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Shotgun proteomics to characterize wastewater proteins

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A R T I C L E I N F O

Method name:
Shotgun Proteomics to characterize wastewater proteins

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Proteomics
Compartmentalized proteome
Wastewater-Based Epidemiology (WBE)
Sewage Chemical-Information Mining (SCIM)
High-Resolution Mass Spectrometry (HRMS)

A B S T R A C T

Classically, the characterization of wastewater components has been restricted to the measurement of indirect parameters (chemical and biological oxygen demand, total nitrogen) and small molecules of interest in epidemiology or for environmental control. Despite the fact that metaproteomics has provided important knowledge about the microbial communities in these waters, practically nothing is known about other non-microbial proteins transported in the wastewater. The method described here has allowed us to perform a large-scale characterization of the wastewater proteome. Wastewater protein profiles have shown to be very different in different collection sites probably reflecting their human population and industrial activities. We believe that wastewater proteomics is opening the doors to the discovery of new environmental and health biomarkers and the development of new, more effective monitoring devices for issues like monitorization of population health, pest control, or control of industry discharges. The method developed is relatively simple and combines procedures for the separation of the soluble and particulate fractions of wastewater and their concentration, and conventional shotgun proteomics using high-resolution mass spectrometry for protein identification.

- Unprecedented method for wastewater proteome characterization.
- Proteins as new potential biomarkers for sewage chemical-information mining, wastewater epidemiology and environmental monitoring.
- Wastewater protein profiles reflect human and industrial activities.

Abbreviations: BSA, bovine serum albumin; HR-LC/MS, liquid chromatography coupled to high-resolution mass spectrometry; PSM, peptide-spectrum match; SCIM, sewage chemical-information mining; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WBE, wastewater-based epidemiology; WWTP, wastewater treatment plant.

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Resource availability:	N.A.

Method details

Background

The analysis by mass spectrometry of small molecules in wastewater is a widely adopted approach for population monitoring by governmental institutions. Typical applications are, for example, monitoring the consumption of tobacco, alcohol or drugs by a community and, more recently, the COVID prevalence using polymerase chain reaction (PCR) [1]. Current development of mass spectrometry allows extending this approach to large molecules such as proteins, as it has been stressed by several authors [1,2]. This would open the window to the monitorization of human health biomarkers already known in clinics, as well as environmental biomarkers. All of this in the context of the sewage chemical-information mining (SCIM) [3].

Previously, we reported for the first time on the peptide and protein components absorbed in a support submerged in wastewater at a wastewater treatment plant (WWTP) [4,5]. We detected proteins from prokaryotic to higher eukaryotic organisms, covering plant, animal, and human proteomes as well. We were able to identify not only major components (albumins, keratins), but also less abundant ones that are known as disease biomarkers. In this study, we used polymer probes to trap the proteins; although an effective method, it required many days to collect the samples and the proteins could be biased by the polymer affinity and the biofilms' formation. Therefore, we centered on developing a strategy that allowed the study of the proteome directly in wastewater samples collected in the inlet of WWTPs [6]. Here we offer an improved method based on it, where we establish the recovery of the soluble and particulate fractions and the process followed for obtaining the peptides.

Laboratory equipment

Apparatus

- Bullet Blender Homogenizer: model Storm 24, Next Advance.
- Centrifuge: model Allegra 25R, Beckman Coulter.
  - Centrifuge rotors: TS-5.1–500 (swinging) and TA-15–1.5 (fixed angle), Beckman Coulter.
- Centrifuge: model MiniSpin, Eppendorf.
  - Centrifuge rotor: F-45–12–11 (fixed angle), Eppendorf.
- Digestor: model Digest Pro-MS, Intavis.
- Electrophoresis gel tank: model Mini-Protean Tetra Cell, Biorad.
- Electrophoresis power supply: model PowerPac 1000, Biorad.
- High resolution mass spectrometer (HRMS): Q Exactive™ HF, Thermo Scientific.
- Thermomixer: model F2.0, Eppendorf.
- Ultracentrifuge: model 90SE, Sorvall Discovery.
  - Ultracentrifuge rotor: SW-28 (swinging), Beckman Coulter.
- Ultra High-Performance Liquid Chromatographic (UHPLC) system: UltiMate 3000 RSLCnano, Thermo Scientific™.
- Vacuum concentrator: Savant SpeedVac SPD130DLX, Thermo Scientific.

Reagents

- mm Zirconium silicate beads (11079101z, BioSpec).
- 10% Sodium dodecyl sulfate (SDS, 71,736–500ML, Sigma-Merck).
- 30% Acrylamide/Bis Solution 37.5:1 (2.7% crosslinker) (161–0158, Biorad).
- Acetonitrile (ACN, 10,001,334, Fisher Scientific).
- Ammonium bicarbonate (ABC, A6141–25 G, Sigma-Merck).
- Ammonium persulfate (APS, GE17–1311–01, Sigma-Merck).
- β-mercaptoethanol (M3148–25ML, Sigma-Merck).
- Bovine serum albumin (BSA, A8022–50 G, Sigma-Merck).
- Bromophenol blue (GE17–1329–01, Sigma-Merck).
- Dithiothreitol (DTT, D5545–1 G, Sigma-Merck).

- Glycerol (GE17–1325–01, Sigma-Merck).
- Glycine (G8898–500 G, Sigma-Merck).
- Iodoacetamide (IAA, I1149–25 G, Sigma-Merck).
- Methanol (MeOH, 15,624,740, Fisher Scientific).
- MilliQ H_2O (Millipore).
- Phosphate-buffered saline (PBS, P4417–100TAB, Sigma-Merck).
- SimplyBlue SafeStain (LC6060, Thermo Fisher).
- Sodium hydroxide (NaOH, S8045–500 G, Sigma-Merck).
- Tetramethylethylenediamine (TEMED, 10,549,960, Fisher Scientific).
- Trifluoroacetic acid (TFA, 302,031–100ML, Sigma-Merck).
- Tris (GE17–1321–01, Sigma-Merck).
- Trypsin (V5280, Promega).
- Tween-20 (P1379–500ML, Sigma-Merck).

Materials and buffers

- 0.5 mL LoBind tubes (10,316,752, Fisher Scientific).
- 1.5 mL LoBind tubes (10,708,704, Fisher Scientific).
- Amicon Ultra-15 Centrifugal Filter Unit 10 kDa cut-off (UFC901024, Sigma-Merck).
- Analytical column: 25 cm x 75 μm, C18, 1.6 μm, Odyssey (DY-25075C18A, Ionopticks).
- Elution plate (40.020, Intavis).
- Reaction plate: (40.010, Intavis).
- Ultracentrifuge tubes 38.5 mL (082,326,823, Izasa Scientific).
- Trap column: Acclaim PepMap100 100 μm x 2 cm nanoViper C18 5 μm 100 Å (164,564, Thermo Scientific).
- Extraction buffer: ACN/milliQ H_2O (1/1, v/v) 0.25% v/v TFA.
- Lysis buffer: 4% SDS, 0.1 M DTT, 100 mM Tris–HCl pH 7.5.
- Reconstitution buffer: 5% MeOH 0.5% TFA.
- Resolving buffer (4x): 1.5 M Tris–HCl pH 8.8.
- Running buffer (10x): 0.25 M Tris–HCl, 1.92 M Glycine, 1% SDS pH 8.3.
- Sample buffer (5x): 0.1 M Tris–HCl, 44% Glycerol, 6% SDS, 0.03% bromophenol blue.
- Stacking buffer (4x): 0.5 M Tris–HCl pH 6.8.

Procedure

Sampling

Twenty four hour composite water samples were collected at the WWTP inlet using refrigerated automatic wastewater samplers that took water samples every 15 min during this period. Samples are collected in the 24 h prior to the beginning on the experiment and stored at 4 ˚C.

Separation of the soluble and particulate fractions

- Place 30 mL of the water sample in an ultracentrifuge tube.
- Ultracentrifuge at 24,000 rpm (~100,000 x *g)* for 60 min at 4 ˚C, Accel = 9, Decel = 9.
- Separate the supernatant from the pellet by pouring the supernatant in a new tube.
- At this point, the procedure can be stopped to continue later on. In this case, store both the supernatant and the pellet at −70 ˚C.

Protein extract preparation

Concentration of the soluble fraction [7]

- Add 2.5 mL of 0.1 M NaOH to the Amicon filter unit and centrifuge in the Beckmann centrifuge (TS-5.1–500) at 4000 x *g* 13 ˚C for 10 min.
- Add 2.5 mL of milliQ H_2O to the filter and centrifuge at 4000 x *g* for 15 min at 13 ˚C.
- Immerse the filter in Tween-20 5% overnight.
- Wash extensively the Tween-20 from the filter by immersing it in milliQ H2O for 1–2 min and gently shaking. Discard the water and repeat this wash four more times. Make sure there is no Tween left (no bright soapy bubbles when the filter is shaken to remove the water). Otherwise, repeat the wash.
- Add 12 mL of milliQ H_2O to the filter and centrifuge at 4000 x *g* for 15 min at 13 ˚C. (x 2)
- Transfer 10 mL of the supernatant from the ultracentrifugation (Step 2.1) to the Amicon filter unit.
- Centrifuge at 4000 x *g* for 15 min at 13 ˚C.

- Transfer the concentrated sample left in the filter (about 500 μL) to a 1.5 mL LoBind tube.
- Evaporate the sample in the vacuum concentrator until dry.
- At this point or before evaporation, the procedure can be stopped to continue later on. In this case, store samples at −70 ˚C.

Lysis of the particulate fraction [8]

- In case the pellet from the ultracentrifugation is stored at −70 ˚C (Step 2.1), thaw it at 4 ˚C.
- Reconstitute the pellet in 30 mL of cold PBS.
- Ultracentrifuge at 100,000 x *g* for 60 min at 4 ˚C, Accel = 9, Decel = 9.
- Note: For the Sorvall ultracentrifuge, we use 24,000 rpm (103,864 x *g*).
- Remove the supernatant to waste.
- Add 250 μL of lysis buffer to the pellet in the ultracentrifuge tube and sonicate in a bath for 5 min.
- Transfer to a new 1.5 mL LoBind tube.
- Add 250 μL of lysis buffer to the ultracentrifuge tube and sonicate in a bath for 5 min.
- Transfer to the previous 1.5 mL LoBind tube.
- Incubate in the Thermomixer for 60 min at 95 ˚C and 800 rpm.
- Let the sample cool down at room temperature.
- Add 500 μL of the sample to 250 μL of Zirconium Silicate beads.
- Let it in the Bullet Blender 3 min at level 8.
- Centrifuge in the Beckman centrifuge (rotor TA-15–1.5) for 10 min at 18,000 x *g* and 22 ˚C.
- Transfer the supernatant to a new 1.5 mL LoBind tube (approx. 400 μL).
- At this point, the procedure can be stopped to continue later on. In this case, store lysed samples at −70 ˚C.

Concentration of the soluble and particulate fractions with SDS-page gels

- Prepare two SDS-PAGE gels (12% resolving, 5% stacking). Use 10 and 5-well combs for the soluble and the particulate fractions, respectively.
- Add 40 μL of the sample buffer 1x with 5% of β-mercaptoethanol to the dried soluble sample.
- Add 7 μL of glycerol and 2 μL of bromophenol blue to a 25% aliquot (approx. 100 μL) of the particulate sample.
- Incubate samples in the Thermomixer at 95 ˚C for 10 min.
- Let samples cool down at room temperature and spin down.
- Load the samples in the gels:
  + Soluble fraction: 40 μL per well in a 10 wells gel.
  + Particulate fraction: 110 μL per well in a 5 wells gel.
  + Standard: 1 μg of BSA per well.
- Run the electrophoresis at 50 V until samples are stacked at the head of the gel (approx. 50 min).
- Wash the gel with milliQ H_2O for 5 min. (x 3)
- Stain the gel with SimplyBlue for 1 hour.
- Wash the gel with milliQ H_2O for 1 hour.
- At this point, the procedure can be stopped to continue later on. In this case, store the gel at 4 ˚C.

In-gel digestion [9] with trypsin

- Excise the bands of concentrated proteins taking as a reference the beginning of the BSA band with a scalpel as shown in Fig. 2, cut them into small pieces (ca. 1mm^3) and place them in different wells of the Digestor reaction plate (use more than one well per sample if necessary).
- Place the reaction plate, the elution plate and the reagents into the Digestor.
- Run the method with the following steps:

  + Wash gel slices with 100 μL of 20 mM ABC pH 7.8 and incubate for 15 min. (x 2)
  + Wash with 100 μL of ACN and incubate 15 min. (x 2).
  + Reduce with 60 μL of 10 mM DTT for 50 min.
  + Alkylate with 60 μL of 55 mM IAA for 30 min.
  + Wash with 100 μL of 20 mM ABC pH 7.8 and incubate for 15 min. (x 3)
  + Wash with 100 μL of ACN and incubate 15 min. (x 3).
  + Add 40 μL of trypsin 3.1 ng/μL and incubate for 10 min.
  + Add 60 μL of 20 mM ABC pH 7.8 without removing the trypsin and incubate for 7 h at 37 ˚C.
  + Extract peptides with 50 μL of ACN/milliQ H_2O (1/1, v/v) 0.25% v/v TFA incubating for 15 min. (x 2)
  + Add 40 μL of ACN/milliQ H_2O (1/1, v/v) 0.25% v/v (TFA) and incubate for 15 min.
  + Add 40 μL of ACN and incubate for 15 min.

- Transfer digested samples to new 0.5 mL LoBind tubes and evaporate to dryness.

Analysis by liquid chromatography coupled to high resolution mass spectrometry (LC–HR-MS)

- Reconstitute samples in 50 μL of 5% MeOH 0.5% TFA.
- Inject 10% of each sample.

+ LC parameters:

- Sampler temperature: 8 ˚C.
- Column temperature: 35 ˚C.
- Solvents: 0.1% formic acid (A) and acetonitrile 0.1% formic acid (B).
- Gradient:

Time (min)	Flow-rate (μl/min)	% B
0	15	3
3	0.3	3
93	0.3	45
95	0.3	95
99	0.3	95
101	0.3	3
105	Stop run	

+ MS parameters:

- Spray voltage: 1500 V.
- Data-dependent mode: 10 MS/MS scans of the 10 most intense signals detected in the MS scan.
- Fragmentation: HCD (27%).
- Full MS (range 375–1600) acquired in the Orbitrap with a resolution of 60,000.
- MS/MS spectra (range 200–2000) obtained in the Orbitrap with a resolution of 15,000.

Database search with proteome discoverer 3.0.1.27

- Processing workflow parameters:

+ Min. precursor mass: 350 Da.
+ Max. precursor mass: 5000 Da.
+ Database: Swiss-Prot (February 2023).
+ Min. peptide length: 6.
+ Precursor mass tolerance: 20 ppm.
+ Fragment mass tolerance: 0.02 Da.
+ Dynamic modifications: Acetyl in N-term, oxidation in M, Met-loss in M and Met-loss+Acetyl in M.
+ Static modifications: Carbamidomethyl in C.
+ Percolator (FDR targets):

· Target FDR (Strict): 0.001 (0.1%).
· Target FDR (Relaxed): 0.005 (0.5%).

- Consensus workflow parameters:

+ Precursor ions quantifier:
· Peptides to use: Unique.
· Precursor abundance based on: Intensity.
· Normalization mode: Total peptide amount.
+ Peptide validator:
· Target FDR (Strict) for PSMs: 0.001 (0.1%).
· Target FDR (Relaxed) for PSMs: 0.005 (0.5%)
· Target FDR (Strict) for Peptides: 0.001 (0.1%).
· Target FDR (Relaxed) for Peptides: 0.005 (0.5%).
+ Minimum peptide length: 6.
+ Protein FDR validator:

· Target FDR (Strict): 0.01 (1%).
· Target FDR (Relaxed): 0.05 (5%).

For the complete list of parameters see Supplementary Material.

Table 1
Collection sites. Population equivalent, population served and water treated at the different WWTPs.

WWTP	Population (thousands)		Water treated [c] (m^3/d)
	Equivalent [a]	Served [b]	
Besòs	2844	1502	322,238
Girona	206	159	43,556
Vic	340	55	22,384

[a] The equivalent population is the population the wastewater treatment plant was designed for. Agència Catalana de l'Aigua (21/11/2022), https://aca.gencat.cat/ca/laigua/infraestructures/estacions-depuradores-daigua-residual/.
[b] The served population is the actual number of people that live in the area whose wastewater the treatment plant receives. https://www.epdata.es/.
[c] https://sarsaigua.icra.cat/.

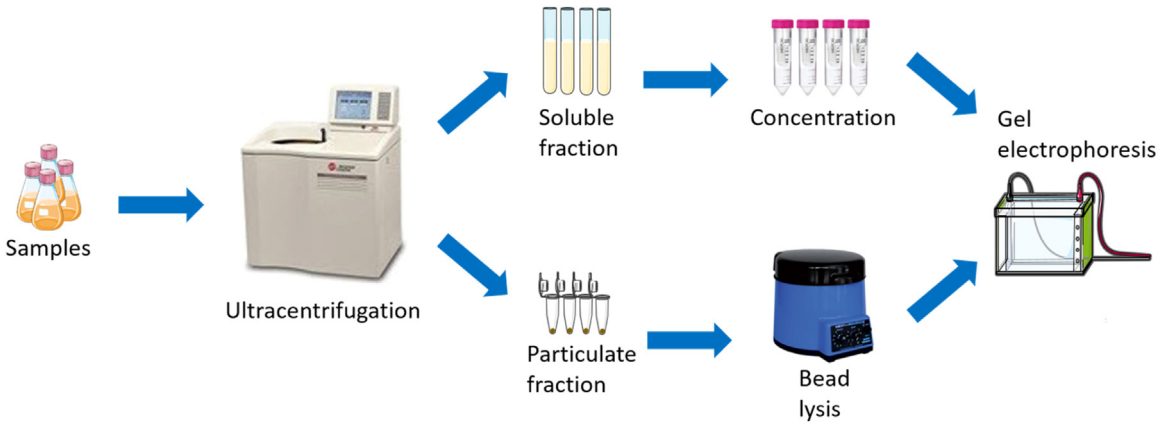

Fig. 1. Outline of the sample fractionation and fraction processing.

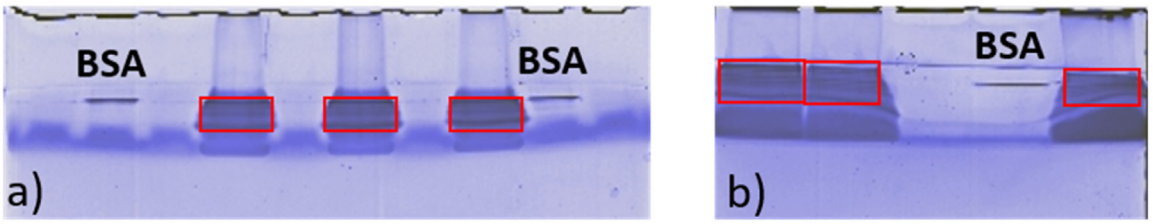

Fig. 2. Bands excised taking the BSA reference for the soluble fraction (a, 10 wells gel) and the particulate fraction (b, 5 wells gel).

Application

Data presented here correspond to the analysis of 24-h composite wastewater samples collected between April and May 2022 at the inlet of 3 different wastewater treatment plants (WWTPs) serving cities of different population and diverse industrial activities (Table 1).

The soluble and particulate fractions were separated by ultracentrifugation and the particulate fraction was disrupted and homogenized mechanically. Solubilized proteins were concentrated in a small band at the head of a polyacrylamide gel (Fig. 1). This band was submitted to the conventional procedures in shotgun proteomics, namely, in-gel digestion and LC-MS/MS analysis followed by database identification (Fig. 3). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [10] partner repository with the dataset identifier PXD042445.

The number of identifications ranged between 244 and 386 for the soluble part and between 488 and 935 in the particulate (Table 2). The proteins and the origin of these proteins in each fraction is very different (Fig. 4). While in the particulate fraction Bacteria proteins dominate, in the soluble fraction the number of Eukaryote proteins increases up to 42% of total protein. In the case of the soluble fractions, the 3 most abundant proteins are amylase enzymes from humans with the double of Peptide-Spectrum Matches (PSMs) than the next most abundant protein. These human amylases are followed by amylase enzymes from murids, human albumin and by albumins from livestock (cow, pig, sheep or rabbit). In contrast, in the particulate fraction, though the most abundant protein is a human elastase (with 4 times less PSMs than the most abundant soluble proteins), it is followed for a mix of proteins from human and different kinds of bacteria.

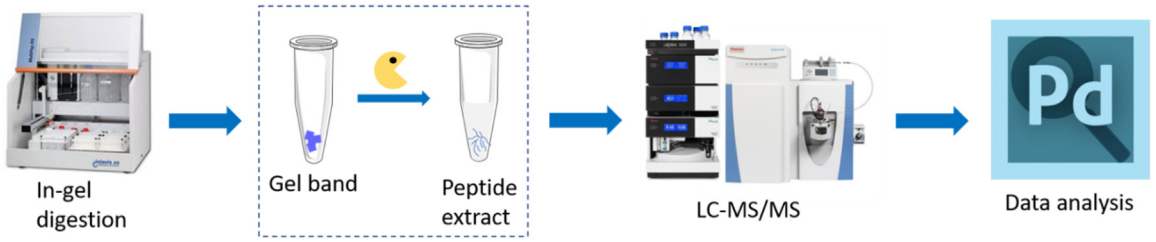

Fig. 3. Outline of the protein digestion and peptide analysis.

Table 2
Number of proteins and peptides identifications by site and fraction (see Supplementary Material for detailed information).

WWTP	Proteins		Peptides	
	Soluble	Particulate	Soluble	Particulate
Besòs	265	935	1183	1894
Girona	244	791	1024	2030
Vic	386	488	1277	1091

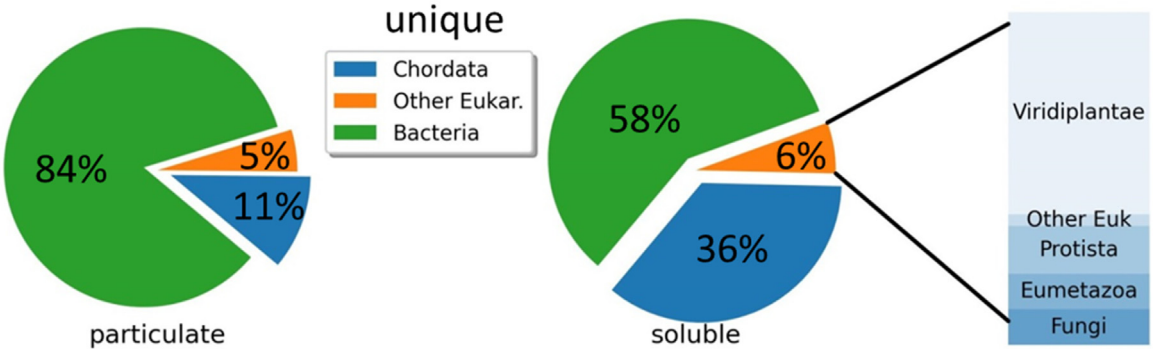

Fig. 4. Distribution of the number of Bacteria and Eukaryote proteins in the particulate and soluble fractions.

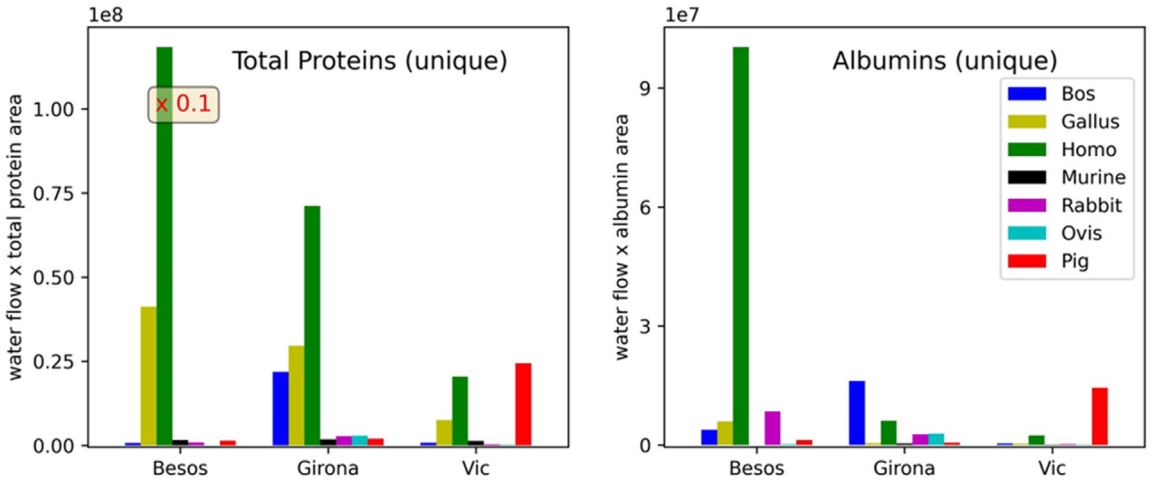

Fig. 5. Distribution by sites of total proteins (right) and albumins (left) from Human, murids and different livestock species. Abundance calculation was made considering only protein unique peptides.

The data allows to characterize the contribution of different species to the wastewater proteome at each site (Fig. 5). In cities with a high population density like Besòs (Barcelona) human proteins are dominant, while in more rural areas, such as Vic, livestock proteins can be more abundant that human proteins. The major contributor to livestock proteins is albumin as reflected in the small difference between total protein and albumin abundances in Fig. 5 for farm animals. This albumin probably comes from the spillage of animal blood and tissue in the wastewater, thus reflecting the activity of the meat industry in these areas.

Conclusions

Wastewater proteomics is an emerging field within sewage chemical information mining and wastewater-based epidemiology. Until now, the study of wastewater was focused on small molecules. To our knowledge, this is the first method developed for the large-scale characterization of proteins in this kind of matrix, overcoming issues as the heterogeneity and complexity of the wastewater, and the interferences from other molecules. We have shown that proteins in wastewater transport information on the human and industrial activities in the urban and rural areas from which these discharges originate.

The method described here has slightly modifications relative to our previous works [6] in order to optimize the efficiency and simplify the resources needed for the analysis. Work is in progress to extend the approach to quantitative targeted methodologies. We believe this method is an effective discovery tool in the search of community biomarkers and a first step to the development of specific test devices for health and environmental monitoring.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ester Sánchez-Jiménez: Methodology, Validation, Investigation, Writing – original draft. **Joaquin Abian:** Software, Formal analysis, Data curation, Visualization, Writing – review & editing. **Antoni Ginebreda:** Conceptualization, Writing – review & editing. **Damià Barceló:** Conceptualization, Supervision, Project administration, Funding acquisition. **Montserrat Carrascal:** Conceptualization, Funding acquisition, Writing – review & editing, Supervision, Project administration.

Data availability

Data are available via ProteomeXchange with identifier PXD042445.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2023.102403.

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