The handle [http://hdl.handle.net/1887/65534](http://hdl.handle.net/1887/65534) holds various files of this Leiden University dissertation.

**Author:** Raus, P.P.M.  
**Title:** Innovative strategies to clinically characterize the human tear proteome: from fundamental exploration to ophthalmological relevance  
**Issue Date:** 2018-09-04
5.1 Bottom-up protein identifications from microliter quantities of individual human tear samples. Important steps towards clinical relevance.

[P. Raus, B. Raghuraman Kumar, M. Pinkse, P. Verhaert (2015) EuPA Open Proteomics 9: 8-13.]

Bottom-up protein identifications from microliter quantities of individual human tear samples. Important steps towards clinical relevance.

Peter Raus1,2, Bharath Raghuraman Kumar2, Martijn Pinkse2, Peter Verhaert2,3,4

1 Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands
2 MIR, Centre for Ocular Surgery and Esthetics, Stationsstraat 129B, 2240 Geel, Belgium
3 Department of Biology & Faculty of Biomedical, Pharmaceutical and Veterinary Sciences, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

ARTICLE INFO

Article history:
Received 17 March 2015
Received in revised form 17 June 2015
Accepted 19 June 2015
Available online 29 June 2015

Keywords:
Human tears
Schirmer strip sampling
Quadrupole Orbitrap
Bottom-up protein identification
Individual sample analysis
Clinical applicability

ABSTRACT

A relatively simple combination of Schirmer strip sampling with straightforward sensitive nanoLC quadrupole-Orbitrap tandem mass spectrometry after a minimum of sample processing steps allows for replicate proteomic analysis of single human tears, i.e., without the requirement for sample pooling. This opens the way to clinical applications of the analytical workflow, e.g., to monitor disease progression or treatment efficacy within individual patients. Proof of concept is provided by triplicate analyses of a singular sampling of tears of a dry eye patient, before and one and two months after minor salivary gland transplantation. To facilitate comparison with the outcome of previously reported analytical protocols, we also include the data from a typical healthy young adult tear sample as obtained by our streamlined method.

With 375 confidently identified proteins in the healthy adult tear, the obtained results are comprehensive and in large agreement with previously published observations on pooled samples of multiple patients. We conclude that, to a limited extent, bottom-up tear protein identifications from individual patients may have clinical relevance.

© 2015 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Since the recent launch, within the Human Proteome Organization (HUPO), of the Human Eye Proteome Project (HEPP) [10,18], tears are among the body fluids which have gained increasing interest as a source of diagnostic markers not only for ophthalmological diseases, but also for systemic and neurological disorders. Whereas accounts of proteins identified from human tears all resulted from the analyses of pooled samples (see advanced LC/MS/MS reports by de Souza et al. [3]; Zhou et al. [15]; Srinivasan et al. [12]; Salvisberg et al. [11]; and the references quoted therein to other earlier (and generally less performant) mass spectrometry based proteomics approaches), we focus on what can be achieved by studying individual tear samples with the latest LC/MS/MS.

In order to be compliant with the envisioned clinical application, we compiled an efficient analytical workflow with minimal sample preparation steps.

We opted for Schirmer strips (instead of capillaries) as most convenient clinician friendly tear sampling tools. On these filter paper strips >20 μl volumes of tear can be easily collected. This minimally invasive form of body fluid collection is highly accepted in the primary healthcare setting and has great potential for use in health screening [9]. As such it is already common use in current ophthalmological practice, e.g., for testing the severity of dry eye disease.

Employing straightforward nanoLC tandem MS by a recently introduced high resolution quadrupole-Orbitrap hybrid system, we demonstrate that it is realistically feasible to perform multiple replicate proteomic analyses (in terms of bottom-up protein identifications) on these microliter sample quantities.

As such the overall sensitivity of this optimized analytical protocol permits intra-individual (unpooled) monitoring of e.g., disease progression or treatment, with several hundreds of relevant data points (protein identifications and relative quantifications) collected for each clinical sample.
As proof-of-concept we monitored tears of a severe case of keratoconjunctivitis sicca before and after surgical treatment. Keratoconjunctivitis sicca, or dry eye syndrome is a very complex multifactorial disease [7], which, as the name indicates, in virtually all cases results in reduced tear volume production, which is reported to be associated with a decreased general lacrimal protein secretion. Very severe cases are uniquely treated by autotransplantation of a minor salivary gland into the eye, a technique originally introduced by Prof. J. Murube and perfectionized over the past ten years [5]. The rationale behind a proteomics analysis of clinically sampled tears is that comparative protein composition analysis of tears from diseased versus treated and/or healthy eyes, may yield medically relevant information regarding both the effectiveness of the treatment and the possible disease etiology.

2. Materials and methods

2.1. Reagents and chemicals

Ammonium bicarbonate, tri(2-carboxyethyl)phosphine (TCEP), iodoacetamide, formic acid, dimethyl sulfoxide, as well as trypsin were from Sigma–Aldrich™.

2.2. Tear collection and sample preparation

Human lacrimal fluids were sampled using Schirmer tear test strips [Haag-Streit, UK], principally as published earlier by Zhou et al. [15] and Srinivasan et al. [12]. For this the paper strip was tenderly placed inside the lower eyelid, after which the subject was instructed to gently close the eye. The moistened strip was removed after a maximum of 5 min. The sampling procedure did not include any anesthetizing eye drops. Both during sampling as well as further strip handling, gloves were worn.

Two different individuals provided the tears samples used in this study (Table 1). Tear samples from an individual diagnosed with severe dry eye syndrome (aqueous deficiency subtype) were collected (with the patient’s consent) at 3 different time points during disease treatment, i.e., before treatment, and 1, and 2 months after surgery (minor salivary gland transplantation). Consistent with the data in the literature [12] the aqeous deficient dry eye typically scored <5 mm of Schirmer strip wetting. For comparative purposes one additional tear sampling of a healthy young adult male volunteer was included in this method evaluation study. Healthy adult tears have been consistently analyzed by the relevant proteomics methods described in the literature [3,15,12,11]. The healthy tear easily moistened >15 mm during sampling.

After sampling, strips were stored in labeled protease-free Eppendorf vials at –20 °C until further analysis. For analysis 2 mm of the wetted part of the filter paper area which had not been in direct contact with the eye ball and conjunctiva (in order to minimize sample contamination with epithelial proteins) was carefully cut from each strip. During sample processing, care was taken to keep the analysis volume to an absolute minimum, to remain maximally compatible with the limited nanoLC injection volume. After transfer to another protease-free microcentrifuge tube the 2 mm ribbon was carefully cut into minute equally sized pieces using clean scissors. The resulting shreds were submerged in 47 µl of 25 mM ammonium bicarbonate (pH 8.0) for 90 min. Reduction of disulfide bonds was achieved by mixing 1 µl of TCEP (×50 stock solution; 10 mM final) with the sample for 30 min. Subsequent alkylation was allowed to occur for 45 min after addition of 1 µl iodoacetamide (×50 stock solution; 20 mM final). Finally overnight protein digestion (RT) was initiated by adding trypsin (sequencing grade; 1 µl of 200 ng/µl stock). Afterwards 5 µl of a mixture of 5% DMSO and 5% formic acid were added to assist resuspension of tryptic peptides.

2.3. Sample analysis

Of each sample 5% of the total reaction volume (2.5 µl) were analyzed by Easy-nLC 1000™ ultra performance liquid chromatography on a 200 mm long in-house packed C18 nano HPLC column (50 µm ID). A 60 min elution gradient (solvent B: 80% acetonitrile, 0.1% formic acid; solvent A: Milli Q: 350 µl/min) was as detailed in Table 2.

Tandem MS analysis was carried out on a Q Exactive Plus™ quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide fragmentation was by high energy collision induced dissociation (HCD), with the MS² settings are summarized in Table 3.

2.4. Data analysis

Spectral files generated (Xcalibur™, RAW format) were analyzed using Proteome Discoverer™ software version 1.4. Multiply charged peptide spectra were deconvoluted to singly charged spectra and deisotoped. The spectral files were then searched against the Uniprot Homo sapiens reference proteome (UP000005640; release Oct, 2014) using the Sequest™ HT algorithm (Thermo Fisher Scientific; parameters see Table 4).

| Table 1 |
| Tear sample donor list (same color row indicates that sample originated from the same individual). |
| Sample code | R | L | Age | Clinical origin |
|-------------|---|---|-----|----------------|
| Y           | R | M | 26  | Healthy volunteer |
| 59          | R | M | 69  | Dry eye patient (untreated eye) |
| 60          | L | M | 69  | Dry eye patient (treated eye, 1 month after surgery) |
| 61          | L | M | 69  | Dry eye patient (treated eye, 2 months after surgery) |

| Table 2 |
| Gradient elution profile (A: Milli Q; B: 80% acetonitrile, 0.1% formic acid). |
| Time (min) | Flow (nl/min) | % A | % B |
|------------|---------------|-----|-----|
| 0          | 350           | 98  | 2   |
| 40         | 350           | 70  | 30  |
| 57         | 350           | 35  | 65  |
| 59         | 350           | 100 | 0   |
| 60         | 350           | 100 | 0   |

| Table 3 |
| Full scan MS and data dependent MS/MS settings of the Q Exactive Plus™ system. |
| Resolution full MS | 70,000 |
| AGC target full MS | 3e6 |
| Maximum IT | 250 ms |
| Scan range | 300–1400 m/z |
| Loop count | 10 |
| dd resolution | 15,000 |
| dd target | 1e5 |
| dd-MS² max IT | 150 ms |
| Isolation window | 2.5 m/z |
| Fixed charge exclusion | 100.0 m/z |
| NCE | 28 2 |
| dd underfill ratio | 0.5% |
| Charge exclusion | Unassigned, 1, >8 |
| Peptide match | Preferred |
| Dynamic exclusion | 30 + |

- 96 -
Peptide spectral matches (PSMs) were validated using Percolator™ with a false discovery rate (FDR) < 1% and a minimum cross correlation score (Xcorr) of 2 as obligatory criteria for confident protein identification.

To reveal (semi) quantitative trends in tear protein abundances we employed the basic spectral counting option (summing PSMs per protein identity) available through the Proteome Discoverer™ software suite (Thermo Fisher Scientific, Bremen, Germany).

3. Results and discussion

Since only 5% of the prepared sample, corresponding to less than 0.5 µl of original tear fluid (equivalent to approximately 4 µg of total protein), was injected for each one hour LC/MSMS run, a triplicate analysis could easily be carried out for all individual samplings.

We here report the obtained number of proteins identified, as well as the (semi) quantitation values of some selected (differential) proteins primarily to illustrate the analysis depth obtainable on single (unpooled) samples. We refer to the Supplemental information for the results of all individual analyses.

We also want to mention that MALDI TOF MS data have been previously reported on individual human tears (e.g., González et al. [6]). These studies are typically limited to mere profiling, lacking direct protein identifications.

3.1. Healthy lacrimal fluid

3.1.1. Protein groups in healthy tears

The combined triplicate analysis yields a total of 375 identified protein groups, based on a total of 8020 peptide spectral matches (2506 unique peptides). In spite of the substantial technical variance of the overall analytical workflow (as illustrated in Fig. 1), out of these, 194 protein groups were consistently identified in all 3 separate measurements.

3.1.2. Proteins identified

The protein identifications agree well with those reported in earlier tear proteome investigations [3,12,15]. Remarkably our number of confidently detected proteins in individual tears (375) does not significantly differ from the number (386) identified from the combined pooled Schirmer strip samples in the LS MS/MS study (linear ion trap-orbitrap hybrid; [12]). In comparison the study of de Souza et al. [3] detected 491 proteins from pooled sets of capillary collected tears, after combining different sample preparation methods, including in gel and in solution digestion. The study by Zhou et al. reported the largest number of human tear proteins so far (1543), by combining the results of the analysis of the equivalent of 400 µg of pooled tears by multidimensional HPLC (SCX followed by nonRP) on a latest generation quadrupole-TOF hybrid. More valuable than mere protein numbers, are the protein identities and (relative) quantities, and with regard to this, our results are highly comparable with the literature studies referenced above. We consistently identified the most abundant tear proteins with the largest number of peptides. As an illustration proteins identified with >100 peptide spectral matches (PSMs) are listed in Table 5. The full lists of protein identifications in all triplicate samples are included as Supplemental data.

3.2. Diseased lacrimal fluids

3.2.1. Protein groups in patient tears

The untreated condition yielded 126 protein groups (2817 PSMs; 788 unique peptides), the one and two month treated samples yielded respectively 161 (4261 PSMs; 1162 unique peptides) and 135 protein groups (3605 PSMs; 894 unique peptides). Considering that these tear analyses were performed on equal volumes of material (equal size wetted Schirmer strip surface areas) as for the healthy samples, these data suggest markedly less (minus 50%) identifiable proteins in the diseased tear. Whether or not this lower number of tear protein identifications is due to an effect of the age difference between the donor of the healthy tear sample and the patient (the amount of tear proteins is known to decrease with age [14]), to a direct effect of the disease, or to potential disease-specific PTMs which were not considered in our typical database search (Table 4), is not assessed by this study. It shall be clear that for such inter-individual comparisons a large cohort of properly matched (sex, age, race, . . .) donors need to be investigated.

Rather than comparing different individuals, it is much more appropriate to compare protein compositions within the very same subject, as we did for the dry eye patient under investigation, the tears of whom were analysed before and at two time points after surgical treatment. Thanks to the fact that following the described workflow, a satisfactory analysis depth can be achieved from unpooled, individual donor samples (see above, healthy young adult male), such intra-individual comparisons now become feasible and yield meaningful results. Although the label-free method for relative protein quantitations has its clear limitations in terms of accuracy, it is clear that this simple cost-effective and sample saving approach does have its merit (see Ref. [4], especially when the purpose is to uncover relative protein abundance trends rather than to reveal accurate up- or downregulation factors. In this respect we find it interesting to note that proteins which, by label-free quantitative evaluation, appear originally

Table 4: SEQUEST HT parameters.

| Database                  | Uniprot Homo sapiens proteins (Oct. 2014 release) |
|---------------------------|----------------------------------------------------|
| Enzyme                    | Trypsin (full)                                      |
| Missed cleavages          | 2                                                  |
| Precursor mass tolerance  | 5 ppm                                              |
| Fragment mass tolerance   | 0.02 Da                                            |
| Dynamic N-terminal modification | Glu > pyro-Glu                                       |
| Dynamic C-terminal modification | Amidation                                          |
| Other dynamic modification | Oxidation of H,M,W                                  |
| Static modification       | Carbamidomethyl C                                  |

Fig. 1. Numbers of protein groups identified by nanoUHPLC quadrupole-Orbitrap MSMS from sub-microliter tear volumes of healthy human volunteer. Triplicate analysis of a single unpooled individually collected tear sample, yielding an overall protein number of 375, with 194 proteins identified consistently in all separate measurements.
unraveling the exact medical/physiological background of what these observations entail is not the scope of this paper, but interestingly, several additional agreements with earlier published semiquantitative data are remarkable.

For example, Zhou et al. [14] described a biomarker panel of proteins the level of which can be correlated with dry eye disease. Although it should be clear that, when considering the number of representative peptides reflecting the protein presence, an inter-individual comparison of the present limited data set (we here show only a single non-age matched healthy sample) cannot be conclusive, it is interesting to note that all 4 biomarker proteins which Zhou reports as downregulated in dry eye disease (i.e., lysozyme, lactotransferrin, lipocalin and prolactin-inducible...
protein) also appear downregulated in the untreated diseased eye compared to the healthy eye (of a considerably younger donor). Moreover, the more appropriate intra-individual comparison seems to indicate that after treatment the amounts of these proteins all show a tendency of getting restored (Table 7).

Of the 8 proteins which Zhou reported as upregulated, protein S100 A9 exhibited the corresponding trend, when comparing the number of peptides between healthy, diseased and treated eyes (Table 7).

In view of the nature of the treatment (minor salivary gland transplantation) it is noteworthy that in the untreated diseased eye, none of the typical proline rich proteins (PROL1 or PRR4) described in tears [15] were detected. Both proteins, which are also known as abundant saliva proteins [1], (re) appear in the treated eye (Table 8).

In the same context we would like to mention the trend observed for lacritin. This is known to be a potent secretagogue for the various tear glands [13], and we previously demonstrated that minor salivary glands secrete substantial amounts of lacritin [2]. This gave rise to the hypothesis that part of the success of the transplantation surgery could be due to the beneficial effect of increased lacritin concentration in the treated eye. The present data are in line with this, as they show a consistent trend when focusing on the peptide spectral matches for lacritin (Table 9). Lacritin is detected with more representative peptides in the healthy versus the dry eye tear, whereas after minor salivary gland transplantation the number of lacritin peptide spectral matches steadily increases from 1 to 2 months post surgery.

Another observation (data not detailed) is that various immunoglobulin chains appear upregulated in the untreated diseased eye (consistent with inflammation), compared to both the healthy and the treated condition.

4. Concluding remarks

It is evident that the high sensitivity and precision as well as the robustness of the analytics described above allows one to observe relevant differences in the protein composition of tears without the need for sample pooling, thus for each sampling individually. Nonetheless, it shall be clear that larger numbers of samples in

---

**Table 7**

| Sample       | UniProt Accession| P06702 | Sum (coverage) | Sum (# proteins) | Sum (# unique peptides) | Sum (# peptides) | Sum (# PSMs) |
|--------------|------------------|--------|----------------|------------------|------------------------|------------------|-------------|
| Healthy      | Protein S100-A9 OS = Homo sapiens GN = S100A9 PE = 1 SV = 1—[S10A9_HUMAN] | 66.67% | 1              | 6                | 6                       | 26               |             |
| Diseased     | Protein S100-A9 OS = Homo sapiens GN = S100A9 PE = 1 SV = 1—[S10A9_HUMAN] | 74.56% | 1              | 8                | 8                       | 32               |             |
| One month   | Protein S100-A9 OS = Homo sapiens GN = S100A9 PE = 1 SV = 1—[S10A9_HUMAN] | 42.98% | 1              | 4                | 4                       | 25               |             |
| treated      | Two months       | Protein S100-A9 OS = Homo sapiens GN = S100A9 PE = 1 SV = 1—[S10A9_HUMAN] | 42.98% | 1              | 4                | 4                       | 27               |             |

**Table 8**

| Sample       | UniProt accession| Sum (coverage) | Sum (# proteins) | Sum (# unique peptides) | Sum (# peptides) | Sum (# PSMs) |
|--------------|------------------|----------------|------------------|------------------------|------------------|-------------|
| Healthy      | Q99935, Proline-rich protein 1 OS = Homo sapiens GN = PROL1 PE = 1 SV = 2—[PROL1_HUMAN] | 33.06% | 1              | 8                | 8                       | 104          |
| Diseased     | Q99935, Proline-rich protein 1 OS = Homo sapiens GN = PROL1 PE = 1 SV = 2—[PROL1_HUMAN] | -    | -              | -                | -                       | -            |
| One month   | Q99935, Proline-rich protein 1 OS = Homo sapiens GN = PROL1 PE = 1 SV = 2—[PROL1_HUMAN] | 33.06% | 1              | 5                | 5                       | 29           |
| treated      | Two months       | Q99935, Proline-rich protein 1 OS = Homo sapiens GN = PROL1 PE = 1 SV = 2—[PROL1_HUMAN] | 31.85% | 1              | 5                | 5                       | 39           |
| Healthy      | Q16378, Proline-rich protein 4 OS = Homo sapiens GN = PRR4 PE = 1 SV = 3—[PRR4_HUMAN] | 28.36% | 1              | 5                | 5                       | 36           |
| Diseased     | Q16378, Proline-rich protein 4 OS = Homo sapiens GN = PRR4 PE = 1 SV = 3—[PRR4_HUMAN] | -    | -              | -                | -                       | -            |
| One month   | Q16378, Proline-rich protein 4 OS = Homo sapiens GN = PRR4 PE = 1 SV = 3—[PRR4_HUMAN] | 20.90% | 1              | 2                | 2                       | 9            |
| treated      | Two months       | Q16378, Proline-rich protein 4 OS = Homo sapiens GN = PRR4 PE = 1 SV = 3—[PRR4_HUMAN] | 23.88% | 1              | 3                | 3                       | 30           |

**Table 9**

| Sample       | UniProt accession| Sum (coverage) | Sum (# proteins) | Sum (# unique peptides) | Sum (# peptides) | Sum (# PSMs) |
|--------------|------------------|----------------|------------------|------------------------|------------------|-------------|
| Healthy      | Extracellular glycoprotein lacritin OS = Homo sapiens GN = LACRT PE = 1 SV = 1—[LACRT_HUMAN] | 43.48% | 1              | 14                   | 14               | 384         |
| Diseased     | Extracellular glycoprotein lacritin OS = Homo sapiens GN = LACRT PE = 1 SV = 1—[LACRT_HUMAN] | 34.78% | 1              | 13                   | 13               | 71          |
| One month   | Extracellular glycoprotein lacritin OS = Homo sapiens GN = LACRT PE = 1 SV = 1—[LACRT_HUMAN] | 43.48% | 1              | 13                   | 13               | 114         |
| treated      | Two months       | Extracellular glycoprotein lacritin OS = Homo sapiens GN = LACRT PE = 1 SV = 1—[LACRT_HUMAN] | 43.48% | 1              | 17                   | 17               | 239         |
combination with extensive standardized (reference) libraries of identities (as well of quantities) of human tear proteins are required to enable clinicians to deduce medically or pharmaceutically pertinent information from nanoUPLC MSMS data acquired according to the above described protocol. As such this paper is not a conventional biomarker discovery study, but purely a proof-of-concept technical note that demonstrates and evaluates the analytical feasibility for identifying medically relevant proteins from individual patient samplings of clinically obtained tear fluid. We moreover show that the workflow allows a differential protein analysis in tear fluid from a single individual during clinical treatment.

Having shown this, we actually have strong reservations whether a bottom-up proteomics approach, as in the typical scientific literature cited above, i.e., semi-quantitative protein identifications per se, is appropriate to provide the physician with the type of data (biomarker (candidate) s) desired. In many cases not the mere presence or absence of a gene product, but rather the (relative) quantities of specifically modified proteoforms and/or protein fragments/peptides will hold the clinically relevant information, and these are generally not distinguishable in bottom-up analyses.

We are, therefore, currently optimizing a complementary (alternative) top-down strategy, which is likewise compatible with the Schirmer strip sampling method, and which focuses on the qualitative as well as quantitative analysis of the many endogenous tear peptides and protein fragments/isoforms which can be detected by nanoUPLC quadrupole-Orbitrap tandem MS. Instead of relying on protein identifications by database searching, the latter approach much more depends on de novo sequencing.

Acknowledgements

The authors thankfully appreciate the stimulating discussions and support by Dr. Dagmar Hoeben and Mr. Lieven De Baere (Allergan N.V., Hoeilaart, Belgium). Also the continued analytical care and assistance by Dr. Markus Kellmann, Dr. Tabiwang Arrey, and Dr. Thomas Moehring (Thermo Fisher, Bremen, Germany) are gratefully acknowledged. Finally we greatly value the encouragement and sponsorship by Mr. Zachary de Silva (http://australian-dryeye.webs.com).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.euprot.2015.06.005.

Reference

[1] F.M.L. Amado, R.P. Ferreira, R. Vitorino, One decade of salivary proteomics: current approaches and outstanding challenges, Clin. Biochem. 46 (2013) 506–517.
[2] R. Crebecq, P. Raus, M. Krishnan, M. Pinke, L. Slaets, G. Laurie, N. Hellings, P. Verhaert, Quantitative comparative proteomics of human tear fluid and labial saliva provides physiological/biochemical evidence supporting salivary gland transplantation for dry eye treatment, Conference Program and Abstract Book, Taormina, Sicily (Italy), Sep. 18–21, in: D.A. Sullivan (Ed.), 7th International Conference on the Tear Film & Ocular Surface: Basic Science and Clinical Relevance, 20132013, pp. 104–105.
[3] G.A. de Souza, L.M.P. de Godoy, M. Mann, Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors, Genome Biol. 7 (2006) R72.
[4] A.M. Falick, W.S. Lane, K.S. Lilley, M.J. MacCoss, B.S. Phinyomark, N.E. Sherman, S.T. Weinstein, H.E. Wirkowska, N.A. Yates, ABREF-PRECOS7, advanced quantitative proteomics study, J. Biomol. Technol. 22 (2011) 21–35.
[5] C. Geerling, J. Mursabe, P. Raus, Minor salivary gland transplantation, in: G. Geerling, H. Brewitt (Eds.), Surgery for the dry eye, Karger, Zürich, Dev. Ophthalmol. (2008) 243–254.
[6] N. González, I. Boro, J.A. Durán, F. Elorza, T. Suárez, Evaluation of inter-day and inter-individual variability of tear peptide/protein profiles by MALDI-TOF MS analyses, MBL. Vis. 18 (2012) 1572–1582.
[7] J. Mursabe, J. Németh, H. Hob, P. Raynal-Hekimian, J. Horvath-Winter, A. Agarwal, C. Baudouin, J.M. Benítez del Castillo, S. Cervenka, L. Chen-Zhu, A. Dicasse, J. Durán, F. Holly, R. Javate, J. Nepp, F. Paulsen, A. Rahimi, P. Raus, O. Shalaby, P. Sieg, H. Soriano, D. Spinelli, S.H. Uguras, G. Van Setten, The triple classification of dry eye for practical clinical use, Eur. J. Ophthalmol. 15 (2005) 660–667.
[8] G.S. Omena, The human eye proteome project, Proteomics 13 (2013) 2375–2376.
[9] J.H.M. Quah, L. Tong, S. Barbier, Patient acceptability of tear collection in the primary healthcare setting, Optom. Vis. Sci. 91 (2014) 452–458, doi:http://dx.doi.org/10.1097/OPX.0000000000000188.
[10] R.D. Sembra, J.J. Engiligh, V. Venkatraman, T.F. Dyrhuland, J.E. Van Eyk, The human eye proteome project: perspectives on an emerging proteome, Proteomics 13 (2013) 2500–2511.
[11] C. Salissova, N. Tajouri, A. Hainard, P.R. Burkhard, P.J. Lalive, N. Turck, Exploring the human tear fluid: discovery of new biomarkers in multiple sicaliursis, Prot. Clin. Appl. 8 (2014) 185–194, doi:http://dx.doi.org/10.1002/prca.2013000053.
[12] S. Simivasan, M. Thangavelu, L. Zhang, K.B. Green, K.K. Nichols, iTRAQ quantitative proteomics in the analysis of tears in dry eye patients, Invest. Ophthalmol. Vis. Sci. 53 (2012) 5052–5055, doi:http://dx.doi.org/10.1167/iovs.11-9022.
[13] T. Vignami, F.Y. Chen, S. Balasubbu, M. Gallup, R.L. McKown, G.W. Laurie, N.A. McNamara, Topical administration of lacritan is a novel therapy for aqueous-deficient dry eye disease, Invest. Ophthalmol. Vis. Sci. 55 (2014) 5401–5409.
[14] L. Zhou, R.W. Beuerman, C.M. Chan, S.Z. Zhao, X.R. Li, H. Yang, L. Tong, S. Liu, M. E. Stern, D. Yan, Identification of tear fluid biomarkers in dry eye syndrome using iTRAQ quantitative proteomics, J. Proteome Res. 8 (2009) 4889–4905.
[15] L. Zhou, S.Z. Zhao, S.K. Koh, L. Chen, C. Vaz, T. Tanawde, K.R. Li, R.W. Beuerman, In-depth analysis of the human tear proteome, J. Proteomics 75 (2012) 3877–3885.