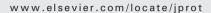
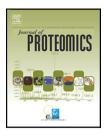


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# The use of a novel quantitation strategy based on Reductive Isotopic Di-Ethylation (RIDE) to evaluate the effect of glufosinate on the unicellular algae Ostreococcus tauri

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#### ABSTRACT

We report a novel stable-isotope labeling strategy for quantitative proteomics analysis. The method consists of labeling N-termini and lysine  $\epsilon$ -amino groups through reductive amination using acetaldehyde. This allows isotope labeling using pairs of either  $^2$ H/ $^1$ H or  $^{13}$ C/ $^{12}$ C without mass spectrum overlap. Our labeling procedure, which is significantly different than that developed for dimethylation, can be completed with little trace of partial ethylation; non-labeled peptides represent less than 0.05% of all peptides. Co-elution of both isotopic  $^{13}$ C/ $^{12}$ C peptide pairs was observed in all cases, simplifying data analysis, which can be performed using standard commercial software such as Mascot Distiller. A  $^{13}$ C/ $^{12}$ C labeled mix in a 1:1 ratio from a complex extract digest of the unicellular algae Ostreococcus tauri, showed a relative standard deviation of less than 14%. This quantitative method was used to characterize 0. tauri in the presence of glufosinate, an herbicide which inhibits glutamine synthetase. Blocking glutamine synthetase significantly reduced the expression of several enzymes and transporters involved in nitrogen assimilation and the expression of a number of proteins involved in various stresses including oxidative damage response were up-regulated.

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#### 1. Introduction

The use of stable isotopic labeling for quantification of proteins or peptides by mass spectrometry has been the basis for several different strategies including in vivo isotopic incorporation such as SILAC or chemical labeling such as isotope-coded affinity tagging (ICAT) [1,2]. ICAT is a versatile approach; however, it is limited to cysteine containing peptides and thus reflects only a fraction of the proteome. SILAC is mostly used for tissue culture cells, although it has been recently applied to mice [3]. In order to overcome these limitations, other chemical labeling methods

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have been developed which globally target N-terminal peptides and  $\epsilon$ -amino groups of lysine (see Regnier et al. [4] for review). One approach has been to label by N-terminal acetylation using acetic anhydride or N-acetoxysuccinimide [4–6]. A major issue of N-terminal acetylation, however, is caused by the charge state reduction, which could affect peptide ionization and fragmentation efficiency.

Other approaches have been suggested including preferential labeling of the  $\epsilon$ -amino group of lysine with o-methylisourea [7]. Although this approach is limited to peptides that contain lysine, it has been reported to increase peptide ionization. Recently, strategies based on isobaric tagging have been introduced, such as TMT and iTRAQ [8,9]. Several challenges are associated with the use of isobaric labeling. One is specific to the ion-trap fragmentation-based instrument which does not capture the lower mass range (1/3 rule) where quantitation based on reporter ions is normally found. This

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problem was solved, albeit at the expense of sensitivity, using ion trap in pulsed Q dissociation mode [10]. For high accuracy instruments such as the Orbitrap, a new "quadrupole like" fragmentation approach has been introduced using the c-trap which allows capturing of fragmented ions of lower mass [11]. In general, the isobaric labeling strategy, when used in data dependant experiments, allows quantification of a given peptide based on a unique time point (i.e. a single MSMS spectra), which could introduce a significant level of noise in the quantitation process.

Recently, Hsu et al. [12] introduced the use of formaldehyde to label the N-terminus and ε-amino group of lysine residues via reductive amination to incorporate dimethyl groups. This approach is inexpensive, applicable for global quantification as all peptides will be labeled and is not limited to a particular type of sample. Moreover, the charge state of the modified peptides is not reduced, thus physico-chemical properties of ions are not significantly altered. However, the isotopic pairing can only be done using <sup>2</sup>H/<sup>1</sup>H, thus resulting in a time lag in the elution profile between isotopic paired peptides. Because of this time lag, data analysis of large and complex samples typical of shotgun proteomic experiments is more challenging. In the present work, we used an isotopic labeling strategy based on primary amine diethylation, in which the heavy isotope of diethyl can be either <sup>2</sup>H or <sup>13</sup>C. Diethylation labeling was introduced for labeling monoamine neurotransmitters [13], but to our knowledge, has not been previously applied to peptide quantitation.

To illustrate the applicability of this novel labeling strategy in a proteomics context, we characterized the effect of glufosinate, an herbicide, on Ostreococcus tauri, a unicellular algae. O. tauri is the smallest known free-living eukaryote, with a cell diameter of approximately 1 µm [14]. The combination of its minimal cell structure and high growth rate makes O. tauri an attractive picoeukaryotic green model organism [15]. The genome of O. tauri has been recently sequenced [16] and found to be 12.5 to 13.0 Mbp, which is significantly smaller than other unicellular green algae, such as Chlamydomonas reinhardtii (120 Mbp) or land plants such as Arabidopsis (125 Mbp).

There is an extensive literature dealing with the influence of herbicides on land plants, but only a few publications that address their impact on aquatic organisms, an egregious omission because these organic compounds often exhibit high water solubility and may easily accumulate in aquatic habitats [17,18]. Glufosinate is known to inhibit glutamine synthetase and is also known to induce formation of reactive oxygen species which could induce photorespiration disturbances. Because of its non-selective nature, glufosinate damages most plants, perturbs the natural equilibrium among soil microorganisms, and has a significant impact on aquatic habitats. Published studies addressing the effects of glufosinate on algae have focused primarily on oxidative stress [17,18]. A proteomics approach has the potential to reveal additional perturbations in addition to oxidative stress associated with glufosinate.

Moreover, in the present work, we have developed a safer strategy for performing reductive amination which used can be broadened to other applications (e.g., peptide dimethylation). We characterize the effect of glufosinate on the O. tauri proteome using Reductive Isotopic Di-Ethylation (RIDE) as the quantitative proteomic strategy. One major finding was that inhibiting glutamine synthetase reduced significantly the expression of several enzymes and transporters involved in nitrogen assimilation (including glutamine synthetase itself). Other groups of proteins involved in oxidative stress were found to be upregulated in presence of Glufosinate. Finally, we demonstrated that our labeling strategy could be applied in quantitative studies of complex proteomics problems.

#### 2. Materials and methods

#### 2.1. Materials

Trypsin (modified, sequencing grade) was purchased from Roche Diagnostics (West Sussex, UK). Iodoacetamide, bovine serum albumin (BSA), Collidine (2,4,6-Trimethylpyridine), 2-Picoline borane (Pic-BH<sub>3</sub>), acetaldehyde, acetaldehyde-<sup>13</sup>C, acetaldehyde-<sup>2</sup>H, sodium cyanoborohydride, hydroxylamine, glufosinate, and trifluoroacetic acid (99% purity sequencing grade) were purchased from Sigma-Aldrich (UK). Formic acid (Suprapure 98–100%), and THP (Tris (hydroxypropyl) phosphine) were purchased from Merck Chemicals (Nottingham, UK). Acetonitrile and water used for LC-MSMS analysis or sample preparation were of HPLC quality (Fisher, UK).

#### 2.2. O. tauri culture

O. tauri strain OTTH0595 was obtained from the Roscoff Culture Collection (RCC 613) and grown in Keller medium (Sigma-Aldrich, UK) dissolved in artificial sea water (Instant Ocean, Aquarium Systems, Sarrebourg, France) adjusted to 30 ppt salinity. Medium was supplemented with antibiotics (Ampicillin 50 μg/mL, Kanamycin 33 μg/mL, Neomycin  $50\,\mu\text{g/mL}$  and Vancomycin  $50\,\mu\text{g/mL})$  and vitamins (B $_{12}$  at  $1 \times 10^{-10}$  M, Biotin at  $1 \times 10^{-9}$  M and thiamine at  $1 \times 10^{-7}$  M). Cells were grown under a 12:12 hour light:dark cycle at 20 °C. Illumination was supplied from fluorescent tubes (FL40SS W/37, Toshiba) with a photosynthetic photon flux density (PPFD) of  $10 \,\mu\text{E m}^{-2}\,\text{s}^{-1}$ , in a plant growth chamber (MRL-350, Sanyo Gallenkamp, Loughborough, UK). Cell proliferation was monitored by OD at 600 nm and by CellTiter (Promega, UK) according to supplier recommendations. O. tauri cultures grown in Keller medium for 7 days were supplemented with different concentrations of glufosinate (GF):  $0 \mu M$ ,  $10 \mu M$ ,  $100 \mu M$ , and  $750 \mu M$ . After five days of treatment with GF, cultures were harvested by centrifugation (3220×g, 10 min, 4 °C) followed by a wash with ice cold PBS. Cells were resuspended in 400 µL lysis buffer containing 8 M urea and 200 mM HEPES KOH, pH 8.0 and disrupted by grinding with acid-washed glass beads (425-600 µm) (Sigma, UK) using a TissueLyser (Qiagen, UK) for 3 min at 30 Hz. Cell debris and insoluble particles were removed by centrifugation (20000×g, 10 min). Protein concentration was measured using the Amido Black Protein Assay [19]. Protein extracts were stored at -20 °C prior to subsequent treatment.

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