Table 1. *4-protmix iTRAQ protein relative quantitation. A: ChipLC coupled to a 6530 Q-ToF and analysed using MASCOT. B: 1200 nanoHPLC coupled to LTQ Orbitrap and analysed using Proteome Discover.*

A	6530 Q-ToF							
	ADH		ENO		GBP		BSA	
iTRAQ ratios	experimental	theoretical	experimental	theoretical	experimental	theoretical	experimental	theoretical
$iTRAQ_{115}/iTRAQ_{114}$	0.88 ± 0.09	0.75	1.68 ± 0.12	1.50	0.44 ± 0.05	0.37	6.50 ± 1.01	6.00
$iTRAQ_{116}/iTRAQ_{114}$	0.23 ± 0.04	0.25	0.33 ± 0.06	0.25	0.25 ± 0.06	0.25	0.40 ± 0.03	0.25
$iTRAQ_{117}/iTRAQ_{114}$	0.53 ± 0.07	0.50	1.00 ± 0.06	1.00	0.25 ± 0.04	0.25	3.99 ± 0.61	4.00

B	LTQ Orbitrap							
	ADH		ENO		GBP		BSA	
iTRAQ ratios	experimental	theoretical	experimental	theoretical	experimental	theoretical	experimental	theoretical
$iTRAQ_{115}/iTRAQ_{114}$	0.89 ± 0.05	0.75	1.87 ± 0.25	1.50	0.52 ± 0.06	0.37	6.21 ± 0.62	6.00
$iTRAQ_{116}/iTRAQ_{114}$	0.24 ± 0.03	0.25	0.25 ± 0.02	0.25	0.26 ± 0.02	0.25	0.17 ± 0.02	0.25
$iTRAQ_{117}/iTRAQ_{114}$	0.59 ± 0.03	0.50	1.19 ± 0.15	1.00	0.38 ± 0.05	0.25	3.93 ± 0.52	4.00
$iTRAQ_{117}/iTRAQ_{115}$	0.68 ± 0.02	0.66	0.70 ± 0.05	0.66	0.72 ± 0.02	0.66	0.65 ± 0.02	0.66
$iTRAQ_{116}/iTRAQ_{116}$	4.02 ± 0.44	3.00	6.88 ± 0.59	6.00	1.90 ± 0.08	1.50	**28.40 ± 6.37**	**24.00**
$iTRAQ_{117}/iTRAQ_{116}$	2.72 ± 0.19	2.00	4.85 ± 0.33	4.00	1.39 ± 0.08	1.00	17.81 ± 4.47	16.00

Table 2. *8-protmix iTRAQ protein relative quantitation measured with LTQ Orbitrap and analysed using Proteome Discover.*

	Eight protein mix (8-protmix)								
	LYS [a]	CYT [b]	GLY [c]	OVA [d]	CAH [e]	MYO [f]	BSA [g]	LAB [h]	theoretical protein ratios
$iTRAQ_{115}/iTRAQ_{114}$	2.15 ± 0.08	2.18 ± 0.09	2.48 ± 0.19	2.54 ± 0.11	2.20 ± 0.08	2.36 ± 0.14	2.19 ± 0.05	1.85 ± n.c.	2.0
$iTRAQ_{116}/iTRAQ_{114}$	2.12 ± 0.11	2.16 ± 0.10	2.20 ± 0.11	2.21 ±0.15	2.19 ± 0.15	2.15 ± 0.43	2.19 ± 0.06	1.93 ± n.c.	2.0
$iTRAQ_{117}/iTRAQ_{114}$	11.54 ± 0.54	11.98 ± 0.74	12.40 ± 1.49	13.97 ± 1.27	12.33 ± 0.24	12.61 ± 0.92	12.14 ± 0.26	8.79 ± n.c.	10.0
$iTRAQ_{117}/iTRAQ_{115}$	5.65 ± n.c.	5.20 ± 0.21	5.25 ± 0.06	5.48 ± 0.40	6.12 ± 0.36	5.52 ± 0.25	5.69 ± 0.31	n.d.*	5.0
$iTRAQ_{116}/iTRAQ_{116}$	1.04 ± 0.04	1.00 ± 0.01	1.10 ± 0.09	1.16 ± 0.07	1.00 ± 0.02	1.13 ± 0.16	1.02 ± 0.04	0.96 ± n.c. [§]	1.0
$iTRAQ_{117}/iTRAQ_{116}$	5.45 ± 0.40	5.43 ± 0.21	5.51 ± 0.28	6.48 ± 1.41	5.74 ± 0.17	5.96 ± 0.65	5.64 ± 0.02	4.56 ± n.c. [§]	5.0

In conclusion, we described two different samples suitable for iTRAQ analysis that can be used to assess the performance of different mass spectrometers and quantitation algorithms. A considerable part of the proteome of a typical biological sample falls within the fold change and dynamic range windows of these two samples.

Evaluation of MS^E based protein quantification methods

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LC-MS based protein quantification methods have recently gained popularity as powerful technologies capable of addressing protein expression analysis in complex samples. As an alternative to isotope labeling methodologies, which are sample-number and sample-type dependant and require special labeling chemistries, label-free approaches afford greater experimental design and less sample

manipulation. A relatively new label-free approach based on MS^E or data independent acquisition mode was applied in this study [1, 2]. Unlike the traditional LC-MS/MS strategy, no precursor ion selection and fragmentation is applied and the acquisition method is based on alternating the collision energy of sequential scans applied to the gas cell between low and elevated states (Figure 1). MS^E based acquisition collects information on all the isotopes of all charge-states of the eluting peptide precursor across the chromatographic peak width. MS^E provides accurate mass data for precursor and product ions which are used for protein identification and also precursor ion intensity measurement for relative protein quantification. Furthermore, absolute protein amount can be estimated by comparing the top three ionising peptides of each protein with a known amount of an internal standard [3]. In this study we evaluated the utility of estimated absolute quantification and comparative analysis of ion currents for the purpose of relative protein quantification on data acquired using the data independent acquisition method. Our results indicate that MS^E based label-free quantification is a robust and a reproducible approach for relative protein quantification in complex samples.

Two mixtures (ECOLI_1 and ECOLI_2) of digested ADH1_YEAST, PGYM_RABIT, ENO1_YEAST and ALBU_BOVIN proteins (Waters Corporation) were prepared at a relative molar ratio for each protein of 1:1, 1:0.5, 1:2 and 1:8 respectively and spiked into a digested E. coli cytosolic protein mixture (Waters Corporation). Data independent acquisition analyses were performed with a nanoAcquity UPLC system interfaced to a SYNAPT HDMS (Waters Corporation) mass spectrometer and samples were run in quintuplicate. Data were processed, searched and quantified, both absolute and relative, with a beta release of ProteinLynx GlobalSERVER v2.4 software. Absolute protein quantification was conducted with a standard protein added to the sample (ADH_YEAST) and its amount provided to the identification software. Absolute protein values were used to express relative quantification results between different samples. The relative quantification software performs a comparison between different samples based on deconvoluted peptide intensities and probabilistic relative protein quantification is subsequently calculated.

In this analysis a total of 525 different proteins were identified (protein FDR=4%), 425 in ECOLI_1 and 428 in ECOLI_2 samples. 257 proteins in ECOLI_1 and 251 in ECOLI_2 were identified in 3 or more replicates. 237 of them were identified in both samples (Figure 2. A, B) and therefore, relatively quantified. Both quantification approaches, comparative analysis of estimated absolute amount and ion current (probability) based, accurately determined the relative protein ratio of the eukaryotic proteins spiked into an E. coli protein matrix (table 1). We also used this approach for the relative quantification of total protein extracts from WT and $E2F2^{-/-}$ T lymphocytes. 736 proteins were identified and 288 were considered for quantification purposes. Obtained results indicate that both quantification approaches equally determine protein expression trends although numerical ratios can vary due to differential handling of protein isoforms and differences in statistical analyses (data not shown). Overall we can conclude that label-free LC-MS^E type of analysis has proven to be a robust and reproducible approach for relative protein quantification in complex samples. Furthermore, relative quantification based on estimated absolute quantification and on ion current comparison consistently measured protein expression ratios.

Table 1. *Expected and experimental ratios obtained for ALBU_BOVIN, ENO1_YEAST and PGYM_RABIT proteins.*

Protein	Expected ratio	Ratio based on absolute quantification	Ratio based on comparative ion current
albu_bovin	8.0	6.95	7.61
eno1_yeast	2.0	1.91	2.04
pgym_rabit	0.5	0.54	0.55

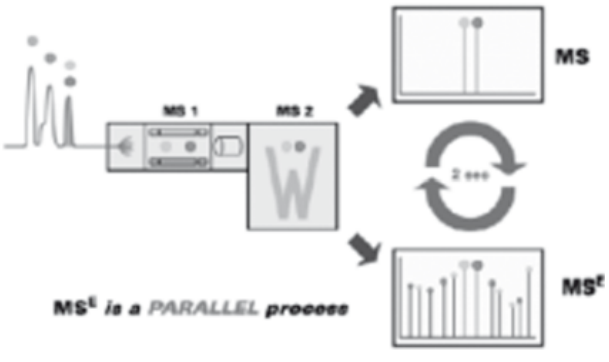

Figure 1. *Schematic of MS^E mode of analysis (figure from Waters Corporation).*

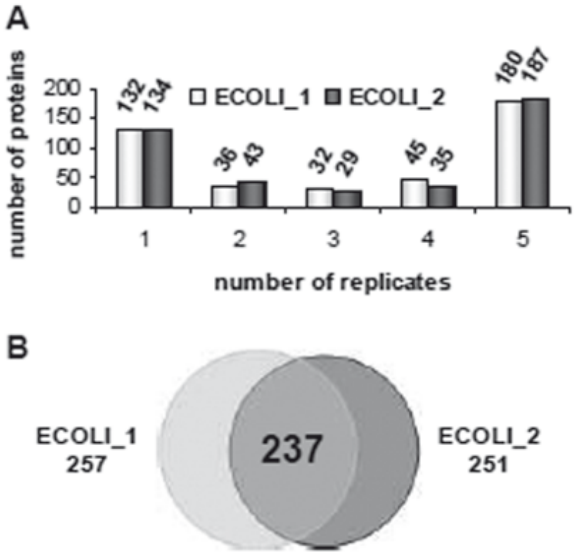

Figure 2. *Graphical representation of identified proteins. A. Number of proteins identified in ECOLI_1 and ECOLI_2 per number of replicates. B. Venn diagram of overlapping proteins identified in 3 or more replicates in ECOLI_1 and ECOLI_2.*

This work was supported by grants from the Basque Government Department of Industry (Etortek and Saiotek to J.M.A.). Proteomics Core Facility-SGIker is supported by UPV/EHU, MICINN, GV/EJ, ESF and ProteoRed agencies.

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Desarrollo de un Modelo Estadístico Universal para Proteómica Cuantitativa Mediante Marcaje Isotópico Estable

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Aunque en la actualidad existen diferentes métodos de marcaje isotópico para la realización de estudios de proteómica cuantitativa, los modelos estadísticos para el tratamiento de los datos obtenidos están todavía muy poco desarrollados. La mayoría de los modelos asumen distribuciones normales de los datos y la existencia de varianzas homogéneas, que no se han demostrado en la práctica. En el laboratorio de J. Vázquez hemos desarrollado recientemente un nuevo modelo estadístico jerárquico de efectos aleatorios para el análisis de los datos de expresión diferencial obtenidos por marcaje con $^{18}O/^{16}O$ y espectrometría de masas de trampa iónica lineal [1]. La validez de este modelo, que considera por separado las fuentes de error debidas a la manipulación de la muestra, la preparación de los péptidos y la cuantificación por el espectrómetro de masas, ha sido demostrada en experimentos masivos de hipótesis nula [1] y ha sido validada en varios modelos biológicos de diferente naturaleza [2]. En el presente trabajo hemos abordado un proyecto cooperativo a gran escala entre varios grupos de investigación con el objetivo de extrapolar dicho modelo estadístico, de forma general, a los métodos de marcaje isotópico más comunes (SILAC y iTRAQ), y a otros espectrómetros de masas.