Isotopic Labeling of Red Cabbage Anthocyanins with Atmospheric $^{13}CO_2$ in Closed Environments

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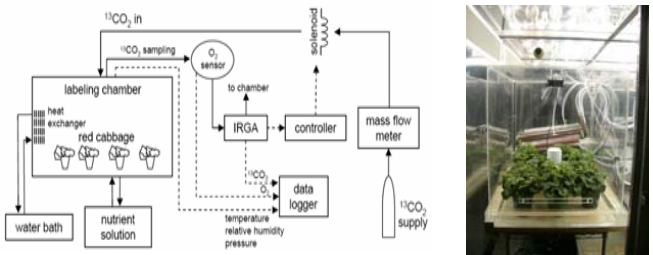


Figure 2. Labeling Chamber

A 650 L acrylic box was seated in a water trough to produce an air-tight seal. Relative humidity and temperature were controlled by coordinated temperature adjustment of the surrounding growth chamber and a cold finger in the box (visible to the rear in the photo). Condensate from the cold finger drained to the water trough. An axial fan circulated air inside the box while an IRGA (PP Systems, WMA-4) calibrated against $^{13}CO_2$ monitored CO_2 concentration and controlled a normally-closed solenoid value, opening below 390 ppm and closing above 400 ppm. The concentration following injection typically increased to about 450 ppm because of delayed mixing. Plants were grown on a calcined clay substrate (Turface) to minimize release of CO_2 from organic material. Both the light cap barrier and the labeling box were constructed from UV-transmitting acrylic, but UV radiation inside the labeling chamber was not controlled or monitored. The photo shows strawberry plants growing inside the box.

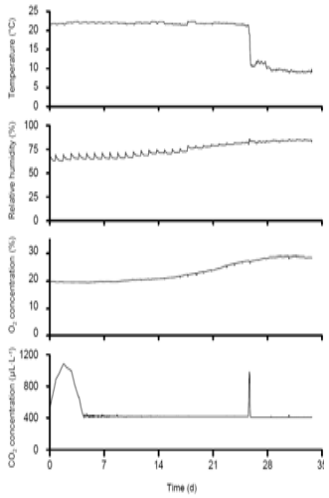


Figure 3. Environmental conditions in the labeling chamber.

Seedlings 9 days post-sowing were transferred to the chamber, supplied with $^{13}CO_2$ at ca. 400 ppm, and subirrigated daily with a complete nutrient solution.

After initial growth at 22° C in constant light (228 µmol m^{-2} s^{-1}; metal halide lamps), anthocyanin accumulation was promoted by diluting nutrient solution to ¼ strength and reducing temperature to 10.5° C at canopy closure (26 days).

Total shoot fresh weight (FW) was 1565 g and root FW was 491 g at harvest.

The initial CO_2 spike resulted from $^{12}CO_2$ respired by root residue from thinned seedlings. The second spike resulted from a brief power failure.

Note the gradual increases in relative humidity and O_2 concentration.

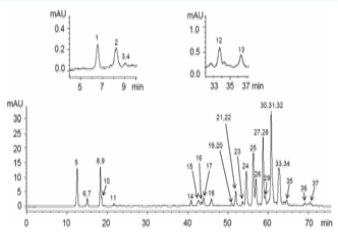


Figure 4. Reverse-phase HPLC chromatograms of ^{13}C-labeled red cabbage anthocyanins.

Freeze-dried and ground red cabbage tissue was extracted in 10% formic acid in methanol using pelargonidin-3-glucoside as an internal standard and separated on a C18 column using a gradient of increasing MeOH in 10% aqueous formic acid with detection at 520 nm. Peak identities are listed in Table 1. Labeling did not affect retention time.

Introduction

Controlled environment agriculture can produce crops with defined phytochemical composition for nutrition research.

Labeling foods to follow the uptake and metabolism of phytochemicals is a specialized example.

Intrinsic labeling is required if the influence of the food matrix is an issue and stable isotopes are preferred for human studies. There are several approaches:

^{15}N can be supplied via fertilizer if a compound of interest contains N.

D_20 can be used in the irrigation water, but isotope effects often limit the proportion of D_20 to 30-50% to prevent extreme growth inhibition. The resulting range of isotopomers make it hard to track labeled compounds for long durations.

As an alternative, we developed a system to incorporate ^{13}C photosynthetically using $^{13}CO_2$ at ca. 99% isotope enrichment that resulted in almost 99% labeling in kale (see Fig. 1; Kurilich et al. J. Agric. Food Chem. 51: 4877, 2003).

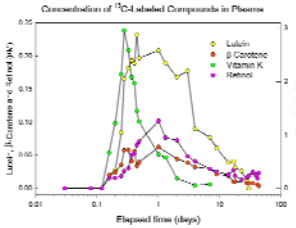
This system was modified to label anthocyanins, flavonoid compounds, that contribute color, photoprotection, and antioxidant activity in plants (See Fig. 5 for structure). Anthocyanins may also provide health benefits in humans, but bioavailability is poor and may be adversely affected by substituents including sugars and aliphatic or aromatic acyl groups (e.g., Charron et al. J. Agric. Food Chem. 55: 5354, 2007). The possibility that anthocyanin breakdown products and/or metabolites are involved in health-promotion needs to be studied. Red cabbage was chosen because it has a large number of variously substituted cyanidins

Figure 1. Labeled kale.

Vitamin K (phylloquinone), β-carotene, lutein and retinol (a β-carotene metabolite) were determined in plasma from the same human volunteer over 46 days following a single serving of kale (400 g).

Labeled compounds appeared rapidly and displayed distinctive kinetics.

Significant levels of labeled β-carotene and retinol were detectable at 46 days.



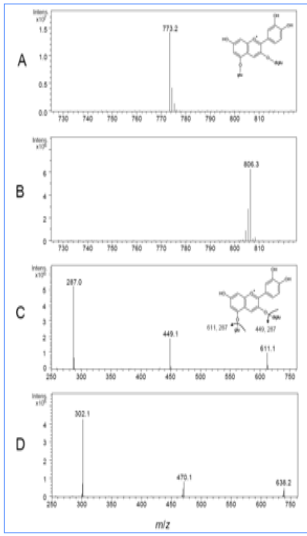


Figure 5. Mass spectra of unlabeled and ^{13}C-labeled cyanidin-3-diglucoside-5-glucoside (peak 5).

A. Structure and [M]+ *m/z* for unlabeled compound.

B. [M]+ *m/z* of ^{13}C-labeled compound.

C. Predicted fragmentation patterns and MS/MS *m/z* fragments of unlabeled compound.

D. MS/MS *m/z* fragments of ^{13}C-labeled compound.

MS was performed with electrospray ionization (ESI) mode using an Agilent ion trap mass spectrometer model SL (for additional details, see Charron et al. J. Agric. Food Chem. 55:5354, 2007). It was assumed based on the literature that fragmentation occurred at glycosidic linkages and that acyl esters were not cleaved from sugars.

Table 1. Anthocyanins in labeled and unlabeled red cabbage

Peak	t_r (*min*)	Carbons	Unlabeled [M]+ (*m/z*)	Labeled [M]+ (*m/z*)	Proposed Identification (based on MS/MS data not shown)
1	6.5	33	773	806	Cyanidin + 1 dihexose + 1 hexose
2	8.2	27	611	638	Cyanidin + 2 hexoses
3	9.1	33	789	822	Delphinidin-3-diglucoside-5-glucoside
4	9.1	27	627	654	Delphinidin-3,5-diglucoside
5	12.6	33	773	806	Cyanidin-3-diglucoside-5-glucoside
6	14.8	42	935	977	Cyanidin-3-(caffeoyl)diglucoside-5-glucoside
7	15.1	27	611	638	Cyanidin-3,5-diglucoside
8	18.2	27	611	638	Cyanidin + 2 hexoses
9	18.3	44	979	1023	Cyanidin-3-(sinapoyl)diglucoside-5-glucoside
10	18.6	43	949	992	Cyanidin-3-(feruloyl)diglucoside-5-glucoside
11	21.7	21	449	470	Cyanidin-3-glucoside
12	34.0	48	1081	1129	Cyanidin-3-(*p*-coumaroyl)triglucoside-5-glucoside
13	36.7	49	1111	1160	Cyanidin-3-glycopyranosyl-(feruloyl)diglucoside-5-glucoside
14	40.9	59	1287	1346	Cyanidin-3-(*p*-coumaroyl)(sinapoyl)triglucoside-5-glucoside
15	42.7	42	919	961	Cyanidin-3-(*p*-coumaroyl)diglucoside-5-glucoside
16	43.4	53	1125	1178	Cyanidin-3-(diacyl)diglucoside-5-glucoside
17	44.0	60	1317	1377	Cyanidin-3-(feruloyl)(sinapoyl)triglucoside-5-glucoside
18	45.9	42	935	977	Cyanidin-3-(caffeoyl)diglucoside-5-glucoside
19	51.0	38	817	855	Cyanidin-3-(sinapoyl)glucoside-5-glucoside
20	51.0	37	787	824	Cyanidin + hexose + pentose + sinapoyl
21	51.9	37	787	824	Cyanidin + hexose + pentose + sinapoyl
22	51.9	53	1141	1194	Cyanidin-3-(caffeoyl)(sinapoyl)diglucoside-5-glucoside
23	53.6	52	1111	1163	Cyanidin-3-(caffeoyl)(feruloyl)diglucoside-4-glucoside
24	54.4	42	919	961	Cyanidin-3-(*p*-coumaroyl)diglucoside-5-glucoside
25	56.3	43	949	992	Cyanidin-3-(feruloyl)diglucoside-5-glucoside
26	56.9	44	979	1023	Cyanidin-3-(sinapoyl)diglucoside-5-glucoside
27	58.7	53	1125	1178	Cyanidin-3-(diacyl)diglucoside-5-glucoside
28	58.7	37	787	824	Cyanidin + hexose + pentose + sinapoyl
29	59.2	51	1081	1132	Cyanidin-3-(caffeoyl)(*p*-coumaroyl)diglucoside-5-glucoside
30	60.7	54	1155	1209	Cyanidin-3-(feruloyl)(sinapoyl)diglucoside-5-glucoside
31	60.7	52	1095	1147	Cyanidin-3-(*p*-coumaroyl)(feruloyl)diglucoside-5-glucoside
32	61.2	52	1111	1163	Cyanidin-3-(caffeoyl)(feruloyl)diglucoside-5-glucoside
33	62.6	55	1185	1240	Cyanidin-3-(sinapoyl)(sinapoyl)diglucoside-5-glucoside
34	62.8	53	1125	1178	Cyanidin-3-(diacyl)diglucoside-5-glucoside
35	64.6	54	1155	1209	Cyanidin-3-(feruloyl)(sinapoyl)diglucoside-5-glucoside
36	69.1	32	655	687	Cyanidin-3-(sinapoyl)glucoside
37	70.4	31	625	656	Cyanidin-3-(feruloyl)glucoside

Conclusions

We describe a system for isotopic labeling of leafy vegetables with ^{13}C and demonstrate successful incorporation of ^{13}C into anthocyanins of preheading red cabbage (*Brassica oleracea* L. var. *capitata*).

Analysis of red cabbage shoot tissue by high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) indicated the presence of 37 anthocyanins, of which fourteen have not previously been described.

35 of 37 compounds were based on cyanidin, but 2 anthocyanins incorporating delphinidin were identified.

Mass shifts representing ^{13}C incorporation into anthocyanins were evident in mass spectra of anythocyanins from labeled tissue and demonstrate successful isotopic labeling into nearly 100% of all carbons.

In some cases (peaks 12, 20, 21, 22, 23, 28, and 32), mass shifts provided unambiguous information on carbon number that facilitated the assignment of sugar and acyl moieties that fragmentation data did not permit.

Major anthocyanins differing in glycosylation and acylation will be isolated and fed to human subjects to study the impact of substituents on anthocyanin uptake and metabolism.