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Regioselective solvent-phase deuteration of polyphenolic compounds informs their identification by mass spectrometry

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ABSTRACT

Liquid chromatography–mass spectrometry (LC–MS) is a highly sensitive tool for the analysis of polyphenolic compounds in complex food and beverage matrices. However, the high degree of isomerism among polyphenols in general often complicates this approach, especially for identification of novel compounds. Here, we explore the utility of mild acid-catalyzed deuterium (MACD) labeling via electrophilic aromatic substitution as a complementary method for informing polyphenolic compound structure elucidation. To prevent hydrolysis of acid-labile glycosidic linkages, optimal reaction conditions that maximize regioselective hydrogen/deuterium (H/D) exchange of aromatic protons while preserving compound integrity were characterized (60 °C, pH 3.0, 72 h). Under these conditions, standard compounds varying in the number and position of hydroxyl, glycosyl, and methyl groups about their aromatic core structure produced distinguishable H/D exchange patterns. The applicability of this method for the analysis of complex mixtures was demonstrated in red wine where the extent of deuterium exchange, together with accurate mass information, led to the putative identification of an unknown compound. The identification was further supported by tandem MS (MS/MS) data, which matched conclusively to the same compound in the Metlin LC–MS/MS library. With the capacity to discriminate between select isomeric forms, MACD labeling provides structural information that complements accurate mass and tandem mass spectral measurements for informing the identification of polyphenolics by MS.

Polyphenolic compounds are secondary metabolites from the phenylpropanoid biosynthetic pathway in plants that are often present in plant-derived foods, beverages and dietary supplements [1,2]. Polyphenolics often demonstrate phytoalexin and antioxidant properties important for promoting abiotic and biotic stress tolerance. In humans, these same properties are likely responsible for the reported health benefits associated with consuming polyphenolics, namely decreased incidence of coronary heart disease, various cancers, and neurodegeneration [3–10].

Of the various polyphenolic classes commonly found in food and beverages (e.g., flavonoids, stilbenoids, chalcones), flavonoids are the most abundant and chemically diverse. According to structure–activity relationship studies, the antioxidant, anti-inflammatory, and substrate binding activities of flavonoids are dependent on a number of key chemical features about their C6-C3-C6 diaromatic flavan core [11–14]. These features, including a hydroxyl group at the C3 position, a ketone group at the C4 position, a double bond between C2 and C3, and the attachment of the benzene ring (B-ring)[1] to either the C2 or C3 position, describe various states of oxidation about the central heterocyclic pyran ring (C-ring) and define the various subclasses of flavonoids, namely flavonols, flavones, isoflavones, flavanones, flavan-3-ols, and anthocyanidins [11–13]. Variable degrees and patterns of glycosylation, methylation, and acylation further increase the structural complexity of dietary flavonoids, which also greatly affects their bioavailability and thus their therapeutic potential [1,15]. In all, more than 7000 unique flavonoid structures have been characterized in sources such as fruits,

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[1] *Abbreviations used:* LC, liquid chromatography; MS, mass spectrometry; DAD, diode array detector; UV, ultraviolet; NMR, nuclear magnetic resonance; H/D, hydrogen/deuterium; D_2O, deuterium oxide; CI, chemical ionization; MACD, mild acid-catalyzed deuterium; CH_3OD, deuterated methanol-*d*; DCOOD, deuterated formic acid-d_2; SPE, solid-phase extraction; MS/MS, tandem MS; CID, collision-induced dissociation; RPLC, reverse-phase LC; UHPLC, ultra-high-performance liquid chromatography; HCD, higher energy collision-induced dissociation; NCE, normalized collision energy; ESI, electrospray ionization.

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vegetables, grains, and their derived beverages, and new compounds continue to be identified with the characterization of novel plant sources and the development of advanced analytical techniques [16].

Due to the complexity of biologically derived samples and the low abundance of many polyphenolics, liquid chromatography–mass spectrometry (LC–MS) is often the analytical platform of choice for characterizing polyphenolics in complex biological mixtures. It is routinely conducted with orthogonal detection methods that provide complementary information for discriminating between the large number of isomeric structures that comprise this class of compounds [17,18]. The most typical approach involves placing an ultraviolet diode array detector (DAD) upstream of the mass analyzer (LC–UV–MS), thereby providing additional information regarding the A-ring and C-ring aglycone structures [17,19–22]. However, although UV absorbance sufficiently discriminates among the various aglycone structures, it is less reliable for assigning the position of methyl, glycoside, and acyl substituents about a given aglycone [17]. Conversely, the coupling of LC–MS with nuclear magnetic resonance (LC–MS–NMR) has shown during recent years to be a powerful technique for unequivocally elucidating polyphenolic structures within mixtures [23]. Although LC–MS–NMR readily discriminates among many regio- and stereoisomers unresolved by UV, it requires MS-compatible deuterated solvents, which not only are costly but also may complicate interpretation of the mass spectrum due to variable rates of deuterium exchange of "active" protons throughout the structure. In addition, NMR spectroscopy is far less sensitive than mass spectrometric analysis, which often limits the complementarity of NMR data to only a small subset of the ionized species.

One alternative, gas-phase hydrogen/deuterium (H/D) exchange, has been used to inform flavonoid structure by characterizing the number and position of acidic flavonoid protons, both at OH groups and at nucleophilic aromatic positions on the A- and B-rings [24–26]. For instance, the Brodbelt group exposed flavonoid ions to deuterium oxide (D_2O) gas in an ion trap and demonstrated how the resulting H/D exchange patterns, as well as exchange kinetics, could be used to discriminate various flavonoids, including stereoisomeric structures [24,25]. Although powerful, the widespread use of their method has been limited because it requires an in-house D_2O gas delivery system into the trap portion of the instrument. By comparison, H/D exchange of flavonoids during ionization in a chemical ionization (CI) source has also been shown to generate variable labeling patterns of various flavonoids, with exchange occurring at all OH groups as well as aromatic protons in positions *ortho* and *para* to OH groups [26]. The utility of this H/D exchange method is also limited because CI is classically coupled to gas chromatography, a mode of sample fractionation not typical for flavonoid analysis. Still, these studies well illustrate the utility of H/D exchange as a complementary technique with MS for profiling polyphenolics in complex mixtures and point to the need for development of more approachable H/D exchange methodologies.

Solution-phase H/D exchange is a potentially useful alternative to gas-phase approaches for characterizing polyphenolics because it does not require specialized equipment and is compatible with liquid chromatographic fractionation of complex mixtures. However, we are not aware of such methods having been described in the current literature. The numerous solution-phase H/D exchange protocols for generating deuterated polyphenolic standards are typically designed for quantification studies and, thus, employ relatively harsh pH and temperature conditions to maximize the completeness of labeling [27–34]. Such conditions are inappropriate for informing the structure of biological flavonoids because they perturb compound integrity via hydrolysis of glycosidic and acyl linkages. Here, we describe offline mild acid-catalyzed deuterium (MACD) labeling of polyphenolic compounds for informing their identification by MS. Importantly, the conditions used for MACD largely preserve compound integrity while producing sufficient H/D exchange information to enhance structural identifications.

Materials and methods

Chemicals

Apigenin, (+)-catechin, daidzein, fisetin, genistein, isoorientin, morin, myricetin, orientin, naringenin, phloretin, piceid, quercetin, quercitrin, rhamnetin, and resveratrol were purchased from Sigma–Aldrich (St. Louis, MO, USA), and astragalin, epicatechin, epicatechin gallate, isoquercitrin, kaempferol, populnin, luteolin, and cynaroside were purchased from Extrasynthese (Genay, France). Solvents were obtained from Sigma–Aldrich and included CHROMASOLV Plus HPLC (high-performance liquid chromatography)-grade acetonitrile, formic acid (>97%), deuterated methanol-*d* (CH_3OD, 99 atom % D), and deuterated formic acid-d_2 (DCOOD, 98 atom % D). D_2O (99 atom % D) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). BioUltra-grade ammonium acetate was also obtained from Sigma–Aldrich. Monovarietal red wine (2009) vinted from cold-climate hardy Frontenac grapes was kindly provided by the University of Minnesota Horticultural Research Center.

MACD labeling

Standard compounds

In general, methanolic stocks (1 mg/ml) of each standard compound were prepared in 2.0-ml microcentrifuge tubes. From these stocks, aliquots were transferred to 1.5-ml microcentrifuge tubes and concentrated to dryness via vacuum centrifugation. The resulting pellets were reconstituted in deuterated methanol (10% of final volume) and diluted to volume with MACD labeling buffer (D_2O containing 10 mM ammonium formate, pH 3.0). The samples were then incubated in the dark for 72 h at 60 °C and then frozen at −20 °C to stop the labeling reaction. Labeling controls were also generated by reacting each compound in nondeuterated buffer (H_2O containing 10 mM ammonium formate, pH 3.0). In addition to these standard conditions, various MACD labeling buffers as well as reaction times and temperatures were also evaluated in the time course of atom % incorporation and compound degradation studies described below (see Fig. 3). Except for the analysis of quercetin and catechin in Figs. 1 and 2 (see figures below in Results and Discussion), respectively, all reactions were conducted in triplicate and analyzed independently.

Red wine

Freshly opened Frontenac wine (40 ml) was poured into a 50-ml conical tube and vigorously vortexed to homogenize the sample. As a labeling control, apigenin, an exogenous flavonoid foreign to red wines, was added to a final concentration of 10 μg/ml. From this stock, two 1-ml aliquots were transferred to 2-ml microcentrifuge tubes and concentrated to dryness via vacuum centrifugation. To generate MACD-labeled wine, one of the resulting pellets was reconstituted in 1 ml of MACD labeling buffer (D_2O containing 10 mM ammonium acetate, pH 3.0) to regenerate the original 1× wine concentration. The other pellet, serving as an unlabeled control, was reconstituted in nondeuterated buffer (H_2O containing 10 mM ammonium acetate, pH 3.0). The two samples were then incubated in the dark for 72 h at 60 °C and subsequently stored at −20 °C to minimize further labeling.

Quercetin: deuterated versus unlabeled

Deuterated quercetin (2 ml, 20 μg/ml), prepared according to the MACD labeling protocol described above, was buffer exchanged via C_{18} solid-phase extraction (SPE; SepPak, Waters) and eluted into acetonitrile containing 20% D_2O. Specifically, the SepPak cartridge was pretreated with acetonitrile (20% [v/v] D_2O), equilibrated with 100% D_2O, loaded with 40 μg of deuterated quercetin in 2 ml of MACD labeling buffer, and washed with 100% D_2O prior to elution with 20% (v/v) D_2O in acetonitrile. The eluted quercetin was subsequently evaporated to dryness under vacuum and reconstituted in 1 ml of cold (4 °C) 10 mM ammonium formate buffer (pH 5.0 in H_2O) to promote the selective H/D back exchange from quercetin's OH groups. Both MS and tandem MS (MS/MS) spectra were collected for deuterated and unlabeled quercetin on a triple quadrupole mass spectrometer (TSQ Quantum Access, ThermoFisher) in the negative ionization mode. The samples, 40 μg/ml each, were infused at a flow rate of 20 μl/min and detected using the following instrument parameters: spray voltage, 3.5 kV; vaporizer temperature, 50 °C; sheath gas, 20 psi; auxiliary gas, 0 psi. Collision-induced dissociation (CID) fragmentation spectra were also collected for each compound using a collision energy setting of 25 eV.

H/D back exchange of catechin during reverse-phase LC–MS and C_{18} SPE?

To evaluate the H/D back exchange of OH groups as well as the A-ring of flavonoids during LC–MS, the flavan-3-ol catechin, a flavonoid with a particularly reactive A-ring for H/D exchange, was MACD labeled as described above. Specifically, 1 ml of catechin (50 μg/ml) in MACD labeling buffer was incubated for 24 h at 60 °C. Half of the resulting deuterium-labeled catechin was buffer exchanged into acetonitrile with 20% D_2O via C_{18} SPE as described above for quercetin. The resulting eluate was concentrated to dryness via vacuum centrifugation and reconstituted in 500 μl of cold H_2O with 10% methanol. Both the non-buffer-exchanged and buffer-exchanged MACD-labeled catechin, together with non-H/D-exchanged catechin (50 μg/ml in H_2O), were subsequently infused into a triple quadrupole mass spectrometer as described above for quercetin. In addition, a 5-μl aliquot of the non-buffer-exchanged MACD-labeled catechin was also analyzed by LC–MS on the same instrument using the following parameters: spray voltage, 3.5 kV; vaporizer temperature, 50 °C; sheath gas, 60 psi; auxiliary gas, 5 psi; mass range, 285 to 300 *m/z*. To promote H/D back exchange of deuterated catechin on column, 90% H_2O with 10% acetonitrile was passed through the column at 400 μl/min for 20 min prior to compound elution in 90% acetonitrile with 10% H_2O. Averaged mass spectra comprising 20 scans were generated for each sample and visually compared.

Time course of atom % incorporation and compound degradation

A standard mixture composed of quercetin-3-rhamnoside (quercitrin), kaempferol-3-glucoside (astragalin), luteolin-7-glucoside (cynaroside), resveratrol-5-glucoside (piceid), and epicatechin gallate was dried via vacuum centrifugation and reconstituted in 70 μl of CH_3OD. The dissolved sample was then diluted with 1330 μl of D_2O to generate a 100-μg/ml compound stock solution. To compare the extent of deuterium incorporation and compound degradation under various acidic conditions, three 40-μl aliquots of the stock solution were diluted 10-fold into D_2O solvent and buffered to pH 3.0 with 10 mM ammonium formate and pH 4.0 and 5.0 with 10 mM ammonium acetate. Exchange reactions at pHs 3.0, 4.0, and 5.0 were conducted at 60 and 90 °C, with an additional set of pH 3.0 reactions also conducted at 30 °C. All exchange reactions were performed in triplicate, and 25-μl aliquots were collected at 2, 4, 8, 24, 48, 72, and 96 h after initiation. A 5-μl aliquot from each time point was subsequently analyzed by reverse-phase LC–MS on an LTQ-Orbitrap XL mass spectrometer (ThermoFisher) using the solvents and flow rate described in the "LC–MS" section below. Solvents were combined to generate the following mobile-phase gradient: 0 to 1 min, 20% B; 1 to 4 min, 20 to 30% B; 4 to 6 min, 35 to 45% B; 6 to 8 min, 90% B; 8 to 10 min, 20% B. Additional instrument parameters are described in the "LC–MS" section below. Atom % incorporation was determined empirically by normalizing the isotope pattern of labeled compounds to that of their unlabeled form. Degradation was monitored for each compound by measuring the change in integrated peak area as a function of time. Details of the approach used may be found below in the "Data analysis" section.

Structure-dependent H/D exchange patterns of MACD-labeled polyphenolic standards

Three stock mixtures (A, B, and C) containing different phenolic compounds were prepared to 100 μg/ml in methanol for each compound. Stock A contained quercetin, phloretin, luteolin-8-C-glucoside (orientin), epicatechin, kaempferol, resveratrol, dihyroxycinnamic acid, and myricetin. Stock B contained luteolin, naringenin, daidzein, fisetin, luteolin-6-glucoside (isoorientin), kaempferol-7-glucoside, quercetin-3-glucoside, and resveratrol-5-glucoside (piceid). Stock C contained rhamnetin, genistein, epicatechin gallate, morin, apigenin, and quercetin-3-rhamnoside. Aliquots (80 μl) of each stock were dried under vacuum, reconstituted in 5 μl of dimethyl sulfoxide (DMSO), and diluted to 80 μl with D_2O to regenerate 100 μg/ml for each compound. Each stock was subsequently subjected to MACD labeling conditions in triplicate by combining 20 μl of each stock with 20 μl of 100 mM ammonium formate (pH 3.0 in D_2O) and 160 μl of D_2O. The reaction mixtures were incubated at 60 °C in the dark; aliquots (20 μl) were removed over time (2, 4, 8, 24, 48, and 72 h) and stored at −20 °C prior to analysis. Samples (5 μl) from each time point and from unexchanged solutions of each stock were subsequently analyzed by LC–MS using an LTQ-Orbitrap XL mass spectrometer and the flow rate and solvents described below in the "LC–MS" section. Solvents were combined to generate the following mobile-phase gradient: 0 to 3 min, 20 to 30% B; 3 to 4 min, 30 to 40% B; 4 to 6 min, 40% B; 6 to 7 min, 40 to 45% B; 7 to 8 min, 45 to 90% B; 8 to 10 min, 90% B; 10 to 13 min, 20% B. The number of H/D-exchanged positions, as well as the atom % incorporation, was determined empirically by normalizing the isotope pattern of labeled compounds to that of their unlabeled form. The details of this analysis can be found below in the "Data analysis" section.

LC–MS

Standard compounds

All LC–MS analyses of standard compounds were conducted by reverse-phase LC (RPLC) on an HSS T3 C_{18} UPLC (ultra-performance liquid chromatography) column (2.1 mm i.d. × 100 mm, 1.8 μm particle size; Waters) using a flow rate of 400 μl/min. Organic solvent gradients were generated with an Accela ultra-high-performance liquid chromatography (UHPLC) pump (ThermoFisher) by combining 10 mM ammonium acetate (pH 5.0 in H_2O; solvent A) and 100% acetonitrile (solvent B) according to the programs described above for each experiment. Unit resolution measurements on quercetin labeling and catechin back exchange were conducted on a triple quadrupole mass spectrometer (TSQ Quantum Access, ThermoFisher) using the instrument parameters described above for the respective experiments. The accurate mass measurements

conducted for all remaining experiments were made on an LTQ-Orbitrap XL using the following parameters: negative mode ionization, 7500 resolution, 500 μs trap fill time, 1 microscan, 4.0 kV spray voltage, 60 psi nitrogen sheath gas, 10 psi nitrogen auxiliary gas, 280 °C capillary temperature, and 90 V tube lens voltage. Data were collected in profile mode across a range of 150 to 650 *m/z*.

Frontenac red wine

Frontenac red wine was analyzed on a hybrid quadrupole-Orbitrap (Q Exactive) mass spectrometer (ThermoFisher) equipped with an Ultimate 3000 UHPLC pump (ThermoFisher). The wine was chromatographically resolved at a flow rate of 300 μl/min on a reverse-phase C_{18} column (HSS T3, 2.1 mm i.d. × 100 mm, 1.8 μm particle size; Waters) by mixing mobile-phase solvent A (10 mM ammonium acetate, pH 5.0) and solvent B (100% acetonitrile) to generate the following gradient: 0 to 2 min, 5% B; 2 to 22 min, 5 to 35% B; 22 to 24 min, 35% B; 24 to 27 min, 35 to 90% B; 27 to 32 min, 90% B; 32 to 32.5 min, 90 to 5% B; 32.5 to 40 min, 5% B. Accurate mass measurements (70,000 resolution) from 200 to 2000 *m/z* were collected in negative ionization mode using the following parameters: 4.0 kV spray voltage, 60 psi nitrogen sheath gas, 10 psi nitrogen auxiliary gas, 50 °C vaporizer temperature, 280 °C capillary temperature. Data-dependent tandem mass spectra of the top 10 most abundant ions (5-s dynamic exclusion) were generated in the higher energy collision-induced dissociation (HCD) cell using a normalized collision energy (NCE) setting of 40%.

Data analysis

Average isotope patterns

Deuterium isotope effects are well known to produce significant shifts in chromatographic retention [35]. To account for differences in isotope peak distributions over a typical peak elution window, isotope patterns were averaged across a retention window, including 9 MS scans about the peak apex for each compound; these were automatically computed and exported in batch using the Processing Setup tool in Xcalibur (version 4.1, ThermoFisher). Individual processing files for each compound were generated using the Quan method with the following generic parameters tailored to each compound: mass range, 6 *m/z* beginning with the quasi-molecular or M + 1 ion for the nondeuterated compound; retention time, ±60 s on either side of the previously observed elution time; signal-to-noise threshold, 3.0. In addition, report templates were generated for each compound's Quan method using the XReport tool within Xcalibur. Spectrum List Tables accessed through the Quan Repeating section were created using the Quan method processing parameters for each individual compound. The resulting .txt report files for each compound were concatenated into a single .txt file and further processed in Microsoft Excel (2007).

Atom % incorporation

To calculate atom % incorporation, an empirical approach was adopted in preference to probabilistic calculations that rely on defined elemental compositions so that the approach could be used with unknown compounds or those with ambiguous elemental compositions. For this approach, the ratio of the monoisotopic ion to the +1 and +2 ions, respectively, was first measured for each compound in its natural abundance (unlabeled) form. These ratios were then used as a template and applied to the isotopic channels of the deuterated compounds, beginning with the monoisotopic ion, to measure the fractional contribution made by each subpopulation of differentially labeled compounds (0D, 1D, 2D, etc.) to the whole. For instance, to measure the relative amount of unlabeled compound remaining postlabeling, the intensity of the monoisotopic ion and the corresponding amounts of signal from the +1 and +2 channels were totaled and divided by the total signal across the isotopic envelope. These intensities were then subtracted from the isotopic envelope to generate an adjusted spectrum. This process was repeated iteratively across the isotopic envelope until the maximum number of labeled sites was found. Using the fractional contribution made by each subpopulation (0D, 1D, 2D, etc.) to the whole, the atom % deuterium incorporation for each labeled compound was calculated by multiplying the ratio of actual-to-maximal number of H/D-exchanged positions by 100.

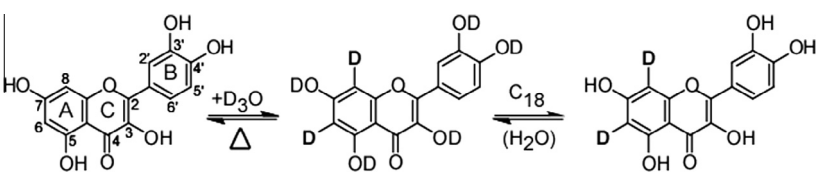
Peak area integration and computing compound degradation

Generation and integration of extracted ion chromatograms (EICs) was performed in batch using the Quan method within the Processing Setup tool in Xcalibur (version 4.1, ThermoFisher). Specifically, five peaks corresponding to the modified forms of five polyphenolics and a peak for the external standard catechin were defined in a single Quan method using similar parameters as described above. In addition, peaks were smoothed using a moving average of 7 scans prior to integration. The resulting values were reported using the Quan Peak Table output in the Non-Repeating section found in the XReport tool within Xcalibur.

To measure compound degradation, the average integrated peak area ($n = 3$) for each of the five modified polyphenolics at each time point was normalized against that of the external standard catechin. The ratio of the normalized signal at each time point relative to that at time point 0 h was then subtracted from 1 to yield the fraction of hydrolyzed compound: 1 – (signal *x* h/signal 0 h). Multiplying the resulting number by 100 provided the % hydrolysis, which was plotted against time to generate the time-dependent hydrolysis profile for each compound.

Results and discussion

Polyphenolic compounds are a chemically diverse and abundant class of plant-derived antioxidants that are commonly characterized in complex biological matrices via MS. To enhance the power of MS for identifying polyphenolics on a metabolomic scale, we optimized a stable isotope labeling method, based on MACD labeling, that introduces deuterium into polyphenolic compounds in a structure-dependent manner. Polyphenolics incubated in mildly acidified D_2O at relatively low temperatures undergo H/D exchange at aromatic protons *ortho* (C6 and C8) to resorcinolic hydroxyl groups (5 and 7 OH) via electrophilic aromatic substitution (Scheme 1) [29]. The hydroxyl groups (OD) coincidently labeled during this process are subsequently back exchanged (OH)



Scheme 1. Mild acid-catalyzed deuterium (MACD) labeling of polyphenolic compound.

following buffer exchange into H_2O while the much less acidic aromatic protons remain stably deuterated.

To demonstrate MACD labeling of polyphenolics, the flavonol quercetin was incubated in acidified D_2O (pH 3.0) for 72 h at 60 °C and then exchanged into H_2O via C_{18} SPE. The overlaid MS spectra of unlabeled and MACD-labeled quercetin indicate heterogeneous labeling of the latter, with an estimated 55% of compounds containing one D incorporation (302 *m*/*z*) and another 42% containing two incorporations (303 *m*/*z*) (Fig. 1A). In addition, when the 303 *m*/*z* ion was subjected to CID MS/MS, only the A-ring containing fragment ions were shifted by 2 *m*/*z* units relative to the fragmentation spectrum of unlabeled quercetin (Fig. 1B). For example, while the $^{0,4}A^-$ fragment shifted from 107 to 109 *m*/*z*, the $^{1,2}B^-$ ion remained unchanged at 121 *m*/*z*. Together, these data indicate that the conditions used for MACD labeling selectively exchange the A-ring aromatic protons at positions C6 and C8 and are not harsh enough to activate H/D exchange at the aromatic protons on the catecholic B-ring.

In light of the selective back exchange of quercetin OH groups following C_{18} SPE, we sought to streamline sample processing by removing the SPE step from the protocol and relying solely on RPLC in protic solvent to induce the desired back exchange. For this purpose, mass spectrometric analysis of MACD-labeled catechin (pH 3.0, 60 °C, 24 h), a flavonoid especially sensitive toward H/D exchange (or back exchange) at its C6 and C8 carbons, was conducted in D_2O labeling buffer or in protic solvent following either C_{18} SPE or inline RPLC (Fig. 2). Compared with unlabeled catechin (289 *m*/*z*; Fig. 2A), the isotope pattern for MACD-labeled catechin infused in D_2O reaction buffer (Fig. 2B) indicated a heterogeneous population of labeled compounds, with the maximally labeled compounds (295 *m*/*z*) corresponding to H/D exchange at all four nonionized OH groups as well as the aromatic positions C6 and C8 on the A-ring. However, when MACD-labeled catechin in D_2O reaction buffer was analyzed by LC–MS in protic solvents, the resulting compounds are largely homogeneously labeled with two deuterium atoms (291 *m*/*z*), suggesting that the OH groups back exchanged while positions C6 and C8 remained deuterated (Fig. 2C). Notably, this isotope pattern was not affected by exposing the labeled compound on-column to variable volumes of protic solvent prior to detection (data not shown), further illustrating the stability of the incorporation at these two positions on the A-ring. A similar labeling pattern was observed for C_{18} SPE-processed MACD-labeled catechin, indicating that RPLC was comparable to SPE for the selective back exchange of polyphenolic OH groups prior to their detection by MS (Fig. 2D).

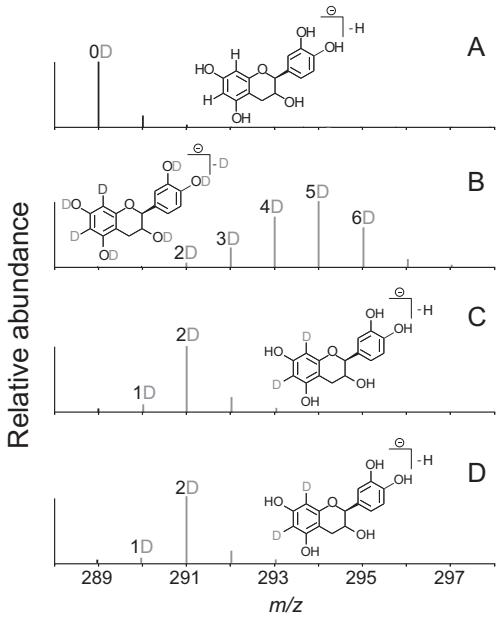


Fig. 2. (−) ESI–MS spectra of unlabeled catechin infused in H_2O (A) and stable isotope-labeled catechin following MACD labeling and infused in D_2O reaction buffer (B), infused in H_2O following C_{18} solid-phase extraction (SepPak) for buffer exchange (C), or following reverse-phase liquid chromatography with the use of H_2O aqueous phase (D).

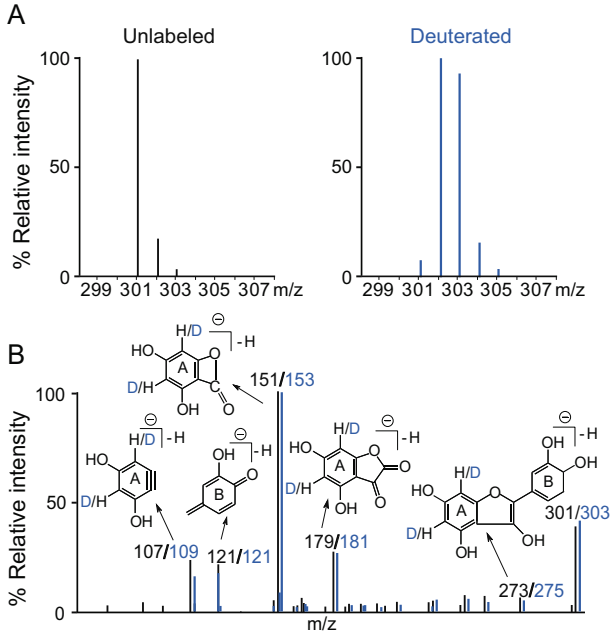


Fig. 1. Mass spectral comparison of unlabeled (black) and stable isotope labeled (blue) quercetin generated via MACD exchange. (A) Side-by-side comparison of the isotope patterns appearing in the (−) ESI–MS spectra. (B) Overlaid MS/MS spectra from the unlabeled and H/D-exchanged compound showing putative structures for select CID fragments according to Fabre and coworkers [38]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

With the goal of using MACD to inform the native polyphenolic constituents in biological samples, we evaluated the impact that various reaction conditions had on both the extent of deuterium labeling and the stability of acid-labile glycosidic linkages for a panel of structurally distinct compounds (Fig. 3). In general, temperature had a much greater impact than pH on deuterium labeling for all compounds, with higher atom % incorporations for all time points at 90 °C as compared with 60 and 30 °C (Fig. 3, solid line). Conversely, pH-dependent labeling effects were observed only for the stilbenoid resveratrol-5-glucoside (piceid), with pH 3.0 producing a modest increase in atom % incorporation over pHs 4.0 and 5.0 at both 60 and 90 °C (Fig. 3D). In addition, whereas the type of sugar conjugated to the flavonoid aglycone appeared to be inconsequential for labeling (Fig. 3A vs. Fig. 3B), the position of the sugar conjugate was not, with the atom % incorporation being greatly reduced at both 60 and 90 °C for the 7-*O*-glucoside of luteolin compared with the 3-*O*-glucoside of kaempferol (Fig. 3B vs. Fig. 3C).

Similar results were observed for compound integrity, with the extent of glycoside hydrolysis, measured by the ratio of glycoside to the external standard catechin, being noticeably greater at 90 °C than at 60 or 30 °C for all compounds (Fig. 3, dashed lines). In addition, a measurable pH effect was also observed, most notably for quercetin-3-rhamnoside, where the most acidic condition (pH 3.0) produced the greatest levels of hydrolysis. Compound structure was also a strong determinant of glycoside hydrolysis, with the type and position of the conjugated sugar greatly affecting

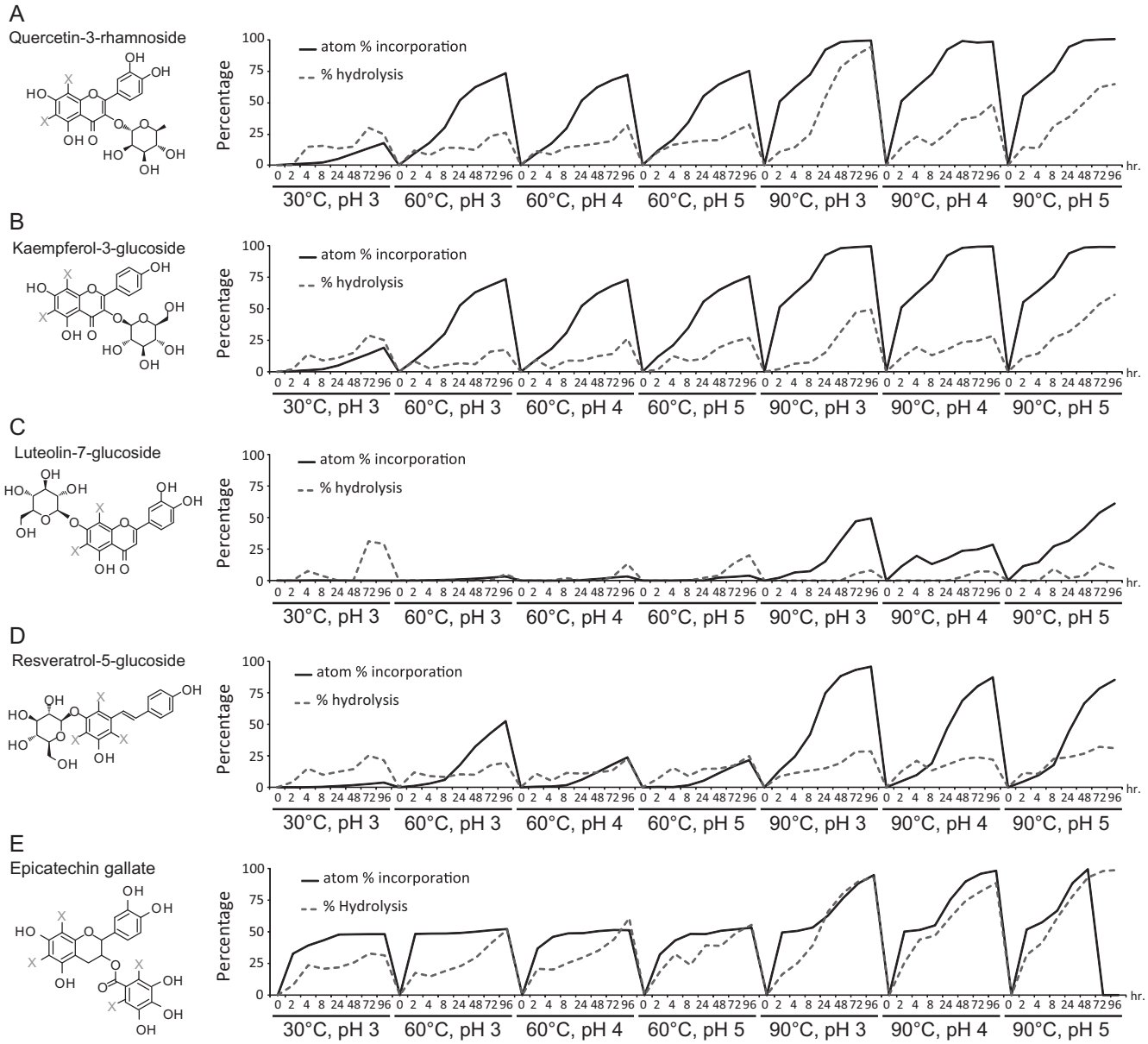


Fig. 3. H/D exchange and glycoside hydrolysis for representative glycosylated polyphenolic compounds under various MACD labeling conditions. Time course plots of atom % incorporation (solid line) and % hydrolysis (dashed line) for quercetin-3-rhamnoside (A), kaempferol-3-glucoside (B), luteolin-7-glucoside (C), resveratrol-5-glucoside (D), and epicatechin gallate (E) are shown on the same *y* axis for seven different temperature/pH combinations. The positions susceptible to stable deuterium incorporation under MACD labeling conditions are designated on each compound's structure with a gray "X".

the integrity of the glycosidic linkage. For example, greater amounts of hydrolysis were observed under most conditions for the flavonol conjugated to rhamnose at the C3 position (quercetin-3-rhamnoside) than the one conjugated to glucose at the same position (kaempferol-3-glucoside) (Fig. 3A vs. Fig. 3B). In addition, comparison of kaempferol-3-glucoside with luteolin-7-glucoside shows the disparate impact that glucoside position has on compound integrity, with the former exhibiting much higher levels of hydrolysis under all conditions (Fig. 3B vs. Fig. 3C). Interestingly, glucose conjugated to the stilbene resveratrol was more resistant to hydrolysis than when the sugar was conjugated to the C-ring but not the A-ring of flavonoids (Fig. 3D vs. Fig. 3B and C).

In addition to glycosylated phenolics, we also monitored deuterium labeling and compound integrity for the acid-esterified flavan-3-ol: epicatechin gallate (Fig. 3E). Compared with the previous compounds, epicatechin gallate was very sensitive to labeling under all conditions tested, with nearly complete labeling (50 atom % incorporation) of the A-ring achieved by 8 h irrespective of temperature or pH (Fig. 3E, solid line). Surprisingly, at 90 °C two protons on the gallate moiety also exchanged (atom % incorporation >50%), showing a slightly faster exchange rate at higher pH. By comparison, only two positional incorporations were observed for the epicatechin aglycone alone at all temperatures, thereby confirming the placement of the additional two exchanged positions for epicatechin gallate on the gallate moiety (data not shown).

Considering that ester linkages, such as the one joining epicatechin and gallic acid, are known to be susceptible to acid-catalyzed hydrolysis, we also investigated the degradation of epicatechin gallate during incubation in the various MACD labeling conditions described above. Similar to the polyphenolic glycosides, epicatechin gallate was degraded largely in a temperature-dependent manner,

with the highest levels occurring at 90 °C, followed by more modest rates at 60 and 30 °C (Fig. 3E, dashed line). Additional pH-dependent degradation at each temperature was negligible. Hence, for all compounds tested, incubation at 90 °C produced both the most robust deuterium labeling of aromatic protons and the most compound degradation, whereas reactions conducted at 60 °C resulted in more modest labeling but also reduced levels of compound degradation.

Based on these findings, we further sought to evaluate how the aglycone structure itself affects H/D exchange patterns under reaction conditions that largely avoid degradation (i.e., 60 °C, pH 3.0, 72 h). A comparison of representative compounds from four different phenolic classes revealed structure-dependent labeling patterns in both the number of exchanged positions (outer bars) and the completeness of labeling (inner bars), as represented by total atom % incorporation (Fig. 4). Following a clear order of reactivity (stilbenoid > chalcone > flavonoid ≫ dihydroxycinnamic acid), the number of exchanged positions generally corresponded with the number of aromatic protons positioned *ortho* and *para* relative to the resorcinolic OH groups (indicated by "X"), whereas the degree of deuterium labeling followed expected trends based on the number and position of ring-activating substituents. Chalcone (phloretin) was the lone exception, undergoing H/D exchange at three positions despite having only two such aromatic protons, a phenomenon likely due to additional labeling of the α-carbons via enolization [36]. Interestingly, deuterating the compounds for shorter periods at higher temperatures (90 °C, pH 3.0) produced time-dependent effects on the number of positions labeled as well as the extent of labeling. For example, whereas the deuterium labeling pattern after 8 h was similar to that observed for the 72-h time point at 60 °C, additional sites of H/D exchange were generated for the flavonoid and chalcone following 24 h of incubation (Fig. 4). However, caution must be taken when incubating compounds at elevated temperatures because additional oxidation and other undesirable side reactions will likely occur.

Given the abundance, high degree of structural variability, and potent antioxidant properties of flavonoids, we sought to further characterize the structure-dependent labeling patterns of this important class of polyphenolic compounds under the conditions described above. Specifically, the deuterium labeling patterns of compounds varying in the number and position of hydroxyl, glycosyl and methyl groups about the three rings were compared following incubation at 60 °C (pH 3.0) for 72 h. In general, compounds with unmodified resorcinol-like OH groups (*meta*-positioned to each other) were deuterated at more positions and with greater atom % incorporation than their modified counterparts (Fig. 5). This discrepancy in labeling pattern was observed in compounds lacking a 5-OH group in the A-ring (Fig. 5B, 1 vs. 2, 3 vs. 4), in compounds glycosylated and methylated at the 7-OH of the A-ring (Fig. 5C, 3 vs. 5, 6 vs. 7), and in compounds such as morin and myricetin where the presence of *meta*-positioned OH groups in the B-ring corresponded to two additional sites of H/D exchange compared with flavonols with phenolic (kaempferol) and catecholic (quercetin) B-ring configurations (Fig. S3 [see online supplementary material], 13 and 14 vs. 6 and 3). These findings were corroborated in a pair of stilbenoids where glycosylation at the 5-OH position resulted in a greatly reduced atom % incorporation compared with the unmodified form (Fig. 5C, 11 vs. 12). In addition to OH groups, glycosylation of aromatic carbons *ortho* to the A-ring OH groups in flavonoids (C6 and C8) also affected deuterium labeling patterns, with C6 and C8 glucosylated luteolin being labeled at one fewer position than the unmodified luteolin compound (Fig. 5D, 8 vs. 9 and 10).

The electronic configuration of the C-ring also affected H/D exchange patterns. For instance, a comparison of three compounds (kaempferol, apigenin, and naringenin), which differ only in their C-ring compositions, revealed that reduction of the double bond between C2 and C3, as seen in the flavanone naringenin, not only

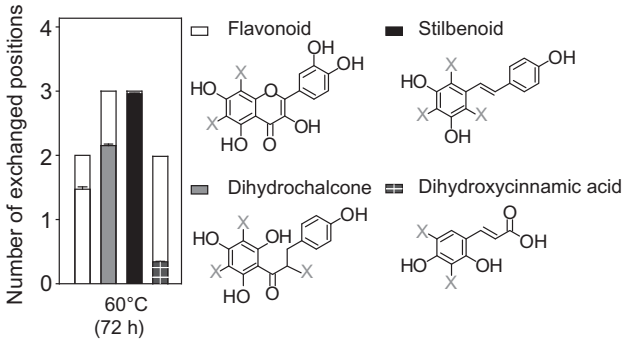


Fig. 4. Comparison of H/D exchange patterns for various phenolic compound classes following MACD labeling at pH 3.0 under different temperature/time reaction conditions. The internal colored bars indicate atom % incorporation for the observed number of exchanged positions indicated by the outer noncolored bars. Error bars for atom % incorporation reflect standard deviations from three replicates. The structures of the compounds examined are presented in the legend, with a gray "X" marking the predicted sites of H/D exchange based on the orientation of substituents about the aromatic rings. Flavonoid: quercetin; dihydrochalcone: phloretin; stilbenoid: resveratrol; dihydroxycinnamic acid: 2,4-dihyroxycinnamic acid.

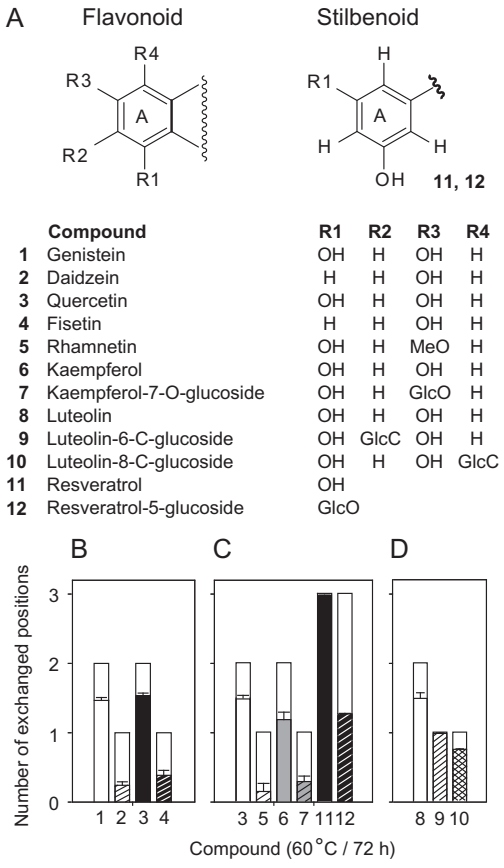


Fig. 5. H/D exchange patterns for select flavonoids and stilbenoids with A-ring compositions (A) that vary based on the presence of an OH group at R1 (B), O-conjugation of the OH group at R3 (flavonoids) or R1 (stilbenoids) (C), and C-conjugation of R2 or R4 (D). MACD labeling was conducted at 60 °C (pH 3.0) for 72 h. The internal colored bars indicate % atom incorporation for the observed number of exchanged positions indicated by the outer noncolored bars. Error bars for atom % incorporation reflect standard deviations from three replicates. MeO, O-methylation; GlcO, O-glucoside; GlcC, C-glucoside.

disrupts the conjugated pi system through the ring but also yields one additional deuterium incorporation into the compound (Fig. 6A). Similar to the chalcone described earlier, this additional site of H/D exchange likely occurred at the α-carbon adjacent to the ketone via acid-catalyzed enolization [36]. Further supporting this assertion is the fact that epicatechin, a compound lacking both a double bond between C2 and C3 and a ketone at C4, produced the same number of exchanged positions as kaempferol and apigenin, compounds with conjugated C-rings (Fig. 6, 19 vs. 6 and 15). In addition to the nature of the C2/C3 bond, the type of substituent attached to the C3 OH group also affected the deuterium labeling pattern of polyphenolic compounds. For example, whereas glycosylation of quercetin's 3-OH group did not change the number of exchanged positions (Fig. 6B), esterification of this same group in epicatechin by gallic acid resulted in two additional sites of H/D exchange (Fig. 6C). These additional sites likely occur at each of the *ortho* carbons on the phenolic ring of the gallate substituent and, according to the atom % incorporation for this compound (54%), are not robustly labeled under the MACD labeling conditions used.

As seen with the representative phenolic compounds discussed previously (Fig. 4), changing the reaction conditions to higher temperature (90 °C, pH 3.0) and shorter incubation periods (8 and 24 h) also altered the observed H/D-exchanged patterns of flavonoids in a compound-specific manner (see Figs. S1–S4 of supplementary material). The greatest change occurred in A-ring modified compounds, where increases in both the number of H/D-exchanged positions and the atom % incorporation rendered the labeling patterns less distinguishable from those of their unmodified counterparts (Fig. S2). This loss of sensitivity to structural variation is most clearly seen in the 24-h reaction data. Together, these data indicate that the H/D-exchanged patterns of flavonoids generated via select MACD labeling conditions are sensitive to structural variations and can be used to complement other mass spectral data to inform compound structure.

To aid in the interpretation of H/D exchange patterns resulting from MACD labeling, we established a set of general principles based on the regioselective deuterium labeling patterns of polyphenolic compounds described. When conducting MACD labeling at 60 °C (pH 3.0) for 72 h, H/D exchange occurs at (i) each aromatic position adjacent to *meta*-oriented hydroxyl groups, such as C6 and C8 in the A-ring of most flavonoids and C2 and C6 of gallic acid conjugates, and at (ii) sp^3 hybridized carbons adjacent (α) to carbonyls, such as C3 in the C-ring of flavanones. However, the number of exchangeable positions is decreased by one if either OH group in the *meta*-oriented pair is modified via methylation or glycosylation or if any of the adjacent aromatic positions is c-glycosylated or dimerized. Paired with accurate mass information, these principles may greatly reduce the complexity in discriminating between isomeric polyphenolic compounds that share similar structures.

To illustrate the potential of MACD labeling for discriminating between isomeric flavonoids in complex mixtures, we applied

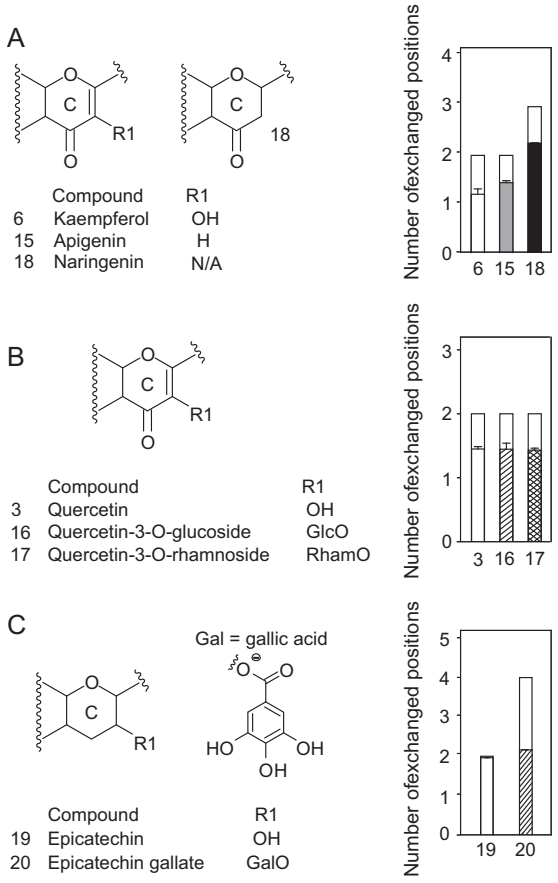


Fig. 6. H/D exchange patterns generated for select flavonoids with C-ring compositions that vary based on the presence of an OH group at R1 or a double bond between C2 and C3 (A), O-conjugation of the OH group at R1 (B), and O-esterification by gallic acid at R1 (C). MACD labeling was conducted at 60 °C (pH 3.0) for 72 h. The internal colored bars indicate % atom incorporation for the observed number of exchanged positions indicated by the outer noncolored bars. Error bars for atom % incorporation reflect standard deviations from three replicates. GlcO, *O*-glucoside; RhamO, *O*-rhamnoside; GalO, *O*-gallate.

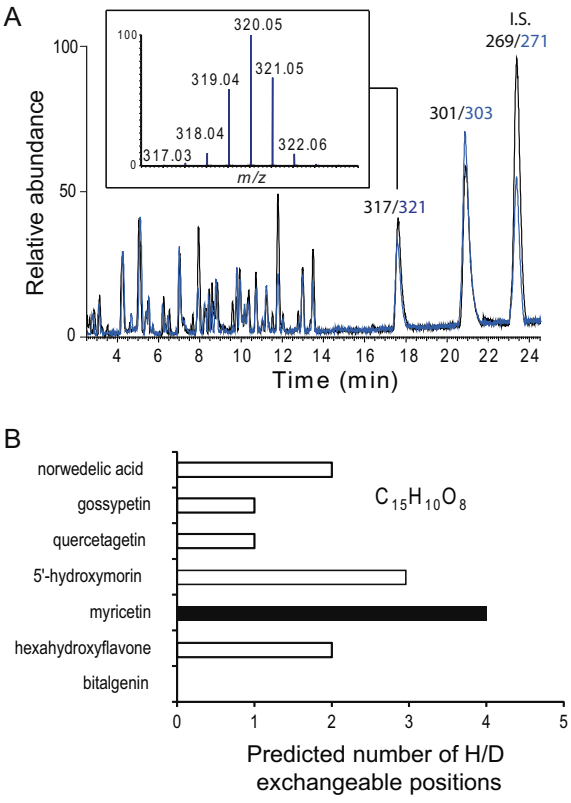


Fig. 7. MACD-labeled Frontenac red wine. (A) Overlaid base peak chromatograms of unlabeled (black) and MACD-labeled (blue) Frontenac red wine with representative labeled ion pairs, including those for the internal standard (I.S.) apigenin, marked with the nominal mass for both the unlabeled and MACD-labeled ions. The isotope pattern for the labeled form of the 317 *m/z* ion is also shown (inset). (B) Predicted number of H/D-exchanged positions for isomeric flavonoids ($C_{15}H_{10}O_8$) in the Arita laboratory flavonoid database that match by mass to the 317.0303 *m/z* (1 ppm mass error) ion [16]. Hexahydroxyflavone: 3,5,8,3′,4′,5′-hexahydroxyflavone. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

our method to the analysis of red wine. As shown in Fig. 7, the overlaid chromatograms of the unlabeled (black) and deuterated (blue) samples are identical, indicating that the complexity of the wine sample was not differentially perturbed by the varying solvent treatments. More important, many of the ions were shifted in mass following MACD labeling, including the internal standard apigenin (269 *m*/*z*) as well as unknowns at 301 and 317 *m*/*z*. That the mass shift varied among the labeled ions (2 vs. 4 Da) suggests that MACD labeling in the wine sample was sensitive to structural differences between compounds. Moreover, the extent of deuterium labeling observed for apigenin is consistent with the value predicted based on its structure, demonstrating that the MACD labeling rules defined using standard compounds apply to the analysis of complex mixtures as well.

Although the base peak for many labeled ions represented the maximally labeled species, in some instances it did not, as seen in the spectrum for the labeled form of the 317 *m*/*z* ion (Fig. 7A, inset). In this case, the prominent +4 isotopologue (321 *m*/*z*), measuring roughly 70% of the base peak ion, clearly indicated the presence of compounds with four exchanged positions. The value of this information for informing the identity of the 317 *m*/*z* ion is demonstrated in Fig. 7B. Of the seven isomeric compounds in the Arita flavonoid database that match based on mass alone (1 ppm mass error), only one (myricetin) is predicted to incorporate four deuterium atoms according to its structure (black bar). Indeed, the fragmentation spectrum of the 317 *m*/*z* ion matched well with the reference spectrum for myricetin in the Metlin LC–MS/MS database, further legitimizing the utility of the MACD labeling data to inform compound structure [37] (Fig. S5).

MACD labeling of polyphenolic compounds is a largely nondestructive chemical labeling strategy that uses electrophilic aromatic substitution to drive H/D exchange at positions *ortho* and *para* to *meta*-oriented phenolic OH groups such as the A-ring of most flavonoids and the B-ring of certain flavonoids. Compared with existing protocols that employ harsher reaction conditions, MACD labeling is much more sensitive to changes in compound structure that alter the electrophilic properties of polyphenolics, including the number, orientation, and modification status of hydroxyl groups. It is especially useful for discriminating between positional isomers of glycosylated flavonoids that are variably modified at positions involved in electrophilic aromatic substitution such as *meta*-oriented OH groups and their *ortho* carbons. Importantly, the sensitivity of the MACD labeling method is sufficient for profiling polyphenolics in complex biological matrices, as demonstrated here in the analysis of red wine, and is expected to largely depend on sample preparation and the analytical limitations of the MS platform employed for analysis. In conclusion, when combined with accurate mass and a comprehensive flavonoid database, the extent of H/D exchange generated from MACD labeling can be used to inform the identification of unknown polyphenolic compounds in complex mixtures. In addition, it should have utility for characterizing the polyphenolic profiles of novel biological samples where it may aid in the structural elucidation of previously uncharacterized compounds.

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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.ab.2014.02.018.

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