SUPPORTING INFORMATION

Assessment of sample preparation bias in mass spectrometry-based proteomics

Frank Klont[†], Linda Bras[‡], Justina C. Wolters[§], Sara Ongay[†], Rainer Bischoff[†], Gyorgy B. Halmos[‡], Péter Horvatovich[†,*]

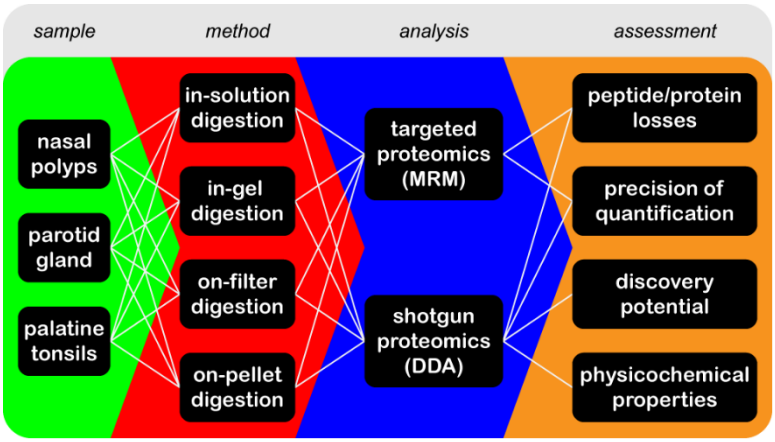
[†]Department of Analytical Biochemistry, Groningen Research Institute of Pharmacy, University of Groningen, 9713 AV Groningen, The Netherlands.

[‡]Department of Otorhinolaryngology, University Medical Center Groningen (UMCG), University of Groningen, 9713 GZ Groningen, The Netherlands.

[§]Department of Pediatrics, University Medical Center Groningen (UMCG), University of Groningen, 9713 GZ Groningen, The Netherlands.

*Corresponding author.

E-mail address: p.l.horvatovich@rug.nl (Péter Horvatovich).



Supplementary Figures:

Supplementary Tables:

Supplementary Methods:

Supplementary Figures

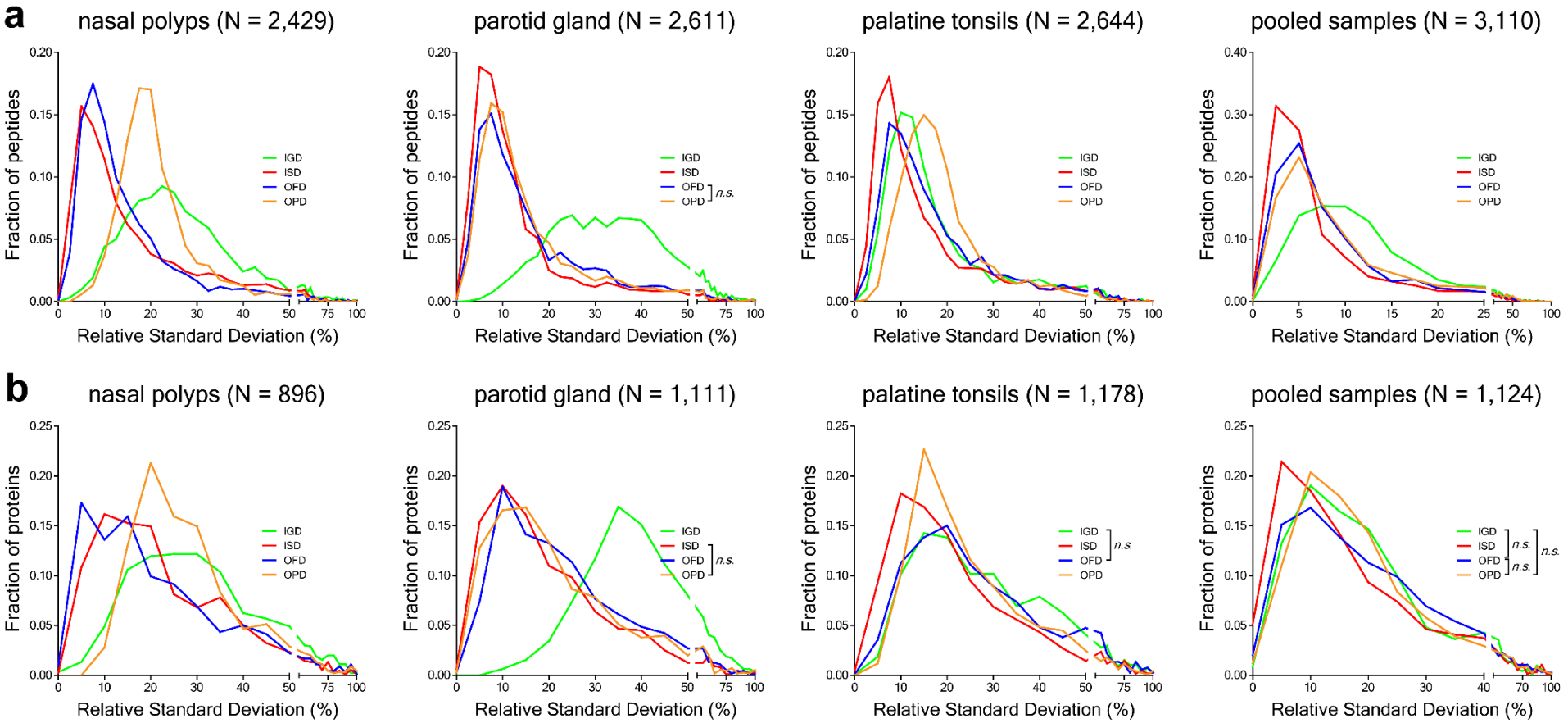


Figure S-1. Performance of the sample preparation strategies based on the methodological quantification (im)precision of (a) peptides and (b) proteins quantified by DDA without median scale normalization. For every tissue and for pooled sample analysis, statistically significant differences ($p < 0.05$, two-tailed Wilcoxon rank-sum test) were found between all strategies, unless specified otherwise in the figure.

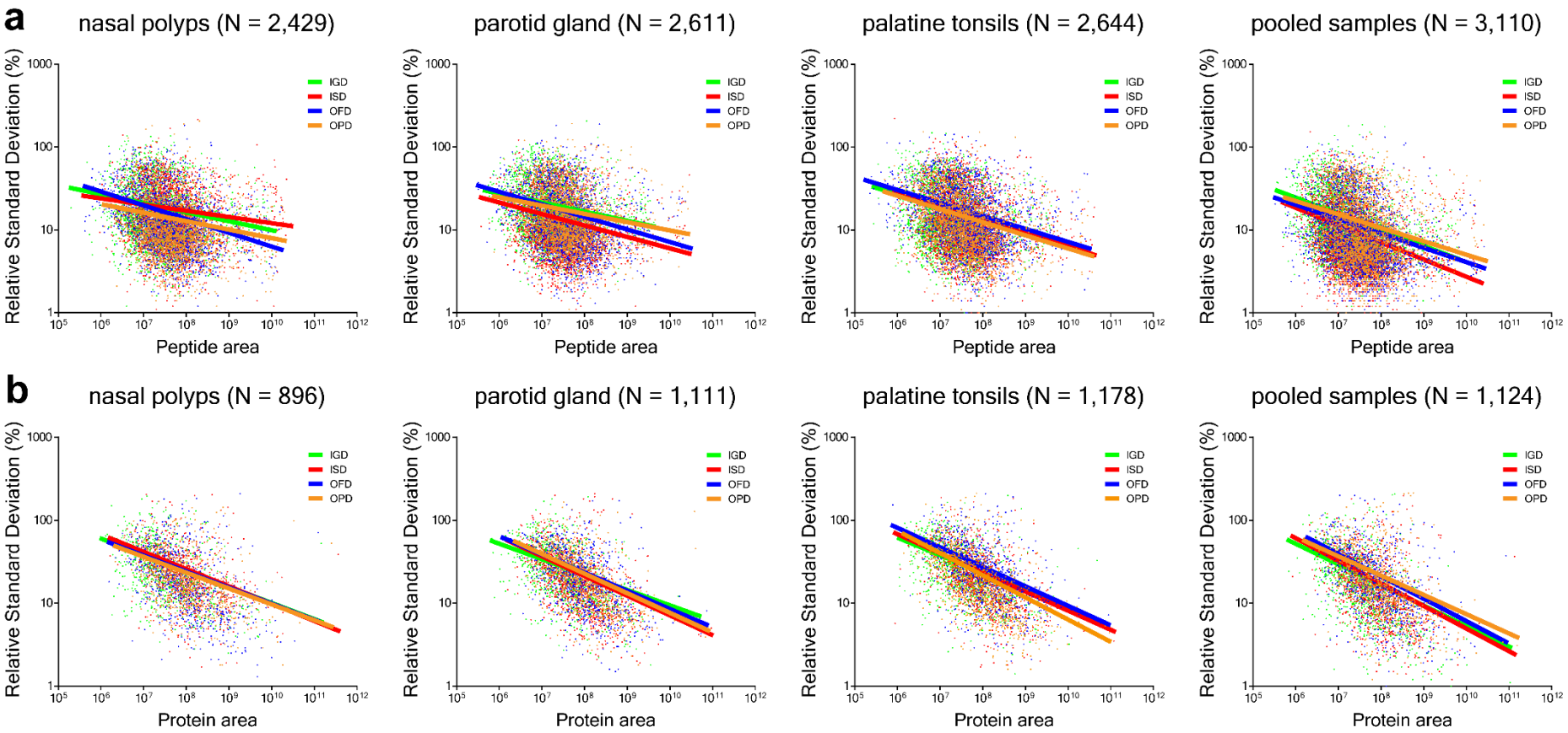


Figure S-2. Correlation between (a) peptide and (b) protein quantities and methodological quantification imprecision using median scale normalized data, as obtained by DDA.

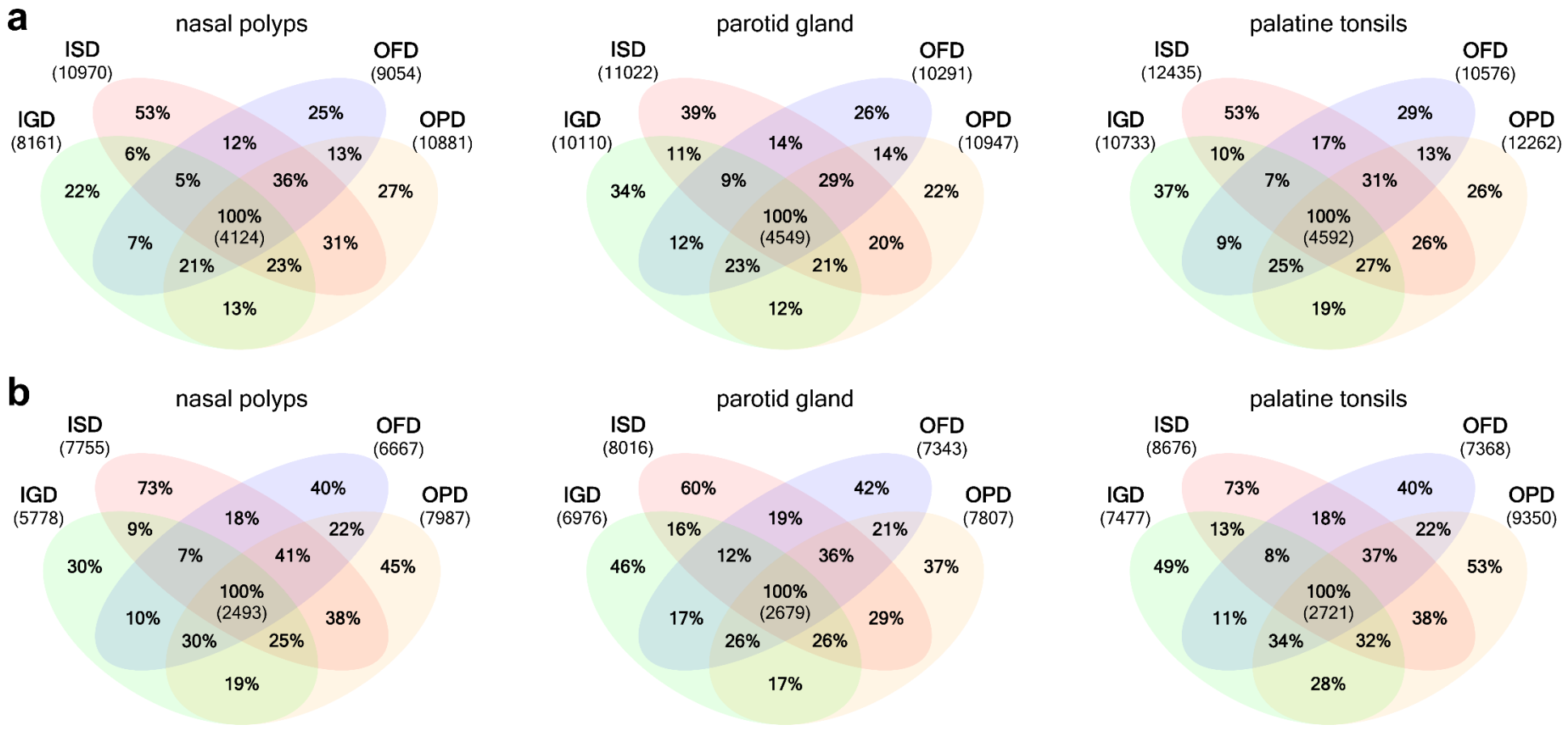


Figure S-3. Venn diagrams of peptides identified in at least (a) four out of five and (b) five out of the five replicates per sample preparation strategy for the different tissues.

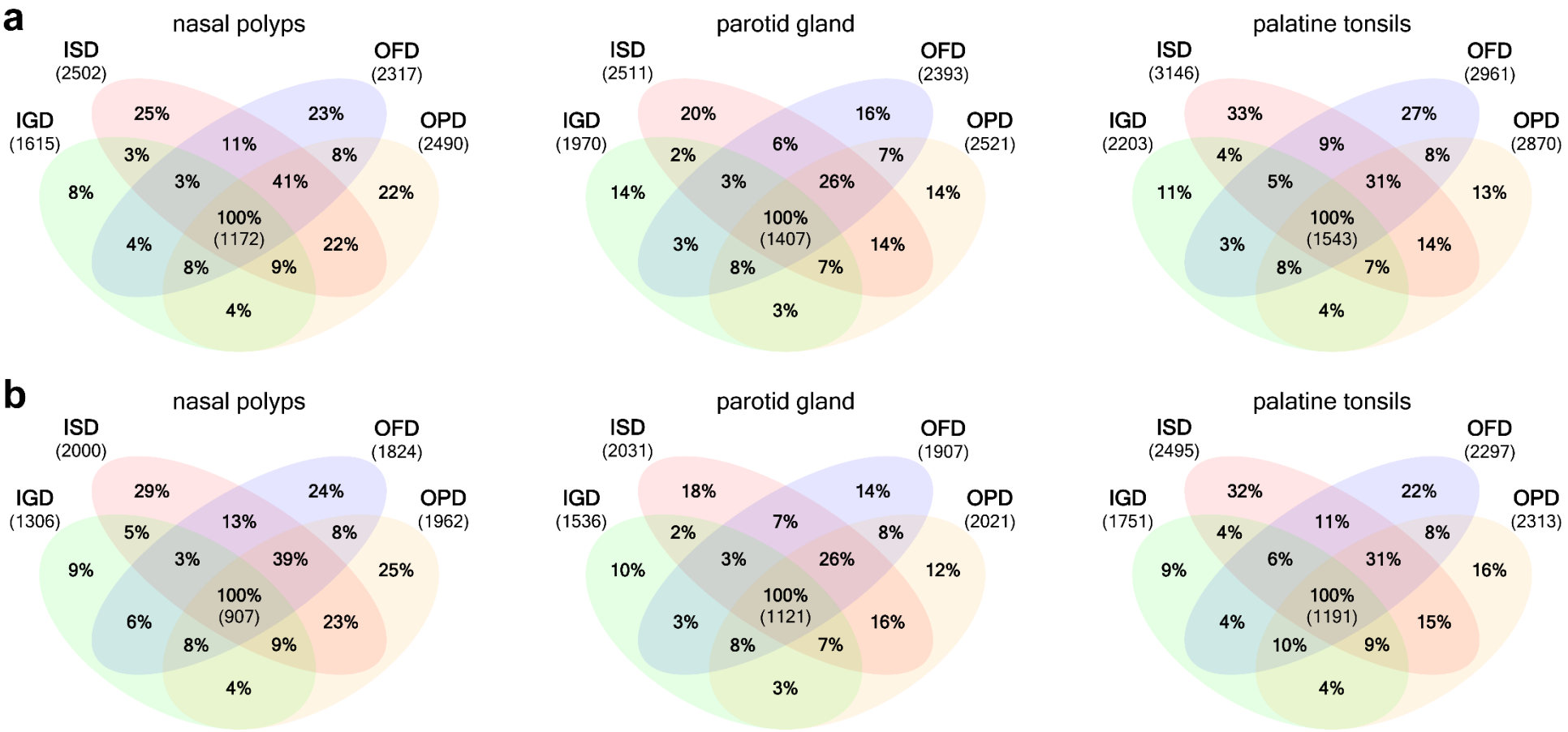


Figure S-4. Venn diagrams of proteins identified in at least (a) four out of five and (b) five out of the five replicates per sample preparation strategy for the different tissues.

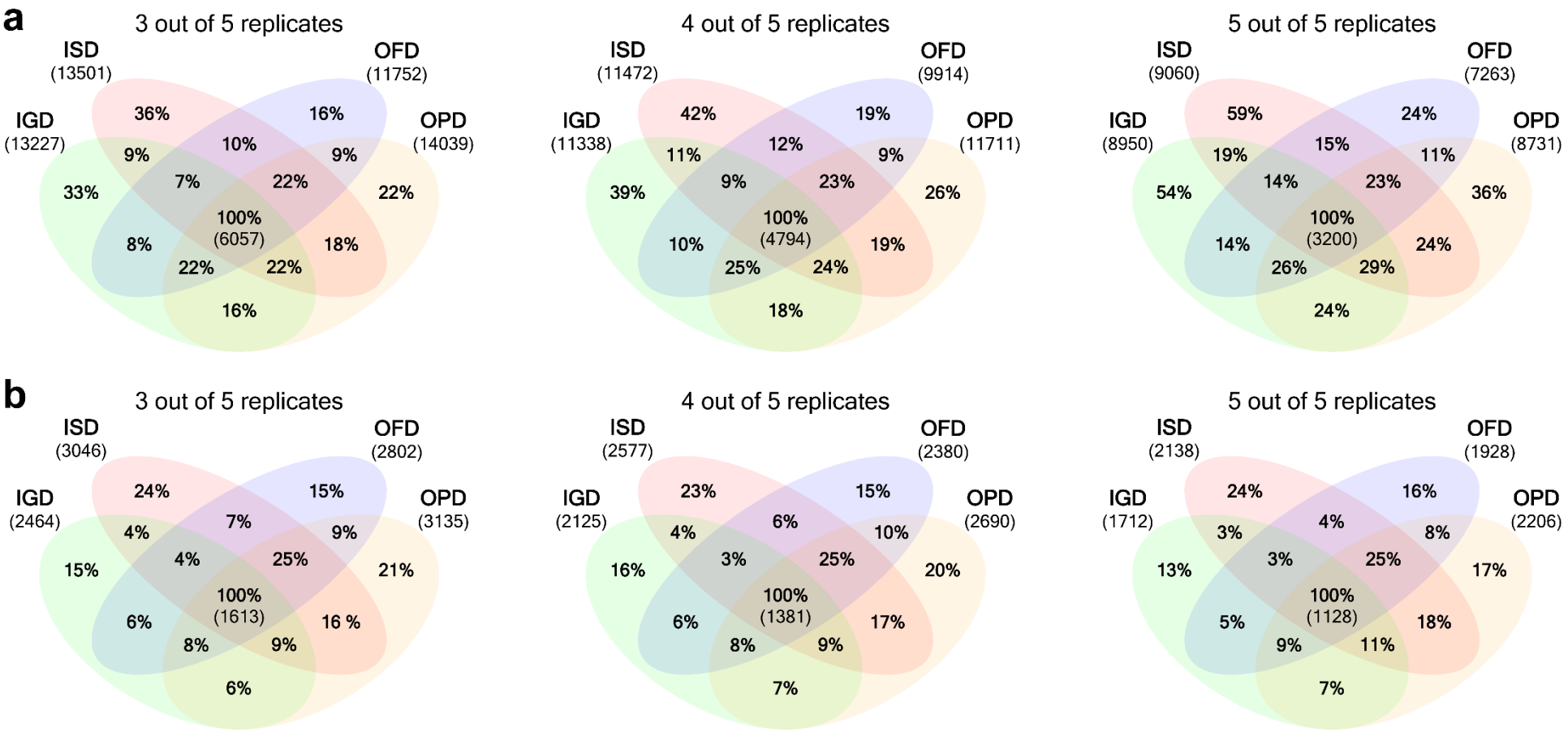


Figure S-5. Venn diagrams of (a) peptides and (b) proteins identified in at least three, four, and five out of the five replicates per sample preparation strategy for the pooled samples.

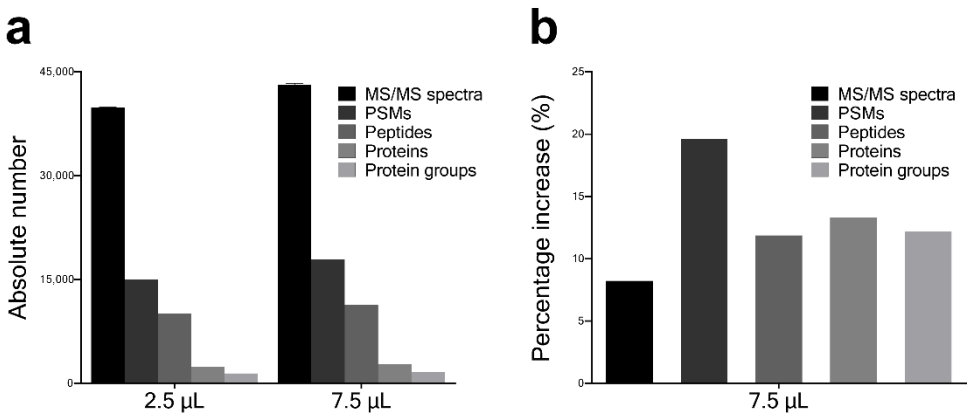


Figure S-6. Effect of increasing the injection volume on the amount of identifications using the IGD pooled sample. (**a**) Event statistics for the 2.5 µL and 7.5 µL injections of the pooled IGD sample (average of duplicate injections). (**b**) Percentage increases of MS/MS spectra and PSMs as well as peptide, protein group, and protein identifications following injection volume triplication (average of duplicate injections).

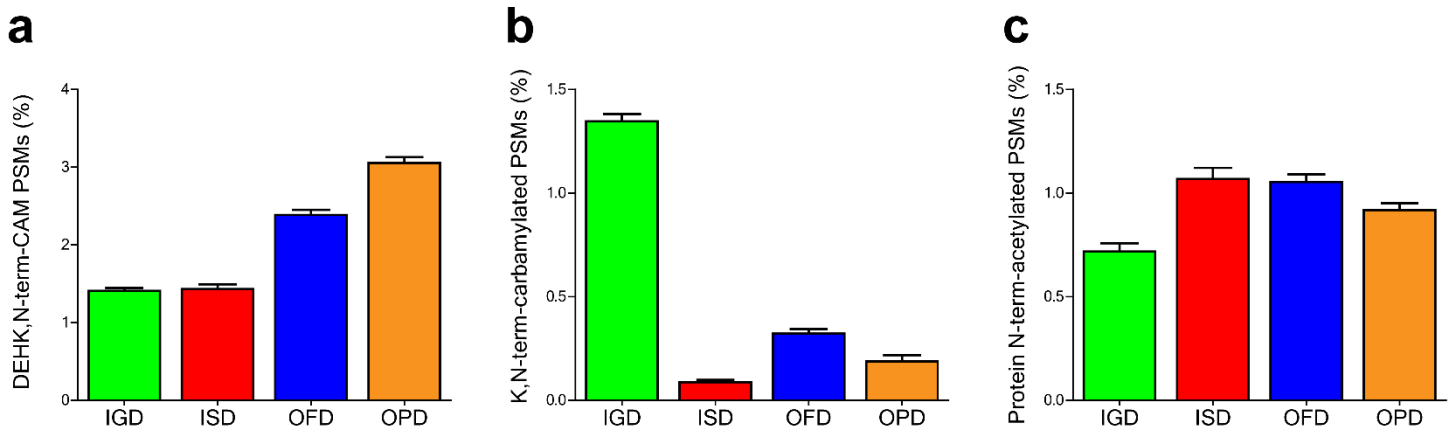


Figure S-7. Potentially relevant results of the combined PEAKS PTM and SPIDER searches for additional PTMs and sequence variants. Proportion of PSMs identified in the pooled samples containing (**a**) carbamidomethyl (CAM)-modified aspartic acid (D), glutamic acid (E), histidine (H), and/or peptide N-terminal (N-term) amino acid residues (relative to the total number of PSMs); (**b**) carbamylated lysines and/or N-terminal amino acids (relative to the total number of PSMs); and (**c**) N-terminally acetylated amino acids of PSMs encompassing the protein's N-terminus (relative to the total number of PSMs).

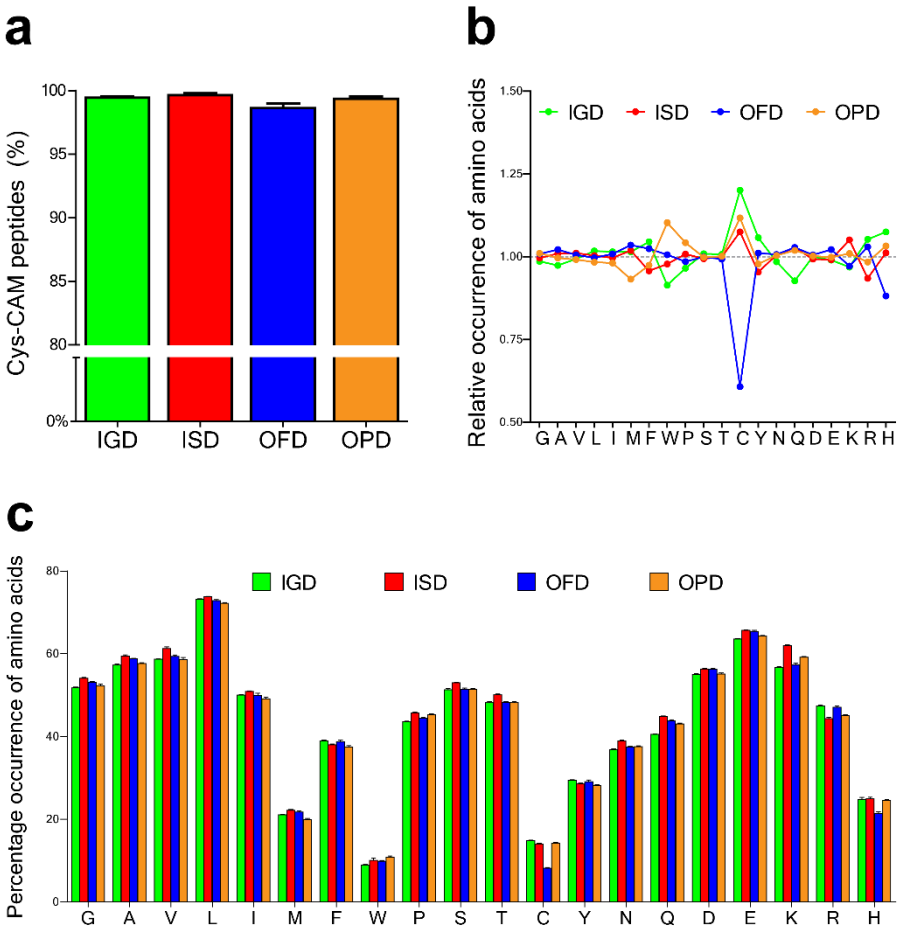


Figure S-8. (a) Percentage of peptides containing carbamidomethylated cysteine residues relative to the total number of identified cysteine carrying peptides for the pooled samples, (b) relative and (c) percentage occurrence of amino acids in the identified peptides for the pooled samples. Data in this graph are based on PEAKS searches using trypsin as protease (≤ 3 missed cleavages), and both cysteine carbamidomethylation and methionine oxidation as variable modifications (≤ 6 modifications per peptide).

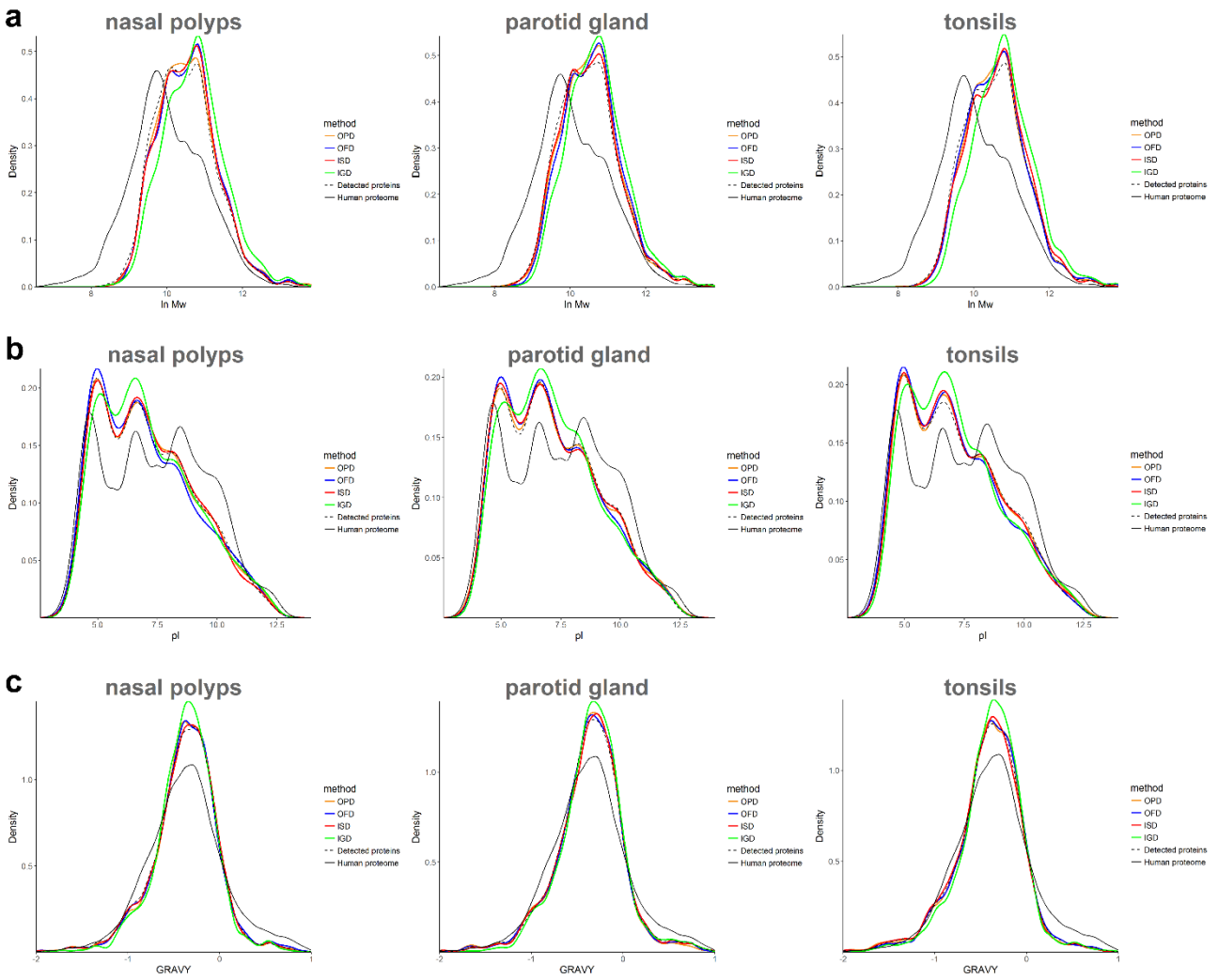


Figure S-9. Distribution of identified proteins according to (a) molecular weight, (b) pI, and (c) GRAVY based on proteins identified in three out of the five replicates per sample preparation strategy for the different tissues. Besides the green, red, blue, and red lines representing IGD, ISD, OFD, and OPD, respectively, graphs feature additional lines for the theoretical distributions of all proteins present in the human reference proteome (straight line) and the distributions of all proteins detected in any of the samples for a specific tissue (dashed line).

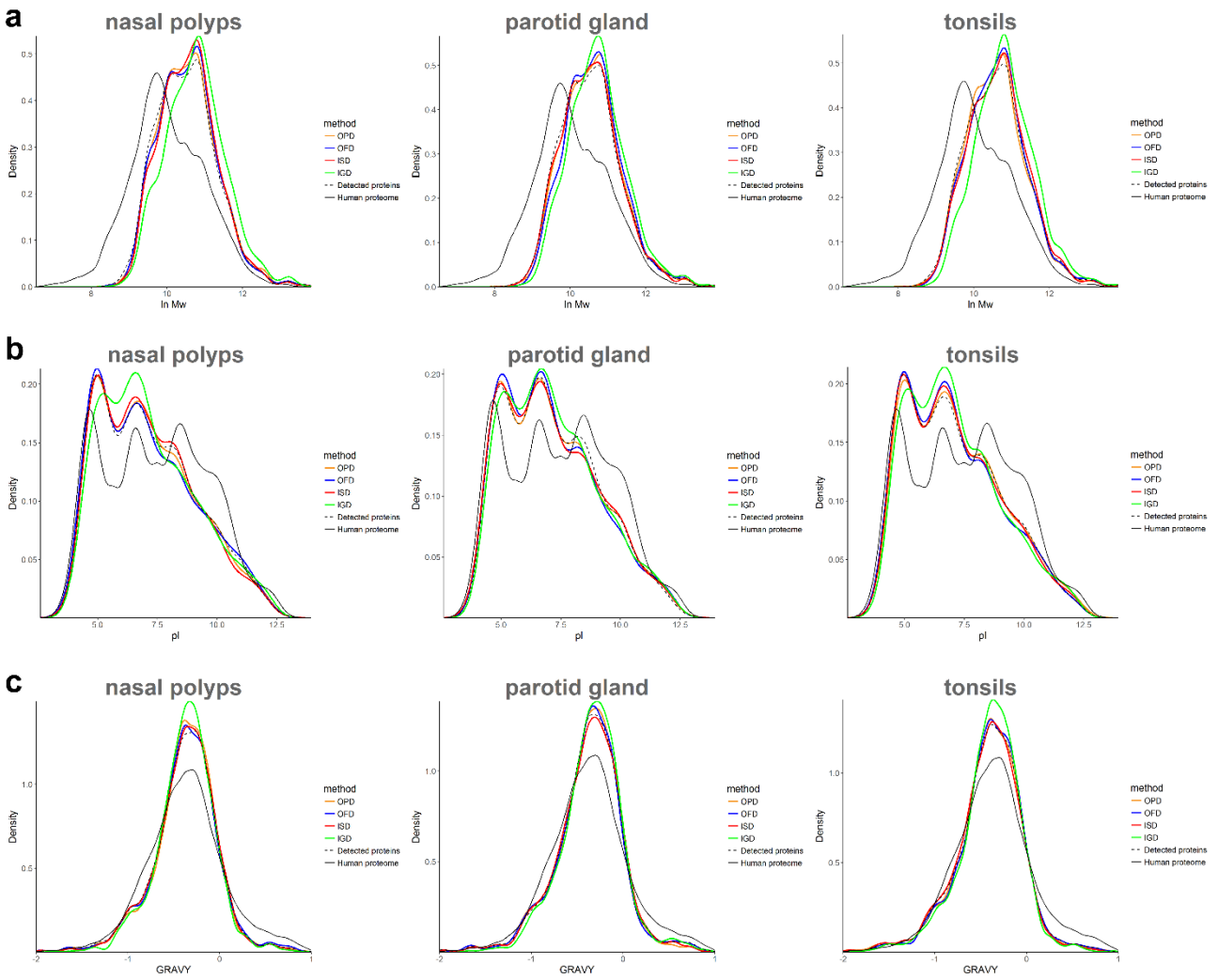


Figure S-10. Distribution of identified proteins according to (a) molecular weight, (b) pI, and (c) GRAVY based on proteins identified in four out of the five replicates per sample preparation strategy for the different tissues. Besides the green, red, blue, and red lines representing IGD, ISD, OFD, and OPD, respectively, graphs feature additional lines for the theoretical distributions of all proteins present in the human reference proteome (straight line) and the distributions of all proteins detected in any of the samples for a specific tissue (dashed line).

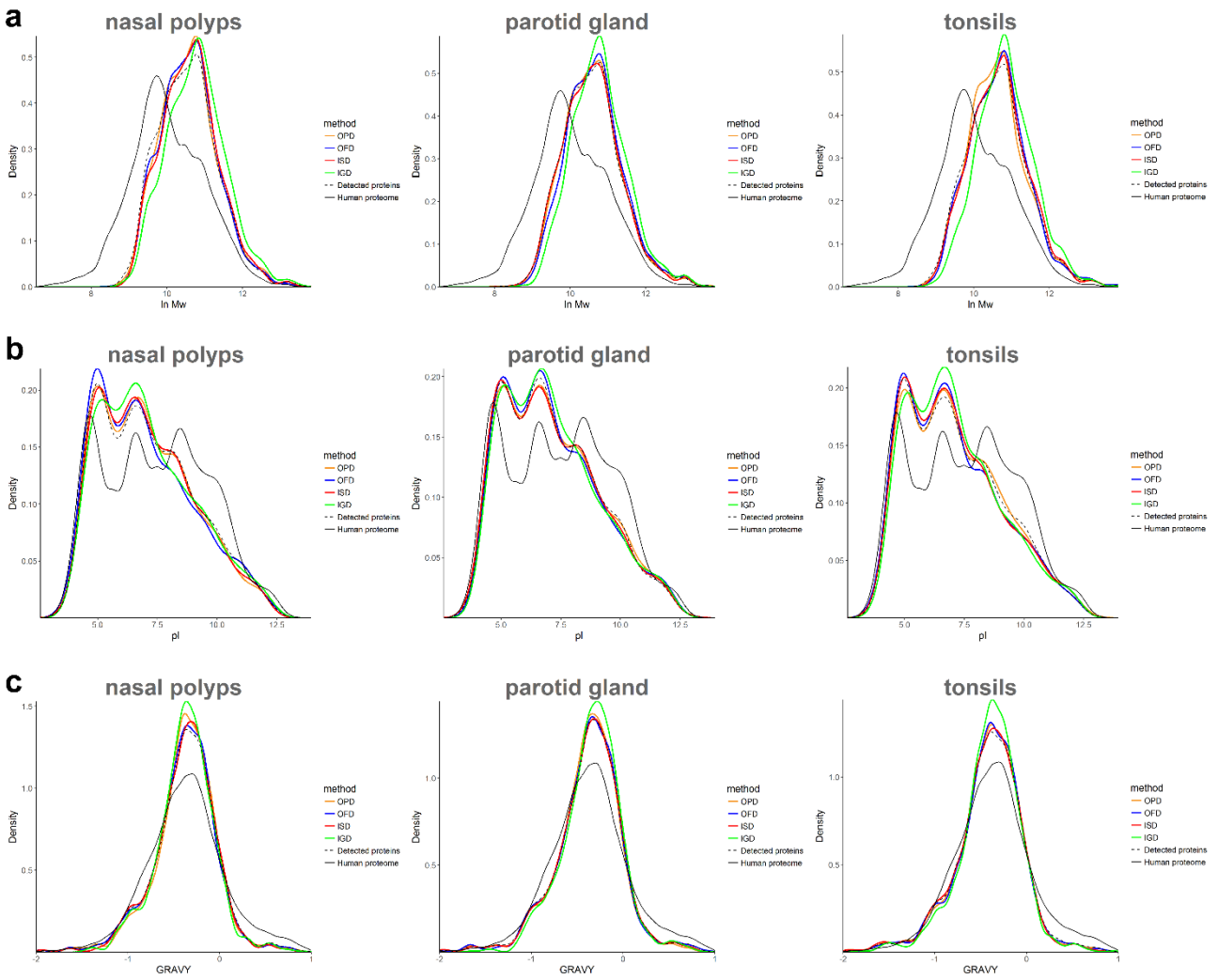


Figure S-11. Distribution of identified proteins according to (a) molecular weight, (b) pI, and (c) GRAVY based on proteins identified in five out of the five replicates per sample preparation strategy for the different tissues. Besides the green, red, blue, and red lines representing IGD, ISD, OFD, and OPD, respectively, graphs feature additional lines for the theoretical distributions of all proteins present in the human reference proteome (straight line) and the distributions of all proteins detected in any of the samples for a specific tissue (dashed line).

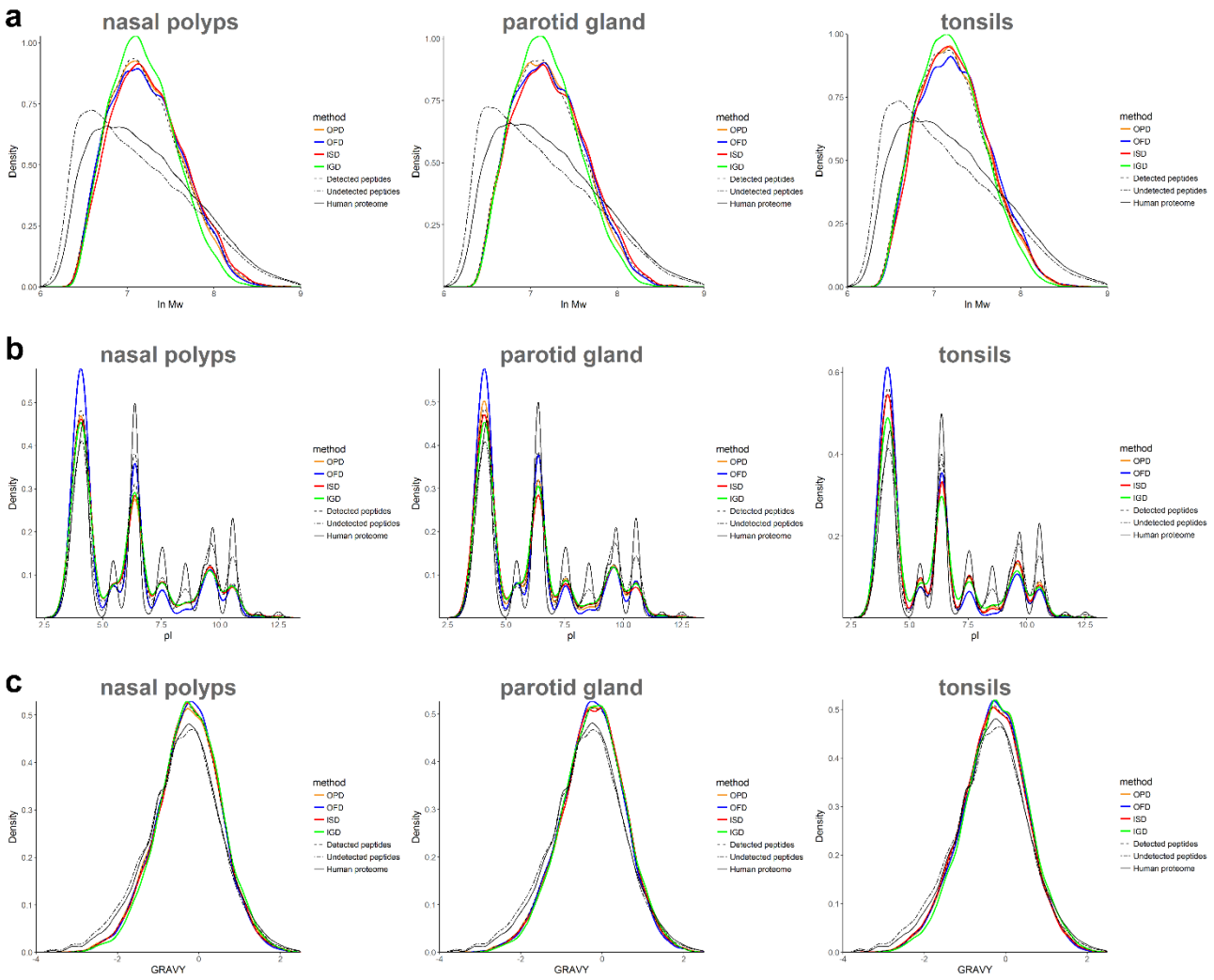


Figure S-12. Distribution of identified peptides according to (a) molecular weight, (b) pI, and (c) GRAVY based on peptides identified in three out of the five replicates per sample preparation strategy for the different tissues. Besides the green, red, blue, and red lines representing IGD, ISD, OFD, and OPD, respectively, graphs feature additional lines for the theoretical distributions of peptides derived from all proteins present in the human reference proteome (straight line), distributions of all peptides detected in any of the samples for a specific tissue (dashed line), and theoretical distributions of undetected peptides (at least five amino acids in length) derived from all proteins detected in any of the samples for a specific tissue (dash-dot line).

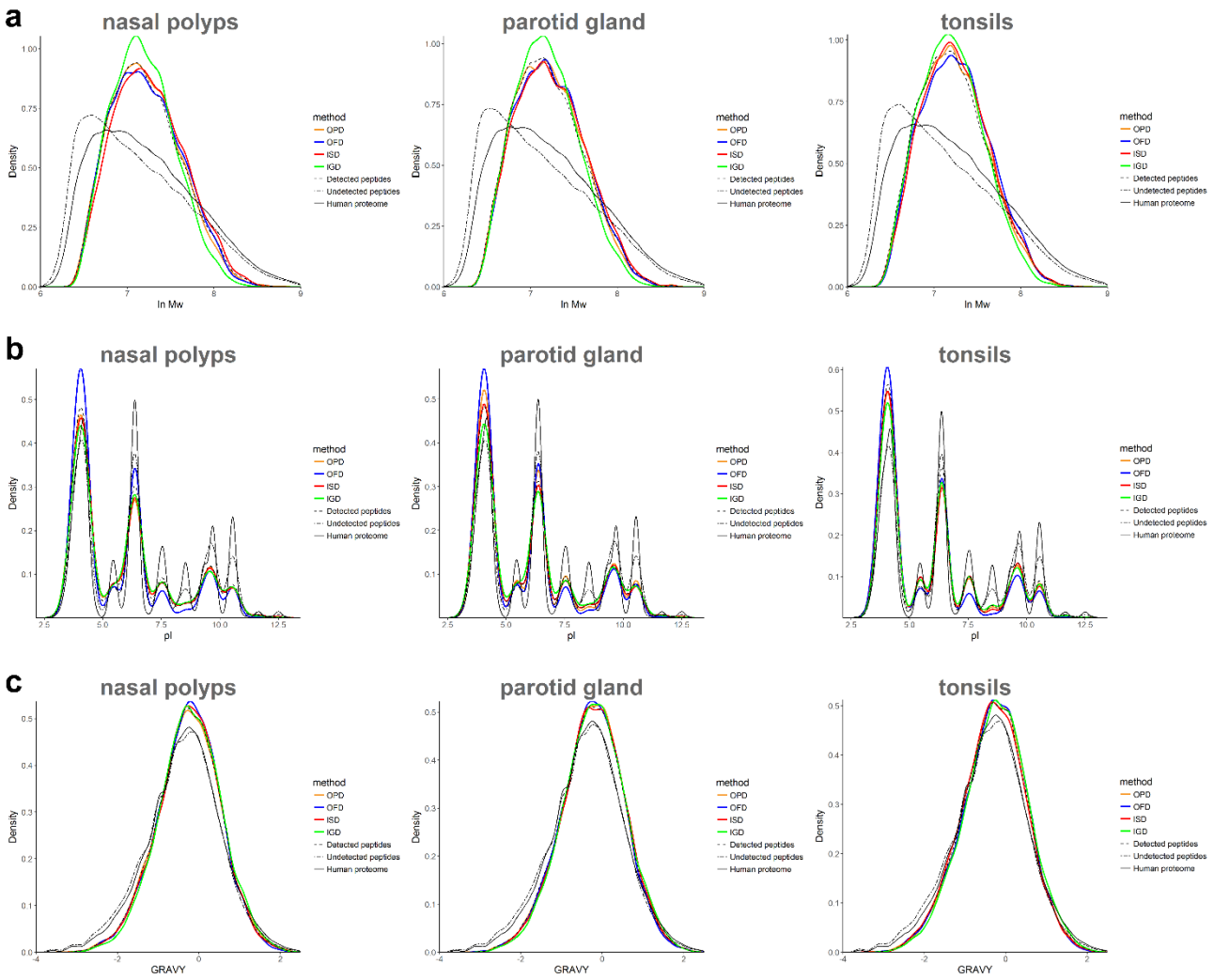


Figure S-13. Distribution of identified peptides according to (a) molecular weight, (b) pI, and (c) GRAVY based on peptides identified in four out of the five replicates per sample preparation strategy for the different tissues. Besides the green, red, blue, and red lines representing IGD, ISD, OFD, and OPD, respectively, graphs feature additional lines for the theoretical distributions of peptides derived from all proteins present in the human reference proteome (straight line), distributions of all peptides detected in any of the samples for a specific tissue (dashed line), and theoretical distributions of undetected peptides derived from all proteins detected in any of the samples for a specific tissue (dash-dot line).

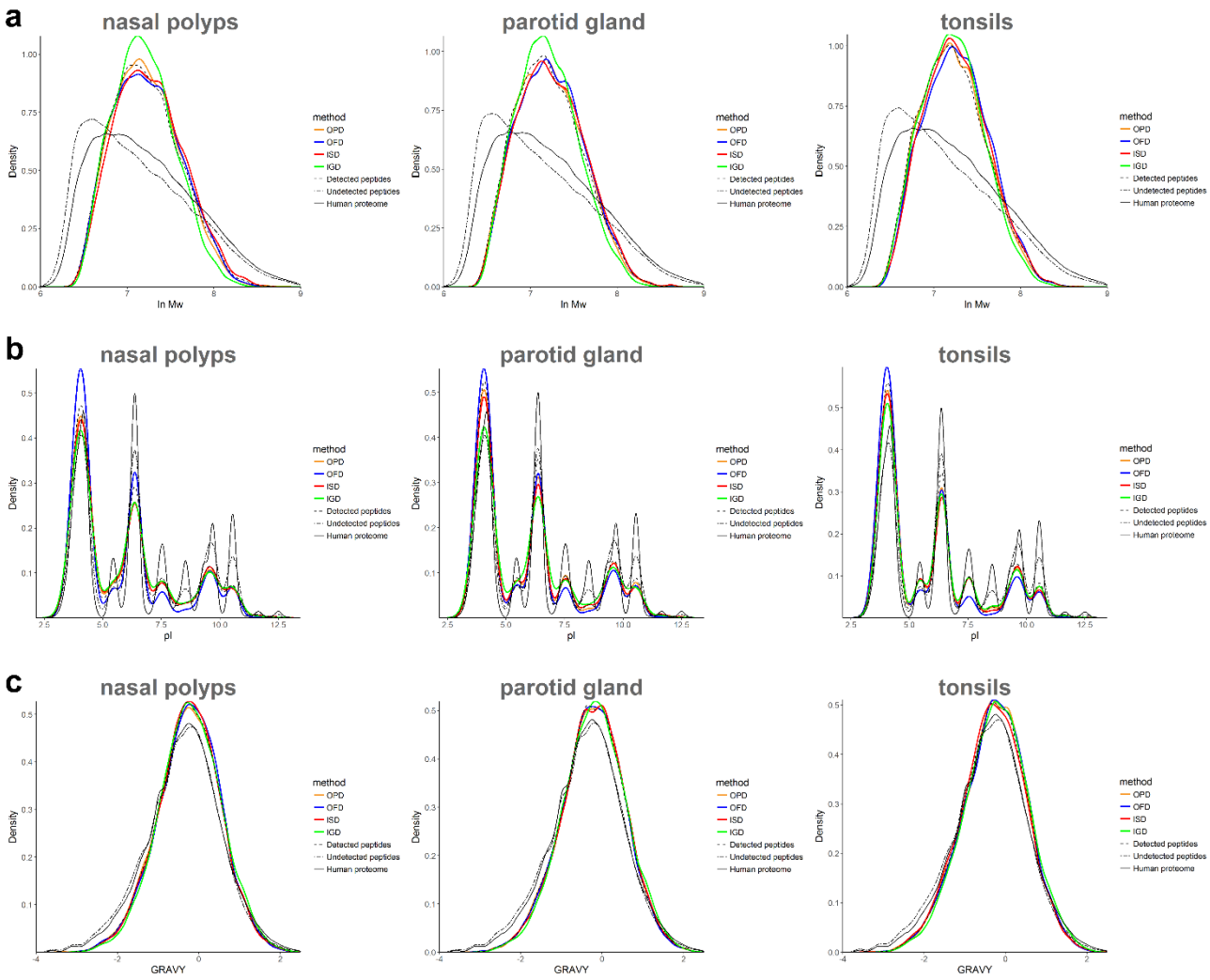


Figure S-14. Distribution of identified peptides according to (a) molecular weight, (b) pI, and (c) GRAVY based on peptides identified in five out of the five replicates per sample preparation strategy for the different tissues. Besides the green, red, blue, and red lines representing IGD, ISD, OFD, and OPD, respectively, graphs feature additional lines for the theoretical distributions of peptides derived from all proteins present in the human reference proteome (straight line), distributions of all peptides detected in any of the samples for a specific tissue (dashed line), and theoretical distributions of undetected peptides derived from all proteins detected in any of the samples for a specific tissue (dash-dot line).

Supplementary Tables

Table S-1. Overview of characteristics for the human nasal polyp, parotid gland, and palatine tonsil tissues that were used for this study.[a,b,c,d]

Tissue type	Cell types/tissue components	Indication of surgery
nasal polyps (NP)	- edematous stroma - epithelial cells (ciliated pseudostratified columnar, transitional, and squamous epithelium) - endothelial cells - inflammatory cells (mainly eosinophils, yet also a minority of T-cells, B-cells, mast cells, neutrophils, and macrophages)	nasal obstruction/ chronic rhinosinusitis
parotid gland (PG)	- epithelial cells (various types of columnar and cubic epithelium) - myoepithelial cells - connective tissue - serous secretory cells (with saliva) - adipocytes	benign salivary gland tumor (in a different part of the gland)
palatine tonsils (PT)	- non-keratinized stratified squamous epithelium - inflammatory cells (B-cells, T-cells, Langerhans cells, macrophages) - reticular cells - endothelial cells	chronic tonsillitis

[a] Junqueira, L. C.; Carneiro, J. *Functionele Histologie*, 11th ed.; Reed Business: Amsterdam, The Netherlands, 2007.
[b] Bailey, B. J.; Johnson, J. T. *Head and Neck Surgery – Otolaryngology*, 4th ed.; Lippincott Williams & Wilkins: Philadelphia, PA, USA, 2006.
[c] Amano, O.; Mizobe, K.; Bando, Y.; Sakiyama, K. *Acta Histochem. Cytochem*. **2012**, 45, 241-250.
[d] Jovic, M.; Avramovic, V.; Vlahovic, P.; Savic, V.; Velickov, A.; Petrovic, V. *Rom. J. Morphol. Embryol*. **2015**, 56, 371-377.

Table S-2. Descriptive statistics for the assessment of method-induced losses of peptides quantified by MRM as well as peptides and proteins quantified by DDA.

		Nasal Polyps				Parotid Gland				Palatine Tonsils				Pooled Samples			
Experiment	Descriptive	IGD[a]	ISD[a]	OFD[a]	OPD[a]	IGD[a]	ISD[a]	OFD[a]	OPD[a]	IGD[a]	ISD[a]	OFD[a]	OPD[a]	IGD[a]	ISD[a]	OFD[a]	OPD[a]
MRM	Median	26.8	100.0	83.7	70.9	29.8	77.8	97.6	94.9	28.1	79.3	100.0	61.5	32.0	79.9	100.0	72.3
(peptides)	Minimum	3.4	21.5	24.9	41.9	3.2	0.6	12.5	40.2	3.6	15.7	26.2	29.5	3.9	26.3	18.5	17.1
	25th %tile	20.8	94.6	67.6	63.8	22.4	61.0	77.3	86.2	22.9	63.8	85.7	54.1	27.0	67.6	99.9	63.7
	75th %tile	32.4	100.0	99.4	77.8	35.0	90.2	100.0	100.0	32.8	100.0	100.0	74.0	37.8	93.6	100.0	83.5
	Maximum	54.3	100.0	100.0	100.0	50.9	100.0	100.0	100.0	53.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0
DDA	Median	39.0	88.7	80.3	84.6	40.2	67.4	95.6	93.7	36.0	93.5	89.9	64.0	43.8	74.3	83.9	98.1
(peptides)	Minimum	0.0	1.1	0.1	0.5	0.1	0.5	0.8	0.2	1.8	0.5	0.8	1.8	1.1	0.6	0.9	1.6
	25th %tile	24.6	59.9	49.2	72.0	26.4	50.3	65.3	77.7	22.6	61.8	57.1	51.9	27.3	51.1	58.9	79.8
	75th %tile	57.0	100.0	100.0	100.0	56.5	84.1	100.0	100.0	52.3	100.0	100.0	79.0	61.3	93.7	100.0	100.0
	Maximum	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
DDA	Median	32.9	100.0	72.6	84.9	38.8	70.2	92.9	96.7	31.3	100.0	82.0	64.2	38.6	77.7	75.3	100.0
(proteins)	Minimum	0.3	1.2	4.9	0.8	0.1	1.2	5.1	4.6	0.8	0.3	3.6	0.9	0.3	0.2	1.2	1.3
	25th %tile	19.0	73.7	52.9	67.6	24.4	53.2	72.8	82.4	17.9	72.2	62.2	49.1	22.8	56.7	58.2	80.0
	75th %tile	50.3	100.0	92.1	100.0	58.1	87.5	100.0	100.0	48.2	100.0	100.0	82.0	60.3	100.0	92.2	100.0
	Maximum	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[a] Descriptive statistics for recovery data are presented as percentages and are based on relative average levels which are obtained by calculating the average level of peptides and proteins detected in all twenty replicates (four strategies, five replicates per strategy) per tissue, followed by setting the highest observed average level to 100%, and by relating the other three average levels to this 100%.

Table S-3. Descriptive statistics for methodological quantification (im)precision assessment of peptides quantified by MRM as well as peptides and proteins quantified by DDA using median scale normalized data.

		Nasal Polyps				Parotid Gland				Palatine Tonsils				Pooled Samples			
Experiment	Descriptive	IGD[a]	ISD[a]	OFD[a]	OPD[a]	IGD[a]	ISD[a]	OFD[a]	OPD[a]	IGD[a]	ISD[a]	OFD[a]	OPD[a]	IGD[a]	ISD[a]	OFD[a]	OPD[a]
MRM	Median	7.6	12.1	6.4	25.0	7.9	6.3	8.6	12.4	9.9	8.1	7.8	15.0	2.8	3.3	2.6	2.3
(peptides)	Minimum	1.3	2.9	1.8	15.1	2.7	1.9	2.4	3.6	4.1	2.0	2.3	6.8	0.5	0.4	0.8	0.6
	25th %tile	5.2	7.2	4.6	22.0	6.2	4.2	6.8	10.3	7.2	6.1	5.2	13.4	1.7	2.0	1.8	1.7
	75th %tile	10.6	23.2	11.0	27.7	11.1	10.0	12.2	15.9	13.3	13.3	10.8	18.7	4.3	5.5	5.4	4.7
	Maximum	33.5	40.4	60.0	62.5	29.9	38.2	52.1	37.9	31.6	41.9	30.1	50.5	35.2	27.2	53.1	41.9
DDA	Median	13.2	12.4	11.4	9.4	14.4	8.9	11.0	11.6	11.8	10.7	11.8	10.7	9.5	5.7	6.7	7.5
(peptides)	Minimum	1.2	0.7	1.2	1.2	1.8	0.5	1.3	0.7	0.9	0.9	1.0	0.6	1.2	0.7	0.3	0.7
	25th %tile	8.2	7.4	7.3	6.4	9.5	5.9	7.0	7.6	7.4	6.4	7.3	6.5	6.3	3.8	4.2	4.5
	75th %tile	23.1	23.6	19.0	15.5	23.6	15.3	21.0	20.6	21.2	20.5	21.7	17.8	16.4	10.7	12.7	15.1
	Maximum	136.1	215.0	182.7	205.9	206.5	121.3	188.4	191.3	148.9	221.5	142.2	153.0	186.4	153.1	132.1	142.9
DDA	Median	22.8	20.8	18.8	18.7	22.2	17.3	19.8	18.8	25.4	19.5	22.9	18.4	16.5	14.5	18.9	16.7
(proteins)	Minimum	2.5	1.7	1.3	1.8	1.8	1.9	1.5	1.8	1.4	0.5	2.0	1.6	0.8	0.9	0.8	0.8
	25th %tile	14.0	13.1	9.9	10.7	13.0	9.9	11.1	11.5	15.0	11.2	13.6	10.1	8.8	7.6	10.1	10.1
	75th %tile	38.8	34.9	33.6	31.1	35.8	30.4	34.3	31.8	40.6	32.0	39.6	31.9	28.1	27.0	31.7	27.5
	Maximum	195.3	205.1	209.4	168.8	192.7	209.5	171.5	165.8	198.6	187.6	208.6	211.0	206.9	197.4	200.5	213.8

[a] Descriptive statistics for quantification precision data are presented as percentages.

Table S-4. Descriptive statistics for methodological quantification (im)precision assessment peptides and proteins quantified by DDA without median scale normalization.

		Nasal Polyps				Parotid Gland				Palatine Tonsils				Pooled Samples			
Experiment	Descriptive	IGD[a]	ISD[a]	OFD[a]	OPD[a]	IGD[a]	ISD[a]	OFD[a]	OPD[a]	IGD[a]	ISD[a]	OFD[a]	OPD[a]	IGD[a]	ISD[a]	OFD[a]	OPD[a]
DDA	Median	24.9	11.5	11.2	19.7	34.0	9.5	12.2	12.0	14.0	11.0	14.0	17.0	11.0	5.1	6.8	7.7
(peptides)	Minimum	1.9	0.8	1.2	4.3	3.7	0.8	1.7	0.8	1.4	1.0	0.9	1.9	1.1	0.5	0.7	0.4
	25th %tile	17.8	6.5	7.2	16.0	24.3	5.9	7.2	7.8	9.8	6.8	8.8	12.8	7.0	3.3	4.1	4.5
	75th %tile	34.3	23.2	18.9	24.8	44.0	15.9	23.3	20.8	23.1	20.3	23.7	23.1	17.4	10.3	12.6	15.3
	Maximum	143.6	214.7	181.8	204.1	198.8	123.0	189.8	192.9	152.7	221.5	141.3	156.5	184.3	154.8	133.7	142.9
DDA	Median	31.0	20.2	18.6	26.2	40.1	17.0	20.5	18.4	27.4	19.1	25.1	22.2	17.5	13.8	18.4	17.2
(proteins)	Minimum	1.9	2.1	0.6	7.7	8.2	1.1	0.7	1.2	1.9	1.3	2.2	3.6	1.8	0.9	0.8	0.9
	25th %tile	20.6	11.8	9.5	19.8	32.5	9.6	11.9	11.3	16.9	11.6	16.4	15.0	10.2	7.2	9.7	10.8
	75th %tile	47.0	33.7	33.1	36.3	51.9	30.2	33.7	31.5	42.3	32.3	41.4	34.5	28.5	26.2	31.3	28.5
	Maximum	192.7	204.4	209.7	178.8	198.3	209.3	174.4	167.4	200.3	187.8	207.9	208.9	207.6	197.5	201.0	213.7

[a] Descriptive statistics for quantification precision data are presented as percentages.

Supplementary Methods

Method S-1. Tissue homogenization & protein extraction

Tissue was homogenized using a CryoMill cryogenic grinder (Retsch) with four 5 mL stainless steel cryogrinding jars each containing two 5 mm stainless steel grinding balls. After pre-cooling the jars for 8 minutes at 5 Hz, four pieces of frozen tissue were placed in each jar and were pulverized following 9 cryogrinding cycles of 3 minutes at 25 Hz with 3 minutes intermediate cooling at 5 Hz. Pulverized tissue was transferred to pre-weighed 2 mL low protein binding microcentrifuge tubes (Eppendorf, #022431102), and an aliquot of 0.1% (w/v) RapiGest (Waters, #186001861) in 50 mM ammonium bicarbonate (ABC) or sodium dodecyl sulfate (SDS)/urea lysis buffer (2% (w/v) SDS, 8 M urea and 100 mM β-mercapto-ethanol in 50 mM Tris/HCl buffer, pH 7.6) was added to get a final tissue concentration of 30 mg/mL. Next, vials were vortex-mixed for 5 minutes and subjected to 3 freeze/thaw cycles (frozen at -80 °C for 10 min & thawed at 30 °C for 10 min). Upon another 5 minutes of vortex-mixing and pelleting debris via centrifugation (10 min; 14,000 $\times$ *g*), the final lysates were collected. Protein concentration was determined using the micro bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, #23235) according to manufacturer's instructions, and lysates were stored at -80 °C until analysis.

Method S-2. In-solution digestion (ISD)

A volume of RapiGest protein extract corresponding to 20 µg of total protein was diluted to 40 µL with 50 mM ABC. Proteins were reduced in 10 mM dithiothreitol (DTT; Sigma-Aldrich, #D9779; 5 µL 90 mM DTT in 50 mM ABC) for 30 min at 60 °C (600 RPM), and were alkylated in the dark in 20 mM iodoacetamide (IAM; Sigma-Aldrich, #I1149; 5 µL 200 mM IAM in 50 mM ABC) for 30 min at 25 °C (600 RPM). After quenching unreacted IAM with a 0.5 molar excess of DTT (5.5 µL 90 mM DTT in 50 mM ABC) for 30 min at 25 °C (600 RPM), trypsin (Promega, #V5111; 1 µL 1 mg/mL trypsin in 50 mM ABC) was added in a final proteinase-to-protein ratio of 1:20 (w/w), and the proteins were digested overnight at 37 °C (600 RPM). Digestion was stopped and RapiGest was hydrolyzed through addition of 2.4 µL 50% (v/v) formic acid (FA) in Milli-Q water (H_2O), and the final peptide mixture was obtained after pelleting debris via centrifugation (10 min; 14,000 $\times$ *g*).

Method S-3. On-pellet digestion (OPD)

SDS/urea protein extract containing 20 µg of protein was diluted to 25 µL with 50 mM ABC. Proteins were precipitated through addition of 50 µL ice-cold 100% (v/v) acetone and two 50 µL aliquots of ice-cold 85% (v/v) acetone followed by centrifugation (5 min; 4 °C; 14,000 × *g*). The supernatant was removed and the precipitation step was repeated. After removing the supernatant of the second precipitation step, the pellet was left to dry by air. Subsequently, proteins were solubilized via pre-trypsination (25 µL 16 µg/mL trypsin in 50 mM ABC) in a final proteinase-to-protein ratio of 1:50 (w/w) for 4 hours at 37 °C (600 RPM). Proteins were reduced with 10 mM DTT (5 µL 60 mM DTT in 50 mM ABC) for 30 min at 60 °C (600 RPM), and were alkylated in the dark with 20 mM IAM (5 µL 140 mM IAM in 50 mM ABC) for 30 min at 25 °C (600 RPM). After quenching unreacted IAM with a 0.5 molar excess of DTT (6 µL 60 mM DTT in 50 mM ABC) for 30 min at 25 °C (600 RPM), trypsin (1 µL 1 mg/mL trypsin in 50 mM ABC) was added in a final proteinase-to-protein ratio of 1:20 (w/w), and the proteins were digested overnight at 37 °C (600 RPM). Digestion was stopped through addition of 1 µL 5% FA (v/v), and the final peptide mixture was obtained after pelleting debris via centrifugation (10 min; 14,000 × *g*).

Method S-4. In-gel digestion (IGD)

The in-gel digestion protocol was based on the "In-Gel Digestion and Sample Cleanup" protocol, as described previously in Wolters *et al*.[a] SDS/urea protein extract containing 20 µg of protein was diluted to 15 µL with 50 mM ABC. Subsequently, 5 µL of NuPAGE LDS Sample Buffer 4× (Thermo Fisher Scientific, #NP0007) was added, and the sample was boiled for 2 minutes. After cooling down to room temperature, the sample was loaded onto a NuPAGE 4-12% Bis-Tris Protein Gel (Thermo Fisher Scientific, #NP0321), and electrophoresis was carried out at 100 V for only 5 minutes allowing the proteins to enter the gel, but preventing their separation. Proteins were localized by staining the gel with Bio-Safe Coomassie Blue G-250 stain (Bio-Rad, #1610786) overnight, and unbound dye was washed away with repeated washes with H_2O. The stained protein band was excised, sliced in pieces of approximately 2 × 2 mm, and was completely destained via repeated washes with 30% (v/v) acetonitrile (ACN) in 100 mM ABC (15 min; 25 °C; 600 RPM). Gel pieces were dehydrated upon washing with 50% (v/v) ACN in 100 mM ABC (15 min; 25 °C; 600 RPM) and 100% ACN (5 min; 25 °C; 600 RPM) followed by drying in an oven at 37 °C (approx. 30 minutes). Next, proteins were reduced in 50 µL 10 mM DTT for 30 min at 60 °C (600 RPM), and, after discarding the DTT solution, alkylated in the dark in 50 µL 20 mM IAM for 30 min at 25 °C (600 RPM). Remaining IAM was discarded, and the gel pieces were dehydrated as described above. Subsequently, gel pieces were reswollen on ice following dropwise addition of digestion buffer containing trypsin (25 µL 40 µg/mL trypsin in 50 mM ABC) in a final proteinase-to-protein ratio of 1:20 (w/w), and the proteins were digested overnight at 37 °C (600 RPM). After digestion, the residual liquid was collected and remaining peptides were extracted in 25 µL 5% (v/v) FA in 75% (v/v) ACN for 20 min at 25 °C (600 RPM). After combining the two volumes, peptides were dried in a CentriVap vacuum concentrator (Labconco) at 45 °C, and the residue was reconstituted in 50 µL 0.1% (v/v) FA to obtain the final peptide mixture.

[a] Wolters, J. C.; Ciapaite, J.; van Eunen, K.; Niezen-Koning, K. E.; Matton, A.; Porte, R. J.; Horvatovich, P.; Bakker, B. M.; Bischoff, R.; Permentier, H. P. *J. Proteome Res*. **2016**, 15, 3204-3213.

Method S-5. On-filter digestion (OFD)

For on-filter digestion, the SDS/urea protein extract was processed according to the "FASP II" protocol, as described previously by Wisniewski *et al*,[a] with minor modifications. An amount of SDS/urea protein extract corresponding to 20 µg of protein was diluted with urea solution (8 M urea in 0.1 M Tris/HCl, pH 8.5) to 200 µL, and this dilution was loaded onto a Microcon Ultracel YM-30 filtration device (Merck Millipore, #MRCF0R030). After centrifugation (15 min; 14,000 × *g*), the concentrate was diluted with 200 µL of urea solution and was centrifuged again (15 min; 14,000 × *g*). Next, 100 µL 50 mM IAM in urea solution was added to the concentrate, the sample was mixed briefly (1 min; 25 °C; 600 RPM), and proteins were alkylated in the dark for 30 min at 25 °C. After centrifugation (15 min; 14,000 × *g*), the concentrate was diluted with 100 µL of urea solution and was centrifuged again (15 min; 14,000 × *g*). This step was repeated twice. Subsequently, the concentrate was diluted with 100 µL of 50 mM ABC, and was centrifuged (10 min; 14,000 × *g*). After repeating this second wash step twice, digestion buffer containing trypsin (40 µL 25 µg/mL trypsin in 50 mM ABC) in a final proteinase-to-protein ratio of 1:20 (w/w) was added to the filter, the sample was mixed briefly (1 min; 25 °C; 600 RPM), and proteins were digested overnight in a wet chamber at 37 °C. Peptides were collected by centrifuging the filter unit (10 min; 14,000 × *g*) followed by an additional elution step with 50 µL 50 mM ABC (10 min; 14,000 × *g*). After combining the two volumes, peptides were dried in a CentriVap vacuum concentrator (Labconco) at 45 °C, and the residue was reconstituted in 50 µL 0.1% (v/v) FA to obtain the final peptide mixture.

[a] Wisniewski, J. R.; Zougman, A.; Nagaraj, N.; Mann, M. *Nat. Methods*. **2009**, 6, 359-362.

Method S-6. Targeted LC-MS/MS analysis

Targeted proteomics analyses were performed using a TSQ Vantage Triple Quadrupole mass spectrometer (Thermo Fisher Scientific) using multiple reaction monitoring (MRM) transitions and settings that have been described previously.[a] Peptide separation was achieved with an UltiMate 3000 RSLC UHPLC system (Dionex) on a 50 cm Acclaim PepMap RSLC C18 analytical column (Dionex, #164540; 2 µm, 100 Å, 75 µm i.d. × 500 mm) which was kept at 40 °C. For targeted analyses, the final peptide mixtures were spiked with pre-digested QconCAT (quantification concatamers; designed to target a set of mitochondrial proteins, details have been described previously[21]) at a level of 1.25 ng per µg of total protein, and were placed in the thermostatted autosampler at 5 °C. A sample volume corresponding to 1 µg of total protein (based on the micro BCA assay) was loaded onto a Acclaim PepMap100 C18 trap column (Dionex, #160454; 5 µm, 100 Å, 300 µm i.d. × 5 mm) using µL-pickup with 0.1% (v/v) FA in H_2O at 20 µL/min. Subsequently, peptides were separated on the analytical column using a 100 min linear gradient from 3 to 60% eluent B (0.1% (v/v) FA in ACN) in eluent A (0.1% (v/v) FA in H_2O) at 200 nL/min.

[a] Wolters, J. C.; Ciapaite, J.; van Eunen, K.; Niezen-Koning, K. E.; Matton, A.; Porte, R. J.; Horvatovich, P.; Bakker, B. M.; Bischoff, R.; Permentier, H. P. *J. Proteome Res*. **2016**, 15, 3204-3213.

Method S-7. Shotgun LC-MS/MS analysis

Shotgun proteomics analyses were performed using an UltiMate 3000 RSLC UHPLC system (Dionex) connected to an Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) operating in the data-dependent acquisition (DDA) mode. A sample volume corresponding to 1 µg of total protein (based on the micro BCA assay) was injected from a thermostatted autosampler (5 °C) onto a Acclaim PepMap100 C18 trap column (Dionex, #160454; 5 µm, 100 Å, 300 µm i.d. × 5 mm) using µL-pickup with 0.1% FA (v/v) in H_2O at 20 µL/min. Peptides were separated on a 50 cm Acclaim PepMap RSLC C18 analytical column (Dionex, #164540; 2 µm, 100 Å, 75 µm i.d. × 500 mm;) which was kept at 40 °C, using a 117 min linear gradient from 3 to 40% eluent B (0.1% (v/v) FA in ACN) in eluent A (0.1% (v/v) FA in H_2O) at a flow rate of 200 nL/min. For DDA, survey scans from 300 to 1,650 m/z were acquired at a resolution of 70,000 (at 200 m/z) with an AGC target value of $3 \cdot 10^6$ and a maximum ion injection time of 50 ms. From the survey scan, a maximum number of 12 of the most abundant precursor ions with a charge state of 2^+ to 6^+ were selected for higher energy collisional dissociation (HCD) fragment analysis between 200 and 2,000 m/z at a resolution of 17,500 (at 200 m/z) with an AGC target value of $5 \cdot 10^4$, a maximum ion injection time of 50 ms, a normalized collision energy of 28%, an isolation window of 1.6 m/z, an underfill ratio of 1%, an intensity threshold of $1 \cdot 10^4$, and the dynamic exclusion parameter set at 20 s.

Method S-8. Data processing

Raw data for the targeted proteomics analyses were processed using the Skyline software (version 3.5), and were furthermore analyzed using Microsoft Excel 2013 (more details on processing of targeted proteomics data have been published previously[a]). Shotgun proteomics data were processed using PEAKS Studio software (version 8.0)[b], and peak lists were searched against the UniProtKB *homo sapiens* 'UP000005640' reference proteome (canonical; 70956 entries; downloaded on December 15, 2016) with trypsin selected as proteinase (≤ 3 missed cleavages), cysteine carbamidomethylation as fixed modification, methionine oxidation as variable oxidation, and allowing ≤ 6 modifications per peptide, ≤ 10.0 ppm precursor mass deviation (using monoisotopic mass), ≤ 0.2 Da fragment ion deviation, and ≤ 1.0% false discovery rates (FDR) for peptide-spectrum matches (PSMs), proteins and peptides for the principal analyses. For evaluation of cysteine carbamidomethylation efficiency, this modification was set to variable; for assessment of protocol-induced asparagine and glutamine deamidation, the corresponding modification was added as variable modification; and any further modification assessment was based on PEAKS PTM (including all 485 built-in modifications) and SPIDER searches on spectra with *de novo* ALC (Average Local Confidence) scores greater than 15%. Label-free quantification using ion counts was performed on the basis of the results of the principal PEAKS search, as described above, followed by further filtering and processing of the data using an in-house developed script in R (version 3.4.0) and R Studio (version 1.0.143). With respect to peptide quantification, peptide areas were summed for peptides with corresponding primary amino acid sequence thus including all PTMs and charge states. For protein quantification, areas of peptides belonging to the same protein group were summed, yet only if they were unique for the corresponding protein group. For both peptide and protein quantification, DDA data was scaled by median scale normalization[26].

[a] Wolters, J. C.; Ciapaite, J.; van Eunen, K.; Niezen-Koning, K. E.; Matton, A.; Porte, R. J.; Horvatovich, P.; Bakker, B. M.; Bischoff, R.; Permentier, H. P. *J. Proteome Res*. **2016**, 15, 3204-3213.
[b] Ma, B.; Zhang, K.; Hendrie, C.; Liang, C.; Li, M.; Doherty-Kirby, A.; Lajoie, G. *Rapid Commun. Mass Spectrom*. **2003**, 17, 2337-2342.

Method S-9. Bioinformatics analysis

Data analysis was performed using R, R studio, Microsoft Excel, and GraphPad Prism (version 5.00). For assessment of (relative) recovery and precision, peptides and proteins that were measured in all 20 samples originating from the same tissue (four sample preparation approaches, five replicates per approach) were included, and average levels as well as coefficients of variation (CV) were calculated for every approach, of which the latter values were used for evaluating the precision of the sample preparation method. For every peptide/protein, the highest average quantity was set to 100% and the other three average quantities were related to this maximum value thereby obtaining relative peptide/protein levels which were used for evaluation of the recovery of the sample preparation approaches.

Assessment of the discovery potential for each of the sample preparation methods was based on comparing the amounts of proteins and peptides identified (in three out of five, four out of five, and five out of five replicates) per method as well as based on comparisons of the trypsin cleavage efficiencies, the fractions of cysteine-containing peptides (relative to the total number of peptides), methionine oxidized peptides (relative to the total number of methionine containing peptides), and asparagine and/or glutamine deamidated peptides (relative to the total number of asparagine and/or glutamine containing peptides) for each method. Furthermore, relevant modifications as pointed out by the PEAKS PTM and SPIDER searches were included as well.

Comparisons of the selected physicochemical properties (*e.g.* molecular weight (MW), isoelectric point (pI), and hydrophobicity, expressed as grand average of hydropathy (GRAVY) scores calculated using the method of Kyte & Doolittle[a]) of the proteins and peptides identified in at least three out of five replicates per sample preparation method were enabled by using the R “Peptides” package (version 2.2, 2017-06-05). For visualization of the corresponding data, density plots were obtained using the R “ggplot2” package (version 2.2.1.9000, 2016-12-30).

[a] Kyte, J.; Doolittle, R. F. *J. Mol. Biol*. **1982**, 157, 105-132.