Journal of Chromatography, 210 (1981) 121–129
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,656

FLUORODENSITOMETRIC DETERMINATION OF POTATO GLYCOALKALOIDS ON THIN-LAYER CHROMATOGRAMS

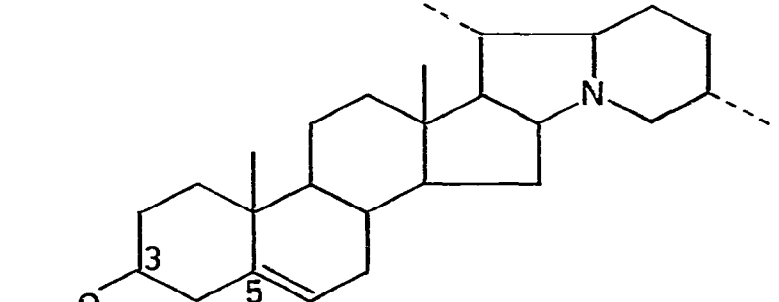
R. JELLEMA*, E. T. ELEMA and Th. M. MALINGRÉ
Laboratory of Pharmacognosy and Galenic Pharmacy, Antonius Deusinglaan 2, 9713 AW Groningen (The Netherlands)
(Received January 16th, 1981)

SUMMARY

Separation and analysis of solanine and chaconine has been achieved by thin-layer chromatography using an optical brightener as detection reagent. The conditions for optimal measurement *in situ* have been established. The method has been applied to the examination of leaf and tuber extracts of *Solanum tuberosum* cultivars. The errors in sample preparation and quantitative analysis range from 2.5 to 5.5%.

INTRODUCTION

The glycoalkaloids are a group of natural substances comprising a steroid base to which are attached one to four sugar molecules. The potato glycoalkaloids solanine and chaconine possess the same aglycone solanidine, but differ in their sugar moiety (Fig. 1). The sugars of solanine are galactose, glucose and rhamnose, which together form the trisaccharide β-solatriose. The trisaccharide part of chaconine is β-chacotriose, which comprises two molecules of rhamnose and one of glucose[1].



	R_1	R_2	R_3
Solanidine	H	-	-
Solanine	D-Galactose	D-Glucose	L-Rhamnose
Chaconine	D-Glucose	L-Rhamnose	L-Rhamnose

Fig. 1. Chemical structures of potato glycoalkaloids.

0021-9673/81/0000–0000/$02.50

Glycoalkaloids are, like saponins, hemolytically active, decrease the surface tension of water and are fungitoxic[2]. Solanine and chaconine also inhibit human plasma cholinesterase[3,4] and have a positive inotropic effect on the heart[5]. The suggestion that glycoalkaloids are teratogenic[6] is not supported by other evidence[7–9], although solanine induces changes in the mitotic cycle of human fibroblast cultures[10].

Potato glycoalkaloids are lethal to animals after intravenous or intraperitoneal administration. Death resulting from solanine or chaconine intoxication is caused by depression of the central nervous system[9,11]. These compounds are also toxic to humans, several cases of solanine intoxication being mentioned in the literature[12–16]. The consumption of damaged potatoes is especially dangerous, because in wounded or green potatoes the glycoalkaloid content is raised[17]. Tubers containing up to 20 mg of glycoalkaloids per 100 g fresh weight are considered to be safe for human consumption.

Several assays are available for the glycoalkaloids. The wet-chemical methods (titrimetry, colorimetry)[18–20] give the total glycoalkaloid content. These assays are also used for the determination of other glycoalkaloids, *e.g.*, solasonine and tomatine. If the content of the individual glycoalkaloids is required, *e.g.*, in breeding programs, screening of *Solanum* species or the evaluation of extraction procedures, then a chromatographic separation is needed before the quantitative analysis. Herb *et al.*[21] separated and determined glycoalkaloids after permethylation by gas–liquid chromatography. The determination by high-performance liquid chromatography avoids the need for derivatization[22,23]. Owing to their poor UV-absorbing properties, the glycoalkaloids are detected at low wavelength (210 nm) or with a RI-detector. This makes the method insensitive and unspecific.

Thin-layer chromatography (TLC) offers the advantage of using a specific and sensitive detection reagent. Cadle *et al.*[24] detected glycoalkaloids using antimony trichloride, which reacts with the $\Delta 5,6$ double bond of solanidine. Dragendorff reagent has also been used for the detection[25]. In both methods the glycoalkaloids are determined by *in situ* measurement of the remission of the spots. Because fluorescence determinations *in situ* are more sensitive and have a more linear response–concentration relation than remission determinations *in situ*[26], we have investigated the application of detection reagents which give fluorescing spots with glycoalkaloids.

PRELIMINARY EXPERIMENTS

Choice of solvent system

For the measurements *in situ* the spots have to be well separated and sharp. We investigated several solvent systems (Table I) comprising mixtures of three or four solvents, which can be divided into three groups: I, organic solvents immiscible with water; II, organic solvents miscible with water; III, water containing an acid or a base. The water content of the mixture determines the migration rate of the compounds[27]. These mixtures are comparable with those used for the separation of saponins or oligosaccharides, which, like the glycoalkaloids, are polar compounds. The hR_F values show that solanine and chaconine are separated by all the mixtures, but sharp spots were obtained only with systems A1 and D1. To avoid the use of two-layer systems, systems A1 and D1 were modified to A2 and D2, respectively.

For qualitative TLC we used systems A1 and 2[28]. In order to remove the

TABLE I

SOLVENT SYSTEMS FOR THE TLC OF GLYCOALKALOIDS

o.l. = Organic layer. $hR_F = 100 \times R_F$. Distance from start to solvent front = 10 cm. All TLC was carried out on pre-coated silica gel plates in unsaturated tanks.

	I	*II*	*III*	*Composition*	hR_F	
					Solanine	*Chaconine*
A1	*n*-Butanol	Formic acid	Water	40:10:50; o.l.	9	15
A2	*n*-Butanol	Formic acid–methanol	Water	75:5:15:7	12	22
B	Chloroform	Methanol	Water	50:50:10	28	42
C	Chloroform	Methanol–glacial acetic acid	–	50:45:5	24	65
D1	Chloroform	Methanol	2% Ammonia	20:20:10; o.l.	16	25
D2	Chloroform	Methanol	2% Ammonia	70:30:5	24	36
E	Ethyl acetate	Pyridine	Water	30:10:30	17	38
F	–	Ethanol	95% Acetic acid	20:60	49	63

solvents after development, the plates were heated for 30 min at 120°C. Under the influence of the formic acid and the heat the glycoalkaloids partly decomposed. In two-dimensional TLC the decomposition products did not react with the detection reagents in the same way as the glycoalkaloids. Therefore we used system D2 for the quantitative TLC.

Choice of detection reagent

Since glycoalkaloids show no absorbance or fluorescence in the visible and UV ranges, reagents are necessary to convert the glycoalkaloids into absorbing or fluorescing substances. The detection reagents which give fluorescing spots are preferred, because the response of such spots is a linear function of the concentration and the response is less dependent on the form of the spots than is the response of absorbing spots. With remission measurements *in situ* the relation between response and concentration is more complex and has to be determined empirically. Also the sensitivity is lower.

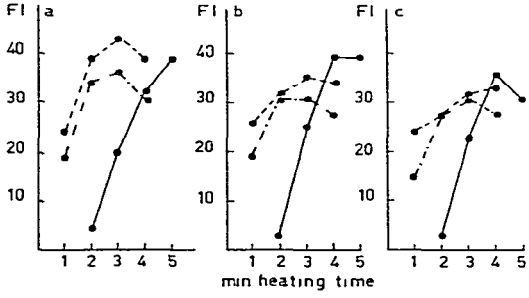


Fig. 2. Fluorescence response of 2-μg solanine spots with 5% sulphuric acid in diethyl ether as detection reagent. a, Measurement directly after cooling of the plate; b, measured after 30 min; c, measured after 60 min. Conditions: Zeiss PMQ II TLC densitometer; excitation, 365 nm (mercury lamp); emission wavelength, 424 nm. ●—●, Plate heated at 80°C; ●--●, plate heated at 100°C; ●-·-·-●, plate heated at 120°C. FI = Fluorescence response in arbitrary peak area units.

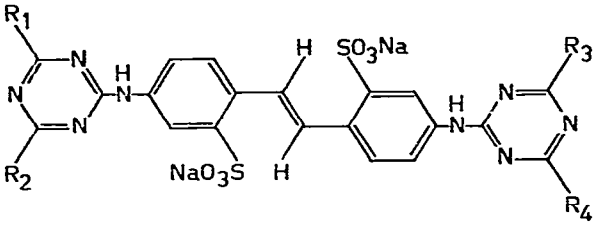


Fig. 3. Structure of the optical brighteners of the stilbenedisulphonic acid type, used as TLC detection reagents for glycoalkaloids. R_1, R_2, R_3 and R_4 are substituents.

First we tried sulphuric acid, a general reagent for steroids. The plates with the glycoalkaloid spots were dipped in a solution of 5% sulphuric acid in diethyl ether, and heated. The glycoalkaloid spots gave a blue fluorescence under long-wavelength UV light (365 nm). The response was dependent on the time and the temperature of the heating, and also on the time between heating and measurement (Fig. 2a, b, c). Maximum fluorescence was obtained with heating at 100°C for 3 min. However, the fluorescence was not stable enough for quantitative work.

Therefore we investigated the application of optical brighteners (OBs), which we have used previously[28,29]. The OBs of the stilbenedisulphonic acid type (Fig. 3) are sensitive and specific detection reagents for glycoalkaloids. In TLC they give blue fluorescing spots with the glycoalkaloids. Fig. 4 shows the fluorescence spectrum. Maximum fluorescence is obtained with an excitation wavelength of 380 nm and an emission filter of 450 nm.

These OBs have *cis–trans* isomers owing to the ethylene bond in the stilbene nucleus[30]. Under influence of light the fluorescing *trans*-form changes into the non-fluorescing *cis*-form. The fluorescence of glycoalkaloid spots was stronger on a plate dipped in an OB solution, kept in the dark, then on a similar plate kept in daylight (Fig. 5). For quantitative work however, the fluorescence was again not stable enough. When the plates were dried for 15 min at 105°C after dipping, the fluorescence was stable for several hours. For quantitative work, all manipulations of the plates between drying after development and placing in the TLC scanner were carried out in the dark. Plates treated in this way and kept in the dark showed the blue fluorescing glycoalkaloid spots even after some months.

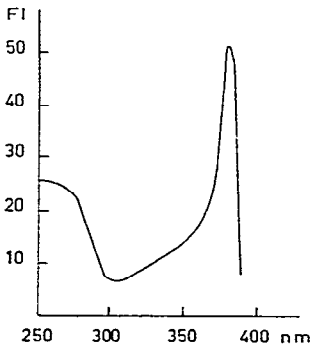


Fig. 4. Fluorescence excitation spectrum of 0.1-μg solanine spot on TLC, dipped in a 0.02% methanolic solution of Blankophor® BA 267%. Emission filter, 450 nm; slit 5 × 1 mm.

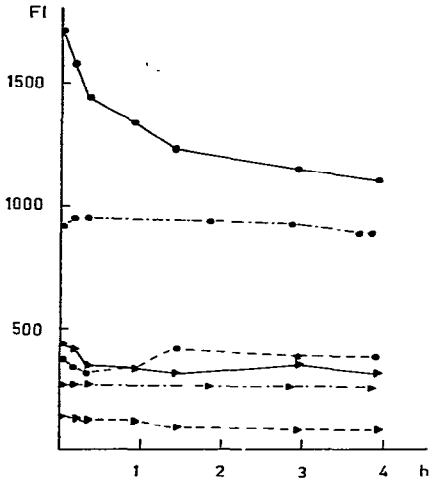


Fig. 5. Variation of fluorescence response with time of 0.5- (●) and 0.1-μg (▲) solanine spots detected with Blankophor® BA 267% dip solution. ———, Dip solution kept in dark; – – –, dip solution kept in daylight; – · – · –, dip solution kept in dark; after dipping the plate was dried for 15 min at 105°C.

EXPERIMENTAL

Reference compounds

A purified glycoalkaloid extract from potato sprouts was separated by open column chromatography (stationary phase, aluminium oxide; mobile phase, water-saturated *n*-butanol) into solanine and chaconine. After recrystallization from aqueous methanol, the identity of the glycoalkaloids was confirmed by TLC and mass spectrometry. The purity was determined by water-free potentiometric titration with perchloric acid. For quantitative TLC, standard solutions in methanol were prepared containing 50, 150 and 250 μg/ml of each compound.

Extraction

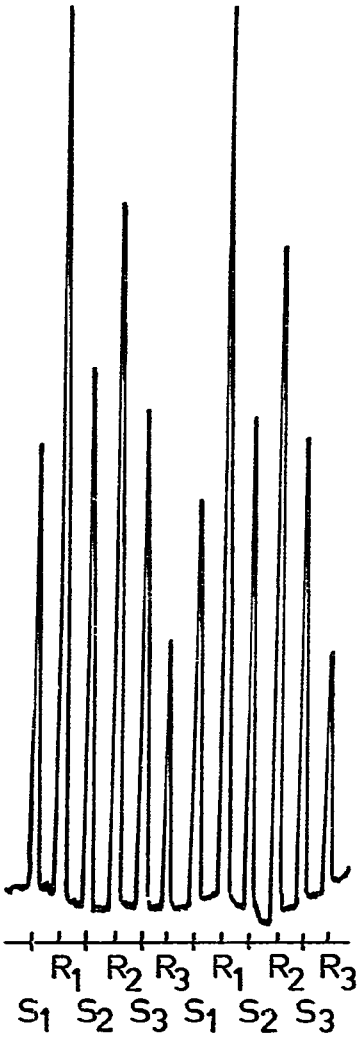
Fifty grams of mashed and frozen potatoes or 10 g of dried and pulverized leaves were refluxed three times with 250 ml methanol, first for 30 min and then twice for 15 min. The methanol extracts were filtered while hot and the methanol was evaporated on a rotary evaporator at <40°C. The residue was dissolved in 5 × 5 ml 5% acetic acid.

This solution was centrifuged (20 min, 3000 *g*), the supernatant decanted and the residue in the centrifuge-tube was washed twice with 5 ml 5% acetic acid and centrifuged. The combined acid solutions were shaken three times with 30 ml diethyl ether. The combined ether extracts were shaken once with 10 ml 5% acetic acid; the resulting acid extract was added to the previous combined acid extracts. All combined acid extracts were made alkaline with concentrated ammonia (25%), and warmed at 70°C for 30 min. After cooling for at least 6 h at 4°C, the precipitated glycoalkaloids were filtered off on a G-3 glass filter, provided with a layer of 1 g Hyflo Super Cel®.

The precipitate was washed with 6 × 5 ml 1% cold ammonia, and dissolved in 100 ml methanol. The methanol was evaporated on a rotary evaporator at <40°C.

The residue was dissolved in methanol to give a concentration of each glycoalkaloid between 50 and 250 μg/ml.

All TLC was carried out on precoated silica gel plates, layer thickness 250 μm (Cat. no. 5721; E. Merck, Darmstadt, G.F.R.). The solvents were of analytical grade. The sample and standard solutions were applied to the plate in duplicate, using the same 2-μl capillary for all solutions. Of the duplicates, one was spotted on the left part of the plate, the other on the right part (Fig. 6). The plate was developed with solvent system D2 over 10 cm, dried for 15 min at 105°C and cooled for another 15 min at room temperature. Subsequent manipulations were carried out in the dark.



C-1

1 SC
63 MD

AR
50125
74474
36905
48218
54374
17053
27221
77519
32642
45482
31002
15062
460080 T

slope	1498×10^2
intercept	13.5×10^2
r	0.9998

Fig. 6. Fluorescence location curve of chromatogram of solanine spots with integration print-out and coefficients of the calibration curve. R_1, R_2 and R_3 = Calibration points of 0.1, 0.3 and 0.5 μg, respectively; S_1, S_2 and S_3 = samples 1, 2 and 3, respectively. SC = Scan number; MD = method, giving peak area; AR = area; T = total area.

Detection

The cooled plate was dipped in a 0.02% methanolic solution of Blankophor® BA 267% (Bayer, Leverkusen, G.F.R.), dried for 15 min at 105°C and cooled for at least 15 min at room temperature.

Densitometry

The fluorescence intensity of the glycoalkaloid spots was measured with a Shimadzu CS 910 TLC scanner with fluorescence attachment. The plates were scanned perpendicular to the direction of development. Instrumental conditions: excitation wavelength, 380 nm; slit, 8 × 0.25 mm; emission filter, 450 nm; scan speed, 20 mm/min. The fluorescence signal was recorded with a Kipp BD 8 flat-bed recorder: sensitivity, 5 mV full scale; paper speed, 10 mm/min. The integrated values of the signal were obtained with a Shimadzu Chromatopec E1A integrator. For each plate the sensitivity of the densitometer was adjusted to full scale deflection of the recorder-pen for the 0.5-μg spot.

RESULTS AND DISCUSSION

With fluorescence measurements *in situ* the relationship between response and concentration is linear only within a limited range of low concentrations. To determine this concentration range we calculated the regression lines for three ranges. Table II shows that for both glycoalkaloids the best fit was obtained for the 0.1–0.5 μg range (correlation coefficient, $r > 0.9994$). The intercept of this line with the ordinate was slightly above the origin. At higher concentrations the calibration graph curved downwards with increasing concentration. The low slope for the 1.0–5.0 μg range was caused by a lower setting of the densitometer sensitivity.

TABLE II

REGRESSION-LINE COEFFICIENTS FOR THREE CONCENTRATION RANGES OF SOLANINE AND CHACONINE

r = Correlation coefficient; res. s.d. = residual standard deviation; n = number of calibration points.

	Concentration range (μg)	*Slope*	*Intercept*	*r*	*res. s.d.*	*n*
Solanine	1.0–5.0	128.1	252.3	0.9798	47.8	5
Solanine	0.2–1.0	896.2	74.0	0.9990	14.8	10
Chaconine	0.2–1.0	441.0	13.2	0.9968	14.7	10
Solanine	0.1–0.5	820.2	5.1	0.9994	7.8	6
Chaconine	0.1–0.5	648.2	1.5	0.9998	3.1	6

To optimize the method we investigated several sources of errors, which influence the reproducibility of the results. Apart from the errors in preparing the sample and the calibration solutions, other errors arise from: 1, application of the spot; 2, the TLC (plate development and detection); 3, the instruments (densitometer and integrator). To avoid errors due to differences in volume between capillaries, all solutions for one plate were applied with the same capillary. Error type 1 is also

influenced by the volume of the spot. When spots were applied with a 5-μl capillary, the relative standard deviation (r.s.d.) of the chaconine peak areas was 3.6%. The same amount of chaconine, applied with a 2-μl capillary, gave a r.s.d. of 2.3%. For solanine, having a lower R_F value, no difference was found. Therefore all spots were applied with a 2-μl capillary.

The large r.s.d.s of the slopes of calibration curves, which were calculated from several plates, indicated that, even under standardized conditions, the plates, development and application of the detection reagent were not reproducible. For solanine (mean slope 1175; $n = 10$) the r.s.d. was 12.7%; for chaconine (mean slope 914; $n = 10$) it was 15.7%. Therefore the fluorescence responses of spots on different plates are not comparable and a calibration graph must be constructed for each plate.

When several spots with the same glycoalkaloid concentration were measured on one plate, the r.s.d. of the peak areas for both glycoalkaloids was 1.3–4.7%. Apart from the error in the spot application, this is caused by irregularities in layer thickness. By applying the spots to the plate according to the data-pair technique[31] the r.s.d. for both glycoalkaloids is reduced to 0.8–2.5%.

The instrumental error was determined by measuring one spot 30 times. The r.s.d. of the peak areas was 0.4–0.7%.

Table III shows the results of the application of the method to three samples. Six extracts were made from each sample and analysed. The r.s.d. contains the error in the extraction, and the values obtained are comparable with those from HPLC and GC methods[21–23] (3.4–10.0%). Studies on these and other potato varieties indicate that the method is sensitive and precise. Because the applied OB is a specific and sensitive TLC detection reagent for glycoalkaloids[28], this method can also be used for the determination of solasodine glycosides, which are important raw materials for steroid drug manufacture, and of tomatine, a fungitoxic glycoalkaloid of *Solanum lycopersicum*, the tomato.

TABLE III

SOLANINE AND CHACONINE CONTENTS OF THREE SAMPLES

r.s.d. = Relative standard deviation.

Sample	*Part*	*Solanine (mg/100 g)*	*r.s.d. (%)*	*Chaconine (mg/100 g)*	*r.s.d. (%)*
Element	Tubers	4.2	3.0	6.7	2.6
Element	Leaves	31.7	4.6	169.5	3.5
Mara	Tubers	14.6	3.3	12.3	5.9

By using transferable calibration factors[32], five instead of three samples can be determined on one plate.

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