# Analysis of Constitutive and Induced Phenolics of *Beta vulgaris* by High Performance Liquid Chromatography

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## ABSTRACT

**Study of the flavonoid phytoalexins betagarin and betavulgarin, compounds induced in leaves of *Beta vulgaris* by infection with *Cercospora beticola*, has been limited by lack of a sufficiently sensitive analytical procedure. Reverse phase high performance liquid chromatography effectively separated the phytoalexins, and provided a highly sensitive technique for their quantitative analysis. Optimal analyses required gradient elution by mixtures of 3% acetic acid and acetonitrile. Modifications of the elution gradient enabled examination of the simple phenolic acids and aldehydes that are found constitutively in many leaf extracts, and often increase with disease or other stress. Other gradient changes provided the best conditions for examination of other sample types or compounds, such as the ferulic amides found in sugarbeet seeds.**

**Additional Key Words:** Sugarbeet, flavonoid, phytoalexin, betagarin, betavulgarin.

Sugarbeet (*Beta vulgaris* L.) responds to infection by the fungus *Cercospora beticola* Sacc. by accumulating in its leaves two flavonoids that are toxic to the pathogen (Geigert et al., 1973; Johnson et al., 1976; Ruppel and Martin, unpublished) and thus are considered to be phytoalexins. These compounds, betagarin (abbreviated B; 5,2'-dimethoxy-6,7-methylenedioxyflavanone) and betavulgarin (BV; 2'-hydroxy-5-methoxy-6,7- methylenedioxyisoflavone), were found in field-grown, *C. beticola* infected sugarbeets in amounts sufficient to be potentially limiting to fungal growth (Martin, 1977). For that work, column and thin layer chromatography (TLC) were used to isolate the phytoalexins; quantitative determinations were made from the ultraviolet

absorbance of eluted TLC spots. It was evident that more sensitive analytical procedures were needed before further studies of phytoalexin accumulation would be practical. The compounds are not appreciably volatile, and as their structures indicate, not readily derivatizable to volatile products; thus, gas chromatography was unsuitable for their separation. However, their structures suggested they should be separable from other phenolic constituents and from one another by high performance liquid chromatography (HPLC). This research was conducted to establish the necessary conditions for HPLC separation and quantitative determination of the sugarbeet phytoalexins B and BV, as the basis for further work on the significance of these compounds in resistance to Cercospora leaf spot disease.

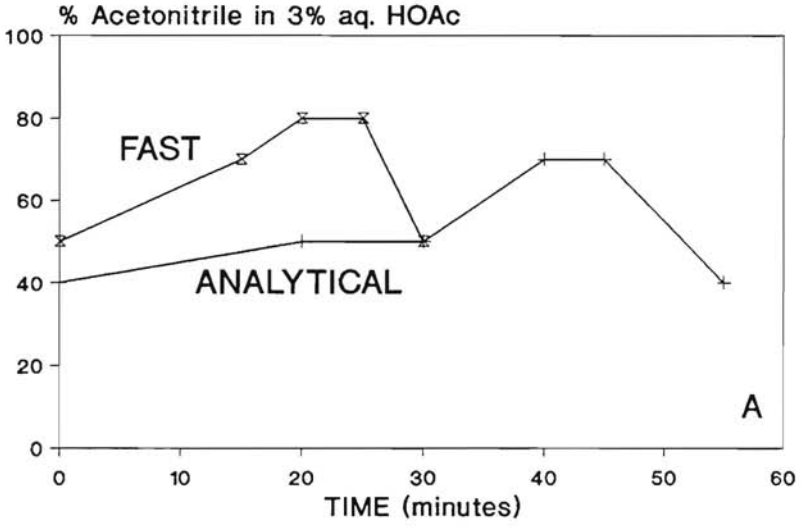
## MATERIALS AND METHODS

HPLC equipment used for this study included a microprocessor-controlled ternary solvent pump (Spectra-Physics SP8700), a Rheodyne 7125 loop injector valve fitted with a 20 μL loop, a C-18 reverse phase column (Spectra-Physics; 4.6 mm X 25 cm, 10 μm packing), and an EM Science-Hitachi L-3000 photodiode array detector (PDAD). PDAD output was directed either to an electronic integrator/recorder (Spectra-Physics SP4270) or to a computer running EM Science-Hitachi D-6000 chromatography data station software. The detector was operated typically at 0.1 absorbance units full scale (AUFS). A guard column (Alltech, 4.6 mm X 1 cm, C18-packed) was connected to the analytical column inlet.

Acetonitrile ($CH_3CN$) was HPLC grade; 3% aqueous acetic acid (HOAc) was prepared with 0.45 μm-filtered glass distilled water. Solvents were degassed with helium before pumping at 0.8 mL/min.

Because the phytoalexins are not commercially available, test samples and standards were separated by column chromatography and TLC-purified as described previously (Martin, 1977). Additional test samples of *C. beticola*-infected sugarbeet leaves were obtained from field plots inoculated with *C. beticola*. Samples were extracted in various ways (e.g., shaking or homogenizing with methanol or diethyl ether) for test purposes, but each was finally evaporated to dryness at room temperature, then dissolved in 0.200 ml 1:1 v/v 3% (aq.) HOAc:$CH_3CN$. Each sample was filtered through a 0.45 μm syringe filter before HPLC.

Seed was collected from field grown, *C. beticola*-infected beet (USDA Plant Introduction #357359, 'Yugo Domasne'). Air-dry seed balls were ground in a Wiley mill to pass a 20-mesh screen. Ground seed meal was extracted 3X with petroleum ether by stirring 15 min. with sufficient solvent to cover; the supernatant was discarded and the defatted product was air dried until it was free-flowing. Defatted seed meal (4.0 g) was extracted by stirring 10 min with enough diethyl ether to cover; the mixture



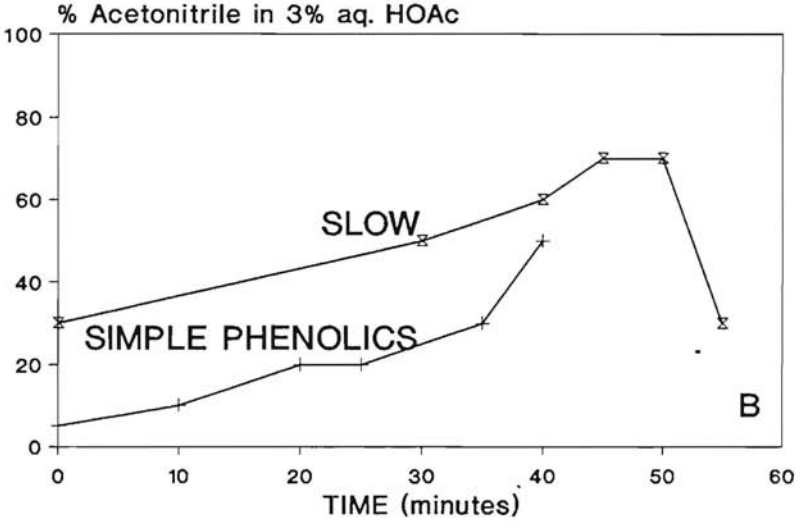




**Figure 1.** HPLC gradients for separation of sugarbeet phenolic constituents. A. Gradients for phytoalexin analysis. B. Gradients for analysis of simple phenolics and ferulic amides.

was filtered through cheesecloth, then through Whatman No. 1 filter paper. The clear filtrate was evaporated to dryness at room temperature in a stream of nitrogen and redissolved in 0.200 ml

of 1:1 v/v 3% (aq.) HOAc:$CH_3CN$.

## RESULTS AND DISCUSSION

Elution of the reverse phase column with fixed-composition (isocratic) mixtures (50:50 vol %) of 3% aqueous acetic acid (Solvent A) and HPLC-grade acetonitrile (Solvent B) could separate

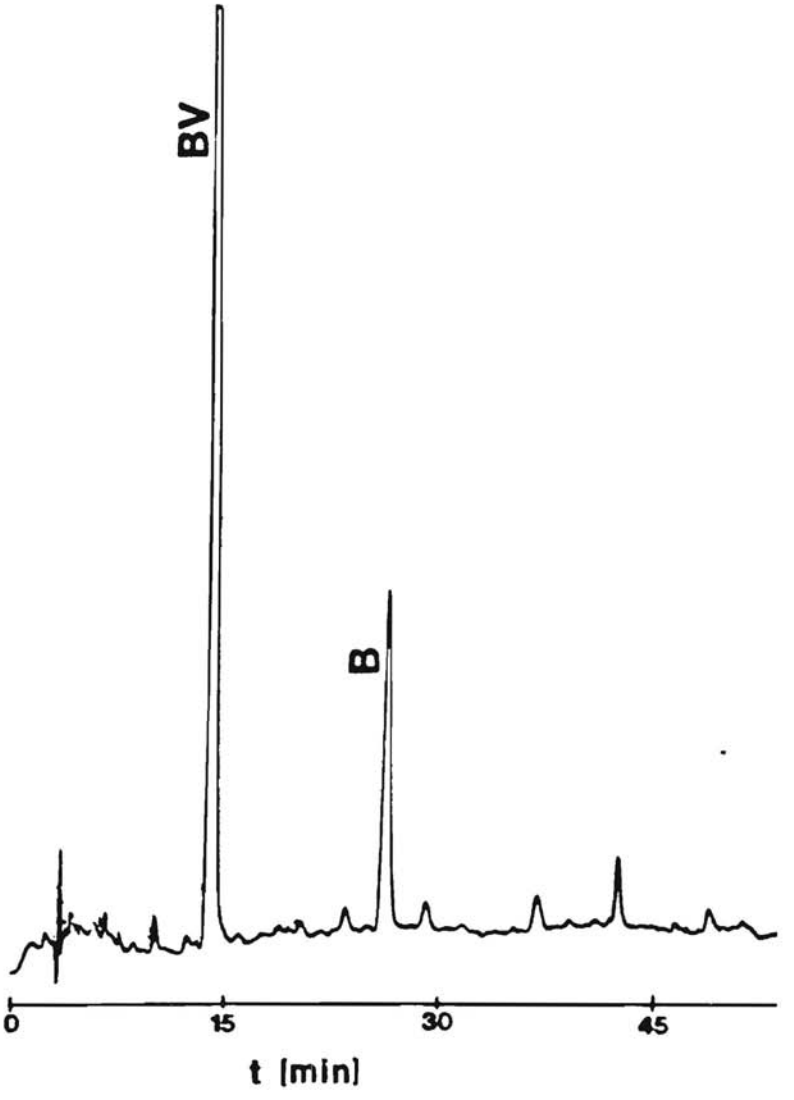



**Figure 2.** Separation of the sugarbeet phytoalexins betagarin and betavulgarin by hplc (analytical gradient). (Recorded at 280 nm, 0.1 AUFS.)

betagarin and betavulgarin from one another, but more satisfactory separations of the phytoalexins from other extract phenolics as well as from one another were achieved by gradient elution with changing proportions of A and B. On reverse phase HPLC the hydroxy- and methoxy-substituted betavulgarin eluted before the dimethoxy-substituted betagarin. Full ultraviolet spectra (200-360 nm) were recorded on the computer at 1.1 second intervals throughout the chromatographic run; these spectral data were examined to confirm compound identity and peak purity. Topographic (pseudo-three-dimensional) display of these data (run time and wavelength as X and Y axes, respectively, with absorbance displayed topographically as the apparent Z axis) was used to optimize separation and fixed-wavelength monitoring.

Single wavelength monitoring at 280 nm was optimal for detection of B and BV, as well as most other typical leaf extract components. Two solvent gradients were selected as optimally separating diverse extracts of *Cercospora*-infected sugarbeet leaves; I refer to these as the ANALYTICAL and FAST gradients, respectively (Figure 1A). With the analytical gradient, suitable for quantitative analysis of most sugarbeet leaf extracts containing phytoalexins, BV and B eluted at approximately 14 and 28 minutes after injection, respectively (Figure 2). The fast gradient, with which BV and B eluted at about 8 and 14 minutes, respectively, was useful for preliminary examination of samples. It also could be used for very uncomplicated samples, such as those having prior chemical separation of the phytoalexins from other leaf extract components.

In some samples, flavanones and isoflavones other than B or BV were recognizable spectrally. Because an isoflavone closely related to BV has been reported from sugarbeet seed (Chiji et al., 1986), I examined an extract from seed balls of a field-grown, *C. beticola* infected annual beet. This test sample did not include any major constituents with spectral characteristics of an isoflavone; however, the ferulic amides described from sugarbeet seed by Chiji et al. (1984) were present. The amides, which were recognizable by their typical ferulic acid-like spectra, were retained on the reverse phase column longer than ferulic acid and related hydroxycinnamic acids. These compounds were obscured by other early eluting compounds on the analytical gradient, but were separated well on a modified, less elutive gradient (SLOW gradient, Fig. 1B).

In the seed sample, and in samples prepared by extracting healthy or diseased sugarbeet leaves in various ways (e.g., by stirring or shaking with absolute methanol or ether) and analyzed with the analytical gradient, a significant group of early-eluting compounds often was observed. Examination of the ultraviolet spectra suggested that these constituents included various simple phenolic acids and aldehydes, phenylpropanoid acids, and some

related phenolic amino acids. A number of HPLC techniques have been described for such compounds (e.g., Wulf and Nagel, 1976; Hardin and Stutte, 1980; Roston and Kissinger, 1982; Banwart, et al., 1985; Wilson, 1985). For sugarbeet extracts, a very slow-eluting gradient beginning at 95:5 (v/v) HOAc:$CH_3CN$ (SIMPLE PHENOLICS in Figure 1B), combined with spectral analysis and comparison with standards, was suitable for investigation of these compounds, the presence and quantity of which vary greatly depending on sample type, sugarbeet cultivar, and presence or lack of eliciting factors such as disease, physical wounding, or other stress. Derivatives of caffeic acid, particularly ferulic acid, frequently were encountered as major leaf extract components. To determine these components in the same HPLC run with the larger and more hydrophobic flavonoids, the simple phenolics gradient (Fig. 1B) can precede the analytical gradient (Fig. 1A).

Reverse phase HPLC is well suited to separation of the phenolic constituents of sugarbeets, whether those components are constitutive or induced by disease or other stress. However, on even the best HPLC systems, retention times are affected by such factors as sample quantitative composition, solvent preparation variables, minor pump variations, and temperature; thus, absolute retention data alone should not be used to identify phenolic components of sugarbeet extracts. The utility of retention data can be increased by the use of an internal standard suitable to the group of compounds under study, with retentions being expressed relative to the standard. Better still, retention data can be used in conjunction with spectral information for confirmation of identity, especially when standards are available. In these ways, most sugarbeet phenolic compounds can be identified at least to a structural group. HPLC- mass spectrometry or isolation of the separated compounds for further spectral analysis may be necessary for conclusive identification.

By the HPLC technique described, the sugarbeet phytoalexins betagarin and betavulgarin can be determined quantitatively, with amounts accumulated in a single *C. beticola*-induced lesion measurable at 0.1 AUFS. Studies of the accumulation of these compounds in diverse *B. vulgaris* cultivars under *Cercospora* infection will be reported subsequently.

## ACKNOWLEDGEMENTS

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