



## Review

## Review: Detection and quantification of proteins in human urine

Sultan Aitekenov<sup>a</sup>, Abdzhappar Gaipov<sup>b</sup>, Rostislav Bukasov<sup>a,\*</sup><sup>a</sup> School of Sciences and Humanities, Department of Chemistry, Nazarbaev University, Nur-Sultan, Kazakhstan<sup>b</sup> School of Medicine, Department of Clinical Sciences, Nazarbaev University, Nur-Sultan, Kazakhstan

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## ABSTRACT

Extensive medical research showed that patients, with high protein concentration in urine, have various kinds of kidney diseases, referred to as proteinuria. Urinary protein biomarkers are useful for diagnosis of many health conditions – kidney and cardio vascular diseases, cancers, diabetes, infections. This review focuses on the instrumental quantification (electrophoresis, chromatography, immunoassays, mass spectrometry, fluorescence spectroscopy, the infrared spectroscopy, and Raman spectroscopy) of proteins (the most of all albumin) in human urine matrix. Different techniques provide unique information on what constituents of the urine are. Due to complex nature of urine, a separation step by electrophoresis or chromatography are often used for proteomics study of urine. Mass spectrometry is a powerful tool for the discovery and the analysis of biomarkers in urine, however, costs of the analysis are high, especially for quantitative analysis. Immunoassays, which often come with fluorescence detection, are major qualitative and quantitative tools in clinical analysis. While Infrared and Raman spectroscopies do not give extensive information about urine, they could become important tools for the routine clinical diagnostics of kidney problems, due to rapidness and low-cost. Thus, it is important to review all the applicable techniques and methods related to urine analysis. In this review, a brief overview of each technique's principle is introduced. Where applicable, research papers about protein determination in urine are summarized with the main figures of merits, such as the limit of detection, the detectable range, recovery and accuracy, when available.

## 1. Introduction

## 1.1. Urine composition

Urine is a readily available liquid for the medical diagnosis of patients. Urine tests are non-invasive procedures that involve no pain or discomfort for patients to determine problems with kidney function since extensive medical research showed that patients, with high protein concentration in urine, have various kinds of illnesses of the kidney, referred to as proteinuria [1]. Thus the precise and simple determination of urinary concentrations of total protein is important for diagnostic purposes. Since urine is a complex matrix, it poses challenges for the analytical determination of proteins and other constituents. Among the reasons that make urine challenging for researchers to analyze are: urine matrix is complex, it consists of various inorganic and organic compounds, from low-molar mass molecules to polymers; urine could contain cells, such as blood cells, or bacteria, which changes the composition of urine in time rapidly; an analytical method for diagnosis

of proteinuria should cover protein presence in urine in a wide range from 0.01 mg/ml to 10 mg/ml. Also, it is important to note that the concentration of protein in urine taken from patients can vary widely depending on dieting, exercising, and time of the day a patient urinated. It is widely accepted that analysis of urea taken throughout a day is the best representation of any illnesses in the human body if any. The so-called – the urine 24-h volume test, that measures the amount of urine the human body produces in a day. Another option is to determine creatinine to protein ratio in urea since creatinine to protein ratio is positively correlated with 24-h volume test for quantifying proteinuria [2,3]. The noninvasive collection of samples and wide range of diagnostic targets found in urine makes urinalysis well suited for point-of-care (PoC) monitoring applications, in which testing is done at the time and place of patient care [4]. Table 1 shows constituents in urine taken from the urine 24-h volume test.

In healthy individuals, Tamm–Horsfall protein (also known as uromodulin) is the most abundant protein in urine (50%), followed by albumin (20%) and immunoglobulin (5%) [6]. Other constituents in the

\* Corresponding author.

E-mail address: [rostislav.bukasov@nu.edu.kz](mailto:rostislav.bukasov@nu.edu.kz) (R. Bukasov).

**Table 1**  
Composition of urine (averages of selected components in 24-h collection test) [5].

Component	Average weight (mg)
Water	1,200,000.0
Urea	24,000.0
Creatinine	1335.0
Uric acid	505.0
Albumin	90.0

urine of either sick or healthy patients include, but not limited to: glucose; low abundant proteins, such as bilirubin, and urobilinogen; cells, such as Erythrocyte, Leukocyte, and other cells; bacteria [7].

### 1.2. Urine as a diagnostic tool

The urine of a healthy individual contains up to 150 mg of protein in total measured throughout a day, of which approximately 20 mg is albumin (human serum albumin) [1]. Albumin excretion of 30–300 mg a day, which is called microalbuminuria, is an early and sensitive marker of diabetic nephropathy [8], cardiovascular and renal disease [9]. The 15–30 mg/L albumin concentration is a critical value that could indicate kidney problems when it is repeatedly exceeded [10]. Though one of the main proteins in urine is albumin, there are thousands of other types of proteins. Furthermore, the removal of albumin from the urine helps to identify the low abundant proteins [11]. Marimuthu et al. by using high-resolution Fourier transform mass spectrometry were able to identify 1823 proteins in the urine of healthy subjects [12]. Using mass spectroscopy and sub-fractionating normal urine by successive steps (vesicle separation, CPLL, and solvent treatments) Santucci et al. were able to identify 3429 individual proteins [13]. All those discovered proteins could be potential biomarkers for diseases. In a review paper by Röthlisberger et al. it was stated that besides albumin, other proteins such as CD14, hh-FABP, BNP/NT-proBNP, NGAL, ORM1 are potential biomarkers of cardiovascular disease [14]. Another biomarker in human urine, Bence Jones protein (BJP), has an important diagnosis and prognosis value for multiple myeloma, cancer formed in a white blood cell called plasma cell [15]. Other types of chemical substances in urine can be important biomarkers as well, for example, urine microRNAs have the potential to be a valid marker for bladder cancer detection [16].

Proteinuria is the main clinical presentation of glomerular diseases (the glomerulus are complex capillary set that are located in the nephrons – renal cells) [17]. The glomerular diseases can be primarily when the disease caused by kidney diseases (such as glomerulonephritis), or secondary, when kidney glomerulus becomes target organ affected by different diseases such as diabetes and cardiovascular diseases, autoimmune and inflammatory disorders, amyloidosis and neoplasms, cancer and many other including genetic disorders [17,18]. The levels of proteinuria, which usually measures in clinical settings, may vary depending on severity of disease and glomerular injury, despite any cause of disease. Any detectable proteinuria in the urine between 30 and 300 mg/24h called albuminuria (or microalbuminuria), above the 300 mg/24h – proteinuria. Depending the amount of proteinuria they considered to named nephritic range proteinuria below 3.5 g/24h and nephrotic range proteinuria when protein loss excess the 3.5 g/24h (heavy proteinuria). The most of the cases, proteinuria caused by cardiovascular diseases limited with nephritic range proteinuria, whereas in renal diseases and cancer the proteinuria may reach nephrotic range proteinuria [19,20]. Since urine is in direct contact with kidneys, analysis of urine is important for diagnosis of renal diseases, such as chronic kidney disease. Several viral infections such as Epstein-Barr virus, hepatitis B and C viruses, herpes zoster, hantavirus, human immunodeficiency virus, dengue fever, COVID-19 and many others also may lead to proteinuria [21,22]. The potential mechanisms of

proteinuria are related to primary affect to glomerulus and/or secondary to autoimmune response to infection [23,24]. Chronic kidney disease (CKD) is a major public health problem. Albuminuria, urinary sediment abnormality and other markers of kidney damage are criteria of CKD, according to international guidelines [25]. Fassett et al. summarized biomarkers for CKD in urine – cystatin C,  $\beta$ -trace protein, NGAL, KIM-1, NAG and many others [26]. A more recent article on this topic is discussed in Ref. [27].

In the United States, it was estimated in 2003 that 11% of the adult population (aged 20 or older) has chronic kidney disease [28]. In a review paper about the prevalence of chronic kidney disease (CKD) by Zhang et al., the authors studied relevant data across America, Europe, Asia, Australia [29]. They reported that the median prevalence of CKD was 7.2% in persons aged 30 years or older. In persons aged 64 years or older prevalence of CKD varied from 23.4% to 35.8%. The authors concluded that worldwide, CKD is becoming a common disease in the general population. In a more recent paper, Wei et al. studied data on kidney damage among elderly people in Wuhan, China [30]. The age-standardized prevalence of kidney damage decreased renal function and proteinuria was 17.2, 13.5, and 5.3%. In the US, patients aged 66 or older have a mortality rate of 111.2 per 1000 due to CKD, compared to 45.2 per 100 due to non-CKD reasons as of 2014 [31]. It remains among the few growing causes of mortality which made CKD the 13th leading cause of death in 2013 [32].

Other renal diseases include diabetic nephropathy, autosomal dominant polycystic kidney disease, paediatric renal disease, acute kidney injury, and renal transplant rejection [33]. Besides renal diseases, urine can also be a useful source of information related to cancers and non-renal diseases. Theodorescu et al. found biomarkers in urine related to urothelial carcinoma, also called transitional cell carcinoma, by the means of capillary electrophoresis coupled with mass spectroscopy [34]. It is the most common type of bladder cancer. Bhasin et al. developed a biosensor device to detect bladder cancer marker DJ-1 in urine at concentration of 10 pM in 1 min [35]. Zhang et al. in their review summarized information about cancer biomarkers in urine [36]. In another work related to non-renal disease, Zimmerli et al. investigated urine samples of patients with coronary artery disease [37]. Rossing et al. identified potential biomarkers for diabetes [38]. Raja et al. investigated into the potential of albuminuria as a biomarker of diabetic complications [39]. Moreover, researchers could diagnose patients with viruses from their urine samples. Yang et al. developed an immunoassay cassette with a handheld reader for HIV urine testing in point-of-care diagnostics [40]. Robles et al. analyzed viruses present in urine from patients with interstitial cystitis [41]. Other viruses in urine described in literature include – Zika virus [42], hepatitis C [43], human papilloma virus [44]. Niedrig et al. wrote a review about usefulness of saliva and urine for the diagnosis of emerging viruses [45]. They concluded that it is important to perform an investigation using non-invasive approaches, such as urine can provide, for the diagnostic of emerging viral diseases.

### 1.3. Clinical urine tests

Urinalysis is an abbreviation for clinical urine tests for diagnostic purposes. A urinalysis (UA) is one of the most common methods of medical diagnosis. There are three basic components to urinalysis [46]:

- Gross/physical examination targets parameters that can be measured or quantified with the naked eye (or other senses), including volume, color, transparency, odor, and specific gravity.
- Microscopic examination. The numbers and types of cells and/or material such as urinary casts can yield a great detail of information and may suggest a specific diagnosis.
- Chemical examination of urine measures quantitatively and qualitatively for pH, blood, nitrite, protein, glucose, ketones, bilirubin, urobilinogen, ascorbic acid.

Conditions for storage of urine samples is an important consideration for chemical analysis, since undesired changes in unpreserved urine occur, such as a decrease of concentration of glucose due to consumption of it by cells or bacteria; a decrease of concentration of bilirubin due to photo-oxidation and etc [47]. The most common form of preservation of urine samples is refrigeration, also chemicals can be utilized too. Remer et al. suggest that high long-term stability and measurement validity for numerous clinical chemistry parameters stored at  $-22\text{ }^{\circ}\text{C}$  without the addition of preservative for human urine can be achieved [48]. Specifically for free-light chains monoclonal immunoglobulin, Pieri et al. suggest to store urine sample at  $+4\text{ }^{\circ}\text{C}$ , if analysis to be made within 72 h, otherwise for longer periods, the refrigeration should be done at  $-80\text{ }^{\circ}\text{C}$  [49]. Moyle et al. found that collection and storage of canine urine samples in clean homopolymer polypropylene (HP), propylene copolymer (PC), or glass containers at  $24\text{ }^{\circ}\text{C}$  for 4 h,  $4\text{ }^{\circ}\text{C}$  for 12 h or  $-20\text{ }^{\circ}\text{C}$  for 72 h is unlikely to result in clinically relevant decreases in measured protein to creatine values [50].

Historically, qualitative or semi-quantitative screening tests for urine protein relied on protein precipitation techniques. Proteins denature upon exposure to extremes of pH or temperature, and the most visible evidence of this is a decrease in solubility. In clinical laboratories, sulfosalicylic acid at room temperature may be used to detect urine protein [47]. This protein precipitation method detects all proteins—albumin and globulins. The analytical signal here is turbidity, which is the cloudiness or haziness of a fluid. This method can not detect protein below 0.05 mg/ml. Modern commercial reagent strips are available for routine protein screening use change in color due to acid-base reaction or formation of complexes between urine constituents and reagent strips material. Some of those reactions are described and compared in Ref. [51]: absorbance at 280 nm, which monitors tyrosine and tryptophan in protein (UV absorbance); the method of Lowry et al. based on complex formation between tyrosine, tryptophan or cysteine and heavy metals using Folin phenol reagent in the presence of copper ions under alkaline conditions (Lowry method) [52]; the Bradford method based on protein dye-binding using Coomassie brilliant blue (CBB method) [53]; the method of Smith et al. based on the formation of a purple complex between bicinchoninic acid and copper ions under alkaline conditions (BCA method) [54]; the method of Watanabe et al. based on protein-dye binding using pyrogallol redmolybdate reagent (PRM method) [55].

To date, different diagnostics methods in clinical settings can be used to ascertain urinary protein and albumin. Urine dipstick test, technique with acidic buffer precipitation and immuno-electrophoresis are commonly used diagnostic methods for urinary total protein quantification [56]. The protein-specific dipstick and immunochemical techniques are the fastest and cheapest way to determine proteins in the urine for diagnostic purposes. Modern automated urine analyzers are based on similar principles and are widely used in clinical routine tests [57]. A urine analyzer is a device used in the clinical setting to perform automatic urine testing. The units can detect and quantify a number of analytes including bilirubin, protein, glucose, and red blood cells. Many models contain urine strip readers, a type of reflectance photometer that can process several hundred strips per hour. Some analyzers can perform up to 240 tests an hour. Prices of urine analysis are relatively low, but they depend on a location/country and they are available from the most clinical laboratories. The high-performance liquid chromatography mostly used for scientific purpose due to its relatively high cost, though relatively inexpensive versions of mass spectrometers are now available [58].

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#### 1.4. Focus of this review

Several review papers are focused on urine proteomics. A review by Albalat et al. focuses on urinary proteins as potential biomarkers for mainly urine diseases, and capillary electrophoresis coupled mass spectroscopy as an instrumental method [33]. The review also gives information on biomarkers on non-renal diseases obtained from the urine. Kalantari et al. state that mass spectrometry as a detection technique is the most common [59]. The paper discusses technical aspects of urinary proteomics, proteomic technologies, and their advantage and disadvantages. As of 2015, several recent experiments are presented there which applied urinary proteome for biomarker discovery in renal diseases including diabetic nephropathy, immunoglobulin A (IgA) nephropathy, focal segmental glomerulosclerosis, lupus nephritis, membranous nephropathy, and acute kidney injury. Decramer et al. in their review paper focus on mass spectrometry-based urinary protein and peptide profiling [60]. The advantages and disadvantages of different mass spectroscopy methods are compared. Applications of urinary proteome analysis to urogenital and non-urogenital diseases are also discussed there.

This review, besides covering mass spectrometry, holds chapters discussing fluorescence spectroscopy, immunoassay, infrared, and Raman spectroscopy that was not given much attention compared from the reviews on urinalysis mentioned in the previous paragraph. This review also covers separation techniques – electrophoresis and chromatography. For each technique, a brief overview of the technique's principle is introduced. Where applicable, research papers about protein determination in urine are summarized with the main figures of merits, such as the limit of detection, the detectable range, recovery, and accuracy. Particularly, electrophoresis, chromatography, immunoassay, and fluorescence spectroscopy chapters are followed by summary tables with analytical parameters taken from individual research papers. It should be noted that not all analytical parameters are given in every paper, for example, sometimes recovery is unknown, and thus is not included in the following tables. Since the volumes used for all techniques are 100–1000 times smaller than a typical urine probe available from one patient for analysis, usually 50–100 mL, urine volume is not introduced in the tables. For instance for proteomics study, a typical sample size for high performance liquid chromatography (HPLC) ranges from few microliters to 0.1 mL [61]. A commercial immunoassay test kits developed by Valle et al. require 10  $\mu\text{L}$  of urine [62]. This review ends with a paragraph about urinary protein biomarkers. Most of those biomarkers were identified by mass spectrometry or immunoassay methods. A summary table of experimental papers on urinary protein biomarkers with related diseases or health conditions is given.

## 2. Separation techniques

### 2.1. Electrophoresis

Electrophoresis is an electrokinetic process, with a long history of development, which separates charged particles (macro-molecules, such as proteins) in a fluid using a field of electrical charge [63]. Particles could be separated based on their charge or their mass. The electrophoretic method is a widely used method in a modern research laboratory, but not so much in a clinical laboratory. Electrophoresis is commonly used for proteins, peptides and nucleic acid analysis [64]. It could be applied as a qualitative or quantitative analytical technique on its own if a reference substance is given, or it could be used as a separation technique for a sample to be further examined by other techniques, such as mass spectroscopy, fluorescence spectroscopy and etc.

Aguzzie et al. successfully used the electrophoretic technique to detect Bence-Jones protein (BJP) in human urine with the limit of detection LOD of 1 mg/L to BJP, and LOD of 3 mg/L to other types of protein [65]. These results were obtained by immunofixation using Dako antisera on cellulose nitrate followed by further staining with gold.

The authors claim that their technique does not require particular skill and is cheap. Such low levels of LOD allowed them to use unconcentrated urine samples, thereby eliminating the problem of the loss of low molecular weight proteins. Usually, though it is often necessary to concentrate the urine before electrophoresis because of the low urinary protein level [6]. This is a time-consuming process. Machii et al. used cellulose acetate membrane electrophoresis followed by colloidal silver staining on the urine of healthy subjects with success [6]. However, when they used silver staining on an agarose gel, urinary protein in healthy subjects was not detected. They estimated that urine has to be concentrated 10 times, furthermore, the pattern of the urinary fraction will change after the concentration procedure is conducted. Giovannoli et al. developed a noncompetitive capillary electrophoresis immunoassay with laser-induced fluorescence detection was used to determine human serum albumin (HSA) in buffer and urine [66]. The injection of an excess of labeled antibody off-line incubated with the analyte allows the surface capture of the free antibody and consequently the immunocomplex detection. In buffer, authors were able to achieve the limit of detection LOD of 0.60 mg/L, with the quantitative range up to 6.6 mg/L. Recovery in the urine matrix was dependent upon sample dilution and HSA added and was as low as  $91 \pm 8\%$ .

One of the variations of the electrophoretic method is called gel electrophoresis, where a gel is used as an anticonvective medium. Particularly polyacrylamide gel PAGE is used for separating proteins due to the uniform pore size provided by the polyacrylamide. Jia et al. used PAGE gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) to show a good correlation ( $R^2 = 0.825$ ) with the pyrogallol red-molybdate (PRM) assay technique used in clinical laboratories for urine samples [67]. Sodium dodecyl sulfate (SDS) coats proteins with a negative charge so that proteins are separated due to their size, not charge. The authors estimated that their method can detect protein as low as 5 mg/L. Tran et al. showed that the use of tube gel electrophoresis for protein separation was further expanded with the invention of gel-eluted liquid

fraction entrapment electrophoresis (GELFrEE) [68]; shown in Fig. 1.

Bessonova et al. in their review paper compared capillary electrophoresis with five on-line preconcentration techniques, including field-amplified sample stacking (FASS), head-column field-amplified sample stacking (HC-FASS), stacking with a polymer solution, dynamic pH junction and large-volume sample stacking (LVSS) with reversed polarity for various analytes including protein in the urine [69]. The use of these preconcentration techniques makes it possible to increase sensitivity in the determination of analytes by a factor of 100 or higher. In another work by Bessonova et al., they found that the lowest LOD was obtained by the LVSS approach, and it was 15 mg/L with UV-detection [70].

Electrophoresis is often used to be followed by another method, such as mass spectroscopy. Wittke et al. used the on-line coupling of capillary electrophoresis (CE) with electrospray mass spectroscopy to distinguish the difference in polypeptide patterns in the urine of healthy individuals and patients with kidney disease [71]. Kiprijanovska et al. used two dimensional gel electrophoresis coupled with mass spectroscopy (2D PAGE – MS) for mapping and identification purposes of the urine proteome [72]. For the same purposes, Nakayama et al. used cellulose acetate membrane electrophoresis coupled with liquid chromatography mass spectroscopy LC-MS [73].

## 2.2. Chromatography

Another widely used separation technique in chemistry is chromatography. In chromatography, a sample is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate due to differential affinities (strength of adhesion, as an example) of the various components of the analyte towards the stationary and mobile phase results. Then resulting constituents can be further analyzed by various

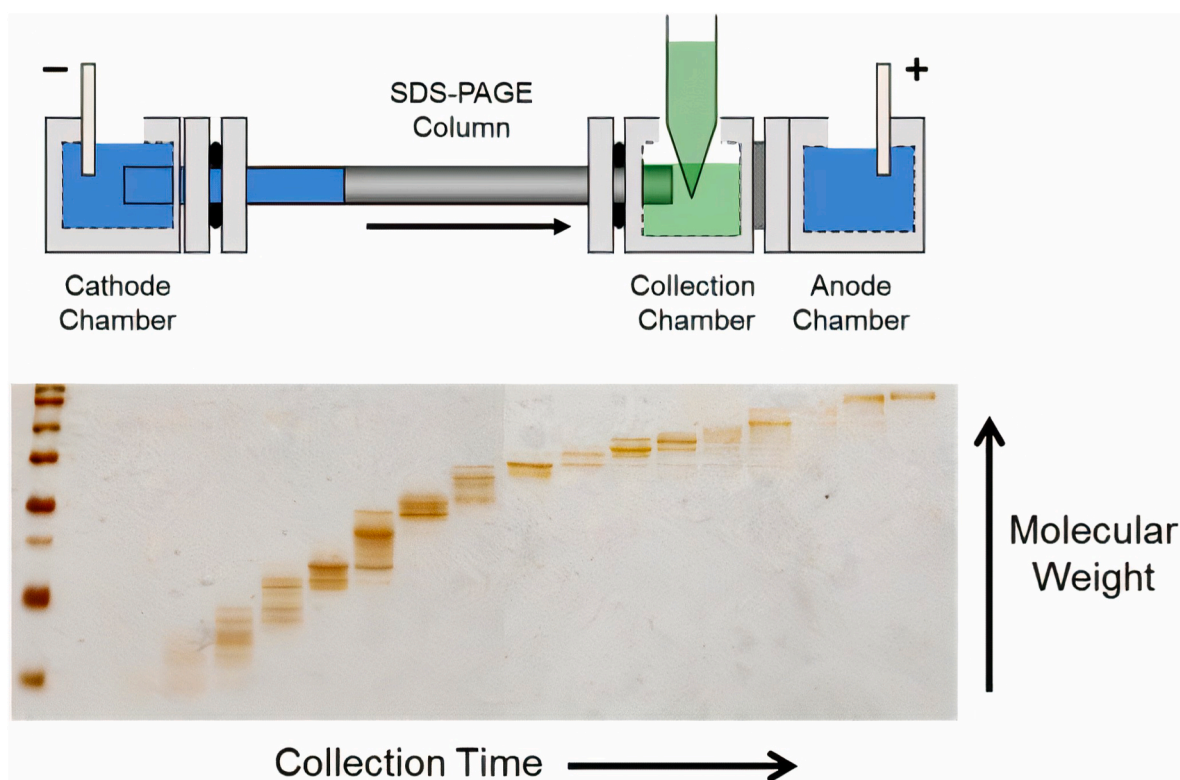


Fig. 1. Diagram of the GELFrEE device [68]. A gel column is utilized to achieve electrophoretic separation of proteins, analogous to SDS-PAGE, which are then eluted into the liquid-phase for manual collection. The fractionation can then be visualized by running a portion of the fractions on a SDS-PAGE gel. Reprinted from Refs. [95].

techniques, such as mass spectroscopy, UV-spectroscopy, fluorescence detection, etc. Depending on the mobile phase methods in chromatography could be classified as gas-chromatography (GC) [74], and liquid-chromatography (LC) [75]. Typically, proteomic sample size ranges from few microliters to 0.1 mL [61].

Ishida et al. quantitatively determined human serum albumin (HSA) in human plasma and urine by utilizing post-column high-performance liquid chromatography (HPLC) with fluorescence detection [76]. Particularly, they used 8-anilino-1-naphthalenesulfonic acid as a fluorophore to bind HSA. The sample size was 1 ml for urine, and the retention time was around 20 min. A linear relationship was observed between fluorescence peak and the amounts of HSA in both plasma and urine, as for the latter matrix, a linear relationship was observed up to 400 mg/L, with the correlation coefficient being 0.998. The LOD for HSA was 0.2 mg/L, while the recovery was  $94.5 \pm 3.8\%$ . The same authors also compared their method with the immunological nephelometric method and obtained the correlation coefficient of 0.990 [77]. Often researchers compare their method with the immunoassay technique that is used in clinical practice. Brinkman et al. in their experiments using the immunonephelometric approach and HPLC determined that HPLC reveals higher values of HSA, especially in the lower concentration range, resulting in a higher prevalence of microalbuminuria [78]. Nephelometry only measures immunoreactive albumin, whereas HPLC measures both immunoreactive and immunoreactive intact albumin, as was found by Comper et al. with the HPLC method [79]. The authors also compared HPLC with radioimmunoassay (RIA), immunonephelometry (IN), and two different methods of immunoturbidimetry (IT).

Owen et al. used a new urinary albumin assay that uses size-exclusion high-performance liquid chromatography with UV-detection [80]. The limit of detection (LOD) of the assay based on a signal/noise ratio of 5 was 3.4 mg/L. The linearity of the HPLC assay was tested in a range of albumin concentrations from 4.3 to 240 mg/L. The maximum deviation from a mean recovery of 100% was 4.6% at a concentration of 66 mg/L. Total imprecision was less than 10% from 16 to 206 mg/L. They also conducted experiments to compare their approach with immunoturbidimetric (ITA) assay, which showed positive proportional bias, which decreased with increasing concentrations of albumin. Singh et al. used liquid chromatography with quadrupole time-of-flight mass spectrometry (Q-TOF, LC-MS) method for the quantification of urinary albumin using a  $^{15}\text{N}$ -isotopically labeled bovine serum albumin as an internal standard in human urine matrix [81]. The limit of detection (LOD) was 4.84 mg/L, while the limit of quantification was 10.5 mg/L. Multiple calibration curves ranging from 4 to 625 mg/L were linear and were reproducible with the coefficient of determination ( $R^2$ ) of 0.999.

Micellar electrokinetic capillary chromatography (MEKC) is a hybrid method that combines chromatographic and electrophoretic separation principles, extends the applicability of capillary electrophoretic methods to neutral analytes [82]. In micellar electrokinetic capillary chromatography, surfactants are added to the buffer solution in a concentration above their critical micellar concentrations, consequently, micelles are formed; micelles that undergo electrophoretic migration like any other charged particle. Glavac et al. utilized MEKC combined with UV-detection of urine samples of patients diagnosed with proteinuria without any sample pre-treatment before analysis [83]. They were able to simultaneously identify albumin (HSA), haemoglobin (HGB), myoglobin (MYO). For all three analytes, linear correlations were obtained with the correlation coefficient ( $R^2$ ) being not less than 0.99. Particularly, for HSA, the limit of detection (LOD) was 0.115 mg/L, the limit of quantification (LOQ) was 0.383 mg/L, a linear range between 1 and 300 mg/L and the recovery was  $101.9 \pm 4.5\%$ . In another work by Wu et al., a MEKC method combining field-amplified sample injection (FASI) with UV-detection has been developed for the analysis of albumin (HSA) and transferrin (TRF) in human urine [84]. Field-amplified stacking or injection is an on-column (on-line) preconcentration technique which is based on the different conductivity between two solution

plugs. For TRF, the authors obtained the following analytical parameters of the method: LOD 0.31 mg/L, linear range 1.00–100 mg/L with  $R^2$  0.994. For HSA: LOD 0.14 mg/L, linear range 0.50–100 mg/L with  $R^2$  0.999. The recoveries of TRF and HSA were 91.1–101.7%.

Jaffuel et al. performed Aquaporin-2 (AQP2) nephrotoxicity biomarker measurement by using liquid chromatography–multiple reaction monitoring cubed mass spectrometry assay (MRM mode in LC-MS/MS) [85]. AQPs regulate numerous downstream effector signaling molecules that promote cancer development and progression [86]. In numerous cancer types, AQP expression has shown a correlation with tumor stage and prognosis. Linearity is observed within the concentration range  $0.5 \cdot 10^{-3}$ – $50 \cdot 10^{-3}$  mg/L, intra and inter-assay precision ranged from 9 to 35% at the lower limit of quantification (LLOQ), and accuracy from 94 to 114%.

Overall, due to complex composition of biofluids, including the urine, liquid chromatography in particular HPLC, which is frequently coupled with MS for detection, remains a common method in bio-analytical laboratories to separate complex mixtures. A review by Novakova et al. states that in order to decrease time for an analysis without compromising resolution and separation efficiency, three main approaches in HPLC exist – the use of monolith columns, LC at high pressures and temperatures, and HPLC at ultra-high pressures [87]. By employing those approaches, time for analysis of biofluids could be done within few minutes up to half an hour. Table 2 below shows a summary table of quantitative methods for application of electrophoresis and chromatography for protein determination in the human urine matrix.

### 3. Detection techniques

#### 3.1. Mass spectrometry

Mass spectrometry is a powerful analytical technique that measures the mass-to-charge ratio of ions. Firstly, molecules in a sample must be ionized, which causes a large molecule to fragment into smaller ions; then those ions are separated by a magnetic field depending on their mass to charge ratio. The results are typically presented as a mass spectrum, a plot of intensity as a function of the mass-to-charge ratio. Usually, mass spectrometry is utilized after pre-treatment of a sample by electrophoresis or chromatography.

Mass spectrometry is a widely used technique for proteomics studies. The proteome is the entire set of proteins that is produced or modified by an organism. Research on urine proteomics is important for the identification purposes of reliable biomarkers that help the diagnosis of disease [90]. In a review by Kalantari et al. on urine proteomics is stated that common techniques for urinary proteome analysis are two-dimensional gel electrophoresis followed by mass spectrometry (2DE-MS), liquid chromatography coupled to mass spectrometry (LC-MS), surface-enhanced laser desorption/ionization coupled to mass spectrometry (SELDI-TOF), and capillary electrophoresis coupled to mass spectrometry (CE-MS) [59]. By using mass spectroscopy protein constituents of urine were identified. Also reviews by Albalat et al. and Decramer et al. focus on mass spectrometry-based urinary protein and peptide profiling [33,60]. The advantages and disadvantages of different mass spectrometry methods are compared. As was mentioned in the introductory part of this review, Marimuthu et al. by using high-resolution Fourier transform mass spectrometry were able to identify 1823 proteins in the urine of healthy subjects [12]. Santucci et al. by using mass spectrometry and sub-fractionating normal urine by successive steps (vesicle separation, CPLL, and solvent treatments) were able to identify 3429 individual proteins [13].

Two ionization techniques in mass spectrometry are commonly used for protein studies: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) [91]. In the ESI approach, ions are produced in an electrospray in which a high voltage is applied to a liquid to create an aerosol. ESI is different from other ionization

**Table 2**

Summary table of quantitative methods for application of electrophoresis and chromatography for protein determination in the human urine matrix (unless otherwise stated in the method section). CV (coefficient of variation) parameters are taken from maximum readings, for Recovery – the range is given.

Method	Analyte	The limit of detection, LOD	Range	Analytical parameters	Experimental parameters	Reference
Electrophoretic with immunofixation	BJP Bence Jones Proteins	1 mg/L	NA	NA	Migration time 33 min and 43 min depending on buffer Voltage 220V	4 Aguzzi et al., 1993 [65]
Electrophoretic with immunofixation	Transferrin, retinol binding protein, b2-microglobulin	3 mg/L (for all)	NA	NA	Migration time 33 min and 43 min depending on buffer Voltage 220V	5 Aguzzi et al., 1993 [65]
HPLC high performance liquid chromatography with fluorescence detection	HSA	0.2 mg/L	0.2–400 mg/L (linear, $R^2 = 0.998$ )	Recovery $94.5 \pm 3.8\%$	Retention time for HSA 16.0 min Fluorescence enhancement factor 1286 TSK gel G2000 SWXL column	6 Ishida et al., 1996 [76]
HPLC with UV-detection	HSA	3.4 mg/L	4.3–240 mg/L (linear)	Recovery $100 \pm 4.6\%$ at 66 mg/L	Zorbax Bio Series GF-250 column	7 Owen et al., 2005 [80]
CZE capillary zone electrophoresis with preconcentration techniques	HSA	15 mg/L	15–1000 mg/L (linear, $R^2 = 0.9840$ )	Sensitivity enhancement factor by large volume sample stacking (LVSS) 67	Voltage 15/25 kV	8 Bessonova et al., 2007 [70]
Capillary electrophoresis immunoassay with fluorescence detection (the buffer matrix)	HSA	0.6 mg/L	0.6–6.67 mg/L (detectable)	Recovery 91–160%	Voltage 12–15 kV	9 Giovannoli et al., 2007 [66]
Q-TOF, LC-MS using a N-15 isotope	HSA	4.84 mg/L	4–625 mg/L (linear, $R^2 = 0.999$ )	LOQ 10.5 mg/L CV intra assay 12.6% CV inter assay 12.2%	Cone voltage for MS 40–80 V	10 Singh et al., 2007 [81]
FASI-MEKC field amplified sample injection MEKC	HSA	0.14 mg/L	0.50–100 mg/L (linear, $R^2 = 0.999$ )	Recovery 91.1–101.7%	Retention time for HSA around 10–12 min Voltage for MEKC 20 kV Sample injection voltage 10 kV	11 Wu et al., 2009 [84]
FASI-MEKC	TRF transferrin	0.31 mg/L	1.00–100 mg/L (linear, $R^2 = 0.994$ )	Recovery 91.1–101.7%	Retention time for TRF around 10–12 min Voltage for MEKC 20 kV Sample injection voltage 10 kV	12 Wu et al., 2009 [84]
LC-MS/MS (MRM mode)	AQP2 aquaporin-2	$5 \cdot 10^{-4}$ mg/L	$5 \cdot 10^{-4}$ – $5 \cdot 10^{-2}$ mg/L (linear)	Accuracy from 94 to 114% CV intra assay 9% CV inter assay 35%	Retention time around 5 min a Symmetry™ C <sub>18</sub> column	13 Jaffuel et al., 2013 [85]
Reversed-phase HPLC with fluorescence detection	Tamm–Horsfall protein	0.35 mg/L	4.5–90.0 mg/L (linear, $R^2 = 0.999$ )	Recovery 100.0–104.2% CV intraday 1.73–2.77% CV interday 2.50–5.35% CV 21.5%	COSMOSIL 5C <sub>18</sub> -MS-II Column	14 Akimoto et al., 2015 [88]
SDS-PAGE sodium dodecyl sulfate–polyacrylamide gel electrophoresis	HSA	5 mg/L	NA	CV 21.5%	Electrophoresis time 5 min Staining time 15 min	15 Jia et al., 2019 [67]
Paper-based analytical device - ion concentration polarization PAD-ICP	HSA	10 mg/L	50–350 mg/L (linear, $R^2 = 0.994$ ) 10–500 mg/L (detectable)	Recovery 93–108% CV 11%		16 Gao et al., 2019 [89]

processes (e.g. MALDI) since it may produce multiple-charged ions, effectively extending the mass range of the analyzer to accommodate the kDa-MDa orders of the magnitude observed in proteins and their associated polypeptide fragments [92]. In MALDI, a sample is dispersed in a certain solid matrix (e.g. aromatic compounds), which then irradiated by the laser that causes electronic excitation of molecules in the sample matrix [93]. Both methods ESI and MALDI produce relatively high molecular ions due to mild fragmentation of those approaches. It should be noted a specific method of MS applied to biomolecules, which is called tandem mass spectrometry, also known as MS/MS. In MS/MS two or more mass analyzers are coupled together using an additional reaction step to increase their abilities to analyze samples. In general, protein identification in mass spectroscopy is performed using either bottom-up or top-down approaches. Bottom-up protein identification methods rely on enzymatic digestion to break down proteins into smaller peptides that are easier to ionize and fragment, resulting in higher sequence coverage [94]. However, in top-down methods, intact proteins are injected into the mass spectrometer and subjected to fragmentation without pre-treatment [95]. Aside from better tracking of protein modifications, an advantage of top-down protein sequencing is that it complements imaging experiments by enabling mass measurement of the intact protein that relates more directly to the MALDI IMS

generated signals [96].

Analysis of a mass-spectrum, a plot of intensity as a function of the mass-to-charge ratio, could be a challenge itself, especially for big molecules like proteins, since they can be divided in many fragments resulting in a complex spectrum. Usually, the mass-spectrum is compared by software with the existing database. Several computationally feasible approaches have emerged for performing protein inference from such data [97].

Mass spectrometry in itself does not allow for quantitative analysis due to the different physicochemical properties of different peptides and proteins [98–100]. However quantification in MS is possible if an internal standard is used, for such there are approaches based on labeling with stable isotopes (such as N-15, O-18), involving artificial labeling of peptides and proteins. On the other hand, there are label-free approaches, in which the samples retain their native isotope composition and are compared between separate measurements. In general, they could be classified into two categories: methods that involve comparing peptide signals at the level of LC-MS analysis; methods that involve counting the number of identified peptides or acquired fragment spectra. Also, it should be noted that label-based methods are relatively laborious while label-free methods are less accurate [98]. An example of quantitative protein determination, in this case – albumin, in the urine

matrix by MS methods is described in a paper by Singh et al. [81]. They used N-15 isotopically labeled albumin as an internal standard. The authors achieved the limit of detection (LOD) of 4.84 mg/L, the limit of quantification of 10.5 mg/L with the linear range being from 4 to 625 mg/L,  $R^2 = 0.999$ . Chen et al. used isotope-labeled peptides as internal standards for quantification of 63 proteins in human urine by multiple reaction monitoring-based mass spectrometry for the discovery of potential bladder cancer biomarkers [101]. Bachmann et al. experimentally compared routine measurements of urinary albumin to isotope dilution tandem mass spectrometry (IDMS) [102]. They reported that median differences between the largest positive and negative biases versus IDMS were 45%, 37%, and 42% in the concentration intervals of 12–30 mg/L, 31–200 mg/L, and 201–1064 mg/L, respectively. Lieske et al. reported their current status on a developing reference method for quantification of urinary albumin by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [103]. They used three different labeled peptides as internal standards to cover the range of clinical values (5–1270 mg/L). Recovery was between 97% and 108% with an average of 103%. In conclusion, quantitative mass spectroscopy assays for peptides and proteins are difficult to implement, and the future will see standardization of MS validation and quality control requirements.

Overall, mass spectrometry coupled with liquid chromatography is a powerful tool for the discovery and the analysis of biomarkers of diseases from biofluid samples, which includes proteomics study of urine as well [104]. Many of urinary proteome biomarkers were discovered by this technique. However, quantitative protein determination represents a challenge by following this approach [105]. Thus, mass spectroscopy could be used for biomarker discovery but not for clinical applications [60]. Another consideration for an analytical technique is the cost of analysis. Practical usage of LC-MS is limited by factors such as instrument size, cost, ease of operation, though, low cost compact mass spectroscopy detectors coupled with cinematographic separation are being developed, their costs equal to around \$50000 [58].

### 3.2. Immunoassay

Immunoassay is a widely used technique in clinical and research laboratories to determine qualitatively and quantitatively macromolecules (usually proteins) generated from organisms [106]. Immunoassay is based on a reaction between an antigen and an antibody, which is found in the adaptive immune system of vertebrates, that includes humans [107]. The reaction is carried out in-vitro (not in a living organism), and the result of it is the formation of a complex antibody-antigen formed by spatial complementary, like lock and key, thus such interactions are highly selective. Antibody-antigen complexes are bounded by weak forces such as electrostatic forces, hydrogen bonds, hydrophobic interactions, and van der Waals forces; which means that such bindings are reversible. The specific piece of the antigen to which an antibody binds is called the epitope. Usually, in analytical papers, the analyte is the antigen while the reagent is the antibody. Immunoassay methods have been widely used in many areas of pharmaceutical analysis such as diagnosis of diseases, therapeutic drug monitoring, clinical pharmacokinetics, and bioequivalence studies in drug discovery and pharmaceutical industries [108]. Conventional immuno-chemical based urinary albumin assays for diabetic patients as well as nondiabetic patients with renal disease, including those who may suffer hypertension and cardiovascular disease, now amount to 100 million assays per year worldwide as of 2004 [79]. The widespread immunoassay methods in the pharmaceutical analysis is attributed to their inherent specificity, high-throughput, and high sensitivity for the analysis of a wide range of analytes in biological samples as well the relatively low cost of the instruments, tools, or the reagents [109].

Based on signal detection immunoassays could be classified as follows:

Immunoassay experiments could be classified into types – competitive and non-competitive [106,109]. The competitive immunoassay

relies on the competition between the antigen of interest (the analyte) and a constant amount of a similar but labeled antigen for a limited amount of specific antibody. When these immunoanalytical reagents are mixed and incubated, the analyte is bound to the antibody-forming an immune complex. This complex is separated from the unbound reagent fraction by physical or chemical separation technique, though sometimes separation is not required. Based on whether the separation step is or is not required, immunoassay methods can be classified into a heterogeneous or a homogeneous assay, respectively. The analysis is achieved by measuring the label activity in either of the bound or free fraction. On the other hand, the non-competitive assay, also called immunometric, uses an excess of labeled specific antibody toward the analyte of interest. It requires two antibodies that bind to non-overlapping epitopes on the analyte molecule. One of the two antibodies is bound to the solid phase, and the second one is labeled and used for detection. Labels commonly used in immunoassays include radioisotopes, fluorophores, and enzymes. For both types, the schematic diagrams are given below (for the competitive – Fig. 2, for the non-competitive – Fig. 3), reprinted from Ref. [109].

- Radioimmunoassay RIA. Detection is based on radioactive labeling of antigen with unstable isotopes such as I-125, C-14, H-3.
- Enzyme immunoassay EIA with sub-categories: ELISA – Enzyme-linked immunosorbent assay; EMIT – Enzyme-multiplied immunoassay technique; MEIA – Microparticle enzyme immunoassay.
- Fluoroimmunoassay FIA
- Chemiluminescence immunoassay CLIA
- Immunonephelometry IN. Detection is based on an antigen-antibody complex scattering light.
- Immunoturbidimetry IT. Detection is based on an antigen-antibody complex blocking light thus increasing the turbidity of the sample.

As it was mentioned in the introductory part, the concentration of albumin higher than 2 mg/L could be indicative of potential kidney diseases [10]. Subsequently for many immunoassays methods for albumin determination in urine the limit of detection (LOD) of magnitude around 1 mg/L would be enough for clinical purposes, thus priority should be given to the parameters such as specificity, accuracy, costs, etc. However for low abundant proteins achieving lower LOD is important [110]. In 1980–1990 common immunoassays for urinary proteins for clinical purposes were developed. Comper et al. compared four immunoassays (RIA, IN, two IT) and high-performance liquid chromatography (HPLC) [79]. The correlation coefficients calculated were high (>0.85) which is indicative of a strong linear relationship between all assays studied. While the correlation was high, the slopes of the regression line were significantly different from 1, it means that certain methods tend to underestimate or overestimate. For example, the authors found that the Beckman immunonephelometry assay consistently gives values approximately threefold lower than the Dade-Behring immunoturbidimetry assay. There have been numerous other studies that have found similar levels of variation between different immunoassays and other techniques, such as in the paper by Shaikh et al. [111]. The authors claim that their LC-MS and HPLC assays both performed poorly at concentrations below 20 mg/L, while immunoturbidimetric assay underestimated albumin compared with LC-MS. Hoofnagle et al. state that immunoassays have four common problems of which a researcher should be aware [112]. Assay performed with one set of reagents does not measure the same concentration as another which results in patients having measurements performed on the same platform for continuity of care. The other three problems results in falsely low or high results depending on the analyte concentration, antibodies used. While immunoassays methods have their shortcomings they are still widely used in clinical practice. Modern immunoassays are actively being developed and some of their analytical parameters are given in the summary Table 3.

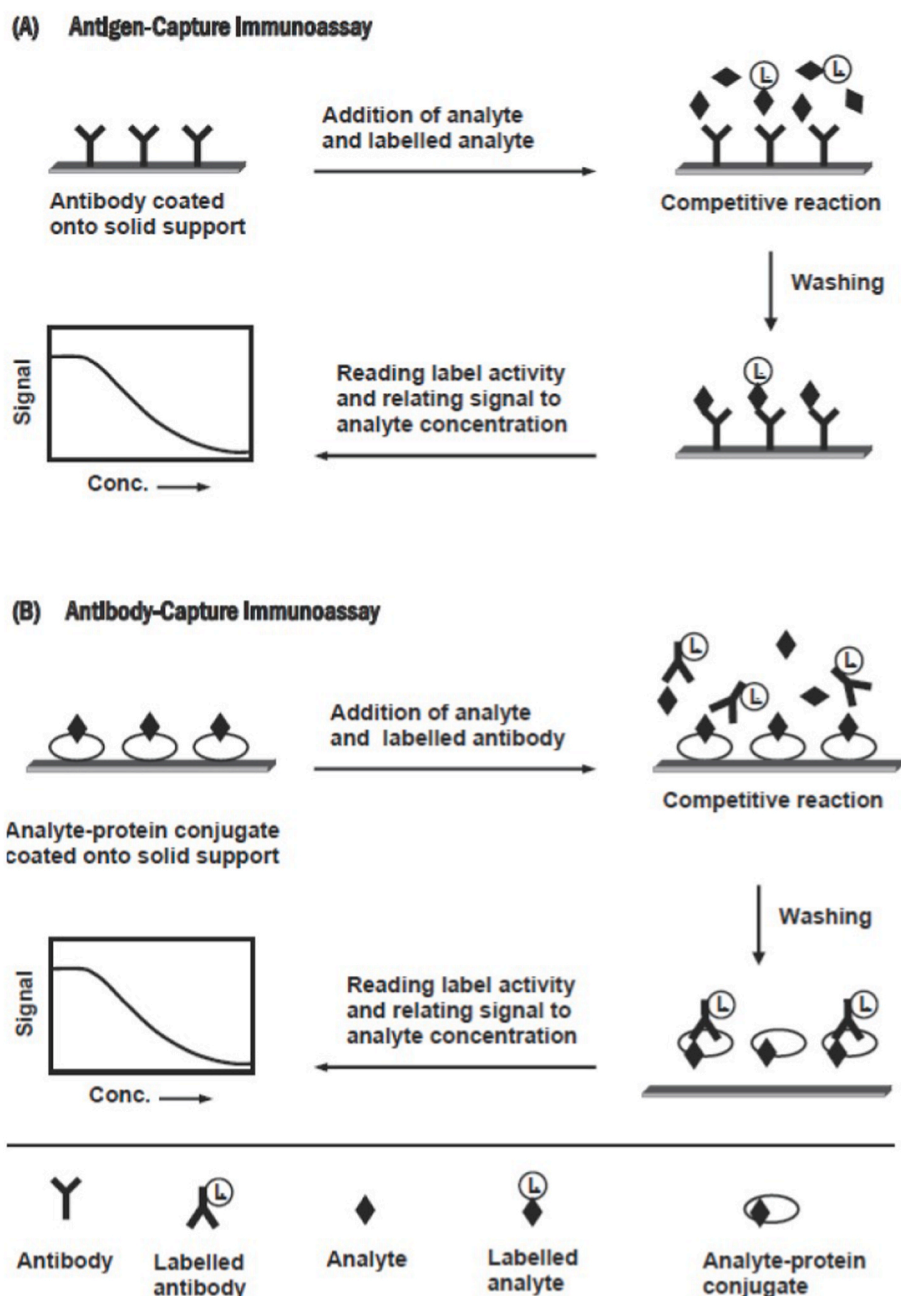


Fig. 2. Schematic diagram for the competitive immunoassays. Reprinted from Ref. [109].

### 3.3. Fluorescence spectroscopy

Fluorescence is a luminescence phenomenon in which a compound emits light after absorption of electromagnetic irradiation [133]. In most cases, the emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation. Often in chemical analysis, a sample is irradiated in UV or visible range, and as a result, fluorescence occurs in the visible and near-infrared region respectively. Fluorescent materials cease to glow nearly immediately when the radiation source stops, unlike phosphorescent materials, which continue to emit light for some time after. Fluorescence spectroscopy is one of the most common techniques with applications in geology, chemistry, medicine, and astronomy [133]. Because of the sensitivity that the method affords, fluorescent molecule concentrations as low as 1 part per trillion can be measured [134]. Fluorescence spectroscopy can be used on its own, and also it is often used in conjunction with high-performance liquid

chromatography as a detector. Often fluorescence detection is used in immunoassays to either label antigen or antibody.

Usually, analyte molecules do not emit strong luminescence for their analytical detection. Hence an analyte usually modified with a fluorophore, or a fluorescent probe, in other words. The fluorophore (or a fluorescent probe) is a fluorescent chemical compound that can re-emit light upon light excitation. A suitable fluorescent probe should have the following characteristics: (i) is conveniently excitable, without simultaneous excitation of the biological matrix, and detectable with conventional instrumentation; (ii) is bright, that is, possesses a high molar absorption coefficient at the excitation wavelength and a high fluorescence quantum yield, (iii) is soluble in relevant buffers, cell culture media or body fluids, (iv) is sufficiently stable under relevant conditions, (v) has functional groups for site-specific labeling, (vi) has reported data about its photophysics, and (vi) is available in reproducible quality [135]. Most common fluorophores are usually organic molecules, that



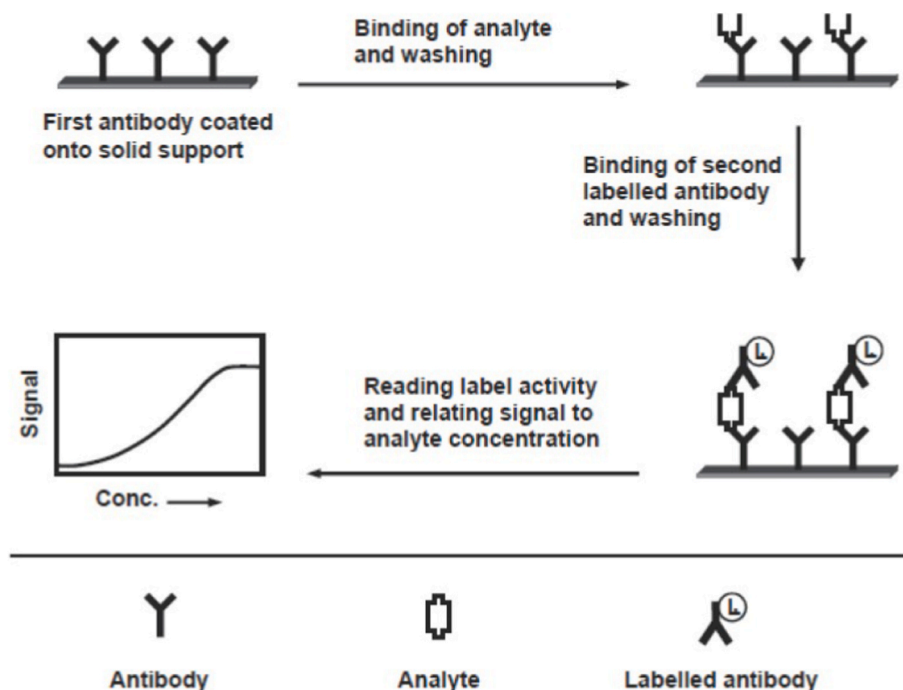


Fig. 3. Schematic diagram for the non-competitive immunoassay. Reprinted from Ref. [109].

typically contain several combined aromatic groups or planar or cyclic molecules with several  $\pi$  bonds. Fluorophores are sometimes used alone, as a tracer in fluids, as a dye for staining of certain structures, as a substrate of enzymes, or as a probe or indicator (when its fluorescence is affected by environmental aspects such as polarity or ions). More generally they are covalently bonded to a macromolecule, serving as a marker (or dye, or tag, or reporter) for affine or bioactive reagents (antibodies, peptides, nucleic acids). Fluorophores are notably used to stain tissues, cells, or materials in a variety of analytical methods, i.e., fluorescent imaging and spectroscopy. Some examples of widely used fluorophores are fluorescein, and by its amine reactive isothiocyanate derivative fluorescein isothiocyanate, methylene blue. Over the last two decades, the role of semiconductor quantum dots (QD) and other types of nanoparticles have grown considerably in bionalysis and bioimaging. Petryayeva et al. conclude that QDs as fluorophores and for other bioimaging purposes offer nontrivial advantages, such as the brightness needed for sensitive detection, the photostability needed for tracking dynamic processes, the multiplexing capability needed to elucidate complex systems or the nanoscale interface needed for biomolecular engineering of novel probes and biosensors [136]. The fluorescent labeling in immunoassays is the common practice (see page 20).

A method of fluorescence, where two light-sensitive molecules, that transfer energy between them, is called fluorescence resonance energy transfer. Fluorescence resonance energy transfer (FRET) involves the transfer of energy from a fluorescent donor molecule via a nonradiative dipole-dipole interaction to an acceptor molecule. The emission of the energy transfer-excited acceptor can be distinguished from donor emission by the use of a longer-wavelength filter (spatial resolution). The fluorescent lanthanide chelates of europium and terbium are attractive FRET donors. The use of a long-lifetime donor fluorophore and a short-lifetime acceptor fluorophore together with pulsed-laser excitation and time-resolved detection (temporal resolution) are effective in reducing the background signal. Qin et al. used time-resolved FRET for the determination of urinary albumin [118]. Particularly, they used, in the competitive homogeneous assay, an albumin-specific monoclonal antibody labeled with a stable fluorescent europium chelate as donor and an albumin labeled with cyanine 5 (Cy5) as acceptor. The limit of detection was 5.5 mg/L with the detectable range

being 10–320 mg/L. Another interesting approach to fluorescence spectroscopy is the fluorescent quenching. Chawjiraphan et al. developed a method to determine HSA in urine and serum by the graphene oxide-mediated (GO) fluorescence quenching aptasensor [132]. When albumin was added to the complex GO with the fluorescence-labeled aptamer, the aptamer detached from the complex to bind albumin, which resulted by an increase in fluorescence intensity, as shown in Fig. 4. The limit of detection was 0.05 mg/L and the detection range is 0.1–14.0 mg/L. Other quantitative works using the fluorescence spectroscopy are summarized in Table 4.

### 3.4. The Infrared and Raman spectroscopies

The absorption of infrared radiation IR excites vibrational transitions of molecules [148]. The infrared spectral region covers wavelengths from 780 nm to 1000  $\mu\text{m}$  which can be further subdivided into the near-infrared region from 780 nm to 2500 nm, the mid-infrared region from 2500 nm to 50  $\mu\text{m}$  and the far-infrared region from 50  $\mu\text{m}$  to 1000  $\mu\text{m}$ . Since vibrational frequency and probability of absorption depend on the strength and polarity of the vibrating bonds, they are influenced by intra- and intermolecular effects [149]. The approximate position of an infrared absorption band is determined by the vibrating masses and the type of bond (single, double, triple), the exact position by electron-withdrawing or donating effects of the intra- and intermolecular environment and by coupling with other vibrations. Information that can be derived from the infrared spectrum:

- Chemical structure of the vibrating group
- Chemical properties of neighbouring groups in a molecule
- Redox state
- Bond parameters
- Hydrogen bonding
- Electric fields
- Conformational freedom

Basically, all polar bonds contribute to the infrared absorption so there is no need to specifically label biomolecules to detect them. However, the infrared spectrum of biomolecules is challenging to

**Table 3**

Summary table of quantitative methods for application of immunoassay for protein determination in the human urine matrix (unless otherwise stated in the method section). CV (coefficient of variation) parameters are taken from maximum readings, for Recovery – the range is given.

Method	Reagent for detection (if any)	Analyte	The limit of detection, LOD	Range	Analytical and experimental parameters	Reference
Fluoroimmunoassay	Fluorescein isothiocyanate	HSA	0.5 mg/L	0.5–20 mg/L (linear $R^2 = 0.99$ )	Total time for experiment 4–6 h Recovery $105 \pm 7\%$	19 Chavers et al., 1984 [113]
Immunoturbidimetry	No reagent	HSA	4 mg/L	4–35.5 mg/L (linear)	Time so screen 15 min Recovery 95%–104%	20 Lloyd et al., 1987 [114]
Immunonephelometry	No reagent	HSA	0.34 mg/L	0.34–43.0 mg/L (detectable)	Automated version 240 samples an hour CV (intraassay) 6% CV (interassay) 9%	21 Marre et al., 1987 [115]
Immunoturbidimetry	No reagent	HSA	2 mg/L	2–260 mg/L (detectable)	CV (interassay) 4.8%	22 Bakker et al., 1988 [116]
Chemiluminescence	Acridinium ester	HSA	0.01 mg/L	0.3–10 mg/L (detectable)	Recovery 90–106%	23 Horton et al., 1989 [117]
Radioimmunoassay	$\text{Na}^{125}\text{I}$	HSA	0.015 mg/L	1.18–14.96 mg/L (detectable)	Recovery 90–106%	24 Horton et al., 1989 [117]
Time resolved Fluorescence Resonance Energy Transfer	Europium chelate (donor)/cyanine 5 (acceptor)	HSA	5.5 mg/L	10–320 mg/L (detectable)	Experiment time 12 min Recovery 103–122% CV (within-run) 6.9–10% CV (between-run) 7.5–13%	25 Qin et al., 2003 [118]
Chemiluminescence enzyme-linked immunosorbent assay based on Avidin–biotin	4-methoxy4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2-adamantane) (AMPPD) to detect enzyme activity	HSA	0.089 mg/L	0.15–15 mg/L (linear $R^2 = 0.9902$ )	Low usage of antibody CV (intra-assay) 10.7% CV (inter-assay) 15% Recovery 112%	26 Zhao et al., 2005 [119]
Chemiluminescence enzyme-linked immunosorbent assay based on fluorescein-isothiocyanate (FITC)	4-methoxy4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2-adamantane) (AMPPD) to detect enzyme activity	HSA	0.089 mg/L	0.15–15 mg/L (linear $R^2 = 0.9906$ )	CV (intra-assay) 10.5% CV (inter-assay) 11.9% Recovery 109%	27 Zhao et al., 2005 [119]
Magnetic two-site immunoassay	Dextran-coated nanoscaled to detect magnetic permeability	HSA	5 mg/L	0–400 mg/L (linear)	Experiment time 6.5 min CV 11%	28 Lu et al., 2006 [120]
Capillary electrophoresis immunoassay with spectrophotometric detection	Hydroxylamine	HSA	0.60 mg/L	0–6.67 mg/L (detectable)	Voltage 12 kV Recovery 91–160%	29 Giovannoli et al., 2007 [66]
Electrochemical immunosensor	$\text{Fe}_3\text{O}_4/\text{Au}$ colloid -modified gold electrode	NMP22 Nuclear Matrix Protein 22	$5 \cdot 10^{-4}$ mg/L	$1.2 \cdot 10^{-3}$ –0.2 mg/L (linear, $R^2 = 0.9932$ )	CV 3.9%	30 Ning et al., 2007 [121]
Resonance scattering (RS) spectral immunoassay	$\text{Cu}_2\text{O}$ for RS	HSA	$7.2 \cdot 10^{-6}$ mg/L	$1.4 \cdot 10^{-5}$ – $4.3 \cdot 10^{-4}$ mg/L	CV 4.5% Recovery 92.1–106.8%	31 Jiang et al., 2009 [122]
Fluoroimmunoassay, a quantum-dot (QS) optical immunosensor (the buffer matrix)	CdSe/ZnS QS	HSA	0.032 mg/L	0.2–200 mg/L (detectable)	Low cost immunosensor.	32 Tu et al., 2012 [123]
Time-resolved fluorescence resonance energy transfer immunoassay	Europium based oligonucleotides (donor)/oligonucleotide(acceptor)	HSA	0.0039 mg/L	0.0039–0.5 mg/L (linear) 0.0039–1 mg/L (detectable)	Experiment time 40 min Tested in serum, salive, urine CV 3%	33 Wang et al., 2012 [124]
Chemiluminescence lateral flow immunoassay	Luminol/enhancer/ $\text{H}_2\text{O}_2$ with silicon photosensor	HSA	2.5 mg/L	2.5–850 mg/L (detectable)	CV 20% Recovery 85–112%	34 Zangheri et al., 2016 [125]
Amperometric immunosensor (the buffer matrix)	Carbon nanotubes/gold nanoparticles screenprinted electrodes	p53 protein	$6.1 \cdot 10^{-4}$ mg/L	$8.4 \cdot 10^{-4}$ –0.437 mg/L (linear)	CV 10% Recovery 84–144%	35 Giannetto et al., 2017 [126]
Piezoelectric immunosensor, label-free immunoassay	Quartz crystal coated with a silver electrode	HSA	0.0095 mg/L	0.1–100 mg/L	Diagnostic sensitivity 98.7% Diagnostic specificity 100%	36 Theansun et al., 2017 [127]
Time-resolved fluorescence immunoassay	Europium (III) and samarium (III)	cystatin-C	$1.26 \cdot 10^{-3}$ mg/L	$0.42 \cdot 10^{-3}$ –0.9563 mg/L (linear, $R^2 = 0.9991$ )	Recovery 99.36% CV 3.2–5.9% (inter) CV 3.4–6.3% (intra)	37 Liu et al., 2017 [128]
Time-resolved fluorescence immunoassay	Europium (III) and samarium (III)	$\beta$ 2-microglobulin	$2.13 \cdot 10^{-3}$ mg/L	$0.86 \cdot 10^{-3}$ –0.9754 mg/L (linear, $R^2 = 0.9998$ )	Recovery 100.18% CV 3.0–6.8% (inter) CV 3.1–8.4% (intra)	38 Liu et al., 2017 [128]
Chemiluminescence immunoassay	Magnetic microparticles using carboxylic acid groups, acridinium	monomeric laminin- $\gamma$ 2	$1.0 \cdot 10^{-5}$ mg/L	$1.0 \cdot 10^{-5}$ –0.02 mg/L (detectable)	Recovery 82.3–96.2%	39 Nakagawa et al., 2017 [129]
Fluoroimmunoassay, optical microchips	Streptavidin-fusion based variants	HSA	0.65 mg/L		Limit of quantification 2 mg/L	40 Semeradtova et al., 2018 [130]
Electrochemical ELISA				$1 \cdot 10^{-3}$ –0.01 mg/L (linear)		

(continued on next page)

Table 3 (continued)

Method	Reagent for detection (if any)	Analyte	The limit of detection, LOD	Range	Analytical and experimental parameters	Reference
	Gold sensor chips with a hair comb design	CFHR1 complement factor H-related 1	$1.29 \cdot 10^{-3}$ mg/L			41 Arya et al., 2018 [131]
Electrochemical ELISA	Gold sensor chips with a hair comb design	NUMA1 nuclear mitotic apparatus protein 1	$0.97 \cdot 10^{-3}$ mg/L	$1 \cdot 10^{-3}$ -0.1 mg/L (linear)		42 Arya et al., 2018 [131]
Electrochemical immunosensor	Gold nanoparticles-platinum nanoparticles-metal organic frameworks	NMP22 nuclear matrix protein 22	$1.7 \cdot 10^{-6}$ mg/L	$5 \cdot 10^{-6}$ - $5 \cdot 10^{-4}$ mg/L (linear, $R^2 = 0.9833$ ) $5 \cdot 10^{-4}$ -0.02 mg/L (linear, $R^2 = 0.9909$ )	CV 1.93% Recovery 96%-106%	43 Zhao et al., 2019 [110]
Fluoroimmunoassay, graphene oxide-mediated fluorescence quenching aptasensor	87-nucleotide ssDNA as aptamer	HSA	0.05 mg/L	0.1-14.0 mg/L (detectable)	Cheap 0.3\$ per reaction	44 Chawjiraphan et al., 2020 [132]
Enzyme fluoroimmunoassay, sandwich-type ultramicroELISA	4-methylumbelliferil phosphate/diethanolamine-Hcl/sodium azide	HSA	$1.44 \cdot 10^{-3}$ mg/L	$1.44 \cdot 10^{-3}$ -0.2 mg/L (detectable)	CV 3.98-4.35% (intra) CV 7.59-8.92% (inter) Recovery 94.26-98.50% Time 1.5 h	45 Valle et al., 2020 [62]

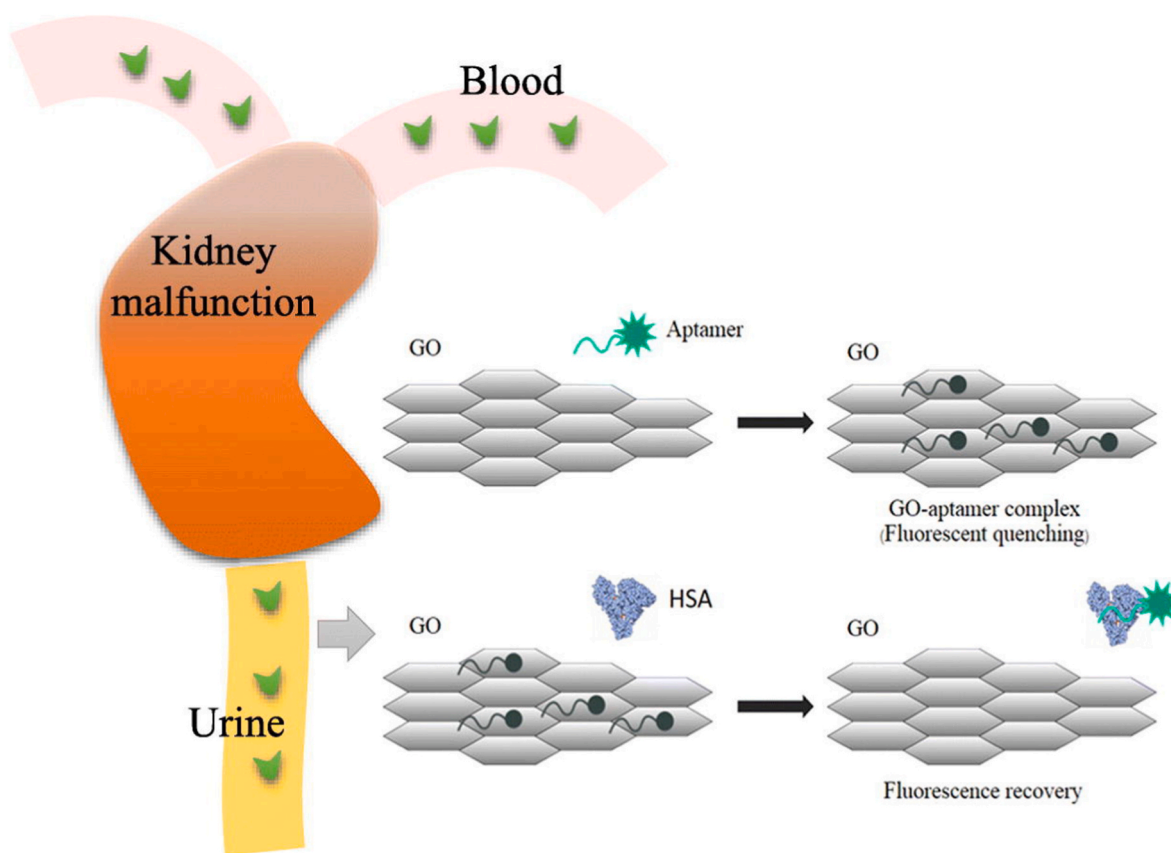


Fig. 4. Schematic of graphene oxide-mediated fluorescence quenching aptasensor for the detection of albuminuria in urine and HSA in human serum. When albumin was added to the complex GO with the fluorescence-labeled aptamer, the aptamer detached from the complex to bind albumin, which resulted by an increase in fluorescence intensity. Reprinted from Refs. [132].

analyze since they are complex with many overlapping bands. Certain mathematical and statistical procedures have to be utilized to analyze the resulting spectrum. The IR spectroscopy is often used to identify organic structures because functional groups give rise to characteristic bands both in terms of intensity and position (frequency). The IR spectra of organic molecules are often interpreted as having two regions: functional group region ( $>1500 \text{ cm}^{-1} = 6.7 \mu\text{m}$ ), and fingerprint region

( $<1500 \text{ cm}^{-1}$ ). In the fingerprint region, there are many troughs that form an intricate pattern that can be used as a fingerprint to determine the compound. Modern infrared spectrometers are usually Fourier transform infrared (FTIR) spectrometers [149]. The FTIR spectrometer works differently than the dispersive spectrometer where a monochromatic light beam is absorbed by a sample. This technique shines a beam containing many frequencies of light at once and measures how

Table 4

Summary table of quantitative methods for application of fluorescence spectroscopy for protein determination in the human urine matrix (unless otherwise stated in the method section). CV(coefficient of variation) parameters are taken from maximum readings, for Recovery – the range is given.

Method	Fluorophore	Analyte	The limit of detection, LOD	Range	Analytical and experiment parameters	Reference
Fluoroimmunoassay FIA	Fluorescein isothiocyanate (FITC)	HSA	0.5 mg/L	0.5–20 mg/L (linear $R^2 = 0.99$ )	Total time for experiment 4–6 h Recovery 105 ± 7%	47 Chavers et al., 1984 [113]
HPLC high performance liquid chromatography with fluorescence detection	8-anilino-1-naphthalenesulfonic acid	HSA	0.2 mg/L	0.2–400 mg/L (linear, $R^2 = 0.998$ )	Retention time for HSA 16.0 min TSK gel G2000 SWXL column	48 Ishida et al., 1996 [76]
Time resolved Fluorescence Resonance Energy Transfer	Europium chelate (donor)/cyanine 5 (acceptor)	HSA	5.5 mg/L	10–320 mg/L (detectable)	Experiment time 12 min Recovery 103–122% CV (within-run) 6.9–10% CV (between-run) 7.5–13%	49 Qin et al., 2003 [118]
Spectrofluorimetry	4-dimethylamino-2,5-dihydroxychalcone (DMADHC)	HSA	0.5 mg/L	1–95 mg/L (linear, $R^2 = 0.99$ )	CV 1.15% Recovery 95.6–103.0%	50 Xu et al., 2005 [137]
Chemiluminescence enzyme-linked immunosorbent assay based on fluorescein-isothiocyanate FITC	4-methoxy4-(3-phosphatophenyl)-spiro-(1,2-dioxetane-3,2-adamantane) (AMPPD) to detect enzyme activity	HSA	0.089 mg/L	0.15–15 mg/L (linear $R^2 = 0.9906$ )	CV (intra-assay) 10.5% CV (inter-assay) 11.9% Recovery 109%	51 Zhao et al., 2005 [119]
Capillary electrophoresis immunoassay with fluorescence detection (the buffer matrix)	Fluorescein 5-isothiocyanate (FITC)	HSA	0.6 mg/L	0.6–6.67 mg/L (quantification range)	Voltage 12–15 kV Recovery 91–160%	52 Giovannoli et al., 2007 [66]
Synchronous fluorescence determination	triphenylmethane acid dye methyl blue	HSA	0.03 mg/L	0.03–266.0 mg/L (linear, $R^2 = 0.9974$ ) 266.0–665.0 mg/L (linear, $R^2 = 0.9911$ )	Recovery 99.0–103.3% CV 2.3%	53 Hou et al., 2007 [138]
Synchronous fluorescence determination	5-Aminosalicylic acid (5-ASA)	HSA	0.552 mg/L	1.60–414 mg/L (linear)	Recovery 100.6–103.7% CV 1.26%	54 Cui et al., 2008 [139]
Spectrofluorimetry	CdS (core)/SiO <sub>2</sub> (nanoparticles)	BSA	0.18 mg/L	0.6–30 mg/L (linear, 0.9998)	Recovery 94–105% CV 2.1%	55 Zhu et al., 2009 [140]
Synchronous fluorescence determination (the buffer matrix)	Methylen blue	BSA	0.0089 mg/L	0.08–40 mg/L (linear, $R^2 = 0.998$ )	CV 1.7%	56 Liu et al., 2010 [141]
Spectrofluorimetry (the human plasma)	Terbium-danofloxacin (Tb3+-Dano)	HSA	5.4 mg/L	13.3–66.5 mg/L (linear, $R^2 = 0.9899$ )	CV (intra-assay) 6.44% CV (inter-assay) 8.08% Recovery 94.3–97.8%	57 Ramezani et al., 2012 [142]
Spectrofluorimetry immunoassay (the buffer matrix)	CdSe/ZnS quantum dots	HSA	0.032 mg/L	0.2–200 mg/L (detectable)	NA	58 Tu et al., 2012 [123]
Time-resolved fluorescence resonance energy transfer immunoassay	Europium based oligonucleotides (donor)/oligonucleotide(acceptor)	HSA	0.0039 mg/L	0.0039–0.5 mg/L (linear) 0.0039–1 mg/L (detectable)	Experiment time 40 min Tested in serum, saliva, urine CV 3%	59 Wang et al., 2012 [124]
Constant-energy synchronous fluorescence	No fluorescent label	HSA	0.007 mg/L	0.1–220 mg/L (linear)	CV 2.2%	60 Madrakian et al., 2015 [143]
Spectrofluorimetry in near infrared region	Based on hemicyanine dye	HSA	1.74 mg/L	27–2750 mg/L (linear, $R^2 = 0.9994$ )	Recovery 107.23–108.79% CV 1.00%	61 Li et al., 2016 [144]
Spectrofluorimetry	poly(thymine) (poly T)-templated copper nanoparticles (CuNPs)	HSA	5.45 mg/L	10–166 mg/L (linear, $R^2 = 0.9976$ )	Recovery 97–101%	62 Chen et al., 2017 [145]
Spectrofluorimetry (the human serum matrix)	CuInZnS	HSA	3 mg/L	5.0–6650 mg/L	Recovery 87.57–94.39% CV 1.96%	63 Gui et al., 2017 [146]
Spectrofluorimetry in near infrared region	Dicyanomethylene-4H-chromene-derived	HSA	1.26 mg/L	1.26–232 mg/L (detectable)	Good agreement with a conventional methods	64 Rajasekhar et al., 2017 [147]
Time-resolved fluorescence immunoassay	Europium (III) and samarium (III)	cystatin-C	1.26 10 <sup>-3</sup> mg/L	0.42 10 <sup>-3</sup> –0.9563 mg/L (linear, $R^2 = 0.9991$ )	Recovery 99.36% CV 3.2–5.9% (inter) CV 3.4–6.3% (intra)	65 Liu et al., 2017 [128]
Time-resolved fluorescence immunoassay	Europium (III) and samarium (III)	β <sub>2</sub> -microglobulin	2.13 10 <sup>-3</sup> mg/L	0.86 10 <sup>-3</sup> –0.9754 mg/L (linear, $R^2 = 0.9998$ )	Recovery 100.18% CV 3.0–6.8% (inter) CV 3.1–8.4% (intra)	66 Liu et al., 2017 [128]
Fluoroimmunoassay, optical microchips	Streptavidin-fusion based variants	HSA	0.65 mg/L	NA	Limit of quantification 2 mg/L	67 Semeradtova et al., 2018 [130]
Fluoroimmunoassay, graphene oxide-mediated fluorescence quenching aptasensor	87-nucleotide ssDNA as aptamer	HSA	0.05 mg/L	0.1–14.0 mg/L (detectable)	Cheap 0.3\$ per reaction	68 Chawjiraphan et al., 2020 [132]
Enzyme fluoroimmunoassay, sandwich-type ultramicroELISA	4-methylumbelliferil phosphate/diethanolamine-Hcl/sodium azide	HSA	1.44 10 <sup>-3</sup> mg/L	1.44 10 <sup>-3</sup> –0.2 mg/L (detectable)	CV 3.98–4.35% (intra) CV 7.59–8.92% (inter) Recovery 94.26–98.50% Time 1.5 h	69 Valle et al., 2020 [62]

much of that beam is absorbed by the sample. Next, the beam is modified to contain a different combination of frequencies, giving a second data point. This process is rapidly repeated many times over a short timespan. Afterward, a computer takes all this data and works backward to infer what the absorption is at each wavelength by a Fourier transform.

Another analytical technique that relies on molecular vibrations is Raman spectroscopy [150]. Raman spectroscopy is a powerful technique to determine vibrational modes of molecules to provide a structural fingerprint, like infrared spectroscopy, by which molecules can be identified. Infrared spectroscopy typically yields similar information because certain molecular vibrations can occur in both techniques but some only detectable in either, which makes them complimentary as well. Also, transitions that have large Raman intensities often have weak IR intensities and vice versa. Raman spectroscopy relies upon the inelastic scattering of photons. The laser light interacts with molecular vibrations, phonons or other excitations in the system, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the vibrational modes in the system. Raman spectra for biomolecules is hard to analyze due to overlapping bands, the same issue as in the IR spectroscopy. Certain mathematical procedures must be employed on Raman spectra to analyze a sample.

Despite difficulties in the analysis of large biomolecules such as proteins, the IR, and Raman spectroscopy make their advances in this regard. For the protein analysis with IR, the MIR region plays an important role since it includes bands that predominantly arise from three conformationally sensitive vibrations arising from the peptide backbone—namely, amide I, amide II, and amide III [151]. Amide group vibrations of the backbone have attracted much attention in protein IR spectroscopy, as they are native to all proteins and inform on secondary conformation and solvation. Such bands include the amide I region, between 1600 and 1700  $\text{cm}^{-1}$ , primarily resulting from C=O stretching vibration, the amide II band, related to CN stretch and NH in-plane bending, the amide III vibration, associated with CN stretching, NH bending, and CO in-plane bending, and, finally, the amide A (NH stretch) band. Conventional IR and Raman methods often lack enough sensitivity. Surface-Enhanced Infrared Absorption Spectroscopy (SEIRAS) [152], and Surface-Enhanced Raman spectroscopy SERS [153–155] improve sensitivity to overcome this problem. Both SEIRAS and SERS utilize the surface plasmon effect from the interaction of light with metallic nanoparticles. SERS has an enhancement factor (of the order of  $10^6$ – $10^{12}$ ) compared to the traditional Raman signal [156]. For SEIRA, the surface-enhancement is comparatively modest ( $\sim 10^1$ – $10^3$ ) [157]. Although the enhancement factor of SEIRAS is smaller than that of SERS, the cross-section for IR absorption is several orders of magnitude higher than the corresponding Raman cross-section. Despite the modest enhancement factor, the SEIRAS technique may be sufficient for many applications [152].

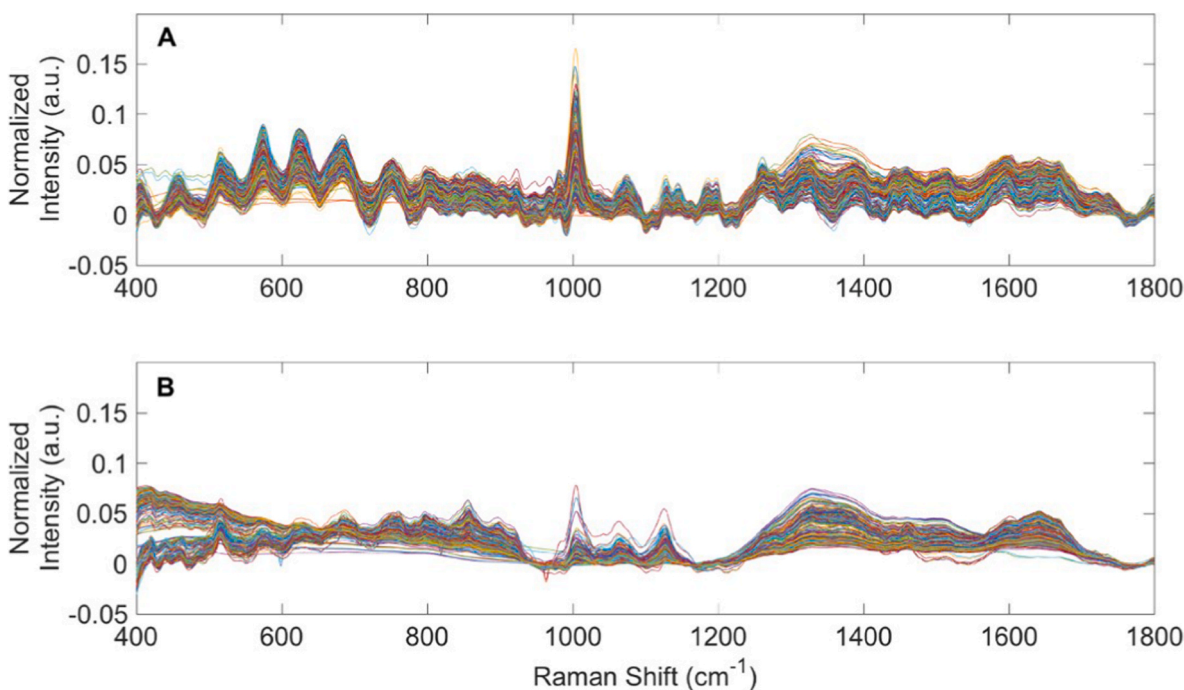
It should be emphasized again that the IR and Raman spectra are rich in data, and mathematical interpretations of those along with the right calibration procedure are important for analysis. Jessen et al. developed a method for simultaneous determination of glucose, triglycerides, urea, cholesterol, albumin and total protein in human plasma by Fourier transform infrared spectroscopy [158]. They put their samples of 40  $\mu\text{L}$  without any reagent treatment or preconcentration into water-thermostated CaF cells to measure transmission. The spectra were recorded between 3100 and 950  $\text{cm}^{-1}$ . In the analysis, the first derivative of the original spectra was used. The first derivative was computed by using the Savitzky–Golay algorithm with 9 smoothing points. For each sample, two IR spectra were recorded and the mean spectrum was subsequently corrected by the subtraction of a water blank spectrum. Since the IR spectra are complex, a calibration procedure is important. The authors, used between 144 and 169 patient samples for each analyte to cover a wide range of concentrations. Jessen et al. compared their results between the FTIR method by other reference methods, which were mainly based on immunoassays. For each analyte, the FTIR spectra are processed by a stepwise selection of wavenumbers giving the

optimal least-squares correlation between the measured IR signal, and the analyte concentration measured by the reference method. The correlation coefficients between the FTIR and the reference method were  $0.87 < R^2 < 1.00$ , and specifically for albumin it was  $R^2 = 0.92$ . For albumin, the calibration range was 20–60 g/L. The authors concluded that the developed FTIR methods use a simple and robust technology to achieve stable and accurate results that meet international quality criteria for the measurement of glucose, triglycerides, urea, cholesterol, albumin, and total protein in human plasma.

Ma et al. synthesized thiourea-functionalized silica nanoparticles and used them for the preconcentration of albumin in urine [159]. The adsorbent with the analyte was then used for near-infrared diffuse reflectance spectroscopy measurement directly and the partial least squares model was established for quantitative prediction. Forty samples were taken as a calibration set for establishing the PLS model and 17 samples were used for validation of the method. The principle of the PLS method is the combination of different signal processing methods and wavenumber regions. The wavenumber regions that produced the best results in cross-validation were selected. The correlation coefficient and the root mean squared error of cross-validation is 0.9986 and 0.43, respectively. The residual predictive deviation value of the model is as high as 18.8. The recoveries of the 17 validation samples in the concentration range of 3.39–24.39 mg/L are between 95.9 and 113.1%. In another work by Hall et al., the authors performed multiple linear least-squares (MLR) and an enhanced partial least-squares (PLS) using this time the second-derivative of the absorbance data to determine analytes, including protein, in unmodified human serum [160]. Pezanti et al. used a polynomial based spectral smoothing method to the urine matrix [161]. In general, all the mentioned works requires no specific reagents, they are fast and are able to quantify multiple analytes with one spectrum.

Premasiri et al. investigated urine by Raman spectroscopy [5]. The result was that since urea is in high concentration, normal Raman spectroscopy is sufficient for analysis of the analyte. However, all other components are in low concentrations requiring the use of SERS. Bispo et al. correlated the amount of urea, creatinine, and glucose in urine from patients with diabetes mellitus and hypertension with the risk of developing renal lesions by means of Raman spectroscopy and principal component analysis [162]. They took Raman spectra of control subjects and patients with illnesses, and then the spectra were submitted to principal component analysis (PCA) followed by discriminant analysis. The essence of PCA is to yield an orthogonal basis in which different individual dimensions of the data are uncorrelated. The discriminating model showed a better overall classification rate of 70%. The same approach was utilized by Almeida et al. to estimate concentrations of urea and creatinine in the human serum of normal and dialysis patients [163]. PCA showed high discrimination between dialysis and normality (95% accuracy). Senger et al. analyzed by the Raman spectroscopy three types of patients: patients receiving peritoneal dialysis (PD) therapy for end-stage kidney disease; patients receiving peritoneal dialysis (PD) therapy for end-stage kidney disease; urine from healthy human volunteers, as shown in Fig. 5 [164]. The authors used the set of mathematical procedures on spectra which included spectral processing (e.g., truncation, baselining, and vector normalization); principal component analysis (PCA); statistical analyses (ANOVA and pairwise comparisons); discriminant analysis of principal components (DAPC); and testing DAPC models using a leave-one-out build/test validation procedure. Their approach was able to identify “unknown” urine specimens as from PD patients or healthy human volunteers with better than 96% accuracy (with better than 97% sensitivity and 94% specificity).

Chamuah et al. used a blu-ray DVD as a SERS substrate for the detection of albumin, creatinine, and urea in urine [165]. The minimum concentration of albumin, creatinine, and urea which can be measured by Raman spectrometer is 0.1 mg/L, 0.2 mg/L, and 0.6 mg/L respectively. The authors investigated correlations between the SERS signal taken from their signature Raman peaks (albumin 1208  $\text{cm}^{-1}$ , creatinine



**Fig. 5.** Raman spectra of PD patient urine and spent dialysate. (A) Averaged, baselined, and vector normalized Raman spectra from 362 urine specimens obtained from patients receiving PD therapy for ESKD. (B) Averaged, baselined, and vector normalized Raman spectra from 395 spent PD dialysate specimens. Reprinted from Ref. [164].

1444  $\text{cm}^{-1}$ , urea 1018  $\text{cm}^{-1}$ ) and the concentration of the respective substance. The linear correlation coefficients ( $R^2$ ) were 0.956 for albumin, 0.976 for creatinine, 0.982 for urea. Dou et al. determined quantitatively concentration of glucose, acetone, urea, and creatinine in the human urine [166]. Their detection limits were 410 mg/L, 400 mg/L, 49 mg/L, and 15 mg/L, respectively. Good correlations were obtained between the concentrations and the Raman intensities with the R values of 0.924 (glucose), 0.987 (acetone), 0.962 (urea), and 0.923 (creatinine), respectively. The authors showed that the excitation wavelength is an important factor to reduce fluorescence of urine, which reduces the signal to noise ratio. The laser with a wavelength of 750 nm performed better than 600 nm, 650 nm, 700 nm. Gaipov et al. have begun to study patients with elevated protein content in urine vs control group (normal protein content) using combination of hybrid Brillouin-Raman spectroscopy and SERS [167].

Chamuah et al. assigned the signature Raman peaks to the corresponding vibrations in analytes [165]:

1. For albumin – peaks at 1208  $\text{cm}^{-1}$  and 1370  $\text{cm}^{-1}$  are attributed to  $\text{SO}_2$  symmetric stretch and CH bond deformation of albumin.
2. For creatinine – the Raman signature peaks are observed at 888  $\text{cm}^{-1}$ , 958  $\text{cm}^{-1}$ , and 1444  $\text{cm}^{-1}$  which could be due to C=O stretching, C–C stretching and  $\text{CH}_3$  anisometric deformation respectively.
3. For urea – a very strong Raman peak at 1018  $\text{cm}^{-1}$  which corresponds to C–N stretching mode.

Throughout is important consideration for laboratory tests. Zhu et al. performed quantitative SERS detection of creatinine in urine, and then compared with a clinically validated enzymatic “creatinine kit” [168]. They wrote that SERS detection process could be completed within 2 min compared with 11 min for the creatinine kit, indicating the practicality of the quantitative SERS technique as high-throughput platform for relevant clinical and forensic analysis.

#### 4. Urinary protein biomarkers

Human urine contains thousands of individual proteins [12,13]. Excessive presence of some of them are associated with certain diseases related not only to renal diseases, but also to cancer, diabetes, and infections. Those molecules that are associated with a certain biological condition are called biomarkers. Most of those biomarkers in human urine were identified by comparing patients with healthy individuals by the means of chromatography or electrophoretic separation followed by mass spectroscopy, or by the immunoassay. Literature review of urinary protein biomarkers with related diseases is summarized in Table 5, corresponding diagnostic sensitivity and diagnostic specificity are given [169]. Some of the listed research papers used Western blot as a technique [170]. Due to the usage of antibodies, this technique was counted as the immunoassay in Table 5. Most of the methods mentioned followed established procedures. To get a general idea of those experimental methods, refer to the previous paragraphs about separation and detection techniques, and Tables 2–4

Below are some observations and conclusions regarding Table 5 about the content of the listed research papers:

- Urinary protein analysis are useful for not only renal diseases. Urine proteomics can be used for detection of at least 15 conditions, including 8 kinds of cancer; 5 kinds of renal complications; and at least 3 different infections. The list of health complications that can be diagnosed from urine analysis is bigger if non-protein substances are included, such as peptides, nucleic acids, low molar mass metabolites, and etc; that can be found in reviews [14,26,27,36].
- New potential urinary protein biomarkers are found primarily by mass spectrometry nowadays. However, further extensive validation on clinically representative population is required. Often it is achieved through the immunoassay, if the appropriate method is developed with a sufficient analytical sensitivity, since the immunoassay methods are expressive, low-cost and quantitative compared to the chromatography coupled with mass spectrometry.

**Table 5**

Protein biomarkers in urine. Sensitivity is the percentage of individuals who have a given disorder who are identified by the method as positive for the disorder. Specificity is the percentage of individuals who do not have a given condition who are identified by the method as negative for the condition.

Method	Urinary Protein Biomarker	Disease	Sensitivity/ Specificity	Reference
LC-MRM/MS SELDI-TOF-MS	Prothrombin urinary glycopeptides <i>m/z</i> 1201 and 1449	Bladder cancer Endometrial cancer, ovarian cancer, cervical cancer	71.1%/75% 100%/91.67%	Chen et al., 2012 [101] Ak et al., 2016 [175]
LC-MS/MS	S100-A9 and Granulins	Hepatocellular carcinoma	NA	Huang et al., 2015 [176]
LC-MS/MS MS, label-free quantitative (LFQ) mass spectrometry, parallel reaction monitoring GeLC-MS/MS analysis, immunoassay – ELISA	leucine-rich alpha-2-glycoprotein LYPD1, LYVE1, PTMA and SCGB1A1 LYVE-1, REG1A, and TFF1	Ovarian cancer Ovarian cancer Pancreatic cancer	NA 92%/78% 76.9%/86.8%	Smith et al., 2014 [177] Sandow et al., 2018 [178] Radon et al., 2015 [171]
LC-MS/MS, reaction monitoring, Western Blot LC-MS, immunoassay – ELISA (commercial kits), Western Blot CE-TOF-MS	FABP5 Flotilin-2, PARK7 CKD273	Prostate cancer Prostate cancer Chronic kidney disease	60%/100% 68%/93% (immunoassay) 33%/83%	Fujita et al., 2017 [172] Wang et al., 2017 [173] Pontillo et al., 2017 [179]
CE-MS	mucin-1	Chronic kidney disease	NA	Zhang et al., 2017 [180]
2D DIGE-MS (2D gel electrophoresis)	preliminary protein profiling for potential biomarkers	Influenza Virus	NA	Prescott et al., 2010 [181]
LC-TOF-MS, immunoassay – ELISA (commercial kits) Immunoassay	$\alpha$ -fetoprotein VEGF, IL-8, MMP-9, MMP-7, survivin and Cyfra 21.1	Hepatitis B Bladder cancer	90.1%/95.4% NA	Zhan et al., 2019 [174] Gogalic et al., 2015 [182]
Immunoassay – Western Blot (commercial kits)	FGFR3 and Cyclin D3	Bladder cancer	73%/90%	Blanca et al., 2016 [183]
Chemiluminescence immunoassay (self made)	monomeric laminin- $\gamma$ 2	Bladder cancer	72%/92%	Nakagawa et al., 2017 [129]
Immunoassay – ELISA (commercial kits)	uCyr61 and uTFF3	Colorectal cancer	75.5%/69.8%	Shimura et al., 2019 [184]
Radioimmunoassay <sup>125I</sup> (commercial kits) Immunoassay – Western blot	TGF $\alpha$ and AFP AQP1 and PLIN2	Hepatocellular carcinoma Kidney cancer	86.7%/NA NA	Tsai et al., 1997 [185] Morrissey et al., 2015 [186]
Multiplex immunoassay (commercial kits) 2DE – Western blot (commercial kits)	HE4, CEA, and TTR clusterin, leucine-rich alpha-2- glycoprotein	Ovarian cancer Ovarian cancer	93.7%/70.6% NA	Lee et al., 2019 [187] Mu et al., 2013 [188]
Multiplex immunoassay (commercial kits)	HSA, KIM-1, MCP-1	Chronic kidney disease	NA	Zhang et al., 2018 [189]
Immunoassay – ELISA (commercial kits) Immunoassay – ELISA (commercial kits) Immunoassay	EGF EGF, MCP-1 HSA, KIM-1, NGAL and MCP-1	Chronic kidney disease Diabetic kidney disease Diabetic kidney disease	NA NA 60%/NA	Ju et al., 2015 [190] Wu et al., 2020 [191] Nowak et al., 2018 [192]
Immunoassay – ELISA (commercial kits)	MCP-1, EGF	Diabetic kidney disease	75.7%/73.9%	Satirapoj et al., 2018 [193]
Immunoassay – ELISA (commercial kits) Immunoassay – ELISA (commercial kits)	AQP5 nonalbumin protein-to-creatinine ratio	Diabetic nephropathy Diabetic nephropathy	85%/88% 92.3%/81.7%	Lu et al., 2016 [194] Kim et al., 2017 [195]
Solid phase immunoassay (commercial kits)	NGAL, urinary type-IV collagen	Diabetic nephropathy	NA	Gaipov et al., 2019 [196]
Immunoassay – ELISA (commercial kits)	$\beta$ 2MG, MCP-1	Autosomal dominant polycystic kidney disease	NA	Messchendorp et al., 2018 [197]
Immunoassay – ELISA (commercial kits)	HGF	Cardiovascular disease	NA	von Scholten et al., 2016 [198]
Immunoassay – ELISA (commercial kits)	EGF, MCP-1	Glomerulonephritis	NA	Chanrat et al., 2018 [199]
Immunoassay – ELISA (commercial kits), immunonephelometry Multiplexed immunoassay	NGAL, KIM-1, Cystatin-C L.infantum biomarkers: Li-isd1, Li-txn1, and Li-ntf2 L.donovani biomarkers: Ld-mao1, Ld-ppi1	Acute kidney injury Visceral leishmaniasis	NA 82.2%/100%	Dubin et al., 2018 [200] Abejion et al., 2019 [201]
Fluorescence emission spectroscopy, synchronous fluorescence excitation spectra	NADH, FAD	Kidney cancer	90%/90%	Atif et al., 2018 [202]

- In total 34 references are included in Table 5; 12 of them utilized mass spectrometry, 24 utilized the immunoassay, and 1 paper – fluorescence spectroscopy.
- Authors of these research papers utilized the mass spectrometry methods with further validation by the immunoassay – [171–174].
- All of the mentioned work (except [129]) in the immunoassay followed manufacturer's protocol, or if a custom immunoassay

was used, other established procedure from their own or previous works.

- ELISA-based immunoassay was the most popular method accounting for 13 references.
- Diagnostic sensitivity and specificity increased if a panel of several biomarkers was considered simultaneously. Though, the panel should be carefully chosen as not all of the components provide increase in diagnostic sensitivity and specificity.

## 5. Conclusion

Urine is a readily available liquid for the medical diagnosis of patients. Urine tests are non-invasive procedures that involve no pain or discomfort for patients to determine problems with kidney function since extensive medical research showed that patients, with high protein concentration in urine, have various kinds of illnesses of the kidney, referred to as proteinuria [1]. Thus the precise and simple determination of urinary concentrations of total protein is important for diagnostic purposes. In this review, we focused on instrumental determination of abundant proteins in urine by electrophoresis, chromatography, mass spectrometry, immunoassay, fluorescence, IR, and Raman spectroscopy. The mentioned techniques are not mutually exclusive, for example, high performance liquid chromatography is usually a separation step followed by mass spectrometry, another example is labelling with fluorophores in immunoassay. Each technique has its own strength to analyze urine for specific information, hence, it is hard to compare them directly, and to find a common denominator for comparison. Overall, number of cited experimental papers that were covered by this review in Tables 2–5: electrophoresis and chromatography – 22 in total and 8 related to HSA detection; mass spectrometry – 17 in total and 5 related to HSA; immunoassay – 47 in total and 17 related to HSA; fluorescence spectroscopy – 22 in total and 19 related to HSA; IR and Raman spectroscopy – 10 in total; all topics – 101 papers. Thus, almost half of the cited literature by the means of immunoassays, which is in a good correlation that immunoassays are widespread in clinics and science.

The limit of detection is important parameter for evaluating an analytical method. In order to develop a method, the limit of detection should take into account which amount of analyte has clinical significance. The 15–30 mg/L albumin concentration is a critical value that could indicate kidney problems when it is repeatedly exceeded [10]. For the corresponding articles discussed above, the limit of detection for electrophoresis, chromatography, mass spectrometry, immunoassay, fluorescence exceeded 2 mg/L for albumin, which is enough for the clinical purpose. For some methods, explained in Ref. [117,119,122,123,127,132,143], the limit of detection does not exceed 0.1 mg/L for albumin in the urine matrix. For low abundant proteins the limit of detection must be obviously much lower. For example, in the article for Aquaporin-2 determination by Jaffuel et al. the limit of detection was  $5 \cdot 10^{-4}$  mg/L [85]. In another work by Zhao et al., the LOD for Nuclear matrix protein 22 was  $1.7 \cdot 10^{-6}$  mg/L [110]. For the IR and Raman spectroscopy methods the LOD might not be so relevant factor. The IR and Raman methods is better characterized by how accurate a particular method can distinguish whether a spectra belongs to a healthy subject or not, as evidenced from works [158,164].

Electrophoresis and chromatography are powerful separation techniques which are accompanied by further detection, usually UV–vis spectrometry or fluorescence. Since the human urine matrix is very complex consisting of inorganic and organic compounds, those techniques are useful for urine analysis [7]. Though certain methods might require preconcentration techniques to enhance sensitivity [69]. High-performance liquid chromatography with mass spectrometry detection is a particularly powerful tool for proteomics studies, as it was able to identify 1823 proteins in urine [12]. Mass spectrometry in itself does not allow for quantitative analysis due to the different physicochemical properties of different peptides and proteins [98–100]. However quantification in MS is possible if an internal standard is used, for such there are approaches based on labeling with stable isotopes (such as N-15, O-18), involving artificial labeling of peptides and proteins, as in work by Singh et al., specifically in the urine matrix [81]. The immunoassay is widely used technique both in clinical practice and in research papers for urine analysis. Though different immunoassay methods can overestimate or underestimate an analyte concentration [79]. They can hurt patients due to inaccurate results [112]. The IR and Raman spectroscopy have advantage of easy preparation of samples to analyze. They require no specific reagents, or no reagents at all, no

preconcentration or dilution, as evidenced from Refs. [158,162,165]. They are rapid. On the other hand, due to overlapping bands of large biomolecules, and the complexity of urine components – hard to analyze their spectra that require mathematical analysis.

Overall, there is no "silver bullet" modern quantification method for the analysis of proteins in urine that has been reported and validated so far. However, at least for the routine clinical diagnostics of kidney problems, applications of relatively direct methods (such as SERS or IR) spectroscopy may expand as instrumentation, substrates (at least SERS substrates), software and computing power for data analysis (peak deconvolution) become progressively less expensive.

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## 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.

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