JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Biosynthesis and Characterization of ^{14}C-Enriched Flavonoid Fractions from Plant Cell Suspension Cultures

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A range of radiolabeled anthocyanins, proanthocyanidins, and other flavonoids were accumulated by cell suspension cultures of two plant species, ohelo (*Vaccinium pahalae*) and grape (a *Vitis* hybrid, Bailey Alicant A), after providing uniformly labeled [^{14}C]sucrose to the medium. Approximately 15% of administered label was recovered in a series of flavonoid-rich fractions varying in composition. Anthocyanins, and monomers to oligomers of proanthocyanidins, were labeled effectively and characterized from both species. Most of the proanthocyanidin oligomers were based on the flavan-3-ols (+)-catechin and (−)-epicatechin. Cyanidin and peonidin glycosides were the dominant forms of anthocyanins in both species. Whereas the predominant form of flavonoids identified from ohelo cell cultures was proanthocyanidins, grape cell cultures produced mostly anthocyanins. The labeled phytochemicals were produced for use in subsequent in vivo animal feeding studies to gauge their bioavailability and accumulation in target organs.

KEYWORDS: Radiolabeling; proanthocyanidins, anthocyanins, flavonoid-rich fractions; ohelo (*Vaccinium*); grape (*Vitis*)

INTRODUCTION

Flavonoids have been implicated in a diverse range of health-related biological functions in human metabolism, including antioxidant capacity, estrogenic activity, protection against DNA damage, modulation of immune system response, inhibition of retinopathy and cataracts, anti-adhesion and antiinflammatory properties, cardiovascular protection, and cancer chemoprevention (*1*−*10*). Both the synthesis and cost-effective synthesis of anthocyanins, proanthocyanidins, and flavonol glycosides have remained elusive to date; therefore, it is necessary to naturally produce flavonoids within plant cells for biological studies (*7*). The typical synergies and additive interactions that potentiate flavonoid bioactivity (for example, in inhibiting platelet aggregation) suggest that these compounds will exert their bioactivities only when harvested or delivered as natural mixtures from plant cell donors (*11*−*14*).

The use of in vitro culture techniques has become a fast and reproducible method to obtain such compounds, often in much higher concentrations than found in the plants themselves. Cell suspension cultures can synthesize high levels of polyphenolics such as anthocyanins and proanthocyanins; the products accumulated in plant cell cultures parallel those produced in vivo in the plant, and mixtures of co-occurring phytochemicals can be more easily harvested from the cultures (*15*−*20*).

Although the higher molecular weight flavonoid molecules (proanthocyanidins) are frequently implicated as beneficial to human health, isolation of specific oligomers has been problematic, in part due to the lack of efficient separation and analytical methods. For biological feeding studies, it is necessary to administer proanthocyanidins as mixtures of monomers and oligomers, the forms in which they naturally occur and are consumed in foods (*21*). However, proanthocyanidin and anthocyanin molecules can be sensitive to routine extraction methods and column chromatography. Larger oligomers are difficult to extract and hard to distinguish using HPLC, particularly when the donor tissue contains a complex profile of polyphenolics (*22*, *23*).

Bioflavonoid uptake, absorption, transport, and metabolic fate have been difficult if not impossible to gauge in many cases.

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10.1021/jf035371o CCC: $27.50
Published on Web 02/13/2004

Relatively sparse knowledge is available concerning accumulation of flavonoids or their metabolic products in various organs of the body, due to the inherent problems of selective monitoring of ingested phytochemicals from a food source in a complex diet. It is also difficult to discern newly absorbed compounds of interest from the background levels already present. This limitation has delayed development of robust dietary or prescriptive guidelines. Early animal studies suggested limited nutritional relevance of flavonoids, as they were believed to be poorly absorbed and rapidly excreted through the bile. However, in recent studies, flavonoids and their metabolites have been detected in plasma and urine of human subjects following consumption of pure compounds and flavonoid-rich foods (*24*−*26*). Mullen et al. (*27*) demonstrated that 26.2% of [2-^{14}C]-quercetin 4′-*O*-glucoside fed to rats was found unmetabolized in the intestine, whereas the remainder had undergone deglycosylation, glucuronidation, sulfation, and/or methylation as it was converted into 18 different metabolites. Vitrac et al. (*28*) incorporated a radiolabeled flavonoid precursor ([^{14}C]phenylalanine) into grape cell cultures and isolated mainly anthocyanins, catechins, and stilbenes (*cis*- and *trans*-resveratrol and their glucosides) using HPLC. Labeled resveratrol was subsequently orally administered in a rodent model revealing bioavailability and organ localization mostly in the unmodified ([^{14}C]-*trans*-resveratrol) form (*29*).

Recently, we isolated a series of proanthocyanidins by vacuum liquid chromatography (VLC) in the form of monomers, oligomers, and mixtures as they occur in situ (*18*, *30*), and developed an enclosed-chamber system for safe production and recovery of a wide range of labeled compounds and mixtures from flavonoid-rich plant cell cultures (*31*). This study investigated the biosynthesis, accumulation, and recovery of intact radiolabeled polyphenolics (especially anthocyanins and complex proanthocyanidins) with a high level of ^{14}C enrichment, after incorporating ^{14}C uniformly labeled sucrose in cell suspension production media.

MATERIALS AND METHODS

General Procedures. Commercial anthocyanin standards (Polyphenols Laboratories, Sandnes, Norway) and proanthocyanidin B1 and B2 (catechin−epicatechin) dimers (Chromadex, Laguna Hills, CA) were used in TLC, HPLC−MS, and NMR analyses to compare color changes, peaks, retention times (t_r), and molecular weights with those of compounds from our cell culture samples. Flavonoid standards included pelargonidin 3,5-*O*-β-diglucopyranoside, quercetin 4′-*O*-β-glucopyranoside, pelargonidin 3-*O*-β-glucopyranoside, cyanidin 3-*O*-β-galactopyranoside, and the 3-*O*-β-glucopyranosides of pelargonidin, cyanidin, delphinidin, petunidin, and malvidin. Stilbene standards included piceid ((*E*)-4,5′-dihydroxy-3′-*O*-glucopyranosylstilbene) and astringin ((*E*)-3,4,5′-trihydroxy-3′-*O*-glucopyranosylstilbene) (Polyphenols Laboratories). Thin-layer chromatography (TLC) was performed using silica gel plates (0.20 μm) (Sigma Chemical Co., St. Louis, MO) with solvent composed of ethyl acetate/methanol/water at a 79:11:10 ratio. Doubly distilled water (DD H_2O) was used throughout all experimental steps.

^{14}C-Labeling of Cell Suspension Cultures. Cell suspension cultures of ohelo (*Vaccinium pahalae*) and grape (a *Vitis* hybrid, Bailey Alicant A) were produced as previously described (*16*, *31*, *32*). Cultures were placed in an enclosed Plexiglas labeling chamber, constructed to provide a safe containment space as labeled polyphenolic compounds were accumulated by the cells (*31*). Uniformly labeled [^{14}C]sucrose ([U-^{14}C]sucrose) with a specific activity of 10 mCi/mmol (374 MBq/mmol) in a crystalline solid form (ICN Biomedicals Inc., Irvine, CA) was used as the source of label delivered to the metabolizing cell cultures. [U-^{14}C]-sucrose stock solutions were prepared in sterile DD H_2O (pH 5.7 for ohelo, 5.8 for grape). The [U-^{14}C]sucrose stock solution was filter-sterilized prior to medium incorporation. Concentrated media containing all components except [^{14}C]sucrose were prepared by bringing the media to 90% of the final volume. The media were then dispensed and autoclaved at 72 and 45 mL per 250 mL flask for ohelo and grape, respectively. After inoculation with established cultures (2.5 mL settled cell volume and 7.5 mL of medium), the stock [^{14}C]sucrose solution was added to ohelo (8 mL) and grape (5 mL) suspension cultures.

To optimize the consistency of production, three separate culture runs were conducted in the labeling chamber for both ohelo and grape cell cultures. For each species, the first run comprised 10 labeled cultures and 1 unlabeled control culture, and the second and third runs comprised 11 labeled cultures and 1 unlabeled control culture. The administered radioactivities per run were 800, 880, and 4083 μCi in ohelo, and 500, 550, and 2712 μCi in grape, in the first, second, and third runs, respectively. The final ^{14}C label concentrations in cell culture suspensions were 0.89 and 0.83 μCi/mL in the first and second runs of ohelo and grape cultures, respectively, and 4.1 μCi/mL in the third run of both ohelo and grape cultures. For each species, the initial two runs tested the system's capacity to produce and accumulate radiolabeled flavonoids using [^{14}C]sucrose. For the third run, a much higher level of labeled sucrose was administered to the culture medium to ensure sufficient enrichment in the labeled flavonoid products for subsequent in vivo animal trials. Products accumulated in the third run for each species were also analyzed by HPLC−MS. Cell suspensions were incubated in the enclosed labeling chamber for 12 and 14 day production runs for ohelo and grape, respectively. All suspension cultures were grown on a 160 rpm rotary shaker at 27 °C under cool fluorescent light at approximately 140 (μmol/m^2)/s.

Harvest and Extraction of Labeled Cells. The cell mass was separated from the liquid medium using Whatman no. 4 filter paper with a light rinse with 10 mL of H_2O. Cells were held at −80 °C until extraction to effect cell lysis and to prevent degradation of phytochemicals. The average fresh cell mass for the three runs was 117 g (ohelo) and 83 g (grape) at the conclusion of the cell culture runs. Cells were extracted five times with 70% aqueous acetone (500 mL, 2 min each at high speed) using a Turbo-twister blender (Hamilton Beach/Proctor-Silex, Inc., Southern Pines, NC). By the third extraction, all the colored materials were extracted from the cells. Performing two additional extractions (70% acetone/water using a high-speed blender) did not release any additional colored material, indicating that five extractions effectively removed anthocyanins from the cell mass. The acetone and some of the water were removed under reduced pressure. The concentrate was then frozen at −20 °C and lyophilized. The average dry extract mass for all three runs was 9.5 g for ohelo and 2.8 g for grape cultures.

Fractionation of Cell Extracts. The dry extracts were fractionated via vacuum liquid chromatography on an HW-40F Toyopearl resin polymer (TOSOH Bioseparation Specialists LLC, Montgomeryville, PA) (*30*). On the basis of TLC analysis, the first fraction (which contained free sugars) was discarded, and the remaining five fractions were combined and lyophilized to yield an average dry mass for runs 1−3 of 5.9 g for ohelo and 1.7 g for grape cultures. This material was then fractionated by vacuum liquid chromatography over type 60, 10−40 μm silica gel with $CaSO_4$ binder (Sigma) (*18*, *30*). Twenty-two fractions of increasing polarity were obtained by vacuum liquid chromatography on silica gel. This procedure was repeated for each of three separate runs for each species. For each of the three runs, the fraction volume was 100 mL for ohelo and 80 mL for grape. Fractions of the first and second runs were concentrated under reduced pressure, and traces of solvent were removed with nitrogen. The remaining solid material was then dissolved in MeOH (10 mL). A 5 μL aliquot from each flavonoid-rich fraction was used for thin-layer chromatography (TLC) (*18*), and another 5 μL aliquot was used to measure the radioactivity in the fraction. Two TLCs were run for each ohelo and grape fraction; one was sprayed with vanillin−HCl reagent and the other with dichromate solution and heated for 10 min at 100 °C. Vanillin−HCl was used as a primary qualitative test to detect the presence of anthocyanins and proanthocyanidins, and the dicromate was used to detect their relative mass in the extracts.

Flavonoid-rich fractions from the third run for each plant species were concentrated in doubly distilled H_2O under reduced pressure to

create a viscous slurry, transferred into glass vials, and stored in the dark at −20 °C. These fractions were kept in slurry form to facilitate immediate use of the labeled fractions in HPLC−MS analysis, and to avoid any potential loss in activity during the dehydration and rehydration steps before use in animal experiments. Redissolving the dried flavonoid-rich fractions in solvents was not easy and never complete; therefore, the potential for inadvertently altering the chemistry and the biological activity of some fractions exists, a possibility we attempted to avoid by keeping third run fractions in slurry form.

Level of Label Enrichment in Fractions. To measure radioactivity in fractions, an aliquot (5 μL) was added to Biosafe II liquid scintillation cocktail (10 mL) (Research Products International Corp., Mount Prospect, IL), and the two components were mixed well before being loaded into the scintillation counter for 2 min runs. Radioactivity was estimated using a Beckman multipurpose liquid scintillation counter, model LS-6500. The amount of ^{14}C label recovered in each fraction was calculated by converting the counts per minute (CPM) into the number of microcuries of label radioactivity. The level of label enrichments (μCi/g) for flavonoid-rich fractions was estimated by dividing the amount of ^{14}C label by the dry mass of each fraction from the first and second runs.

Composition of Flavonoid-Rich Fractions from Silica Gel Chromatography. The 22 fractions after silica gel vacuum liquid chromatography from the third run for both ohelo and grape were used for this analysis. On the basis of TLC, fractions with similar profiles were combined into larger fractions. For ohelo, seven major fractions were made by combining fractions 1−4, 5−7, 8−10, 11−13, 14−16, 17−19, and 20−22. For grape, six major fractions were made by combining fractions 1−5, 6−8, 9−11, 12−15, 16−19, and 20−22. These fractions were concentrated under reduced pressure to a viscous slurry, which resulted in final volumes of 2.1, 10, 10.5, 19, 18, 17, and 5.7 mL for ohelo, and 2.4, 2.3, 7.5, 18, 17, and 3.7 mL for grape. As noted above, these were not completely concentrated to avoid loss of biological activity. A 100 μL aliquot of each was diluted with 500 μL of doubly distilled H_2O (standard dilution) and used for HPLC−MS.

The HPLC−MS analyses were made with an LCQ Deca XP mass spectrometer (Thermo Finnigan Corp., San Jose, CA), MS version 1.3 SRI, electrospray ionization (ESI) in the positive ion mode (m/z 150−2000), with a photodiode array (PDA) detector (200−600 nm), version 1.2, autosampler version 1.2, and Xcalibur software for data processing. The HPLC separations were carried out on a C_{18} reversed-phase column (150 × 2.1 mm i.d., particle size 5 μm, 90 Å) (VYDAC, Western Analytical, Murrieta, CA). The mobile phase consisted of H_2O with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A step gradient of 0%, 5%, 50%, 80%, 100%, and 0% of solvent B at 3, 4, 30, 42, 50, and 58 min, respectively, a flow rate of 200 μL/min, and an injection volume of 10 μL were employed. The volume injected contained approximately 150 μg for ohelo or 100 μg for grape samples. Subsets of the concentrated slurries above were diluted 30 times or 100 times with H_2O and analyzed by HPLC−MS to ensure that polymerization did not occur as an artifact of the mass spectrometric analysis.

NMR spectra were obtained on a Varian Unity Inova 500 spectrometer (Varian Instruments, Palo Alto, CA) at a proton frequency of 500.078 MHz, using a 5 mm proton dedicated probe at 298 K. Spectra were acquired over a sweep width of 8 kHz, using at least 16 90° (7.3 μs) pulses and 65K points, giving an acquisition time of 4.1 s. No additional recycle delay was used. Samples (1 mL) from the concentrated slurries above (fractions 8−10 and 17−19 for ohelo and 9−11 and 16−19 for grape) were concentrated to dryness (average of 100 mg for ohelo and 60 mg for grape flavonoid-rich fractions) and used for NMR analyses. Samples were dissolved in dimethyl sulfoxide (DMSO-d_6; 2 mL) (Cambridge Isotope Laboratory, Inc., Andover, MA).

RESULTS AND DISCUSSION

Cell Growth and ^{14}C Partitioning. To investigate whether enclosure of cell cultures in the labeling chamber adversely affected productivity, parallel cell suspensions were grown at the same time in a standard growth room using a rotary shaker at 160 rpm and 27 °C, under cool white fluorescent lamps providing approximately 140 (μmol/m^2)/s irradiance; the same conditions were applied to labeled cultures contained in the labeling chamber. In addition, a control culture (no ^{14}C source added) was included within the labeling chamber for each cell type. Enclosure in the chamber did not adversely affect cell growth, as evidenced by a direct comparison of fresh cell mass produced per flask (an average fresh cell mass per flask of 10.5 g for ohelo, and 7.3 g for grape, n = 3 runs). These results assured us that using the labeling chamber was not detrimental to culture productivity. Since the vehicle for ^{14}C delivery to metabolites was [U-^{14}C]sucrose, it was expected that label would be incorporated into any product or structure in the cells that utilized sugar as a carbon source. The proportions of ^{14}C label incorporated into the cells, released as CO_2 through respiration, or remaining in the medium were presented previously (*31*).

Table 1. Fraction of Dry Mass (g) in Each of the 22 Fractions from Silica Gel Chromatography for Ohelo and Grape Cell Cultures

ohelo cell culture				grape cell culture			
no.	run 1	run 2	%[a]	no.	run 1	run 2	%
1–3	0.0038	0.0069	5.9	1–3	0.0032	0.0064	14.0
4	0.1568	0.4342		4	0.1134	0.1017	
5	0.1737	0.3299	21.3	5	0.1310	0.0713	
6	0.3407	0.3469		6	0.0129	0.0284	3.1
7	0.5018	0.4961		7	0.0058	0.0123	
8	0.5129	0.6581	19.6	8	0.0067	0.0286	
9	0.2472	0.2282		9	0.0111	0.0305	5.8
10	0.1228	0.2411		10	0.0285	0.0445	
11	0.0932	0.3439	15.2	11	0.0195	0.0414	
12	0.0984	0.4555		12	0.0239	0.0310	21.6
13	0.1242	0.4462		13	0.0263	0.0507	
14	0.2361	0.4992	30.2	14	0.0352	0.0689	
15	1.0881	0.5549		15	0.1861	0.2365	
16	0.5156	0.2061		16	0.3058	0.3358	53.1
17	0.0826	0.2509	6.9	17	0.1949	0.4524	
18	0.0314	0.2192		18	0.1681	0.0740	
19	0.0077	0.1125		19	0.0557	0.0299	
20	0.0074	0.0274	1.0	20	0.0104	0.0138	2.3
21	0.0121	0.0158		21	0.0044	0.0132	
22	0.0078	0.0286		22	0.0147	0.0143	
total	4.3643	5.9016	100.0	total	1.358	1.6856	100.0

[a] Percentages of corresponding recombined dry mass fractions for runs 1 and 2.

Composition of Flavonoid-Rich Fractions from Silica Gel Chromatography: TLC Analysis. Vacuum liquid chromatography on a silica gel column of the material derived from Toyopearl separation yielded 22 fractions from each run, the last 18 of which were flavonoid-rich fractions of increasing polarity. The total dry mass of these fractions was 4.4 and 5.9 g for ohelo and 1.4 and 1.7 g for grape in the first and second runs, respectively (**Table 1**). For ohelo, the greatest amount of dry mass was accumulated in fractions 14−16 (30.2%) followed by fractions 5−7 (21.3%). In contrast, grape accumulated the greatest mass in later fractions (53.1% in fractions 16−19). These results indicate that there were large differences in metabolite accumulation and composition between the cultured cells from the two different plant species.

Thin-layer chromatography was used routinely at each stage of fractionation to monitor the chromatographic separation of phenolic compounds. Extracts of ohelo cell cultures produced purple-blue-colored spots under visible light that corresponded to anthocyanin pigments. Previous reports suggested that this cell culture line produces primarily cyanidin-based compounds (*33*, *34*). The same phenomenon (purple-blue spots under visible light) was observed with grape cell cultures, but in a much wider

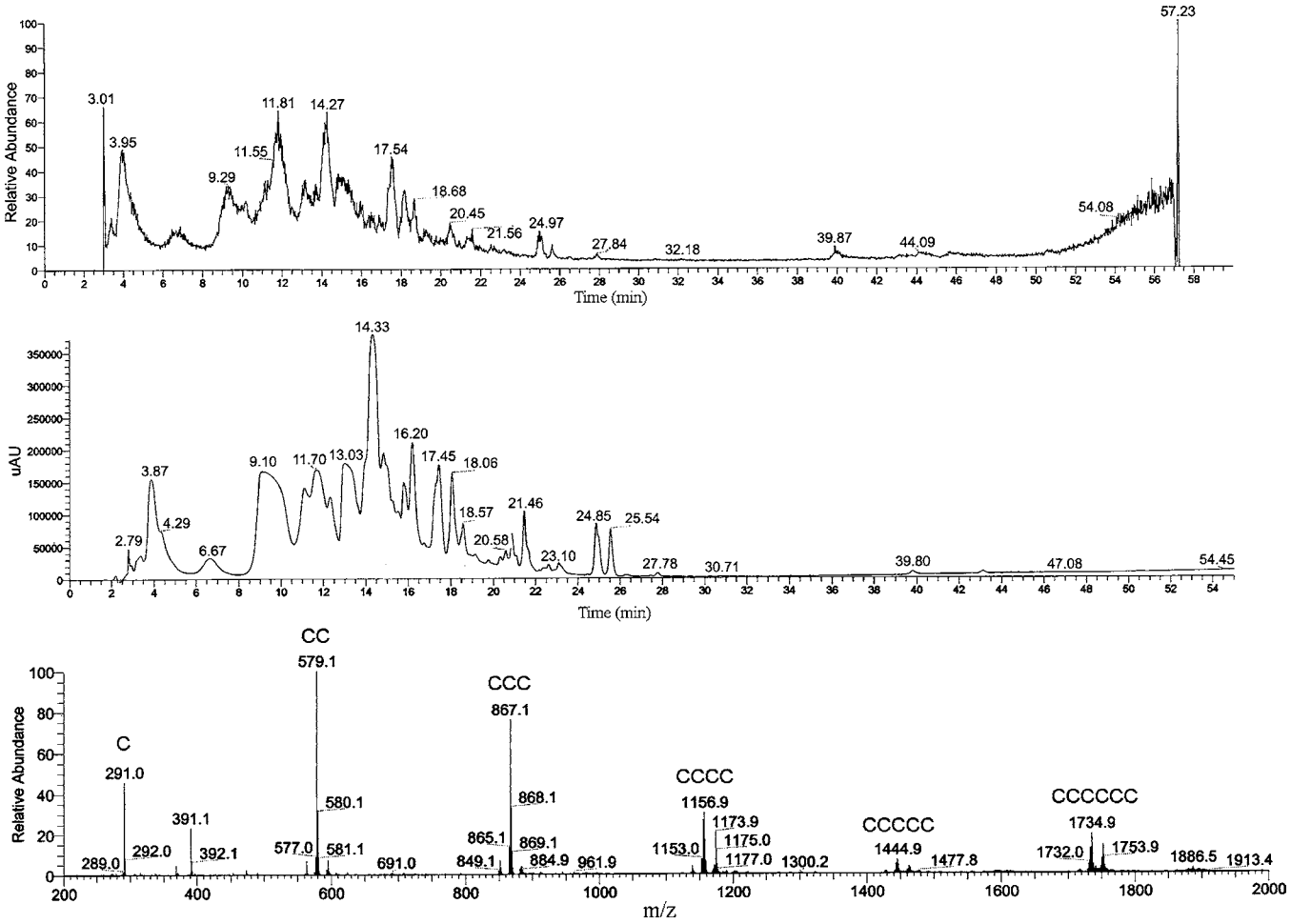

Figure 1. Representative HPLC–MS output of fractions 5–7 for ohelo cell culture extracts including the total ion current (top), photodiode array chromatogram (middle, 200–600 nm), and ESI full mass spectrum (bottom, *m*/*z* 200–2000). C = catechin or epicatechin.

range of fractions. A number of anthocyanins have previously been reported from grape species and their cell cultures (*15*, *19*, *32*).

The presence of proanthocyanidins in these cultures was established by visualizing TLC plates with vanillin−HCl reagent. Vanillin reacts with free monomers and terminal flavan-3-ols in the presence of acid. In addition, acid reacts with the extenders of proanthocyanidin molecules, resulting in colored adducts (*35*, *36*). The presence of both anthocyanidins and proanthocyanidins in a few fractions from ohelo and grape cell cultures led to difficulties in interpreting the results of the vanillin−HCL reagent. TLC analysis, using the intensity of the black color formed following the application and development of dichromate reagent, indicated that the ohelo cells produced more types and amounts of proanthocyanidins than grape cells.

Composition of Flavonoid-Rich Fractions: HPLC−MS and NMR Analysis. HPLC−MS and NMR were used to characterize proanthocyanidins and anthocyanins present in the flavonoid-rich fractions from the third run from each cell line. For ohelo cell cultures, a series of proanthocyanidins ranging from monomers to dodecamers were detected. The proanthocyanidin monomers were mainly (+)-catechin and (−)-epicatechin with *m*/*z* 291. These compounds were previously reported in an approximate 1:6 ratio in ohelo (*V. pahalae*) cell culture extracts (*18*). To estimate the relative ratio of the proanthocyanidins and anthocyanins present in the fractions for this study, the HPLC−MS peaks plotted from each major fraction were measured in millimeters and summed, and each peak was expressed as a percentage of the total sum. This approximate estimation was used to gauge the relative importance of phenolic compounds present in the fractions. Therefore, it is noted that the data on flavonoid quantification were used to illustrate the relative abundance on the basis of MS peak heights. On the basis of this estimation, monomers (*m*/*z* 291, 15%), dimers (*m*/*z* 579.1, 32%), and trimers (*m*/*z* 867.1, 24%) of proanthocyanidin based on catechin and epicatechin were the major components of fractions 5−7 of ohelo. Small MS peaks of proanthocyanidin tetramers, pentamers, and hexamers were detected in these fractions. The HPLC−MS spectrum showing the total ion current chromatogram from *m*/*z* 200 to *m*/*z* 2000 from this major fraction (fractions 5−7) and the corresponding photodiode array detector response at 200−600 nm is shown in **Figure 1**. The major peaks in the total ion current spectrum (TIC MS) were detected within the first 30 min of each HPLC−MS run. These peaks corresponded to compounds with maximum wavelength absorption of 200−600 nm. This indicates that the majority of the fraction mass was phenolic compounds. The same pattern was also observed with HPLC−MS output for grape below. The ^{1}H NMR spectroscopic data were in accordance with previously reported ^{1}H NMR, ^{13}C NMR, and mass spectrometric data obtained from extracts from this cell line (*18*). Small MS peaks corresponding to gallocatechin/epigallocatechin (16 units greater than the proanthocyanidins based on catechin/epicatechin) and afzelechin/epiafzelechin (16 mass units less) were present in these fractions. With the later fractions, increasingly higher peaks coresponding to these compounds were observed. These results indicate that a large number of proanthocyanidins based on catechin and epicatechin monomers with various

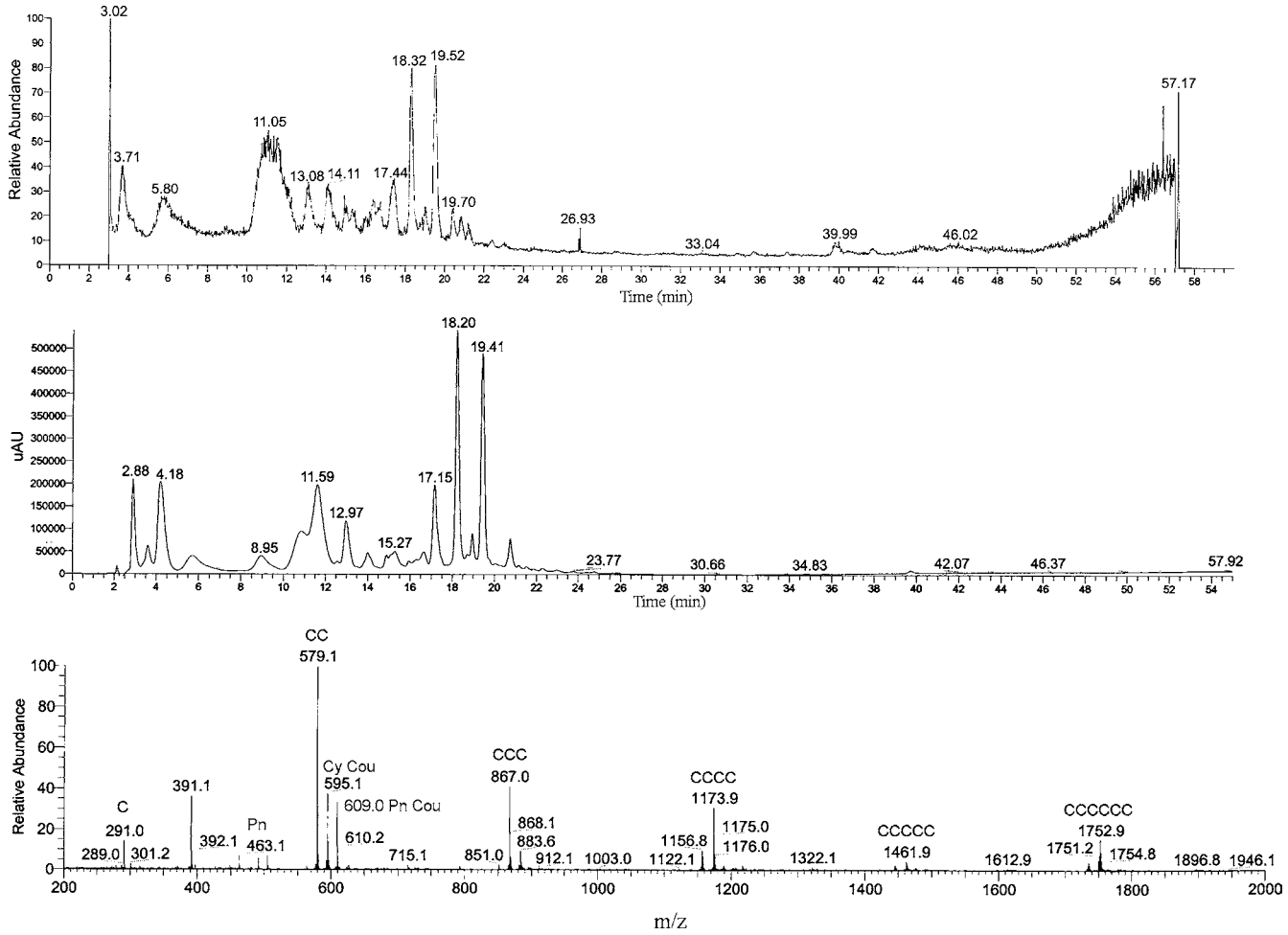

Figure 2. Representative HPLC–MS output of fractions 5–8 for grape cell culture extracts including the total ion current (top), photodiode array chromatogram (middle, 200–600 nm), and ESI full mass spectrum (bottom, *m*/*z* 200–2000). C = catechin or epicatechin. Pn = peonidin 3-*O*-glucoside (*m*/*z* 463.1). Cy Cou = cyanidin 3-*O*-*p*-coumarylglycoside (*m*/*z* 595.1). Pn Cou = peonidin 3-*O*-*p*-coumarylglycoside (*m*/*z* 609.0).

chemical structures can be reliably produced in plant cell cultures. For example, the peak at t_R = 9.3 min from fractions 5−7 from ohelo contained mostly catechin/epicatechin (*m*/*z* 291) and a trimer (*m*/*z* 867.1), although the subsequent peak at t_R = 11.7 min contained mostly dimers, tetramers, and hexamers. The peak at t_R = 14.3 min had trimers and hexamers, the peak at t_R = 15.3 min contained dimers, trimers, tetramers, pentamers, and hexamers, and the peak at t_R = 17.5 min also had dimers, trimers, tetramers, pentamers, and hexamers. Because these oligomers eluted at different retention times, they must be oligomers of different chemical configurations.

All subsequent ohelo fractions derived from silica gel chromatography contained a larger percentage of proanthocyanidin oligomers. For example, fractions 8−10 contained a series of oligomers similar to those of fractions 5−7 above, but with an increased ratio of the higher MW oligomers. Dimers (11%), trimers (18%), tetramers (20%), pentamers (17%), and hexamers (15%) comprised most of fractions 8−10. The next major fractions (11−13) were composed mainly of pentamers (21%) and hexamers (28%) and contained relatively small MS peaks of the dimers and trimers. Fractions 14−16 contained increasing proportions of the higher MW oligomers. A relatively small MS peak of a quercetin glucoside (*m*/*z* 465, t_R = 20.25 min) was found in fractions 8−10. Ohelo suspension culture was previously reported to produce quercetin (*18*).

Small MS peaks which corresponded to anthocyanins were found in fractions 8−22 including cyanidin 3-*O*-glucoside (*m*/*z* 449), petunidin 3-*O*-glucoside (*m*/*z* 479), peonidin 3-*O*-glucoside (*m*/*z* 463), and cyanidin 3-*O*-*p*-coumarylglucoside (*m*/*z* 596). Many *Vaccinium* species have been reported to synthesize these types of anthocyanins (*33*, *34*, *37*). Overall, this cell culture was found to produce mainly proanthocyanidins in addition to minor quantities of anthocyanins in the isolated flavonoid-rich fractions.

The retention times (minutes) for the standards used in this study were as follows: piceid (17.5), pelargonidin (11.8), cyanidin (15.5), astringin (17.4), quercetin (19.8), dimer (5.8). The mixture of pelargonidin, cyanidin, delphinidin, petunidin, and malvidin glycoisides had retention times of about 11, 12, 13, 14, and 16 min, respectively. A quercetin glucoside with *m*/*z* 465 was distinguished from anthocyanins with the same mass by comparing the UV spectra and t_R values. The quercetin glucoside eluted at 19.8 min, whereas delphinidin glucoside, also *m*/*z* 465, eluted before 16 min. Samples were run using the same HPLC−MS method conditions; however, when the mass range was set at a maximum of *m*/*z* 4000, the modified procedure revealed that oligomers up to dodecamers are actually present in the ohelo cell culture fractions, although in minor amounts.

Grape cell cultures accumulated more anthocyanins and fewer proanthocyanidins than ohelo cell cultures. Fractions 1−5 contained mostly lipids and catechin or epicatechin monomers and only small amounts of proanthocyanidin dimers. Proanthocyanidin dimers (39%) and trimers (16%) comprised a major portion of fractions 6−8. Fractions 9−11 had 12−13% dimers and trimers with minor quantities of tetramers and pentamers

Table 2. Incorporation of ^{14}C Label in the 22 Fractions from Silica Gel Chromatography for Ohelo and Grape Cell Suspension Cultures

	amt of label (μCi) (%)[a]					
	ohelo cell cultures			grape cell cultures		
no.	run 1	run 2	run 3	run 1	run 2	run 3
1–3	0.0 (0.0)	0.0 (0.0)	0.2 (0.0)	0.0 (0.0)	0.2 (0.2)	0.1 (0.0)
4	0.7 (0.7)	1.3 (1.1)	8.6 (1.4)	1.0 (1.6)	1.0 (1.3)	10.8 (2.2)
5	6.4 (6.0)	7.4 (6.5)	40.1 (6.7)	3.3 (5.2)	3.4 (4.5)	6.1 (1.3)
6	10.0 (9.4)	8.2 (7.2)	31.0 (5.2)	1.1 (1.7)	2.9 (3.0)	3.0 (0.6)
7	14.2 (13.4)	11.3 (10.0)	29.3 (4.9)	0.7 (1.1)	1.6 (2.1)	3.5 (0.7)
8	12.8 (12.0)	14.1 (12.4)	41.8 (7.0)	0.7 (1.1)	2.4 (3.2)	6.1 (1.2)
9	8.3 (7.8)	5.3 (4.7)	33.8 (5.6)	1.2 (1.9)	2.5 (3.3)	10.2 (2.1)
10	4.1 (3.9)	5.6 (5.0)	34.1 (5.7)	3.0 (4.8)	4.6 (6.1)	15.8 (3.2)
11	3.3 (3.1)	7.2 (6.4)	26.1 (4.4)	2.1 (3.3)	3.1 (4.1)	18.3 (3.7)
12	3.4 (3.2)	7.6 (6.7)	24.8 (4.1)	2.3 (3.7)	1.7 (2.2)	21.1 (4.3)
13	3.0 (2.8)	8.9 (7.9)	47.5 (7.9)	2.6 (4.1)	3.5 (4.7)	21.6 (4.4)
14	6.3 (6.0)	10.7 (9.4)	46.0 (7.7)	2.9 (4.6)	5.0 (6.6)	31.8 (6.5)
15	22.6 (21.3)	14.5 (12.8)	29.9 (5.0)	13.5 (21.5)	17.3 (23.0)	38.9 (7.9)
16	9.8 (9.2)	4.9 (4.3)	34.7 (5.8)	17.0 (27.1)	11.9 (15.9)	35.6 (7.3)
17	1.5 (1.4)	2.5 (2.2)	48.4 (8.1)	5.8 (9.2)	8.6 (11.5)	80.1 (16.3)
18	0.2 (0.2)	2.1 (1.8)	52.5 (8.8)	2.2 (3.5)	3.3 (4.4)	87.6 (17.9)
19	0.0 (0.0)	1.2 (1.1)	35.5 (5.9)	2.7 (4.3)	1.4 (1.9)	73.6 (15.0)
20	0.1 (0.1)	0.4 (0.4)	21.2 (3.5)	0.6 (1.0)	0.4 (0.5)	21.8 (4.4)
21	0.0 (0.0)	0.0 (0.0)	10.5 (1.8)	0.2 (0.2)	0.2 (0.2)	3.7 (0.7)
22	0.0 (0.0)	0.1 (0.1)	3.5 (0.6)	0.1 (0.1)	0.4 (0.6)	0.7 (0.1)
total	106.6	113.6	599.3	62.7	75.2	490.4
percentage of label recovery	13	13	15	13	14	18
av of three runs		14			15	

[a] The initial number of ^{14}C microcuries administered to cell cultures was 800, 880, and 4083 μCi in ohelo and 500, 550, and 2712 μCi in grape in the first, second, and third runs, respectively. Values in parentheses are percentages of ^{14}C label recovered in each fraction.

in comparison to fractions 6−8. Two large peaks corresponding to cyanidin 3-*O*-*p*-coumarylglucoside (*m*/*z* 595, t_R = 18.2 min) and peonidin 3-*O*-*p*-coumarylglucoside (*m*/*z* 609, t_R = 19.4 min) with maximum wavelength absorbance of 280 and 525 were observed in fractions 6−8 of grape extracts (**Figure 2**). An output of the HPLC−MS spectrum of fractions 6−8 showing the total ion current, photodiode array response at 200−600 nm, and mass scan from *m*/*z* 200 to *m*/*z* 2000 is shown in **Figure 2**. Subsequent fractions contained smaller amounts of proanthocyanidins, but increasingly large amounts of anthocyanins. The most abundant anthocyanins from grape fractions were peonidin 3-*O*-glucoside (*m*/*z* 463) and cyanidin *p*-coumarylglucoside (*m*/*z* 595). Peonidin 3-*O*-glucoside was a significant component (19%, 45%, 32%, and 39%) of fractions 9−11, 12−15, 16−19, and 20−22. These fractions contained most of the mass of proanthocyanidins and anthocyanidins from silica gel vacuum liquid chromatography of grape (**Table 1**). Fractions 6−8 and 9−11 each contained 15% cyanidin 3-*O*-*p*-coumarylglucoside. Several other anthocyanins such as cyanidin 3-*O*-glucoside were identified in grape fractions, but in minor quantities. The identification of anthocyanins in this study was in agreement with recent reports (*38*).

An HPLC−MS peak with *m*/*z* 391 corresponded to the stilbene glucoside piceid previously reported from grape cell cultures. However, because this mass was similar to that of the ubiquitous plasticizer bis(2-ethylhexyl) phthalate (*m*/*z* 391), it was difficult to distinguish the two conclusively in all fractions. In fractions 9−11 in grape, there was an unambiguously large MS peak (*m*/*z* 391) that was confirmed to be piceid. Grape cell cultures (*Vitis vinifera*) have been reported to produce stilbenes such as resveratrol (*20*, *43*).

^{14}C Label Enrichments in Flavonoid-Rich Fractions. Overall from the three labeling runs, an average of 14% or 15% of the administered radioactivities was recovered in flavonoid-rich fractions of ohelo and grape cell cultures, respectively. This was comparable to previous studies, using [^{14}C]-L-phenylalanine as a radioactive precursor in grape cell suspension cultures, in which 15% incorporation into stilbenoid compounds was reported (*28*). In another previous report, 20% incorporation of [^{14}C]-L-phenylalanine into bibenzyl compounds was achieved using *Marchantia* cell suspension cultures (*39*).

In the first and second runs of ohelo and grape, most of the recovered ^{14}C label was incorporated into fractions 5−17 from vacuum liquid chromatography on silica gel. However, in the third run, when the initial label concentration in the production medium was much higher (4.1 μCi/mL), much of the label was recovered in later (higher molecular weight, proanthocyanidin-rich) fractions (fractions 8−20) for both species. The amount of ^{14}C label incorporated into the flavonoid-rich fractions of ohelo and grape in the first two labeling runs (with the same level of initial label administered) was consistent. The percentage of total label recovery was 13%, 13%, and 15% for ohelo, and 13%, 14%, and 18% for grape, in the first, second, and third runs, respectively (**Table 2**). The total amount of ^{14}C label recovered in the third run was about 5 and 7 times higher for ohelo and grape, respectively, compared with the average of the first two runs. These results indicate that the labeling system and production were reproducible and predictable, and that the same procedure can be applied to similar labeling runs to obtain a required incorporation. The amount of ^{14}C label incorporated was directly proportional to the mass of flavonoid-rich fractions in the first and second runs. Correlation (r^2), calculated using the SAS program (*40*), between dry mass and the amount of ^{14}C in flavonoid-rich fractions was highly significant ($P < 0.0001$) in each run from both ohelo and grape cell lines. The correlation coefficients were 0.97 and 0.89 for ohelo, and 0.84 and 0.77 for grape, in the first and second runs, respectively.

The level of ^{14}C label enrichment, which is a function of the number of microcuries of ^{14}C label and mass of the flavonoid-rich fractions, varied among fractions from silica gel chromatography and between the two plant cell lines. The fractions containing proanthocyanidins and anthocyanins (fractions 5−20)

had an average level of ^{14}C label enrichment of 22 and 71 μCi/g for ohelo and grape for the first two runs, respectively. However, label enrichment was projected to be equivalent to 110 and 497 μCi/g in ohelo and grape fractions in the third run (4.1 μCi/mL), respectively. Similar levels of ^{14}C label enrichment were reported by Vitrac et al. (*28*) (260−350 μCi/g) and Deprez et al. (*41*) (200−500 μCi/g), although higher concentrations of ^{14}C-radiolabeled phenylalanine (5 μCi/mL) were used in those reports. The labeled flavonoid-rich fractions of ohelo and grape possessed different proanthocyanidin and anthocyanin profiles. Whereas ohelo cultures produced a range of labeled proanthocyanidins with smaller amounts of accompanying anthocyanins, grape cultures synthesized much greater quantities of anthocyanins and only modest quantities of proanthocyanidins, on the basis of the dry mass of fractions, and the detected flavonoids from HPLC−MS analysis. These fractions are uniquely labeled with different combinations of phytochemicals that have been reported to exhibit antioxidant and anticancer effects (*18*, *42*−*44*).

Synergistic effects are considered an important factor in determining the bioactivity of polyphenolics; therefore, these flavonoid-rich fractions are suitable candidates for investigating the bioactivity of natural co-occurring mixtures. The presence of both anthocyanins and proanthocyanidins in the fractions may help to elucidate the potentiation effects created when different flavonoids interact to exert a biological effect. Interactions between potentiating compounds in a mixture are commonly recognized in the pharmacology field and therefore should be evaluated before further fractionation and purification is conducted. The difficulties of identifying specific bioactive flavonoid entities may be due in part to the multitude of structures involved and to a loss of efficacy when interacting components are separated. Additionally, it is also recognized that natural combinations of phytochemicals often exhibit enhanced bioactivity in comparison to single compounds (*12*).

In conclusion, we report the biosynthesis of radiolabeled proanthocyanidins and anthocyanins of a wide range of molecular weights, ranging from anthocyanins to proanthocyanidin monomers, such as catechin and epicatechin, to more complex oligomers, using cell suspension cultures of two plant species. Vacuum liquid chromatography on silica gel permitted rapid fractionation of relatively large amounts of extracts (up to 20 g), shortening the time that sensitive compounds are exposed to chromatographic supports and air, and minimizing potential modification of bioactivity (*45*). The use of a labeling chamber in our study offered a means of safe and efficient labeling, and allowed reproducible results in radiolabeling of unique classes of natural plant phytochemicals. These labeled phytochemicals will be used for in vivo studies of the bioavailability, metabolic fate, and site of accumulation in animal target organs.

ACKNOWLEDGMENT

This work was supported by USDA/IFAFS Grant No. 00-52101-9695, NIH NCCAM Purdue-UAB Botanical Center for Dietary Supplement Research (P50 AT-00477), and NIH NCCAM Grant No. AT00404-01. This work was also supported in part by the U.S. Department of Agriculture, Agricultural Research Service, under Cooperative Agreement No. 58-6250-6-001. The contents of this paper do not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government. We appreciate the donation of grape cells by Dr. Michael Shuler, Cornell University.

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Received for review November 20, 2003. Revised manuscript received January 1, 2004. Accepted January 2, 2004.

JF035371O