# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF STEROIDAL SAPOGENINS

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The field of plant cells in suspension cultures is one which is undergoing rapid expansion, mainly in connection with its actual use for plant propagation and breeding, but also because of its potential for the production of secondary metabolites (1). The greatest interest has been shown in alkaloids and steroids. Diosgenin and related steroidal sapogenins are well-known starting compounds for the manufacture of pharmaceutically important steroids and are obtained commercially principally from the tubers of various strains of *Dioscorea*. It was shown (2, 3) that diosgenin has been produced by plant cells in suspension cultures, and we are currently investigating the metabolic pathways involved in the synthesis of this compound in *Dioscorea deltoidea*.

An assay of steroidal sapogenins, obtained by acid hydrolysis of *Agave*, was performed by the use of high-performance liquid chromatography (hplc) (4). However, these compounds had to be derivatized with benzoyl chloride before their separation on a Lichrosorb RP-8 column with 80% aq. acetonitrile as eluent. The present contribution describes the application of hplc in the quantitative analysis of diosgenin and other sapogenins without preparation of derivatives prior to separation. The method is simple, rapid, and accurate and offers the advantage that the underivatized separated compounds can be used for further analyses. By this method, the diosgenin present in plant cells can be readily isolated, identified, and quantitatively estimated.

## EXPERIMENTAL

GROWTH CONDITIONS.—*Dioscorea deltoidea* A-51 cells (obtained from E. J. Staba) were inoculated into 500 ml Erlenmeyer flasks containing MS medium (5), supplemented with 2,4-dichlorphenoxyacetic acid (0.1 mg/liter), nicotinic acid (1 mg/liter), thiamine HCl (10 mg/liter), pyridoxine HCl (1 mg/liter) and inositol (100 mg/liter). Glycine was omitted from the growth medium. Growth proceeded with an agitation rate of 100 rev/min at 28°.

SAMPLE PREPARATION.—Lyophilized plant cells (100 mg) were refluxed for 2 hr in 2N HCl, filtered, washed with water, and lyophilized for 4 hr. The dried cells were extracted with chloroform for 3 hr at 60°. The chloroform extract was filtered (0.46 μm filter; Millipore Corp.) and evaporated to dryness. The residue obtained was dissolved in a small volume of chloroform.

CHROMATOGRAPHY.—Analyses were performed with a Varian model 5000 liquid chromatograph, equipped with a refractive index detector (Varian), a high-pressure injection valve (20 μ liter, Varian) and an integrator (Varian model CDS-111L). A strip-chart recorder (Varian model 9176) was used at a chart speed of 0.25 cm/min. Analyses were performed on a stainless steel (4 mm x 30 cm) Micropak MCH-10 column (RP-$C_{18}$; Varian). Elution was carried out with a solvent mixture containing 83% acetonitrile, 10% methanol and 7% chloroform at a flow rate of 1 ml/min. All solvents used were of spectrophotometric grade (Aldrich). Gas-liquid chromatography (glc) (6) and thin-layer chromatography (tlc) (7) were performed according to published procedures.

## RESULTS AND DISCUSSION

Fig. 1 illustrates a typical chromatographic separation of four steroidal sapogenins: hecogenin (H), tigogenin (T), diosgenin (D), and stigmasterol (S) in order of decreasing polarity. Cholesterol was also injected into the hplc apparatus (retention time of 50 min) and was separated from stigmasterol (not shown). Better separation of diosgenin and tigogenin was obtained when the proportion of acetonitrile in the eluent solution was increased. However, a higher proportion of acetonitrile resulted in an increase in the retention times for stigmasterol and cholesterol. This caused an increase in peak width leading to a loss in sensitivity and

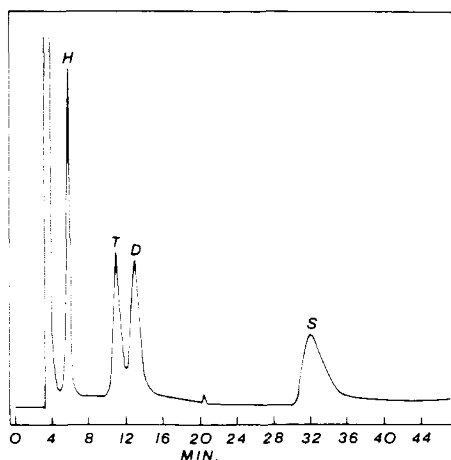


FIG. 1. HPLC—chromatogram of standard mixture (30 μg each) of hecogenin (H), tigogenin (T), diosgenin (D) and stigmasterol (S). Column: Micropak MCH-10, solvent, 83% acetonitrile, 10% methanol and 7% chloroform; flow-rate. 1 ml/min, range 1 of r.i. detector and attenuation 2.

precision of detection of these sterols. To overcome these problems, the flow rate could be gradually increased from 1 to 3 ml/min, 15 min after injection of the sample (not shown).

A linear response between the peak area and the amount of diosgenin was observed between 0 to 40 μg.

The analytical method was tested in an experiment on *D. deltoidea* A–51 cells grown in a suspension culture. The compound producing the major peak had the same retention time as diosgenin.

The presence of diosgenin in the chloroform extract of the cells was further confirmed by glc and tlc analyses, where the isolated compound corresponded exactly to authentic standards of diosgenin.

When *D. deltoidea* A–51 cells were grown on sucrose (30 g/liter) as the carbon source, 1.7 and 2.0% (of dry weight of cells) of diosgenin were produced after 24 and 34 days of growth, respectively. The results of the quantitative analysis of diosgenin by the method described in the present contribution were similar to those obtained by us when samples of the chloroform extracts of *D. deltoidea* A–51 were analyzed according to the method described by Higgins (4), where steroidal sapogenins are chromatographed as the benzoyl chloride derivatives.

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