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Note

Separation of amines, guanidines and hydroxycinnamic acid amides by ion-exchange chromatography

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For the continuation of a study on amine metabolism in barley seedlings, it was necessary to develop a method for separating *p*-coumarylagmatine and its antifungal dimers (the hordatines A and B) and their glycosides (hordatines M) (Fig. 1)[1,2]. Di- and polyamines can be separated by the widely used procedure of automated ion-exchange chromatography[3] with detection by fluorescence after reaction with *o*-phthalaldehyde[4]. Although coumarylagmatine and the hordatines cannot be detected by the *o*-phthalaldehyde system, the guanidino groups in these compounds will form an orange chromogen with the Sakaguchi reagent. An amine analyser was therefore designed with the column eluate divided between two systems, using respectively *o*-phthalaldehyde derivatisation for amines and the automated Sakaguchi reaction for guanidines. Similar automated Sakaguchi systems have been described in the separation of more acidic unconjugated guanidino compounds[5,6]. Elution of the hordatines from a cation-exchange resin requires the use of buffers of higher pH and ionic strength than are necessary for the separation of the polyamines. This may be due to hydrogen bonding of the aromatic rings to the polystyrene resin.

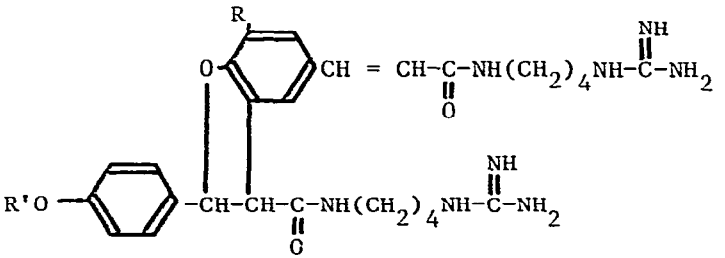

Fig. 1. Structures of the hordatines, antifungal dimers of coumarylagmatine found in barley seedlings.
1 Hordatine A: R = H, R′ = H
2 Hordatine B: R = OCH_3, R′ = H
3 Hordatines M: R = OCH_3 or H, R′ = D-glucopyranosyl.

EXPERIMENTAL

Sample preparation

For preparation of extracts, barley shoots were soaked for 18 h in 4 volumes 18

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TABLE I

BUFFER COMPOSITION AND ELUTION CYCLE

Buffer	*pH*	*Composition*	*Temperature (°C)*	*Duration (min)*	*Compounds eluted*
1	3.0	0.2 *M* Na_3 citrate 0.1 *M* NaCl	56	24	Acidic and neutral amino acids
2	5.6	0.2 *M* Na_3 citrate 0.1 *M* NaCl	56	30	Basic amino acids
3	5.6	0.2 *M* Na_3 citrate 2.0 *M* NaCl	75	39	Polyamines
4	10	0.2 *M* $NaHCO_3$ 2.5 *M* NaCl 0.08 *M* NaOH	75	27	Coumarylagmatine
5	13	0.05 *M* NaCl 0.13 *M* NaOH	95	30	Hordatines A and B
6	13	0.45 *M* NaCl 1.17 *M* NaOH	95	30	Hordatines M

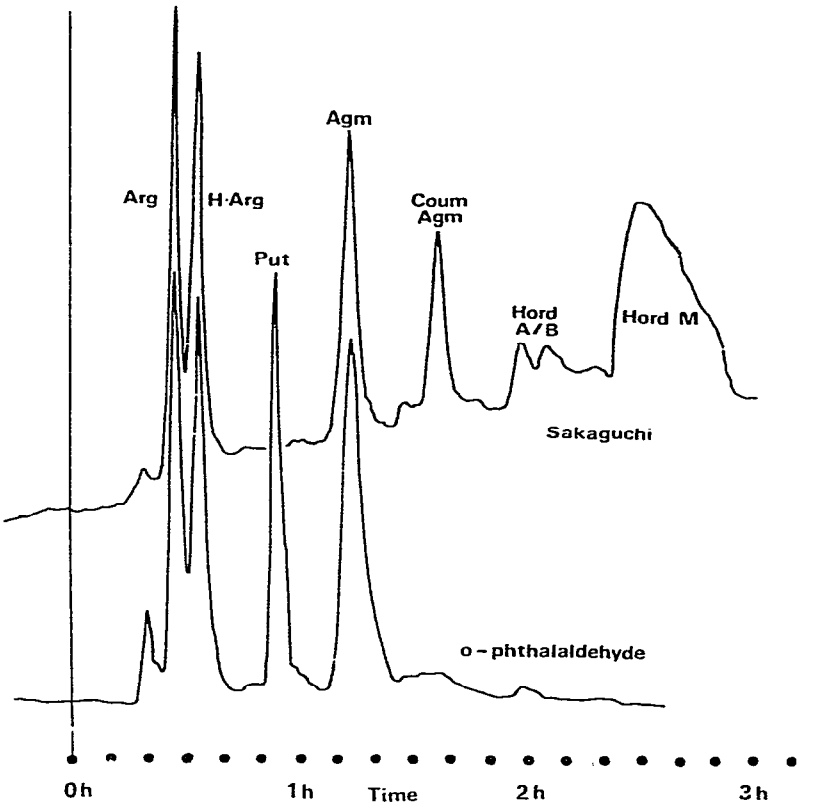

Fig. 2. Separation of amino acids, amines and guanidines by the amine analyser, with detection by *o*-phthalaldehyde and Sakaguchi reagents. Arginine (Arg), L-homoarginine (H·Arg), putrescine (Put) and agmatine (Agm) were authentic standards. Coumarylagmatine (Coum.Agm) and the hordatines (Hord) were extracted from the shoots of barley seedlings.

M acetic acid at 4°C. The filtrate was evaporated to dryness at less than 40°C, dissolved in 0.1 volume water and clarified by centrifugation.

Chromatographic procedure

A sequence of 6 buffers was developed for optimal resolution of the relevant compounds (Table I). All buffers contained Hibitane (10 mg/l) to prevent microbial growth and were passed through a 0.22-μm Millipore filter. Buffer 1 was pumped for 15 min to equilibrate the column prior to sample injection. A maximum sample volume of 0.25 ml was injected, containing 0.1 μmole L-homoarginine as an internal standard. This is eluted immediately following arginine (Fig. 2). Buffers were selected by a six-port automated rotary valve and passed through the column at 0.5 ml/min by a Milton Roy minipump. The back-pressure fell from 4 to 1.4 MPa during the cycle. The 13 × 0.4 cm stainless-steel column was packed with 8–9 μm Durrum DC4A sulphonated polystyrene cation-exchange resin. Column temperature was regulated in 3 steps at 56°C, 75°C and 95°C by immersion in a programmed water-bath. The

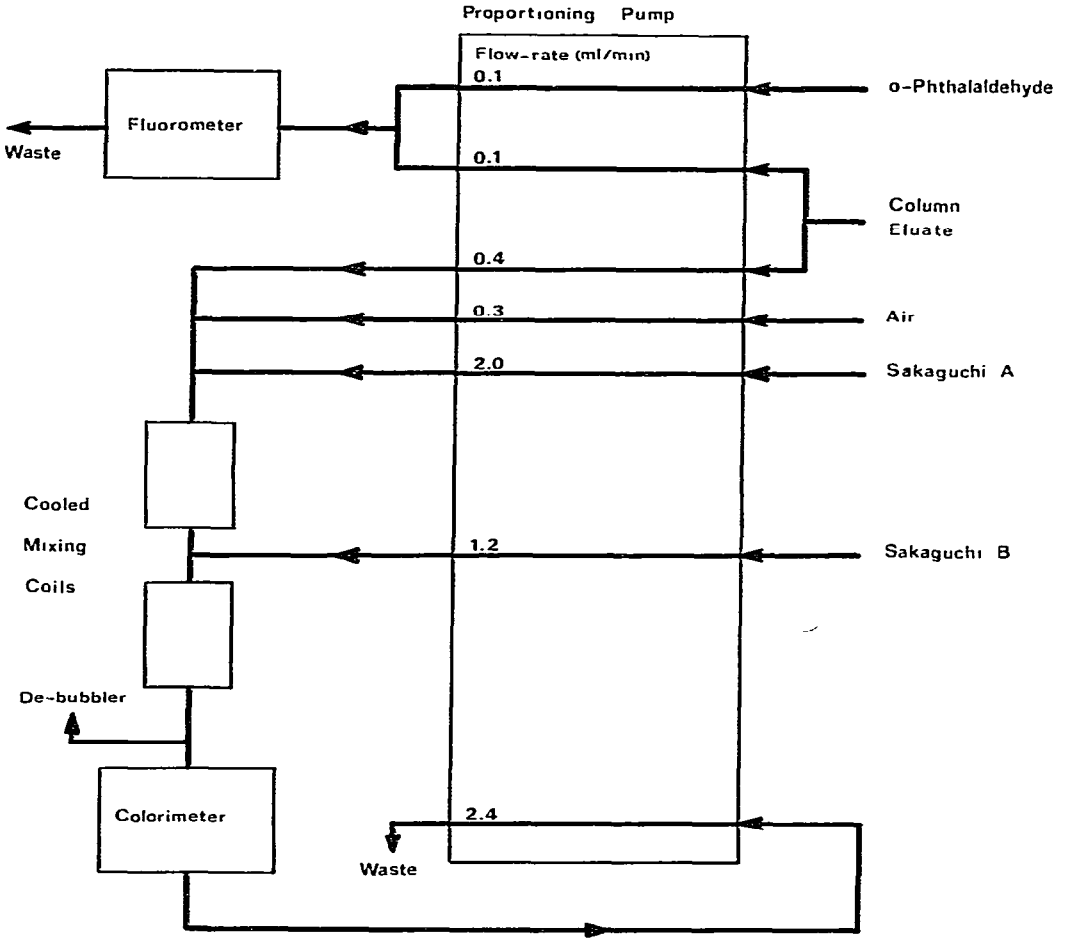

Fig. 3. Post-column flow diagram for the amine analyser.

elution cycle was controlled by a programmer which operated the buffer supply valve and regulated the column temperature.

Detection systems

The column eluate was divided between the two detection channels and the reagents added by a proportioning pump (Fig. 3). *o*-Phthalaldehyde reagent[4] was mixed at an equal flow-rate (0.1 ml/min) with 20% of the eluate, and the fluorescence monitored after a 2-sec delay in a Gilson Spectra/glo fluorometer equipped with a 40-μl flow cell (excitation 360 nm, emission 455 nm). *o*-Phthalaldehyde reagent was stored at 4°C under nitrogen. The remainder of the column eluate (0.4 ml/min) was segmented with air bubbles (0.3 ml/min) and mixed with 8-hydroxyquinoline sulphate (3.4 m*M* in 3 *M* NaOH, 2 ml/min, Sakaguchi A) in a coil at 4°C. N-Bromosuccinimide (8.4 m*M*, 1.2 ml/min, Sakaguchi B) was then added and mixing was completed in a further coil at 4°C. The air bubbles were removed and the flow fed to a colorimeter for monitoring at 495 nm.

CONCLUSIONS

The response of the fluorescence channel is linear between 10 pmole and 1 μmole of standard amine injected. However in the present work this detection system was considerably attenuated since the Sakaguchi channel is of much lower sensitivity and is limited to a linear range of 10 nmole to 0.25 μmole.

Separation of the compounds related to hordatine metabolism could be achieved in *ca.* 3 h with little sample preparation (Fig. 3).

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