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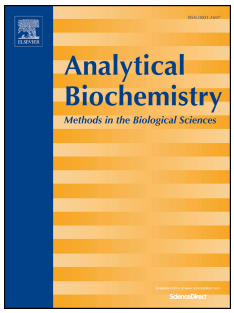

Comparison of methods to isolate proteins from extracellular vesicles for mass spectrometry-based proteomic analyses

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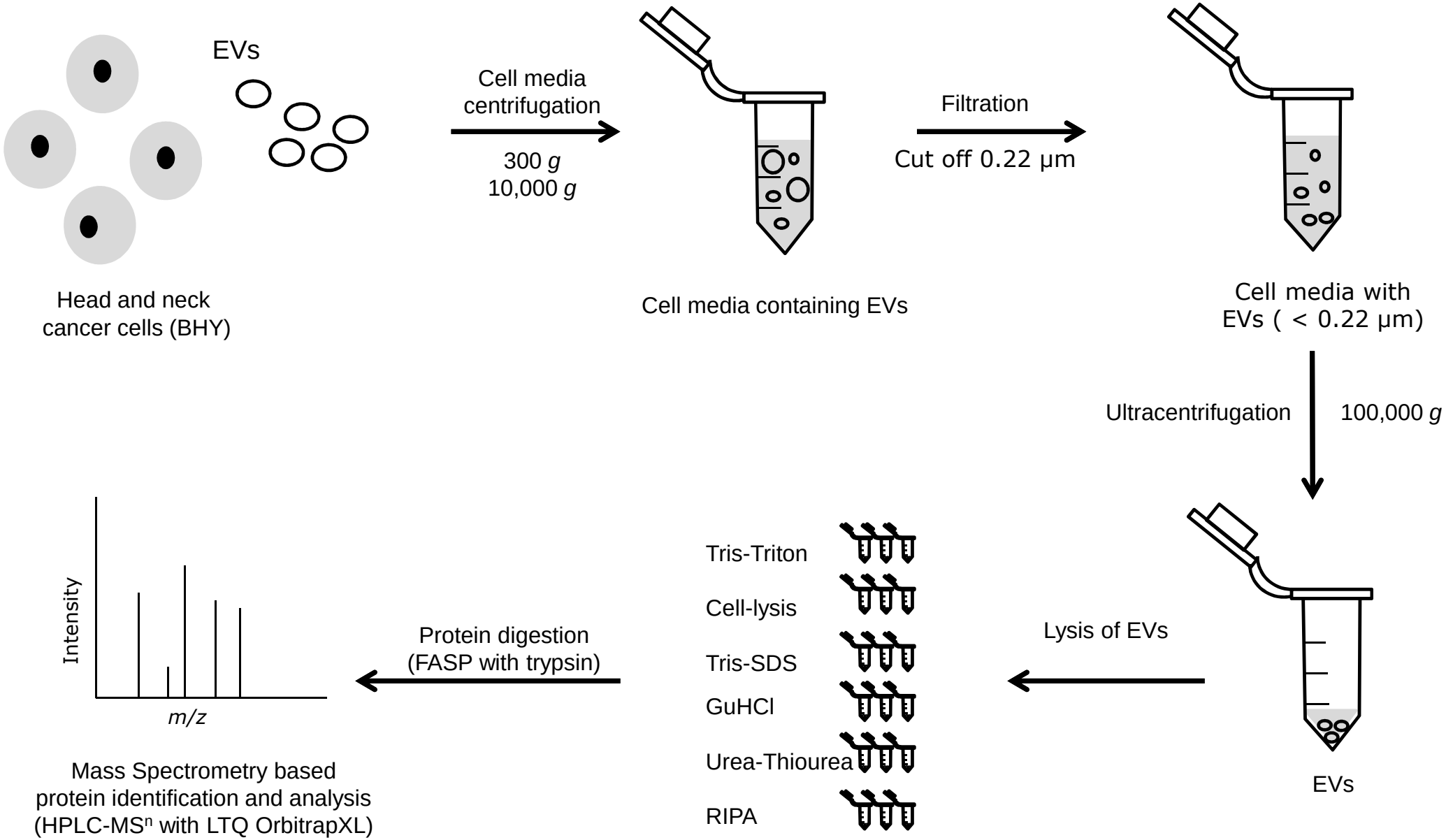
EVs
Head and neck cancer cells (BHY)
Cell media centrifugation
300 g
10,000 g
Cell media containing EVs
Filtration
Cut off 0.22 μm
Cell media with EVs (< 0.22 μm)
Ultracentrifugation
100,000 g
EVs
Lysis of EVs
Tris-Triton
Cell-lysis
Tris-SDS
GuHCl
Urea-Thiourea
RIPA
Protein digestion (FASP with trypsin)
Intensity
m/z
Mass Spectrometry based protein identification and analysis (HPLC-MSⁿ with LTQ OrbitrapXL)

Comparison of methods to isolate proteins from extracellular vesicles for mass spectrometry-based proteomic analyses

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Abstract

Extracellular vesicles (EVs) are cell-derived membrane-bound organelles that have generated interest as they reflect the physiological condition of their source. Mass spectrometric (MS) analyses of protein cargo of EVs may lead to the discovery of biomarkers for diseases. However, for a comprehensive MS-based proteomics analysis, an optimal lysis of the EVs is required. Six methods for the protein extraction from EVs secreted by the head and neck cell line BHY were compared. Commercial radioimmunoprecipitation assay (RIPA) buffer outperformed the other buffers investigated in this study (Tris-SDS, Tris-Triton, GuHCl, urea-thiourea, and commercial Cell-lysis buffer). Following lysis with RIPA buffer, 310 proteins and 1469 peptides were identified using LTQ OrbitrapXL mass spectrometer. Among these, 86 % of proteins and 72 % of peptides were identified in all three replicates. In the case of other buffers, Tris-Triton identified on average 277 proteins, Cell-lysis buffer 257 proteins, and Tris-SDS, GuHCl and urea-thiourea each 267 proteins. In total, 399 proteins including 74 of the top EV markers (Exocarta) were identified, the most of the latter (73) using RIPA. The proteins exclusively identified using RIPA represented all Gene Ontology cell compartments. This study suggests that RIPA is an optimal lysis buffer for EVs in combination with MS.

Introduction

Extracellular vesicles (EVs) is a broad term that includes all cell-derived membrane-bound organelles that range in size from exosomes (30 - 150 nm) [1,2] to microvesicles (50 - 1000 nm) [3], and apoptotic vesicles (1000 - 4000 nm) [4]. They are secreted by all forms of organisms from prokaryotes to eukaryotes [5], by tumour and non-tumour cells [6], and they have been observed in body fluids including blood plasma [7], saliva [8], amniotic fluid [9], and cerebrospinal fluid [10]. They hold a cargo of proteins [11], lipids [12], metabolites [13], and nucleic acids [11,14]. Interest in the function of EVs has grown rapidly in recent years as they play a role in diverse cellular functions such as tumour metastasis [15], intercellular communication [16], and the delivery of RNA and proteins into cells [17]. They may even propagate diseases such as neurodegeneration by carrying aggregated proteins [18]. The EV cargo can function as a biomarker as it reflects the physiological condition of the source [19]. A change in the protein cargo was observed in exosomes after exposure to different cellular stressors, including ionizing radiation [11,20,21]. An increase in the concentration of exosomal lipopolysaccharide-binding proteins (LBPs) was reported in patients with non-small-cell lung cancer compared to healthy controls [22]. Consequently, using an efficient method for extraction of the EV cargo is essential. As proteins are central players in the cell, this study concentrates in elucidating the protein cargo in EVs. The isolation and detection of EV proteins depends on the efficiency of the lysis process, particularly of the disruption of the membrane/lipid bilayer, thereby releasing the internal components. In addition, the ability to keep the released proteins soluble is essential [23]. The selection of the optimal buffer for EV lysis is difficult based on current literature as the number of proteins identified varies depending on the cell line in question [24], their isolation method [25], and the characteristics of MS strategy used [26]. To overcome these problems, we tested six different methods to determine the optimal lysis and extraction buffer, based on the number of proteins identified after lysis of EVs.

Materials and Methods

Cell culture

The head-and-neck cancer cell line BHY (DSMZ no.: ACC 404) was cultivated in Dulbecco's modified Eagle medium (DMEM, Gibco®, Invitrogen™, Karlsruhe, Germany) with high glucose, 2 mM L-Glutamine and sodium pyruvate, supplemented with 10 % exosome-depleted fetal bovine serum (FBS, GE Healthcare, Amersham, U.K.). Prior to cultivating the cells, the exosomes present in the FBS were removed by centrifugation (100,000 *g*, 4 ^{0}C, 14 h). The cells were incubated in a humidified atmosphere at 37 °C and 5 % CO_2. All ultracentrifugation steps were performed on an Optima L-90K ultracentrifuge (Beckman Coulter, California, United States) with a type 70 Ti Rotor (Beckman Coulter).

Isolation of EVs

An adapted protocol was used to isolate the EVs [27] where 6×10^6 BHY cells per T175 cell culture flask (Grenier Bio-One, Kremsmuenster, Austria) were seeded in 25 mL exosome-depleted medium and incubated for 72 h. EV isolation was performed from a pool of 230 mL conditioned medium obtained from $1{,}81 \times 10^8$ cells. The collected medium was successively centrifuged at 300 *g* (4 °C, 10 min) and 10,000 *g* (4 °C, 30 min) to remove cells and cellular fragments. For the removal of microvesicles and apoptotic bodies larger than 200 nm, the supernatant was passed through a filter with a pore size of 0.22 µm. EV sedimentation was performed with three successive ultracentrifugation steps (100,000 *g*, 4 °C, 2 h). Each centrifugation step was followed by removal of supernatant and resuspension of the EV-pellet in PBS using quick vortexing. After the final centrifugation step, the supernatant was discarded and the EV pellets were resuspended in 115 µL PBS (Merck KGaA, Darmstadt, Germany) and sonicated on ice-water at 4 °C for 2 min before a storage at 4 °C. The isolated EVs had a mean diameter of 195 nm and mode diameter of 143 nm (Supplementary Material Figure 1), characteristic of exosome-like EVs [11].

Nanoparticle tracking analysis (NTA)

For quantification and size distribution of the isolated EVs, they were analysed with the NanoSight LM10 microscope (Malvern, Herrenberg, Germany). The EV suspension was diluted 1:1000 with ultrapure H_2O to an optimal particle concentration of 15-50 particles per frame and was measured three times for 30 s.

Lysis of EVs

Identical concentrations of protease and phosphatase inhibitors were used for all experiments, where one tablet each of cOmplete™ EDTA-free Protease Inhibitor (Sigma Aldrich, Schnelldorf, Germany) and PhosSTOP™ (Sigma Aldrich, Schnelldorf, Germany) was dissolved in 10 mL of each of the lysis buffers tested. Ultrapure water (resistance $\geq$ 18.2 MΩ·cm^{-1}) from PureLabflex (ELGA Labwater, Wycombe, United Kingdom) was used. The lysis buffers and EVs were kept on ice at all times unless otherwise stated. The lysis for all buffers was performed from the same pool of EVs and for each lysis, 2 µL of EV-suspension (~ 2.02×10^9 particles in PBS, Supplementary Material Figure 1) was used.

Urea-thiourea lysis

An adapted protocol [28,29] was used for urea-thiourea lysis of EVs. Fifty µL of urea-thiourea buffer containing 2 M urea (GE Healthcare, Amersham, U.K.), 7 M thiourea (GE Healthcare, Amersham, U.K.), 30 mM Tris.HCl pH 7.5, (Calbiochem, Darmstadt, Germany) was added to EVs and placed on ice. The EVs were dounce-homogenized using an Eppi-Pistille (Schuett-Biotec, Goettingen, Germany) with 20 up and down movements on ice and placed on ice-cold sonication bath for six times for 10 s with 10 s intervals. During the intervals they were placed on ice-water to reduce overheating.

Radioimmunoprecipitation assay (RIPA) and Cell-lysis buffer (CLB) lysis

RIPA buffer (Thermo Fisher Scientific, Illinois, USA) containing 25 mM Tris.HCl pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, and 0.1 % SDS was used. Cell-lysis buffer (CLB, Cell Signaling Technologies, Massachusetts, USA) contained 20 mM Tris.HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, and 1 µg/mL leupeptin. The EVs were lysed adapting to the manufacturer's instructions[30,31]. The EVs were placed in 50 µL RIPA or CLB and incubated at 4 °C for 30 min and placed in an ice-cold sonication bath for 30 s. This step was followed by a gentle mix on ice for 15 min.

Tris-SDS lysis

The EVs were lysed with Tris-SDS according to an adapted protocol [32,33]. The EVs were added to 50 µL SDS-Tris buffer [2 % (w/w) sodium dodecyl sulfate SDS (Calbiochem, Darmstadt Germany), 20 mM Tris.HCl pH 8 (Calbiochem, Darmstadt Germany] and were heated at 95 °C for 5 min. The EVs were placed in an ice-cold sonication bath six times for 30 s with 30 s intervals on ice to reduce overheating.

Tris-Triton lysis

For lysing the EVs with Tris-Triton buffer [25 mM Tris.HCl (Calbiochem, Darmstadt, Germany, 120 mM NaCl (Merck KGaA, Darmstadt, Germany), pH 7.5, 1 % Triton-X 100 (SigmaAldrich, Schnelldorf, Germany)], 50 µL of the buffer was given to the EVs before incubation on ice for 4 h with a quick vortex once every hour as described previously [20].

Guanidium chloride (GuHCl) lysis

GuHCl (Sigma Aldrich, Schnelldorf, Germany) was solubilized in water at 35 °C to a concentration of 6 M and 50 µL was used to lyse the EVs. The EVs in GuHCl buffer were vortexed for 2 min. The samples were placed in a thermomixer (25 °C, 1000 rpm, 20 min) and vortexed for 2 min, then in an ice-cold sonication bath for four times á 30 s. The samples were cooled between the sonication steps by placing them on ice-water for 2 min. The steps were adapted according to manufacturer's instruction[34].

Protein concentration determination

The protein concentration for samples containing urea was determined by Qubit® fluorimeter (Thermo Fisher Scientific, Darmstadt, Germany) according to the instruction manual. For other samples, protein concentration was determined using bicinchoninic acid assay (BCA) as in the instruction manual (Thermo Fisher Scientific, Darmstadt, Germany) using bovine serum albumin (BSA) as a standard on an Infinite M200 Spectrophotometer (Tecan GmbH, Crailsheim, Germany).

FASP digestion of proteins

A modified version of filter aided sample preparation (FASP) protocol [35,36] was used to digest 10 µg of each EV lysate. Briefly, the proteins were diluted in 100 - 500 µL UA buffer (8 M urea in 0.1 M Tris.HCl),

reduced using 1 µL of 1 M dithiothreitol (30 min, 60 °C) and alkylated using 10 µL of 300 mM iodoacetamide (30 min, dark) before being centrifuged (14,000 *g*, 10 min, room temperature) through a 30 kDa cut-off filter (Vivacon 500, Sartorius, Goettingen, Germany). The proteins were then washed thrice with UA buffer and twice with 50 mM ammonium bicarbonate and proteolysed on the filter for 2 h at room temperature using 1 µg Lys-C (Wako Chemicals, Neuss, Germany) and for 16 h at 37 °C using 2 µg trypsin (Promega, Mannheim, Germany). The peptides were collected by centrifugation (10 min, 14,000 *g*), acidified with 0.5 % trifluoroacetic acid (TFA), and stored at -20 °C.

High-performance liquid chromatography online coupled to tandem mass spectrometry (HPLC-MS/MS)

All samples were thawed and centrifuged for 5 min at 4 °C. The HPLC-MS/MS analyses were performed as described previously [37,38] on an LTQ OrbitrapXL mass spectrometer (Thermo Scientific™, Bremen, Germany) coupled to an Ultimate3000 HPLC. Each sample was automatically injected and loaded onto a C18 PepMap100 trap column (300 µm i.d. × 5 mm, 100 Å Thermo Scientific™, Bremen, Germany). After 5 min, the peptides were eluted and separated on an analytical column (75 µm i.d. × 25 cm, nanoEase MZ HSS T3 Column, 100 Å, Waters, Milford, U.K.) using a linear 80 min acetonitrile gradient (5 % to 25 %) acetonitrile in 0.1 % formic acid at 250 nL/min flow rate. The top 10 most abundant peptide ions were selected for fragmentation by collision induced dissociation in the linear ion trap if they showed an intensity of at least 200 counts and if they were at least doubly charged. During fragmentation a high-resolution (60,000 full-width half maximum) MS spectrum was acquired in the Orbitrap with a mass range from 300 Da to 1500 Da and a dynamic exclusion of 60 s. Normalized collision energy of 35 was used.

Label-free proteomic analysis

The identification of proteins was performed with Proteome Discoverer™ 2.2 (Thermo Scientific™, Bremen, Germany) with a SEQUEST HT search algorithm using the SwissProt *Homo Sapiens* database (TaxID=9606, version 2017-10-25, 73,931 proteins). A precursor mass tolerance of 20 ppm and fragment mass tolerance of 0.5 Da were used. A decoy database search was performed with the cut off for false discovery rate set to 1 %. Only proteins with at least one unique peptide were considered. Peptides with

a maximum of two miscleavages and a size ranging from 6 to 150 amino acids were searched. Oxidation (methionine) and deamidation (asparagine, glutamine) were allowed as dynamic modifications, and carbamidomethyl (cysteine) were considered as static modifications. Intensity-based protein abundances were normalized on 'total peptide amount'. The normalization is based on peptide abundances of a MS-file to the summed peptide abundances of all MS-files.

Venn diagram and Gene Ontology (GO) analysis

A web-based tool (http://www.interactivenn.net/ retrieved date: 13.03.2019) was used to compare the proteins identified with different buffers [39] and gene ontology (GO) analyses were performed with PANTHER (v.14, www.pantherdb.org) [40]. In both cases, proteins identified in every replicate (n=3) were used.

Immunoblotting

A standard western blotting protocol was used for immunoblotting where 10 µg lysed-EVs and RIPA-lysed cell lysate were used. Additionally, 8 µL of EVs (8.08×10^9 particles), without pre-lysis, was used as a control as well. Antibodies directed against GAPDH (sc-47724, Santa Cruz, California, U.S.A.), Calnexin (sc11397, Santa Cruz California, U.S.A.), ALIX (2171, Cell Signaling Technologies, Massachussetts, U.S.A), TSG101 (GTX70255, GeneTex), and CD9 (sc13118, Santa Cruz, California, U.S.A.), were applied. Horseradish peroxidase (HRP) conjugated secondary antibodies (anti-mouse or anti-rabbit) and enhanced chemiluminescence (ECL™) reaction kit (GE Healthcare, Amersham, U.K.) were used and detection was with a chemiluminescence Alpha Innotec reader (Biozym, Niedersachsen, Germany).

Statistics

There were three technical replicates performed for each buffer. Data are presented as mean ± standard deviation (SD) of three replicates. The paired Student's *t*-Test was applied to the number of identified proteins/peptides with each buffer compared to RIPA. In the case of coefficient of variation (CV) studies,

only proteins identified in every replicate were taken and a two-tailed *t*-test was performed with the assumption that unequal variances exist between the samples. Significance was considered at $p < 0.05$ (*) and $p < 0.0001$ (****).

Results

The numbers of proteins and peptides identified after EV lysis using different methods are listed in Table 1. The highest number of proteins (310 ± 6) and peptides (1469 ± 39) were identified after lysis with RIPA buffer. The RIPA lysis was not statistically significantly better than Tris-Triton as far as the numbers of identified proteins and peptides were concerned but it was significantly better in this respect to all other buffers ($p < 0.05$, paired *t*-test) (Table 1). There was no significant difference in the average number of peptides per protein between RIPA and the other buffers. In terms of repeatability, 86 % of proteins and 72 % of peptides were identified in all three replicates when the EVs were lysed with RIPA buffer. Repeatability was lower for all other buffers (80 - 85 %) (Table 1).

The known EV constituent proteins CD9, Alix, and TSG 101 were identified in all lysis conditions used (Supplementary Material Figure 1). Of the top 214 proteins that are identified most commonly in exosomes [41], altogether 74 proteins were identified in this study. Of these, RIPA scored the highest number of identifications (73) when compared to CLB (65), GuHCl (62), Tris-SDS (66), Tris-Triton (69), and urea-thiourea (62).

<u>Differences in protein identification with different buffers</u>

A principal component analysis (PCA) was created based on the intensities of the identified proteins and peptides using the Proteome Discoverer 2.2 software. The three technical replicates representing one buffer condition clustered nicely in all cases (Figure 1). The clusters representing CLB, Tris-SDS, and Tris-Triton clustered close together whereas those representing RIPA, GuHCl, and urea-thiourea were further apart. The most dense cluster was observed in the case of Tris-SDS whilst the most scattered one represented CLB.

A box plot diagram representing the range of the coefficient of variation (CV) values for protein abundances within technical replicates is presented in Figure 2. The smallest average CV was observed when EVs were lysed with Tris-SDS (7.56 %), being significantly lower than when EVs were lysed with Tris-Triton (9.59 %), GuHCl (9.87 %), CLB (13.3 %), urea-thiourea (12.2 %), or RIPA (12.1 %).

A Venn diagram representing proteins identified by different buffers revealed that 155 proteins were

shared in all lysis conditions (Figure 3). Fourteen proteins were identified exclusively using RIPA, 4 using GuHCl or Tris-SDS, 3 using CLB or urea-thiourea, and 1 protein was identified exclusively using Tris-Triton.

When RIPA buffer was compared against other buffers individually, 48 proteins were identified exclusively with RIPA compared to Tris-Triton, 57 compared to CLB, 49 compared to Tris-SDS, 63 compared to GuHCl, and 58 compared to urea-thiourea buffer. These proteins exclusively identified with RIPA were subjected to gene ontology (GO) "cellular component" analysis using following categories: membrane (GO:0016020), cell junction (GO:0030054), supramolecular complex (GO:0099080), protein-containing complex (GO:0032991), organelle (GO:0043226), extracellular region (GO:0005576) and cell (GO:0005623). The analysis revealed that proteins representing all categories were more efficiently identified using RIPA in comparison to other buffers (Figure 4). Furthermore, RIPA was notably more efficient than GuHCl in isolating membrane proteins with 12 additional membrane proteins identified between the two methods. However, 31 proteins were identified in the EV-proteome that were not identified with RIPA (SM Figure 2). The GO analysis of these proteins revealed that they belonged to categories: protein-containing complex (GO:0032991), organelle (GO:0043226), extracellular region (GO:0005576) and cell (GO:0005623).

Discussion

Proteins in circulating EVs may prove to be suitable biomarkers of disease and therapy response [42]. It is essential that the changes in the EV protein cargo truly reflect the biological conditions and are not influenced by technical limitations. In this study, we have systematically compared commonly used methods to isolate proteins from EVs. The highest number of proteins were identified after EV lysis with RIPA buffer that contains a combination of ionic (1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate) and non-ionic detergents (1 % Nonidet™ P-40). Also EV lysis using Tris-Triton proved to be efficient as it did not score statistically significantly worse than RIPA if the number of identified proteins was concerned. Our results are in agreement with a previous study, showing that EVs were relatively efficiently lysed by SDS or Triton-X 100 [43]. It also correlates to another study showing the highest EV protein amount after extraction using Triton and similar hydrophobic detergents when compared with other ionic or zwitterionic detergents [44].

The PCA plots represent the 'relatedness' between different replicates of the same extraction buffer. A particular lysis procedure is more repeatable if the co-ordinates for different replicates lie close to each other. If the co-ordinates for replicates extracted by different buffers are far apart from each other, it represents the identification of different sets of proteomes. We show here that different protein isolation methods result in different sets of EV protein identification, depending on the lysis buffer.

Variability in MS-based proteomics arises from different factors. In a study performed using human brain, protein extraction from tissues was a major contributor for coefficient of variation (CV), being responsible for 72 % of CVs. This was followed by variances due to instrumentation and stability during a two-week experimental period (24 %), and by variability in the digestion step (3 %) [45]. The CV between technical replicates of EV proteomes in this study ranged from 7.56 % (Tris-SDS) to 13.3 % (CLB). Although the average CV for RIPA buffer was 12.1 %, the median CV was relatively low (9.3 %) suggesting that that lysis with RIPA buffer is a robust and reliable method. Moreover, using RIPA buffer, 16 % more proteins were identified compared to Tris-SDS buffer.

When the different lysis buffers were compared, 155 proteins were commonly identified in all lysis conditions. At least three times more proteins were identified exclusively using RIPA than with any other buffer. On a closer inspection when RIPA was compared to other lysis buffers individually, 55 proteins on average were identified exclusively using RIPA. The GO analysis showed that several membrane proteins were present only among the proteins identified exclusively with RIPA and not identified

amongst proteins identified exclusively with other buffers (SM Figure 2). Membrane proteins are known to be hydrophobic, leading to resistance against isolation and digestion [46]. The combination of ionic and non-ionic buffers, as present in RIPA, could be the reason for the observed exclusive identification of membrane proteins. As EVs have a high composition of membrane proteins due to their small size and high surface area to volume ratio, this could provide a particular advantage for the use of RIPA in the case of EVs [47].

Conclusion

Taken together, this study shows that RIPA buffer, having a combination of both ionic and non-ionic detergents, results in the highest number of identified EV peptides and proteins in MS-based identification. One of the most important points in mass spectrometry (MS) related study is that of normalization. The number of proteins identified needs to be normalized based on the number of EV particles used for lysis, the amount of proteins isolated after EV lysis, the amount of peptides obtained after protein digestion, and the amount of peptides injected into the liquid chromatography (LC) column. Equal number of EV particles was used for different lysis firstly, and same quantities of proteins were digested. After digestion, similar amounts of peptides were injected into the LC column. For further analysis, the proteins were normalized in the Proteome Discoverer software with 'total peptide amount', where the individual peptide abundances for a single run is normalized to the sum of peptide abundances in all of the runs.

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Supplementary Material

The characterisation of EVs by nanoparticle tracking analysis is provided in SM Figure 1. The accession numbers of proteins identified in EVs with different buffers can be observed in SM List 1 and the complete list of EV proteome in SM List 2.

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Data availability

Raw MS-data are available at: http://dx.doi.org/doi:10.20348/STOREDB/1135

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Table 1. Proteins and peptides identified in EVs after lysis with different buffers. The common proteins or peptides indicate the proteins/peptides that were identified in every technical replicate (n=3).

Lysis buffers	Proteins identified		Peptides identified		Average number of peptides per protein
	Mean ± SD	Common	Mean ± SD	Common	
Tris-Triton	277 ± 25	227 (81.9 %)	1310 ± 120	927 (70.7 %)	4.73 ± 0.03
CLB	257 ± 7*	214 (83.1 %)	1191 ± 65*	802 (67.3 %)	4.70 ± 0.15
Tris-SDS	267 ± 12*	227 (85.1 %)	1225 ± 37*	900 (73.5 %)	4.93 ± 0.33
GuHCl	267 ± 3*	221 (82.9 %)	1319 ± 25*	985 (74.7 %)	4.89 ± 0.06
Urea-Thiourea	267 ± 8*	224 (83.9 %)	1246 ± 40*	892 (71.6 %)	4.62 ± 0.04
RIPA	310 ± 6	266 (85.6 %)	1469 ± 39	1053 (71.7%)	4.81 ± 0.09

* $p < 0.05$ (two-tailed paired Student's *t*-test compared against RIPA)

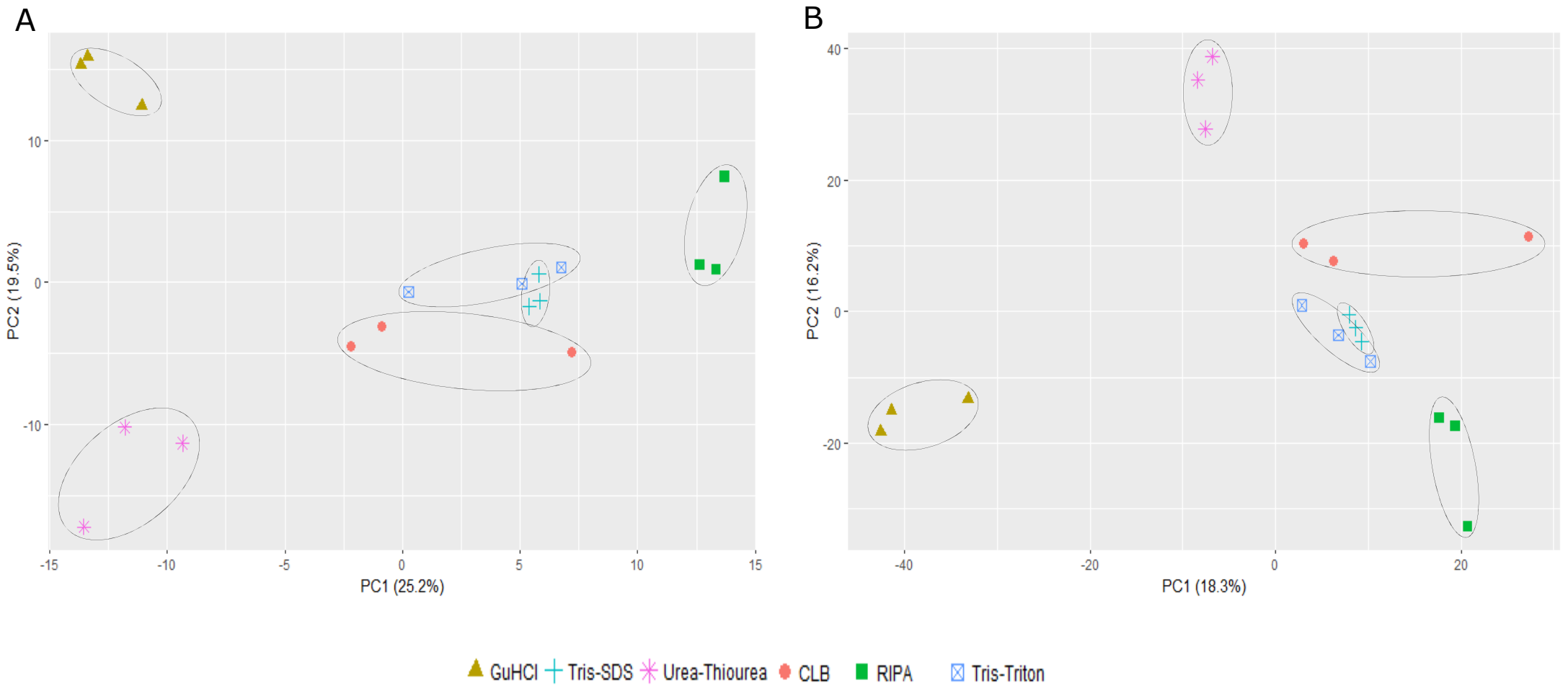

Figure 1. Principal component analysis (PCA) of different buffers for lysis based on (A) proteins and (B) peptides in EVs. PCA replicates for each buffer are grouped and indicated by ellipses.

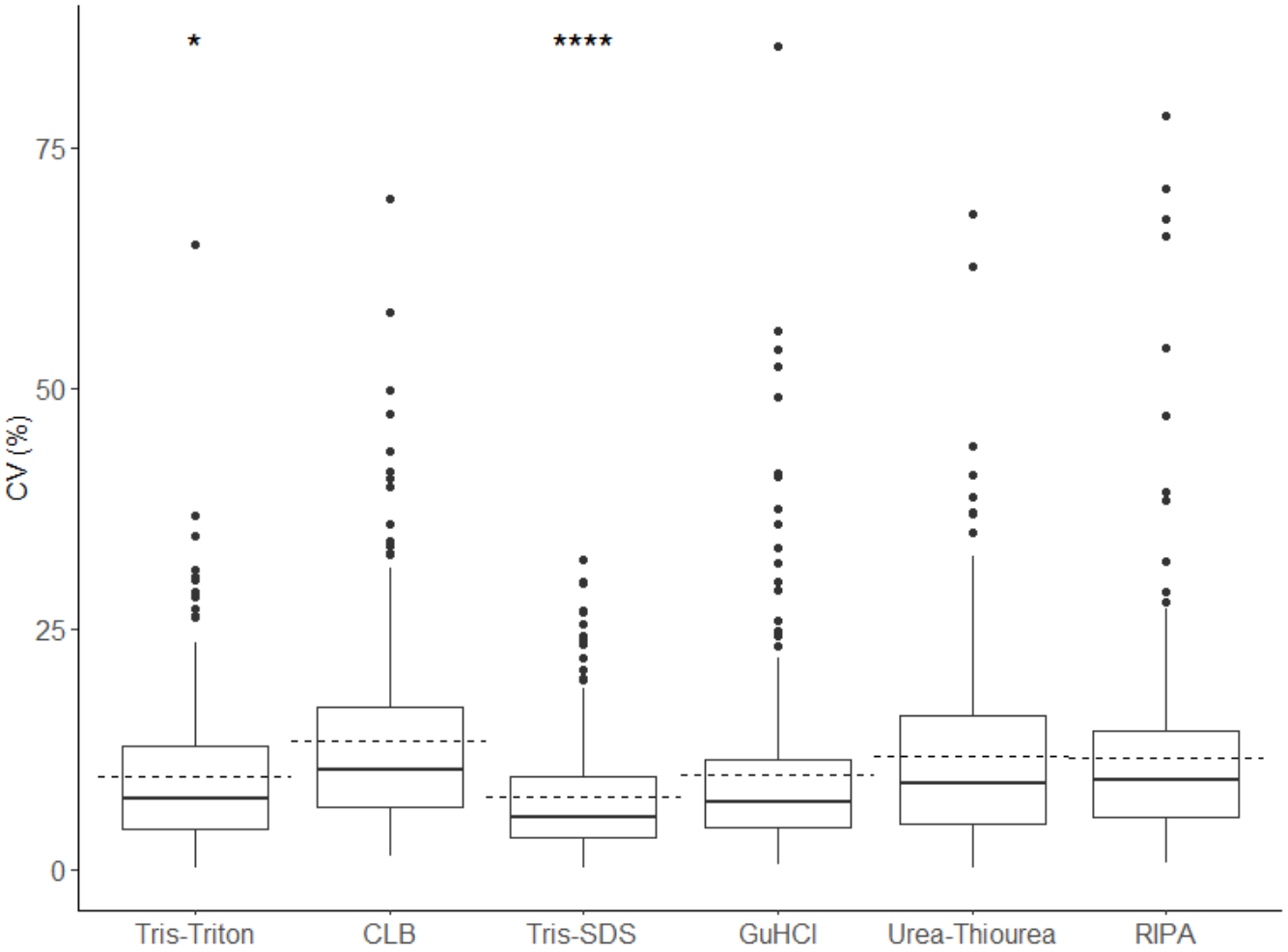

Figure 2. A box plot diagrams representing the coefficient of variation percentages (CV %) of protein abundances within technical replicates in EVs. Only proteins identified in every replicate (n=3) were considered. Fifty percent of the values lie inside the box and continuous lines represent 1st, 2nd, and the 3rd quartile whereas the dotted line represents the statistical mean. Outlier proteins, with CV higher than 100 %, were removed.
* $p < 0.05$, ****$p < 0.0001$ (two tailed Student's *t*-test compared against RIPA, assuming unequal variances between the samples)

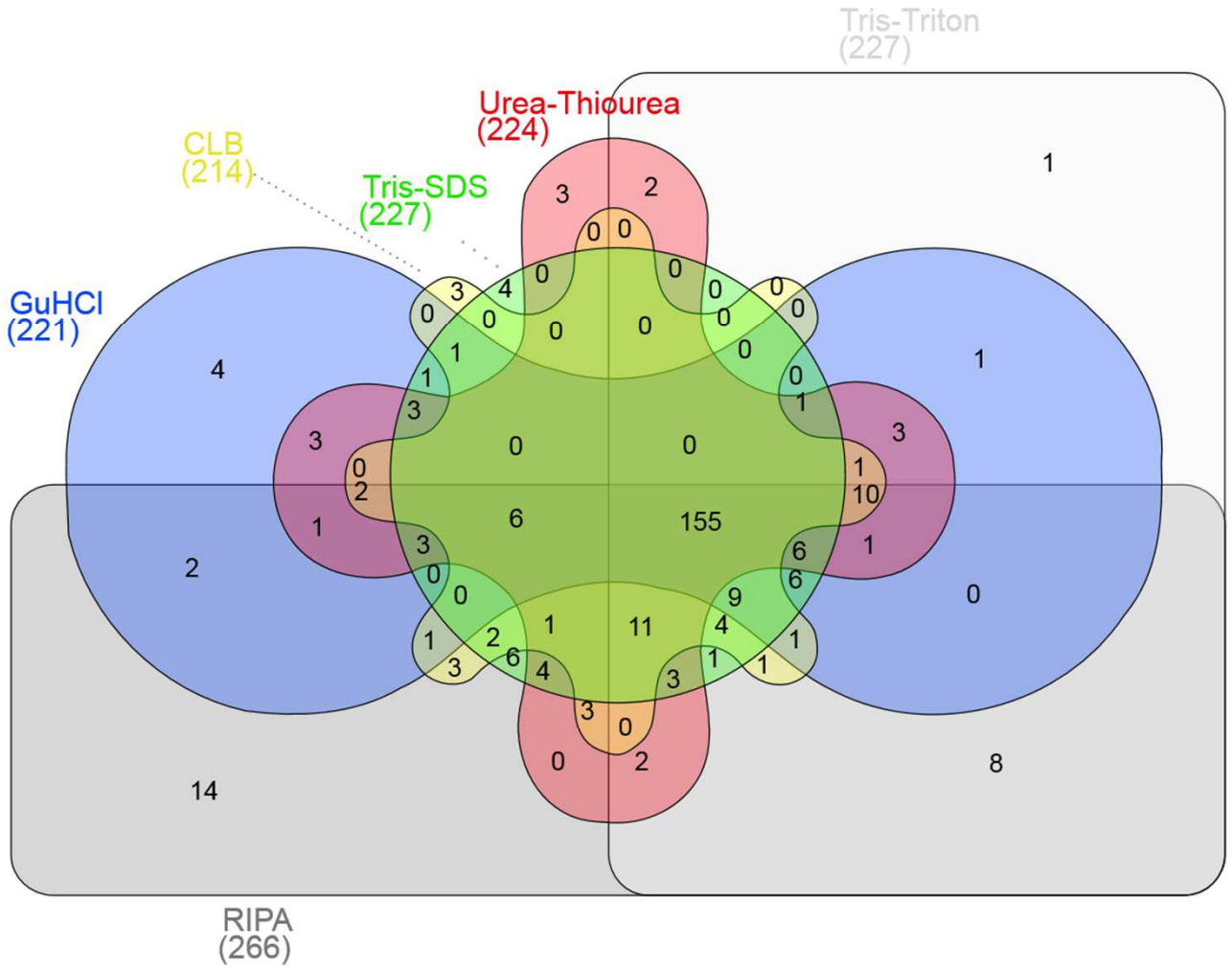

Figure 3. A Venn Diagram representing the proteins identified by the particular buffers. Only proteins identified in every replicate (n=3), marked by numbers inside brackets below the buffers, were considered. There were 155 proteins identified by all buffers combined whereas 14 proteins were identified exclusively by RIPA buffer, 4 each by GuHCl and Tris-SDS, 3 by CLB and urea-thiourea, and 1 protein was identified exclusively by Tris-Triton.

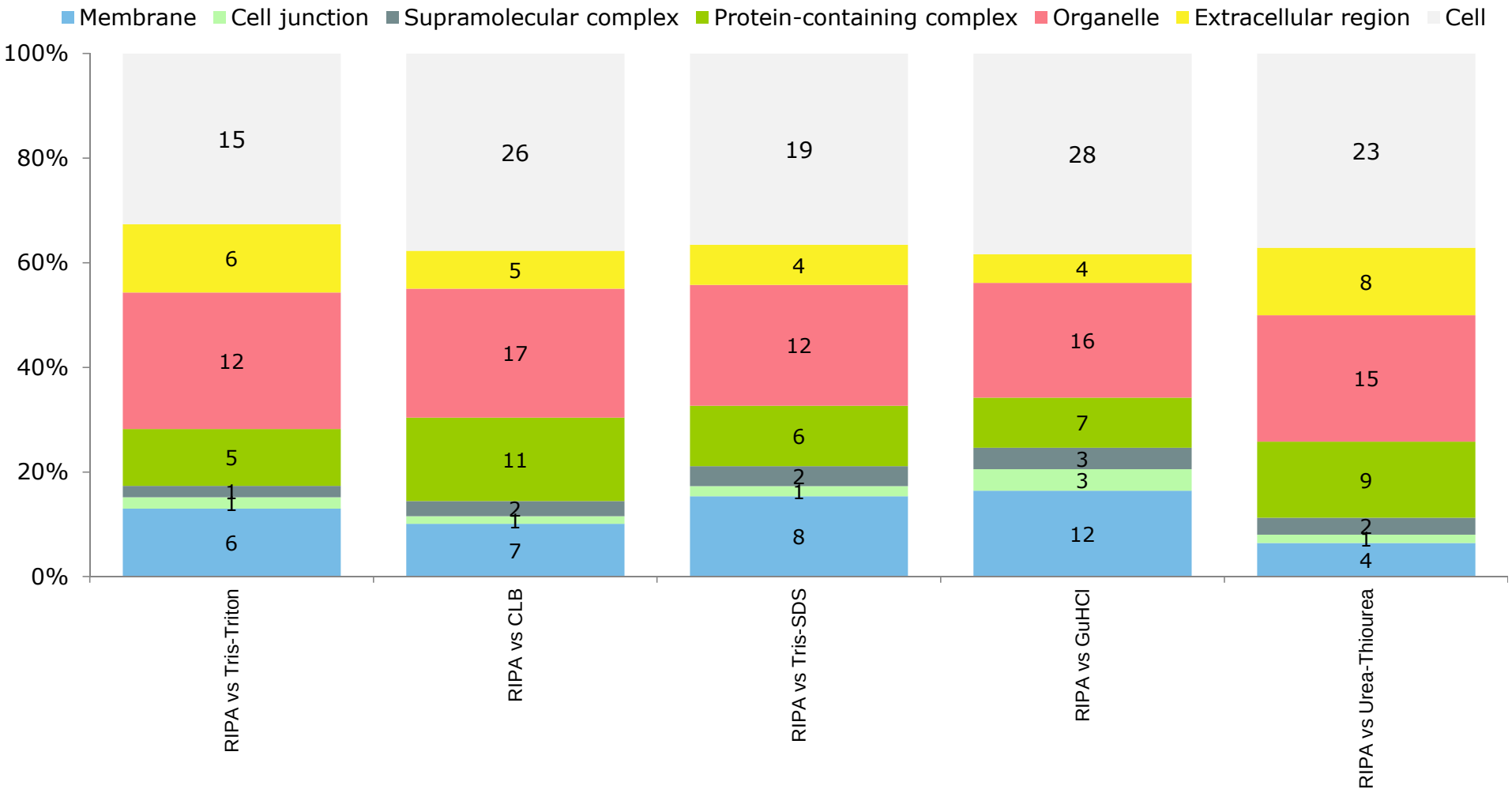

Figure 4. Gene ontology (GO) analysis (www.pantherdb.org) of proteins identified exclusively with RIPA compared against other buffers (Tris-Triton, CLB, Tris-SDS, GuHCl, and Urea-Thiourea). Only proteins identified in every replicate (n=3) were considered. RIPA identified 48 proteins exclusively compared to Tris-Triton, 57 compared to CLB, 49 compared to Tris-SDS, 63 compared to GuHCl, and 58 compared to urea-thiourea. The numbers inside the bar charts represent the 'component hits' of the identified proteins when 'Cellular Component' analysis was performed. Proteins belonging to cellular component: membrane (GO:0016020), cell junction (GO:0030054), supramolecular complex (GO:0099080), protein-containing complex (GO:0032991), organelle (GO:0043226), extracellular region (GO:0005576) and cell (GO:0005623) were identified.

- Six different protein extraction techniques for extraction of proteins from extracellular vesicles (EVs) were tested
- 399 proteins, including 74 of top EV-markers (Exocarta), were identified
- Most number of proteins (310 ± 6) were identified after the EVs were lysed with RIPA buffer.