

NOTE

A Reverse-Phase HPLC Method for Tricin Separation from Wheat Leaves

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The agronomic, ecological, and dietary roles of flavonoids are being increasingly recognized (Harborne 1991). The flavonoid profile of the Gramineae family is dominated by apigenin and luteolin *C*-glycosides and tricrin *O*-glycosides (Harborne 1988). Tricin (Fig. 1) is a characteristic grass flavone (Harborne and Hall 1964) isolated in small amount from Khaple wheat leaves by Anderson (1932). It is found mostly in the combined form (Harborne and Hall 1964). Four mono- and diglycoside derivatives have been described in seven *Triticum* species (Harborne et al 1986).

Although protection of primary metabolism from light damage is the first function assigned to flavonoids, a role in antiherbivory defense is suggested as a secondary function (Harborne 1991). The inhibition by tricrin of the feeding activity of the aphid *Myzus persicae* (Dreyer and Jones 1981) and of boll weevil (*Anthonomus grandis*) (Miles et al 1993, 1994) supports this hypothesis. Other biological activities have been reported for tricrin. It was isolated from a fraction enriched in antileukemia activity of *Spartina cynosuroides* (Bhattacharyya et al 1978) and it has also been reported to have effect on guinea pig intestine (Bickoff et al 1964).

As part of our studies on wheat flavonoids we developed a method for tricrin isolation. Tricin was identified by negative electrospray mass spectrometry and by UV-Vis absorption spectroscopy.

MATERIALS AND METHODS

Plant Material

Wheat leaves of field grown plants were collected at three stages of the growth cycle. Leaf number five and flag leaves were collected when they were fully expanded at the upper level of the canopy. Flag leaves were also collected when they started to senesce. Leaves were oven-dried at 60°C and ground.

Extraction and Purification Procedures.

Tricin was isolated from senescing flag leaves. Leaf powder was extracted with MeOH in a sonic bath at a proportion of 1 mL of MeOH per gram of leaf powder. After centrifugation, the methanolic extract was filtered through a 0.22- μ m micro-spin filter (Lida, Kenosha, WI) before HPLC injection. A Nucleosil 120, C-18, 5 μ m, 250 \times 10 mm semipreparative column (Tracer, Barcelona, Spain) was used to separate tricrin by hand-collection of the eluent. After removing the MeOH from the collected tricrin fraction by evaporation, tricrin was concentrated on Sep-Pak C-18 columns (Waters, Milford, MA) and rechromatographed to ensure purity.

An extraction procedure was also established for routine analysis. Wheat leaf powder (0.1 g) was extracted three times with 10 mL of methanol in a sonic bath for 10 min. Extracts were pooled and

methanol was evaporated in vacuum. Residue was redissolved in 1 mL of HPLC-grade methanol and filtered through a 0.22- μ m micro-spin filter (Lida). A volume of 40 μ L was injected on a Nucleosil 120, C-18, 10 μ m, 250 \times 4.6 mm, analytical column (Tracer).

Apparatus

A Waters 600E gradient system liquid chromatograph, equipped with an automatic injector Waters WISP, a diode array absorbance detector (Applied Biosystems ABI 1000S, Perkin-Elmer, Barcelona, Spain), and a column oven (model 480, Kontron Inst., Milan, Italy) were used. The data from the detector were acquired and processed with a Kontron PC Integration Pack. A Hettich Mikroliter centrifuge (Hettich, Tuttingen, Germany) was used in the preparation of the samples. The spectra of the collected chromatographic peaks were recorded using a UV-Vis scanning spectrophotometer (UV-2101PC, Shimadzu Corp., Osaka, Japan). A Platform II quadrupole mass spectrometer, fitted with a pneumatically assisted electrospray ionization source (Micromass, Manchester, UK) was used to assess the molecular mass of the target chromatographic peak. Two syringe pumps (Phoenix 20, CE Instruments, Milan, Italy) provided an accurate continuous micro-flow and an injection valve (7125, Rheodyne, Cotati, CA) was used to introduce the liquid sample into the continuous flow to the source.

HPLC Conditions

A binary solvent elution system included: eluent A = 20 mM H₃PO₄ in deionized water, and eluent B = methanol, HPLC grade. The elution program started with A-B (100:0), 0–2 min linear gradient to A-B (50:50), 2–5 min linear gradient to A-B (45:55), 5–20 min isocratic with A-B (45:55). The flow rate was 1.5 mL/min. The temperature of the oven thermostat was set at 30°C. Stabilization with eluent A during 10 min was conducted before every run. A volume of 40 μ L of sample was injected and the absorbance was recorded at 350 nm.

ESI-MS Conditions

An aliquot of 20 μ L of the isolated peak was injected into the mass spectrometer. Methanol and water 1:1 (v/v) acidified with 0.05% trifluoroacetic acid was the carrier used at a rate of 20 μ L/min. Nitrogen gas was used as both drying gas and nebulizing gas at rates of 400 and 20 L/hr, respectively. The source was set in negative ion mode with the following conditions: probe tip at 3.50 kV,

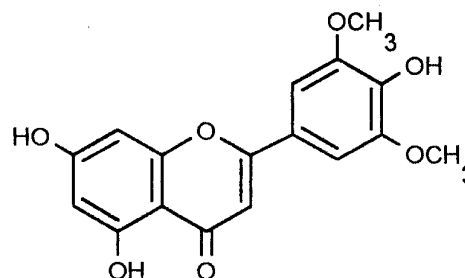


Fig. 1. Chemical structure of tricrin.

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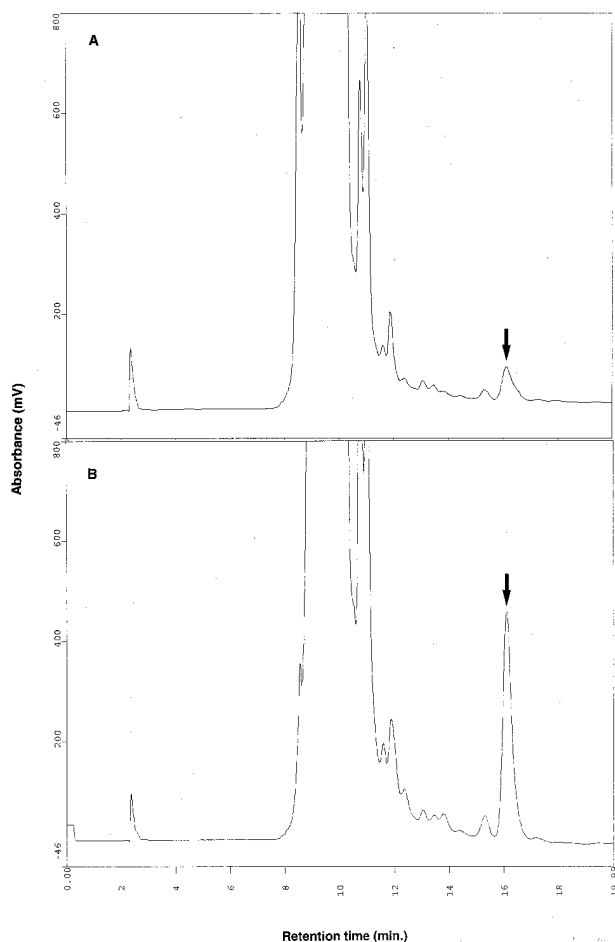


Fig. 2. Chromatogram obtained from crude extracts of wheat leaves running the program of elution with the analytic column as described in text for extract from leaf number five (A) and extract from flag leaf (B). Absorbance recorded at 350 nm. Arrows indicate triclin peak at retention time 16.2 min.

counterelectrode at 1.00 kV, cone at 60 V, and source temperature at 80°C. Data acquisition was recorded by scanning from 50 to 60 amu during 1 sec with an interscan time of 0.2 sec, and by using Mass-Lynx software.

UV-Visible Absorption Spectroscopy

The classical procedure of Mabry et al (1970) was applied to elucidate peak identity. Spectra of purified compound were scanned from 200 to 500 nm in MeOH (spectrophotometric grade) and in MeOH plus shift reagents NaOMe, AlCl₃, AlCl₃/HCl NaOAc, and NaOAc/H₃BO₃ using methanol as the reference. Purified compound was dissolved adjusting the concentration to give a major peak absorption close to 1 absorbance unit.

Tricin

UVλ_{max}^{MeOH} nm: 240sh, 270, 301sh, 349; +NaOMe 263, 274sh, 327, 419; +AlCl₃ 259sh, 277, 303, 370, 394; +AlCl₃/HCl 259sh, 278, 303, 362, 387; +NaOAc 269, 320, 412; +NaOAc/H₃BO₃ 270, 305sh, 351, 418sh.

RESULTS AND DISCUSSION

A clear separation of a pure peak of triclin from other wheat leaf flavonoids was obtained running the program of elution with the analytical column as shown in the chromatogram in Fig. 2a,b. The mass spectrum of the purified peak at 16.2 min in the chromatogram shows clearly the M-1 trace at 329 amu and another signal

at 145, which corresponds to the adduct methanol-trifluoroacetate anion from the liquid carrier (background). The molecular mass of triclin (C₁₇H₁₄O₇) is 330.29 amu, and the M-1 has the expected value. The triclin identity was confirmed by the spectral data

The relative concentration of triclin on upper canopy leaves of wheat plants was lower in early stages of development (extracts from leaf number five) (Fig. 2a) as compared to later stages of development (extracts from flag leaf) (Fig. 2b). Differences between leaves in the concentration of flavonoids have been reported for other cereal species: field oat plants showed higher concentrations of two flavonoids in primary leaves than in later leaves (Effertz and Weissenböck 1976, Popovici and Weissenböck 1976). Flavonoid concentration in leaves is also influenced by leaf age, as shown in barley leaves in which flavonoid concentrations have been reported to decrease when leaves are near senescence (Liu et al 1995).

In addition to the antiherbivory activities, allelopathic effects of flavonoids and their influence on soil ecology (Rao 1990) are interesting topics for the improvement of agronomic practices and for utilization of residues. The simple method for triclin isolation we describe here can be a helpful tool for these studies and for the elucidation of triclin significance in wheat plants.

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LITERATURE CITED

- Anderson, J. A. 1932. The yellow coloring matter of Khaple wheat, *Triticum dicoccum*. Can. J. Res. 7:285.
- Bhattacharayya, J., Stagg, D., Mody, N. V., and Miles, D. H. 1978. Constituents of *Spartina cynosuroides*: Isolation and ¹³C NMR analysis of triclin. J. Pharm. Sci. 67:1325-1326.
- Bickoff, E. M., Livingston, A. L., and Both, A. N. 1964. Tricin from alfalfa isolation and physiological activity. J. Pharm. Sci. 53:1411-1412.
- Dreyer, D. L., and Jones, K. C. 1981. Feeding deterrence of flavonoids and related phenolics towards *Schizapus graminum* and *Myzus persicae*: aphid feeding deterrents in wheat. Phytochemistry 20:2489-2493.
- Effertz, B., and Weissenböck, G. 1976. Dynamik der flavonoid-akkumulation in primärblattgeweben von *Avena sativa* L. Ber. Deustch. Bot. Ges. 89: 473-481.
- Harborne, J. B. 1988. The Flavonoids. Advances in Research Since 1980. Chapman and Hall: London.
- Harborne, J. B. 1991. Flavonoid pigments. Pages 389-429 in: Herbivores: Their Interactions with Secondary Plant Metabolites, 2nd ed. Vol. I: The Chemical Participants. G. A. Rosenthal and M. R. Berenbaum, ed Academic Press: San Diego, CA.
- Harborne, J. B., and Hall, E. 1964. Plant polyphenols XII. The occurrence of triclin and of glycoflavones in grasses. Phytochemistry 3:421-428.
- Harborne, J. B., Boardley, M., Fröst, S., and Holm, G. 1986. The flavonoids in leaves of diploid *Triticum* species (*Gramineae*). Pl. Sys. Evol. 154:251-257.
- Liu, L., Gitz Iii, D. C., and McClure, J. W. 1995. Effects of UV-B on flavonoids, ferulic acid, growth and photosynthesis in barley primary leaves. Physiol. Plant. 93:725-733.
- Mabry, T. J., Markham, K. R., and Thomas, M. B. 1970. The Systematic Identification of Flavonoids. Springer-Verlag: New York.
- Miles, D. H., Tunsuwan, K., Chittawong, V., Hedin, P. A., and Kokpol, U. 1994. Boll weevil antifeedants from *Eleocharis dulcis* Trin. J. Agric. Food Chem. 42:1561-1562.
- Miles, D. H., Tunsuwan, K., Chittawong, V., Kokpol, U., Choudhardy, M. I., and Clardy, J. 1993. Boll weevil antifeedants from *Arundo donax*. Phytochemistry 34:1277-1279.
- Popovici, G., and Weissenböck, G. 1976. Änderungen des flavonoidmusters während der ontogenese von *Avena sativa* L. Ber. Deustch. Bot. Ges. 89:483-489.
- Rao, A. S. 1990. Root flavonoids. Bot. Rev. 56:1-84.

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