,87	✗	⌄
18	UI saponin	11,48	✗	⌄
19	Saponin class IV(3) +2 hexoses +2 desoxyhexoses + 1 pentose	10,79	✗	⌄
20	Saponin class IV(3) +2 hexoses +2 desoxyhexoses	11,48	✗	⌄
21	Saponin class IV(3) +2 hexoses +3 desoxyhexoses	11,56	⌄	⌄
22	Saponin class IV(3) +2 hexoses +1 desoxyhexose + 1 pentose	11,65	✗	⌄
23	Saponin class IV(3) +2 hexoses +1 desoxyhexoses	11,76	✗	⌄
24	Saponin class III(3) +1 pentose +1 hexose +3 desoxyhexoses	11,93	✗	⌄
25	Saponin class IV(3) +2 hexoses +3 desoxyhexoses + acetyl group	12,37	✗	⌄

metabolites mainly eluting between 4 and 7 min (zone A), and less polar metabolites eluting from 7 to 10 min (zone B) (Fig. 5). The chromatogram was dominated by four peaks eluting in the B zone.

The main HRMS features, and the corresponding putative identifications are reported in Table 1 where retention times, experimental *m/z* values in positive and negative mode, mass error and main fragments are listed.

Chromatogram zone A showed the presence of low intensity peaks, including some sinapic acid derivatives, kaempferols and some aromatic alcohol glycosides. Chromatogram zone B included various steroidal saponins four of which represented the major chromatogram peaks. Peak attribution has been detailed in the Supporting Information.

3.2.6. *SEC AS-LC-MS analysis of Allium lusitanicum*

Before SEC AS-LC-MS analysis the crude extract was analyzed by LC-MS in order to verify the presence of the metabolites listed in Table 1.

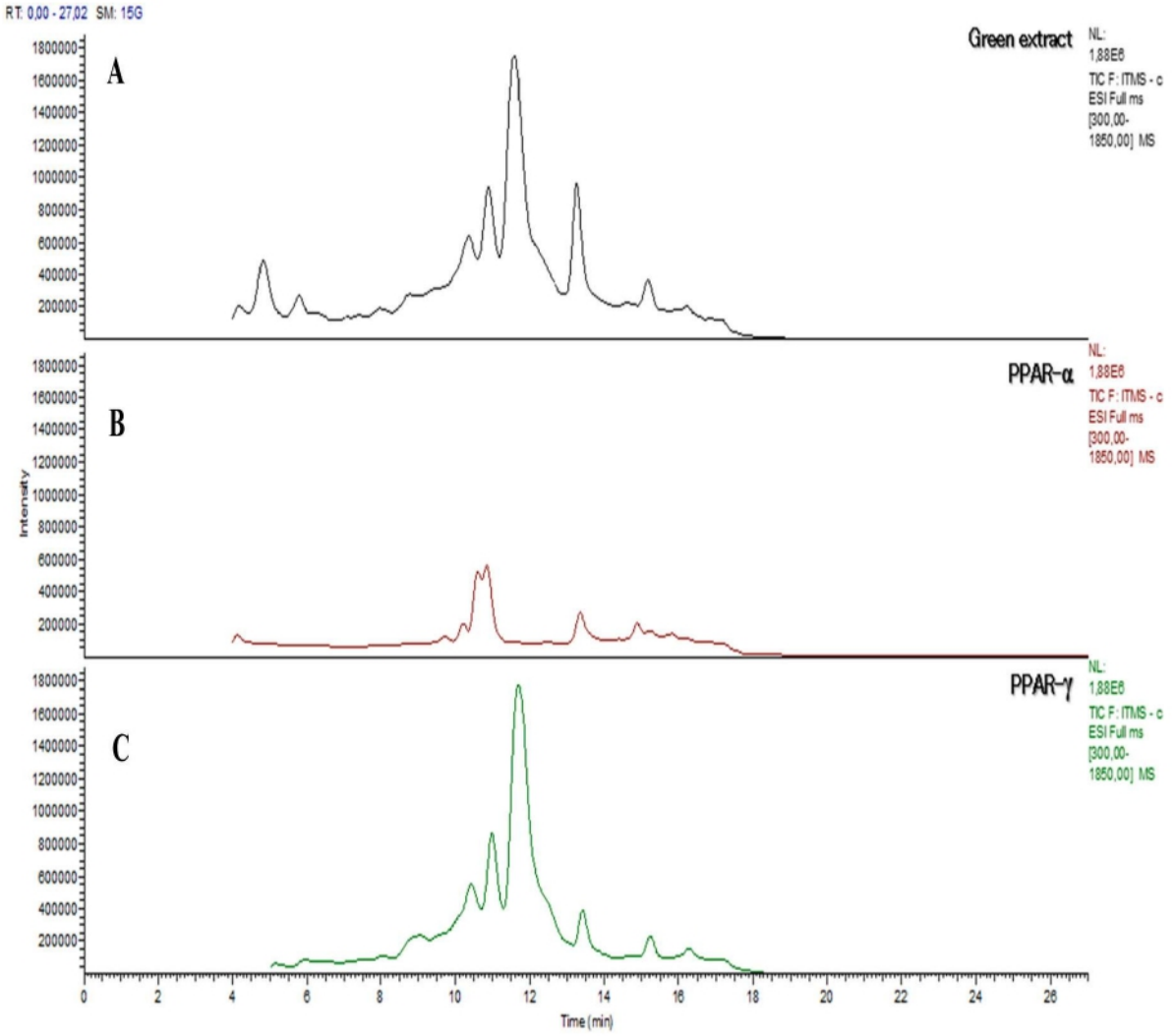

Fig. 6. LC-MS full scan chromatograms of (A) *Allium lusitanicum* crude extract; (B) *Allium lusitanicum* crude extract fished by PPARα and (C) *Allium lusitanicum* crude extract fished by PPARγ. Chromatographic conditions in paragraph 2.3.2.

The high-resolution extract fingerprinting was used for metabolites identification by the extraction of each mass-to-charge value (*m/z*) reported in Table 1. As expected, all the compounds were found, and the retention time order was respected compared to the LC-ESI-HRMS analysis.

The SEC AS-LC-MS system described in paragraph 2.3.2 was then used for the analysis of *Allium lusitanicum* extract after incubation with the LBDs of the γ and α isoforms.

More in detail *Allium lusitanicum* extract was incubated with PPARγ and PPARα isoforms and injected in the SEC column aiming to selectively isolate and collect in the trap column the peak attributable to the receptor-metabolite complexes, thus discarding to the waste all the unbound components of the matrix. After trap column desalting, bound ligands were diverted to the reverse-phase column, where the analytes were separated using the gradient elution and detected by MS. Peak annotation was based on *m/z* (negative and positive ionization modes). Fished ligands and the corresponding obtained retention times are reported in Table 2.

As an example, the LC-MS full scan chromatogram obtained by the direct injection of the plant extract into the C_{18} trap and analytical columns is represented in Fig. 6, in comparison with the full scan MS trace after selective fishing by PPARα (Fig. 6B) and PPARγ (Fig. 6C) where it can be observed a reduction of the total ion current, in particular for the PPARα isoform. The incubation of PPARγ with *Allium lusitanicum* provided the most remarkable results in respect to PPARα receptor since more ligands were found to bind the isoform. Notably, most of the fished compounds were saponins, known PPARγ ligands. Conversely, few ligands were found to bind to the α isoform, including sinapic acid and kaempferol (Table 2). Fig. 7 displays the extracted ion chromatogram of a representative saponin (compound 17) in the crude extract and after the fishing by the two PPAR isoforms. As shown saponin 17 was not fished by PPAR α (chromatogram B) while the fishing was obtained after incubation with PPAR γ (chromatogram C).

4. Conclusions

In this work a new SEC-AS-MS method has been developed for the affinity screening of new PPARα and PPARγ ligands. The system proved to be highly specific as confirmed by saturation binding experiments using two specific ligands for the two isoforms.

The results obtained upon application of the analytical system to a methanolic extract of *Allium lusitanicum* remarked the specificity of the fishing method, which provided an indirect validation of the analytical platform since most fished compounds were saponins, well known in literature as PPARγ ligands.

It must be pointed out that like all fishing methods in heterogeneous matrices the method can fail for ligands that are present in low concentrations for sensitivity reasons and for low affinity compounds.

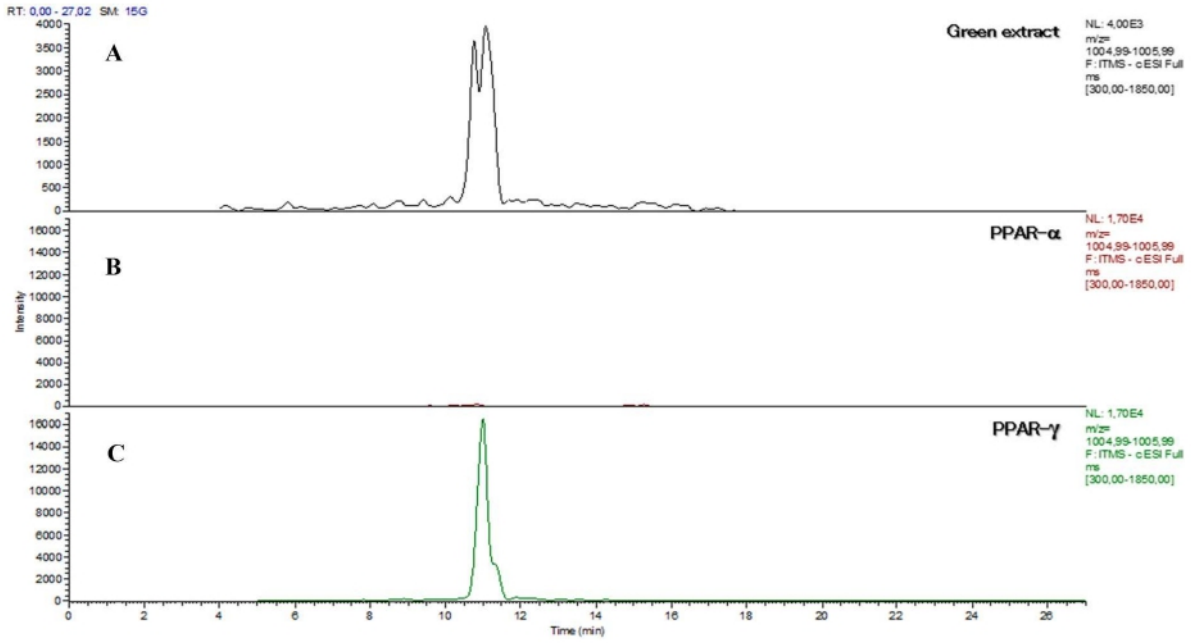

Fig. 7. *Allium lusitanicum* extract in negative ion mode: (A) extracted ion chromatogram for saponin class IV(4) + 2 hexoses + 1 desoxyhexose + acetyl group, m/z = 1005.492 in the crude extract; (B) extracted ion chromatogram for saponin class IV(4) + 2 hexoses + 1 desoxyhexose + acetyl group, m/z = 1005.492 fished by PPAR α; (C) extracted ion chromatogram for saponin class IV(4) + 2 hexoses + 1 desoxyhexose + acetyl group, m/z = 1005.492 fished by PPAR γ.

Next steps will include the integration of the two chromatographic dimensions in view of automation to improve the throughput and productivity of the AS-MS platform for the identification of new selective metabolites targeting PPARα and PPARγ.

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CRediT authorship contribution statement

G. De Soricellis: Writing – original draft, Methodology, Investigation, Data curation. **F. Rinaldi:** Writing – original draft, Methodology, Investigation, Data curation. **S. Tengattini:** Investigation. **C. Temporini:** Investigation. **S. Negri:** Investigation, Data curation. **D. Capelli:** Investigation. **R. Montanari:** Investigation. **H. Cena:** Resources. **S. Salerno:** Writing – original draft. **G. Massolini:** Resources. **F. Guzzo:** Resources, Investigation. **E. Calleri:** Writing – original draft, Resources, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.Enrica Calleri.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2024.342666.

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