Research Article

Isotope-labeled differential profiling of metabolites using N-benzoyloxysuccinimide derivatization coupled to liquid chromatography/high-resolution tandem mass spectrometry

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RATIONALE: An isotopic labeling strategy based on derivatizing amine-containing metabolites has been developed using light ($^{12}C_6$) and heavy ($^{13}C_6$) N-benzoyloxysuccinimide reagents for semi-targeted metabolomic applications.

METHODS: Differentially labeled samples were combined and analyzed simultaneously by liquid chromatography/high-resolution tandem mass spectrometry (LC/HR-MS/MS) to compare relative amounts of amine-containing metabolites. The selectivity of the reaction was determined with model metabolites and was shown to also be applicable to thiol and phenol moieties. The potential for relative quantitation was evaluated in cell extracts and the method was then applied to quantify metabolic perturbations occurring in human cultured cells under normal *vs.* oxidative stress conditions.

RESULTS: A total of 279 derivatized features were detected in HL60 cell extracts, 77 of which yielded significant concentration changes upon oxidative stress treatment. Based on accurate mass measurements and MS/MS spectral matching with reference standard solutions, 10 metabolites were clearly identified. Derivatized compounds were found to have diagnostic fragment ions from the reagent itself, as well as structurally informative ions useful for metabolite identification.

CONCLUSIONS: This simple derivatization reaction can be applied to the relative quantitation of amine-, thiol- and phenol-containing compounds, with improved sensitivity and chromatographic peak shapes due to the increased hydrophobicity of polar metabolites not readily amenable to reversed-phase LC/MS analysis. Copyright © 2015 John Wiley & Sons, Ltd.

The goal of metabolomic studies is to analyze low molecular weight endogenous metabolites present in a biological sample and track concentration changes across different samples.[1,2] In comparison with other 'omics' approaches, these analytes are arguably more chemically diverse, and there is currently no universal platform capable of measuring all metabolites in a sample.[3] Liquid chromatography coupled to mass spectrometry (LC/MS) is one of the most popular techniques for this type of analysis.[4] Features, i.e., signals characterized by a given m/z-RT (retention time) pair, exhibiting significant changes are typically filtered using statistical tools such as multivariate analysis (e.g., principal component analysis and partial least-squares discriminant analysis).[5] Once statistically interesting features are found, the chemical structure of the corresponding compounds should be elucidated.[6,7]

Quantitation of these metabolites is often also desirable; however, this is not straightforward. Absolute quantitation relies on a reference standard solution linking the analyte signal to a corresponding concentration. For this purpose,

stable isotope labeled internal standards (IS) can be spiked into samples, with the advantage of having the same ionization efficiency and chromatographic elution behavior as their non-labeled counterparts. Nevertheless, isotopically labeled compounds are not available for every analyte. It is possible to use a limited subset of IS for each chemical class,[8] but this solution is not ideal since ionization efficiencies and matrix effects are not comparable for all analytes.[9]

Another option uses relative quantitation to determine metabolite changes between two samples (i.e., differential analysis). This concept relies on sample derivatization with a reagent that is available in two isotopic forms: one (heavy) form (or SIL analog) and one light (unlabeled) form. Several isotope-labeling strategies have been developed to derivatize specific chemical groups, including amines, carboxylic acids, and aldehydes and ketones.[10,11] Amine-containing metabolites have very diverse chemical structures (e.g., amino acids, polyamines, catecholamines, nucleotides) and play essential biochemical roles. Reagents used for amine isotope labeling fall into several classes: aldehydes,[12,13] dansyl chloride and derivatives,[14,15] pentafluorophenyl-activated esters of poly(ethylene glycol),[16] methyl acetimidate,[17] and N-hydroxysuccinimide derivatives.[18–21] Nevertheless, most of these approaches have been investigated for targeted analysis. Metabolomic applications has been

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demonstrated with dansyl or dansyl-like derivatives of amine metabolites, using a $^{13}C_2$ analog as the heavy reagent.[14,15,22,23]

In this work, an isotope-labeling strategy based on an *N*-hydroxysuccinimide ester reagent with a heavy reagent incorporating six ^{13}C atoms into a benzyl ring has been developed. Following the evaluation of this approach using a set of standard metabolites and cell extracts, the method was applied to a cellular model of oxidative stress to demonstrate its potential for differential analysis.

EXPERIMENTAL

Materials

Benzoic acid, (ring-$^{13}C_6$)-benzoic acid, formic acid (>98%), 2-aminoisobutyric acid (AIBA), *N*-acetylcysteine (NAC), adenosine (Ado), adenosine 5′-diphosphate (ADP), adenosine cyclic 2′,3′-monophosphate (AMPc), alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), citrulline (Cit), cystathionine, cysteine (Cys), γ-aminobutyric acid (GABA), glutamic acid (Glu), glutamine (Gln), glutathione (GSH), glutathione disulfide (GSSG), glycine (Gly), histidine (His), hydroxykynurenine (Kyn-OH), hydroxyproline (Pro-OH), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), ornithine (Orn), phenylalanine (Phe), phosphorylethanolamine, proline (Pro), serine (Ser), taurine (Tau), threonine (Thr), tryptamine (Trpmn), tryptophan (Trp), tyrosine (Tyr) and valine (Val) were obtained from Sigma-Aldrich (Oakville, ON, Canada). *N*-Hydroxysuccinimide was purchased from Alfa Aesar (Ward Hill, MA). *N,N*′-Dicyclohexylcarbodiimide, ethyl acetate and hexane were purchased from Acros Organics-Fisher Scientific (Ottawa, ON, Canada). Acetonitrile (ACN), monopotassium phosphate and hydrogen peroxide (30%) were from Caledon Laboratory Chemicals (Georgetown, ON, Canada). Methanol was obtained from EMD (Gibbstown, NJ, USA) and ultrapure water was supplied by a Synergy® UV purification system (Millipore, Billerica, MA, USA). Disodium tetraborate decahydrate was obtained from Alfa Aesar (Ward Hill, MA, USA). Potassium chloride, sodium chloride and anhydrous dipotassium phosphate were from Anachemia (Rouses Point, NY, USA). The HL-60 promyelocytic human cell line was acquired from the American Type Culture Collection (Manassas, VA, USA). Iscove's modified Dulbecco's medium and Trypan blue solution (0.4%) were from Gibco (Life Technologies, Burlington, ON, Canada). Fetal bovine serum (FBS) was from HyClone (Thermo Scientific, Logan, UT, USA). Penicillin-streptomycin glutamine solution was purchased from Wisent (St-Bruno, QC, Canada).

Derivatization reagents (*N*-benzoyloxysuccinimide and *N*-benzoyl-ring-$^{13}C_6$-oxy-succinimide) were synthesized in-house (method described in the Supporting Information). Two standard solutions of model metabolites (400 μM each) were prepared by mixing individual stock solutions into two standard mixes (A and B).

Cell culture

HL-60 cells were cultured in Iscove's modified Dulbecco's medium containing 20% (v/v) FBS, 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, at 37°C

under a humidified atmosphere containing 5% CO_2. The stock suspension was diluted to a concentration of 5×10^6 cells per mL.

Differential analysis/oxidative stress experiments

Aliquots (0.4 mL) of HL60 cells (5×10^6 cells/mL) (n = 5) were treated with either 20 μL of water (control), or 20 μL of a 20 mM H_2O_2 solution (treated), for 20 min at 37°C with gentle mixing. Metabolites were extracted (see below) and cell extracts were evaporated to dryness. Control series were derivatized with light and heavy reagents, whereas treated samples were derivatized with light reagent only. Derivatized extracts were mixed 1:1 (light/heavy), resulting in five control:control and five treated:control samples. Quality control samples were prepared by pooling equal aliquots of control samples.

Metabolite extraction

Cell culture samples were centrifuged (3500 rpm, 5 min at 4°C) and supernatants were discarded. Pellets were then washed twice with 1 mL cold phosphate-buffered saline (PBS). To the resulting pellets, 300 μL MeOH/H_2O 1:1 (v/v) was added and samples were sonicated (8 min). Ice-cold MeOH (300 μL) was then added, samples were centrifuged (14000 rpm, 8 min at 4°C), and 550 μL of supernatant was retrieved into a new tube. An additional 200 μL of MeOH/H_2O 1:1 (v/v) was added to the pellet, and 200 μL of resulting supernatant were combined with the previous extract (total volume of 750 μL). Extracts were evaporated to dryness prior to derivatization.

Relative quantitation experiments

Five extracts were created each using four aliquots of 1 mL HL60 cell suspensions (5×10^6 cells/mL), subsequently divided into multiple aliquots representing extracts from 1, 2 or 5 million cells each. Light and heavy labeled samples were mixed in 1:1 proportions to prepare five series of the following mixtures (light:heavy ratios): 1:1, 1:2, 1:5, 2:1, 2:2, 5:1, 5:5.
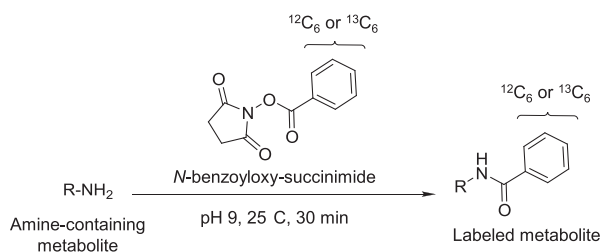
Derivatization reaction

Dried samples were reconstituted in 120 μL of derivatization solvent (50 mM borate buffer containing 5% ACN, pH 9), followed by the addition of 20 μL *N*-benzoyloxysuccinimide (light or heavy, 20 mM in ACN), and incubated for 30 min at 25°C, while mixing (Fig. 1).

LC/MS analysis

Reversed-phase liquid chromatography was performed on a Shimadzu Nexera ultra-high-performance liquid chromatography (UHPLC) system with mobile phases A (H_2O) and B (ACN), each containing 0.1% formic acid. Separation was achieved using a Cogent C18 column (150 × 2 mm, 4 μm), with a Phenomenex SecurityGuard C18 (4 × 2.0 mm) guard column, at 40°C, using a gradient elution as follows: 5% B (0–2 min), 25% (6 min); 65% (17 min); 90% (18–20 min), at a flow rate of 0.4 mL/min.

The LC system was coupled to a hybrid quadrupole-time-of-flight mass spectrometer (AB Sciex Triple TOF® 5600, Concord, ON, Canada) using a DuoSpray™ ion source operated in positive electrospray mode. Source conditions were: source voltage 5000 V, curtain gas 35 psi, temperature

Figure 1. Derivatization reaction using benzoyloxysuccinide reagent, with example shown with free amine group being labeled.

500°C, and nebulizer and drying gases (GS1/2) both 50 psi. The declustering potential was 60 V and MS/MS experiments used collision-offset voltages of 30 ± 10 V. TOF-MS spectra (*m/z* 110–1000,250 ms accumulation time) were acquired, followed by MS/MS experiments (*m/z* 50–860, 100 ms accumulation time), using information-dependent acquisition and dynamic background subtraction, with the five most intense precursor ions. Follow-up experiments for metabolite identification used an inclusion list of selected precursor ions, with an MS/MS range of *m/z* 50–625 and 200 ms accumulation time, for increased sensitivity and higher quality MS/MS data. Mass calibration was performed automatically every four injections, using a mix of standards from *m/z* 121 to 922 for TOF-MS, and, in MS/MS mode, using product ions of reserpine (MH$^+$) ranging from *m/z* 74 to 609.

Data processing and statistical analysis

Raw LC/MS/MS data was processed using Metabolite Pilot™ (version 1.5, AB Sciex) for peak picking and peak-pair finding, using a custom differential isotope-filtering tool. LC/MS filtering criteria were: peak width ≥2.5 s, intensity ≥100 counts per second (cps), and sample-to-control intensity ratio ≥3, *m/z* tolerance of 25 ppm, and XIC width of 25 mDa. The differential isotope filtering step was performed to find peak pairs with *m/z* differences (Δ*m/z*) of 6.0201 and 12.0402, for singly and doubly labeled compounds, respectively, using a set of control cell extracts derivatized in a 1:1 ratio. This peak list was then exported into Microsoft Excel 2010 and further annotated and filtered to remove adducts and multiply charged species or multimers. Accurate peak integration of remaining features was performed using MultiQuant 2.1, and average peak area ratios (light/heavy derivative) were compared using a two-sided *t*-test. All calculations were performed in Excel, including *t*-statistics, adjusted degrees of freedom and resulting *p*-value.

For structural elucidation of metabolites, underivatized accurate masses were calculated and subjected to database searching (METLIN[24,25] and HMDB[26,27]). Metabolite identification was achieved by retention time (RT) and HRMS/MS spectral matching, with derivatized commercially available reference standards.

RESULTS AND DISCUSSION

Derivatization reaction

The derivatization reagent was designed to target amine groups (Fig. 1), since succinimidyl esters are activated carboxylic acids commonly used to form amide bonds.[28] They are highly reactive electrophiles towards primary and secondary amines (nucleophilic substitution). To be applicable for differential analysis, light and heavy versions of the reagent were synthesized, the heavy one containing six ^{13}C atoms on the aromatic ring. In the present study, the 6 Da difference
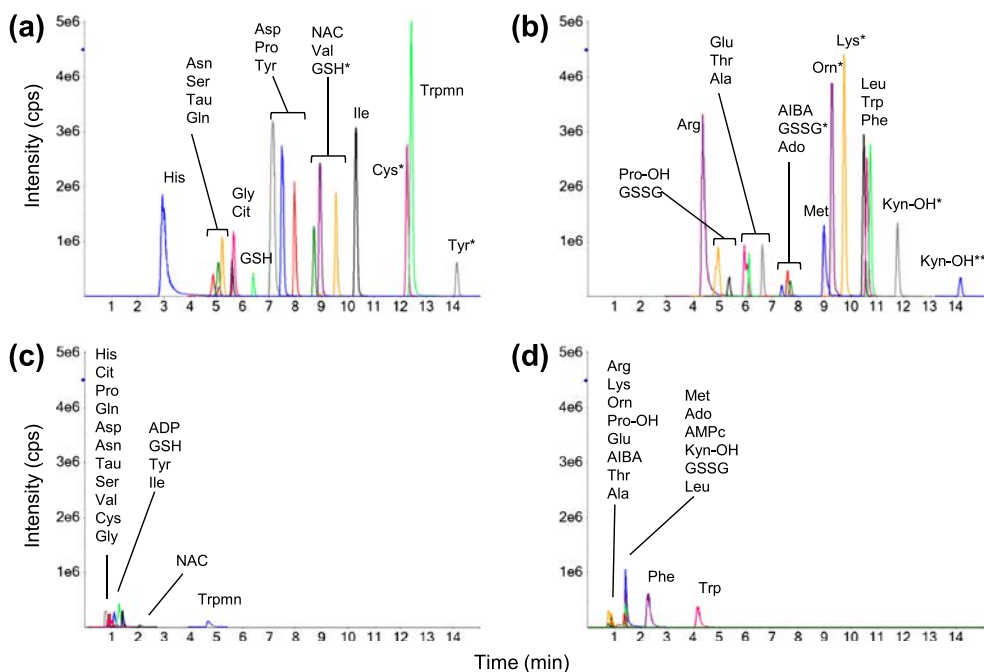

Figure 2. Extracted ion chromatograms of model metabolites in standard solutions containing 8.5 μM of each metabolite: (a) standard mixture A, derivatized; (b) standard mixture B, derivatized; (c) standard mixture A, underivatized; (d) standard mixture B, underivatized. *: doubly derivatized metabolite. **: triply derivatized metabolite.

was chosen so as to introduce a highly unique isotope pattern for filtering purposes when analyzing complex samples.

The other aspect considered in the design of the heavy reagent was the nature of the isotope introduced. Given the structure of organic compounds, labels usually include ^{2}H or ^{13}C. While the use of the former is more widespread and typically cheaper, it also has the potential drawback of extensive isotope effects in RP-LC.[29] Typically, deuterated analogs have weaker hydrophobic interactions than their non-deuterated counterparts, and therefore exhibit earlier retention times in RP-LC.[29,30] Therefore, complete co-elution of light and heavy derivatives is rarely possible.[12,20,31] This lack of co-elution would be detrimental to both relative quantitation and metabolite peak finding. In this study, peak pairs (light/heavy derivatives) were systematically inspected for proper co-elution and no isotope effects were observed.

A set of standard metabolites was used to characterize the selectivity of the reaction. Two standard mixes (A and B) were prepared to alleviate possible isobaric interferences. Derivatives of amine-containing metabolites were obtained, and the presence of multiple amine groups led to the formation of multiply labeled metabolites. The only notable exception was the case of aromatic amines, which did not react with the reagent, given that the nitrogen lone-pair of such amines is delocalized into the aromatic system (adenine moiety). Also amide groups do not react, since they exhibit more acidic behavior than their amine counterparts, therefore are much less reactive. On the other hand, the reaction was also found to occur with free thiols (Cys, NAC, GSH) and phenols (tyrosine, hydroxykynurenine).

RP-LC of endogenous metabolites can be quite challenging, because they are often very polar small molecules. The limitation of RP-LC is illustrated in Fig. 2 by the extracted ion chromatograms of a set of underivatized model metabolites: 31 of the 33 metabolites eluted earlier than 1.5 min, and would therefore have considerable matrix effects in biological samples. Moreover, elution of a large number of compounds in a short time window would impair peak finding algorithms, bias quantitation (presence of multiple interferences), limit MS/MS triggering in IDA experiments as well as the use of retention time matching. The benzyl group in the derivatization reagent increases chromatographic retention and therefore improves metabolite separation. The derivatized metabolites also exhibit much higher signals than their underivatized counterparts.
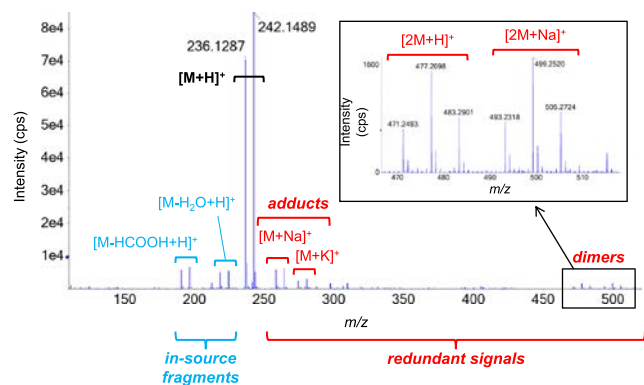
Figure 3. TOF-MS spectra of Leu illustrating typical redundant species observed.

Table 1. Representative data from a subset of 10 model metabolites

		His	Tau	Gln	Gly	Glu	Pro	Val	Ile	Leu	Phe
m/z (theoretical)		260.1035	230.0487	251.1032	180.0661	252.0872	220.0974	222.1130	236.1287	236.1287	270.1130
m/z (measured)		260.1022	230.0479	251.1033	180.0649	252.0883	220.0969	222.1128	236.1287	236.1287	270.1133
Δ *m/z* (ppm)		-5.1	-3.5	0.5	-6.5	4.4	-2.1	-1.0	0.1	0.1	1.0
RT (min)		3.2	5.6	5.4	5.8	6.2	7.6	9	10.4	10.6	10.9
Peak area ratios ($^{12}C_6$/$^{13}C_6$) (%CV, n=5)	cell extract ratios 1:1	0.95 (10.3%)	0.92 (4.5%)	0.92 (3.6%)	0.78 (3.8%)	1.08 (4.2%)	0.96 (5.8%)	0.90 (3.4%)	0.92 (3.1%)	0.91 (4.9%)	0.92 (5.5%)
	2:2	0.91 (12.5%)	0.90 (11.1%)	0.86 (11.0%)	0.72 (9.0%)	1.03 (6.3%)	0.85 (13.3%)	0.83 (10.0%)	0.84 (10.8%)	0.85 (10.1%)	0.86 (9.7%)
	5:5	0.91 (2.6%)	0.87 (3.6%)	0.90 (6.8%)	0.75 (2.1%)	1.03 (4.0%)	0.89 (9.1%)	0.86 (6.1%)	0.87 (5.2%)	0.88 (4.8%)	0.91 (7.9%)
Slope	1:5 to 5:1	0.8143	1.0145	1.0090	0.7564	1.0177	0.9514	0.9443	1.0016	0.9782	0.9913
r²		0.99859	0.99863	0.99921	0.99966	0.99960	0.99913	0.99951	0.99931	0.99933	0.99917

For the 31 derivatized model metabolites, the median of peak intensity ratio (derivatized/underivatized) was 9.1. This ratio was >30 for over 25% of them, with a maximal value close to ~400 for Cys (doubly derivatized).
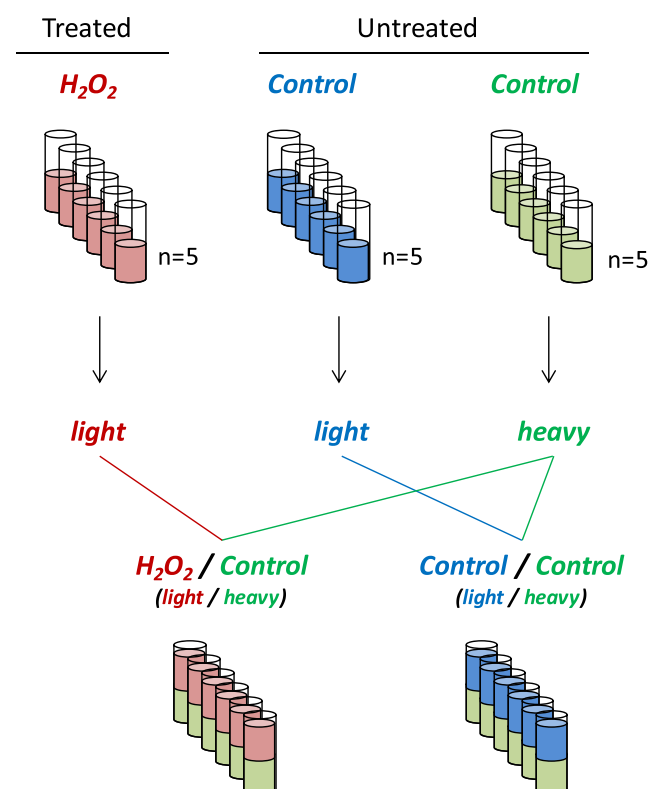
Peak picking and pair finding

A generic peak-picking step yielded an initial list of LC/MS peaks. This list can then be filtered to find peak pairs having the same RT and 6.020 or 12.040 Da differences for singly and doubly derivatized compounds, respectively. The resulting list was then annotated to remove redundant features from cluster or adduct ions. An example of the multiple ion species seen in the mass spectrum for a given

Figure 4. Experimental design of the differential analysis for oxidative stress study in cells.

metabolite is shown for leucine in Fig. 3. In this case, the pair at *m/z* 236.1287 and *m/z* 242.1489 would be retained in the filtered list but minor adduct and cluster ions can easily be removed prior to differential analysis of metabolites.

Relative quantitation

Cell extracts were employed to evaluate that signal ratios between heavy and light derivatives could effectively track concentration ratios. Other research groups have investigated the quantitative performance of their strategies with standard solutions of model metabolites (i.e., non-biological samples).[12,14,15,17,20] The experimental design relied on mixing known amounts of derivatized metabolites (light and heavy) in various ratios to generate a calibration curve. This, however, does not directly ascertain the ability of the derivatization reaction to track quantitative changes reliably in specific biological samples. The experimental design chosen to test the present approach employed five independent pooled cell extracts, each being divided into different aliquots. A representative sample was used to identify singly derivatized features, leading to a final list of 115 derivatized peak pairs (after removing redundant features, including adducts and in-source fragments). Average peak area ratios (light/heavy) were determined and coefficients of variations (CVs) across the five sets were calculated to estimate the reproducibility of the method, accounting for sample variability related to extraction, derivatization and LC/MS analysis. The median CV was 15.3%, with a large proportion (75%) having CVs less than 25%.

The agreement between experimental measurements (mean peak area ratios) and expected values (known concentration ratios) was estimated using several parameters. First, coefficients of determination (r^2) measured how well values correlate, of which 92% of features exhibited $r^2 \geq 0.95$. The slopes of the corresponding linear regressions were also compared (ideal slope of 1), and 70% yielded a slope between 0.7 and 1.3. Lastly, the best estimation of accuracy is arguably the bias (i.e., relative difference) between known concentration ratios and mean peak area ratios, where in the case of the 115 peak pairs, the median bias was –5.7%. Representative results for 10 model metabolites detected in cell extracts are presented in Table 1, illustrating the individual calculated peak area ratios from 1:1, 2:2 and 5:5 extract ratios and their associated %CVs, as well as linearities (slope and r^2) from 1:5 to 5:1 extracts ratios.

Table 2. Identified metabolites with significant changes following H_2O_2 treatment of HL60 cells

|	Metabolite	Formula (underivatized)	# Labels	Fold change	*p*-value
1	Taurine	$C_2H_7NO_3S$	1	−2.6	2.48e-06
2	Glutamic acid	$C_5H_9NO_4$	1	−2.1	1.06e-05
3	Cystathionine	$C_7H_{14}N_2O_4S$	2	−2.0	1.00e-05
4	Glutathione (GSH)	$C_{10}H_{17}N_3O_6S$	2	−1.9	2.72e-02
5	γ-Aminobutyric acid (GABA)	$C_4H_9NO_2$	1	−1.8	3.26e-03
6	Phosphorylethanolamine	$C_2H_8NO_4P$	1	−1.6	1.72e-04
7	Methionine	$C_5H_{11}NO_2S$	1	−1.6	3.81e-03
8	Glycine	$C_2H_4NO_2$	1	−1.6	2.72e-04
9	Tyrosine	$C_9H_{11}NO_3$	2	−1.5	5.01e-03
10	Methionine sulfoxide	$C_5H_{11}NO_3S$	1	4.0	2.61e-04

Differential analysis

The present method was applied to evaluate its ability to track metabolic perturbations in biological samples. HL60 cells were treated with H_2O_2 to mimic oxidative stress. This metabolic perturbation was employed to test the derivatization approach, not necessarily to accurately model a given pathological process. The experimental design is illustrated in Fig. 4. The amount of cells per sample (2×10^6) was chosen in the range previously investigated during the quantitative evaluation of the method. A set of quality control (QC) samples (5 pooled derivatized controls extracts) was

analyzed at the beginning, in the middle and at the end of the sequence and showed no significant deviations in RT over the course of the analytical run.

The pooled QC sample was also used for data processing in order to identify feature pairs. The search was performed for singly and doubly derivatized species, and resulted in a raw list of 398 feature pairs. This list was inspected and redundant features were removed, including sodium and potassium adducts, dimers, trimers, and doubly charged species. Dimers, trimers and doubly charged species were identified by manually inspecting MS spectra and were only removed if the monomeric feature was also identified. Interestingly, dimers
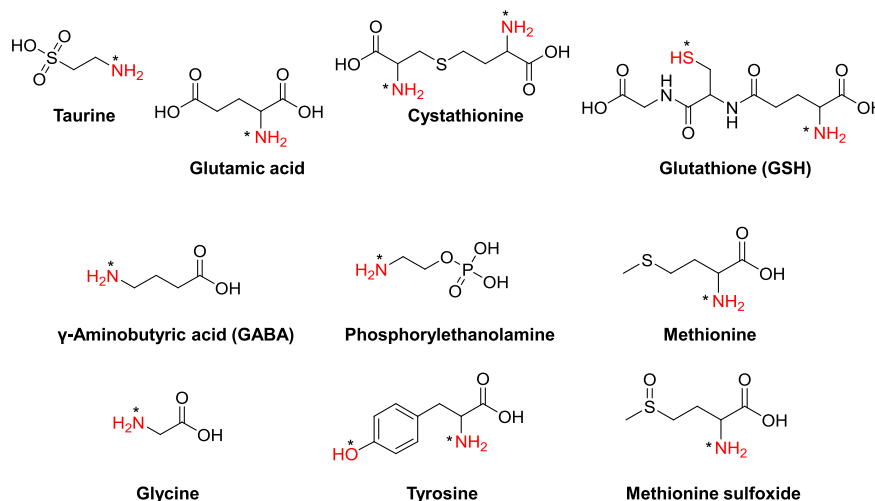
Figure 5. Metabolites, identified by retention time and MS/MS spectral matching with authentic standards, found to change in HL60 cells upon H_2O_2 treatment. Site of benzyl label derivatization indicated by *.

Table 3. LC/MS/MS data (retention times and fragment ions) for identified metabolites changing in H_2O_2-treated HL60 cells

Metabolite	RT (min)	Precursor m/z ^{12}C	^{13}C	51/55	77/83	105/111	Fragment ions
Glycine	5.9	180.0655	186.0856	+	+	+	**134.0604, 162.0614**
γ-Aminobutyric acid	7.1	208.0968	214.1169	+	+	+	72.0478*, 86.0613*, 90.0572*, **149.0657, 191.0790**
Taurine	4.3	230.0481	236.0682	+	+	+	**148.0755**
Phosphorylethanolamine	3.1	246.0526	252.0727	+	+	+	**148.0749, 149.0780**
Glutamic acid	6.3	252.0866	258.1067	+	+	+	102.0563*, 130.0508*, **160.0748**, 188.0695, **206.0823, 234.0767**
Methionine	9.5	254.0845	260.1046	+	+	+	56.0532*, 61.0140*, 104.0538*, 130.0660, **160.0761, 206.0815, 208.0795**, 236.0737
Methionine sulfoxide	6.0	270.0794	276.0995		+	+	56.0527*, 104.0503, 131.0155*, 130.0654, 149.0259*, **160.0755, 206.081, 252.0685**
Tyrosine	14.4	390.1336	402.1738	+	+	+	119.0494*, **240.1020, 344.1286, 372.1237**
Cystathionine	9.7	431.1271	443.1673		+	+	114.0384*, 142.0326*, 146.0608, **160.0698**, 174.0554, 192.0653, **206.0814**, 218.0636, 220.0429, 226.0537, 238.0538, 240.0689, 246.0587, 263.0851, **264.0691**, 281.0961, 309.0912, 385.1220, 413.1168, 431.1275
Glutathione	9.9	516.1435	528.1837		+	+	142.0322*, 170.0270*, **180.0475**, 199.0534*, 216.0653, 227.0485*, 234.0762, 245.0603*, **265.0648**, 283.0753, 291.0803, 303.0981, 319.0752, 360.1203, 376.0974, 394.1075, 395.1055, **413.1170**, 441.1119, 516.1451

*Denotes fragments without benzyl label. Top three most intense fragments shown in bold.

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and trimers exhibit a characteristic isotopic distribution based on the combination probabilities of light (m) and heavy (M) derivatives (monomers). For instance, dimers give rise to three peaks distanced by 6.020 Da with intensity of ratios 1:2:1, corresponding to: $[m+m+H]^+$ (first peak), $[M+m+H]^+$ and $[m+M+H]^+$ (second peak), $[M+M+H]^+$ (third peak). Similarly, dimers and trimers could be observed as sodium and potassium adducts. After removing these features, the resulting list consisted of 279 feature pairs, used to calculate peak area ratios in all samples.

The mean of peak area ratios for each feature was compared between the two sets of samples (control/control and treated/control, $n = 5$) using a two-sided *t*-test. A feature was qualified as discriminating if the *p*-value associated to the *t*-test was <0.05 and if the control/control mean was changing by at least 50%. A final list of 77 statistically significant features fell within these criteria.
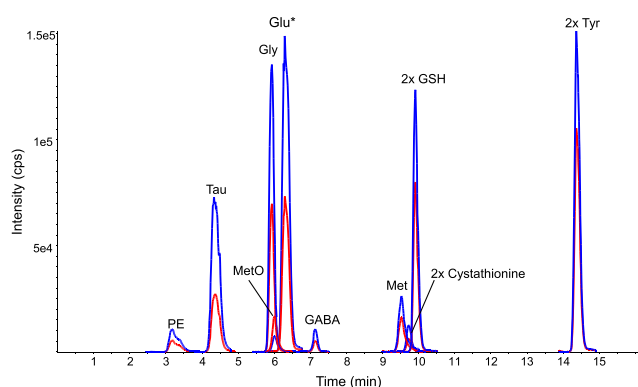

Figure 6. Overlaid extracted ion chromatograms for the ten derivatized metabolites found to be significantly changed after H_2O_2 treatment, from a representative sample with treated (light, in red) and control (heavy, in blue) cells. 2× indicates double-derivatization and * denotes that the glutamic acid peak has been reduced 4-fold for clarity.

Structural elucidation and metabolite identification

Once features of interest were noted as statistically relevant, the next step was to identify metabolites, which usually represents the most challenging part in metabolomic workflows. For the 77 discriminating features found, the monoisotopic mass of the native (neutral, underivatized) metabolite was first determined by subtracting the mass of the label moiety, namely 105.03403 Da (C_7H_5O) for singly and 209.06025 ($C_{14}H_9O_2$) Da for doubly derivatized metabolites. The resulting mass was entered in METLIN and HMBD databases to search for possible chemical structures of known metabolites within a mass tolerance of 5 ppm, which yielded a list of 38 putative metabolites. The high resolution (~25,000) and proper mass calibration of the TOF-MS setup enabled accurate mass measurements, crucial for structural elucidation of unknown metabolites. Nevertheless, metabolite identification based solely on accurate mass is relatively limited, since it simply yields a putative list of metabolites. The second key element used for structural elucidation and to refine metabolite database results was the MS/MS spectra. Moreover, MS/MS data were used to confirm that an ion corresponded effectively to a derivative, based on diagnostic fragment ions found to be characteristic of the label moiety and having a 6.0201 Da difference between the light and heavy labels, including cyclobutadienyl cation ($C_4H_3^+$, *m/z* 51.0229 / $^{13}C_4H_3^+$, *m/z* 55.0363), phenylium ion ($C_6H_5^+$, *m/z* 77.0391/$^{13}C_6H_5^+$, *m/z* 83.0593), benzoyl cation ($C_7H_5O^+$, *m/z* 105.0340/$^{13}C_6CH_5O^+$, *m/z* 111.0542).

Finally, when available, metabolites changing significantly with H_2O_2 treatment were identified by comparison with commercially available standards. Ten discriminating metabolites were clearly identified based on RT and MS/MS matching with standard derivatized metabolites. There was a significant decrease in taurine, glutamic acid, cystathionine, GSH, GABA, phosphorylethanolamine, methionine, glycine, and tyrosine, as well as a significant increase in methionine
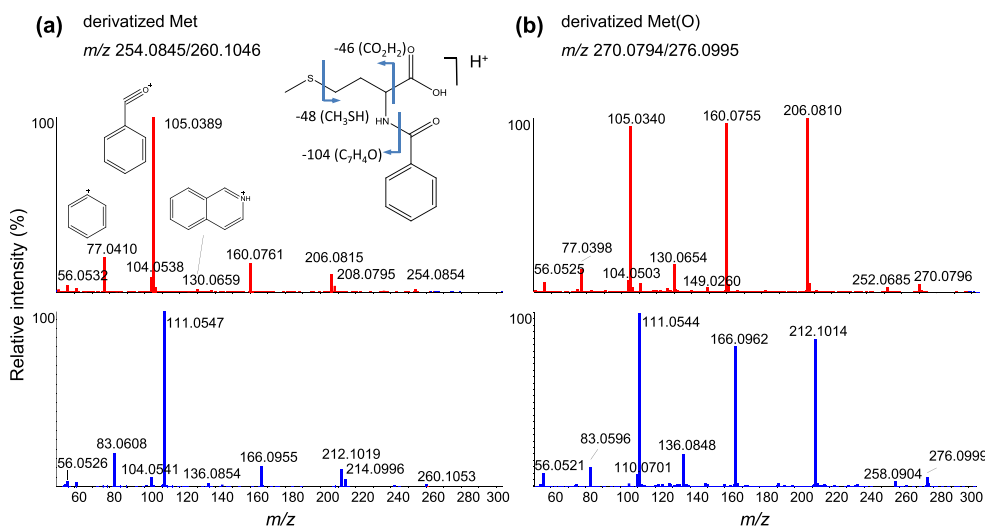
Figure 7. High-resolution MS/MS spectra of light and heavy derivatized methionine (a) and methionine sulfoxide (b) from a representative cell extract sample. Proposed fragmentation pathways are shown on the structure of derivatized methionine and a few specific product ions are drawn within the MS/MS spectrum of light-derivatized Met.

sulfoxide. Table 2 lists the resulting identified metabolites found to be significantly changed in the oxidative stress cell model, along with corresponding fold changes and p-values. The structures of these identified metabolites are shown in Fig. 5, illustrating the primary amine, thiol and phenol label sites seen for these compounds. LC/MS/MS data, including retention times, exact masses (m/z) of derivatized metabolites, diagnostic (label-related) as well as structurally informative fragment ions, are listed in Table 3. Figure 6 shows the overlaid light- and heavy-labeled metabolite extracted ion chromatograms for all ten identified species found to be significantly changed after H_2O_2 treatment, from a representative sample with treated (light) and control (heavy) cells. All derivatized metabolites yielded at least two of the diagnostic ion pairs for benzyl-labeled species as well as some structurally informative ions. MS/MS matching was performed based on accurate mass (±10 ppm) as well as relative intensities between fragment ions. As a representative example, high-resolution MS/MS data is shown for methionine (Met) and methionine sulfoxide (Met (O)) in Fig. 7, along with the proposed fragmentation reactions from both label-specific ions as well as structurally informative ions.

CONCLUSIONS

A novel isotope-labeling strategy was developed for differential metabolomics analysis, using N-benzoyloxy-succinimide. The derivatization reaction was selective towards amine, phenol and thiol groups, with possibilities for multiple derivatization on certain metabolites. In comparison with their unlabeled counterparts, derivatized metabolites exhibit significant increases in sensitivity and retention time, allowing sufficient retention for polar metabolites and satisfactory separation of isomers (e.g., Leu and Ile). The reagent was prepared in two isotopic forms (light and heavy), the heavy version containing six ^{13}C atoms. In contrast with deuterium-containing isotopic analogs, the presence of ^{13}C prevented isotope effects and therefore ensured optimal relative quantitation. The quantitative performance was investigated with an experimental design using biological samples (cell extracts). Method accuracy was investigated by comparing 115 singly labeled feature pairs for peak area ratios (light/heavy derivative) in cell extracts with known concentration ratios, and an accuracy median bias of –5.7%. In addition, precision (CV) of peak area ratios (n = 5) was <25% for over75 % of the peak pairs.

The method was applied to a cellular model of oxidative stress to assess its application for characterizing metabolite changes in biological samples. Cell cultures (n = 5) treated with 1 mM H_2O_2 for 20 min and untreated cell cultures (n = 5) were, respectively, derivatized with the light and heavy reagent. Seventy-seven features were deemed discriminant (p <0.05, change >50%). Structural elucidation was carried out based on accurate mass determination, MS/MS spectra, and comparison with standard model metabolites, resulting in ten discriminating metabolites being clearly identified, with fold-changes ranging from –2.6 to +4.0.

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