Journal of Chromatography, 331 (1985) 285–293
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 846

DETERMINATION OF THE TOTAL C_{27}-STEROIDAL ALKALOID COMPOSITION OF *SOLANUM* SPECIES BY HIGH-RESOLUTION GAS CHROMATOGRAPHY

W. M. J. VAN GELDER
Foundation for Agricultural Plant Breeding, SVP, P.O. Box 117, 6700 AC Wageningen (The Netherlands)
(Received April 12th, 1985)

SUMMARY

A method was developed for determining the total C_{27}-steroidal alkaloid composition of tubers of *Solanum* species. The substances studied included the $\Delta 5$- and 5α-aglycone pair solanidine and demissidine. High-resolution gas chromatography (HRGC) enabled the separation of solanidine and demissidine and of all other C_{27}-steroidal alkaloids studied. Current procedures for extraction of potato samples and a recently developed two-phase hydrolysis technique were used. No compounds interfering with the resolution of the alkaloids were found to be present in the potato extract, and no derivatization of the C_{27}-steroidal alkaloids was required. The chromatographic variables were optimized. Relative response factors for the C_{27}-steroidal alkaloids are presented. The contents of solanidine glycosides determined by a colorimetric method and by HRGC were in good agreement, the correlation coefficient being 0.976. The HRGC method described enables for the first time determination of the total C_{27}-steroidal alkaloid composition in a single analysis.

INTRODUCTION

The *Solanum* glycoalkaloids α-solanine and α-chaconine are naturally occurring toxicants common to commercially available potatoes. They belong to the class of solanidine glycosides, being comprised of the C_{27}-steroidal alkaloid solanidine and a sugar moiety (galactose + glucose + rhamnose and glucose + rhamnose + rhamnose, respectively). The low levels of 2–15 mg per 100 g fresh weight which are normally present in potatoes[1,2] are generally regarded to present no health hazard to the consumer. Elevated levels (>28 mg per 100 g fresh potato) of solanidine glycosides have been associated with poisoning and death of animals and man[3–5].

In potato-breeding programmes, wild and primitive *Solanum* species are used to introduce certain traits such as resistance to disease, pests or cold. Together with these traits, very high levels of solanidine glycosides or other types of *Solanum* glycoalkaloids such as demissine, tomatine, solamargine, solasonine, etc. (Fig. 1), may be transmitted from the wild species to hybrid progeny. The genetic and environmental control of potato glycoalkaloids has been reviewed[3,6,7].

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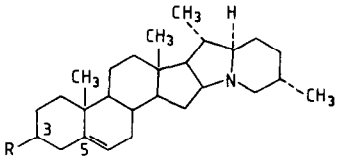
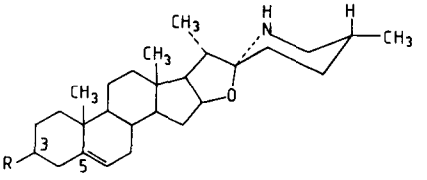


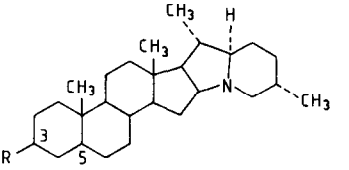
22α H, 25β H-solanid-5-en-3β-R

Solanidine :R = -OH
Solanthrene :Δ3, R = -
Solanine :R = glu-gal-rham
Chaconine :R = rham-glu-rham



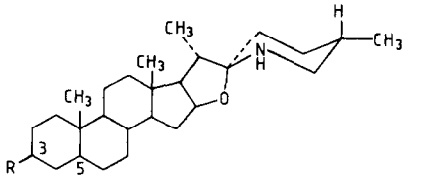
(25R)-22αN-spirosol-5-en-3β-R

Solasodine :R = -OH
Solasodiene :Δ3, R = -
Soladulcidine :5α; R = -OH
Solasonine :R = glu-gal-rham
Solamargine :R = rham-glu-rham



5α, 22αH, 25βH-solanidan-3β-R

Demissidine :R = -OH
Demissine :R = xyl-glu-gal
glu



(25S)-22βN-5α-spirosolan-3β-R

Tomatidine :R = -OH
Tomatidenol :R = -OH;Δ5,6
Tomatine :R = xyl-glu-gal-glu

Fig. 1. Chemical structures of *Solanum* C_{27}-steroidal alkaloids and derivatives: glu = D-glucose; gal = D-galactose; rham = L-rhamnose; xyl = D-xylose.

Before being used as parents in a breeding programme, accessions of wild species have to be analyzed in order to identify and quantify their glycoalkaloids. When hazardous compounds such as solasonine or solamargine, containing the teratogenic aglycone solasodine[8,9], or glycoalkaloids with unknown toxicity are present the hybrid progeny have to be monitored too. Few procedures for determining the total C_{27}-steroidal (glyco)alkaloid composition have been described. These include thin-layer chromatography (TLC), gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC); for a review of glycoalkaloid methodology see Coxon[10]. The applicability of these techniques is limited by the physico-chemical properties of the (glyco)alkaloids. Therefore combinations of chromatographic procedures are used for the separation, identification and quantification of these compounds resulting in laborious and time-consuming analyses[11–16]. The aim of this study was to develop a method for determining the total C_{27}-steroidal (glyco)alkaloid com-

position of *Solanum* species. Such a method should be comprehensive, quantitative and efficient enough for routine incorporation into a breeding programme[6,7,17].

Preliminary experiments showed that, in respect of detection and quantification of *Solanum* alkaloids, GLC compares favourably with HPLC and TLC, and gas chromatography of aglycones compares favourably with that of glycosidically bound alkaloids[18]. Therefore the possibilities of GLC and of high-resolution gas chromatography (HRGC) for directly chromatographing aglycones was investigated. HRGC appeared to be the obvious technique for determining the C_{27}-steroidal alkaloid composition, enabling simultaneous separation and quantitation of individual *Solanum* alkaloids, including $\Delta 5$- and 5α-alkaloid pairs. Current procedures for extraction and sample clean-up and a recently developed two-phase hydrolysis technique are used. No further clean-up and no derivatization are required prior to HRGC of the C_{27}-steroidal alkaloids.

EXPERIMENTAL

Apparatus

A Packard 439 microprocessor-controlled gas chromatograph was used, equipped with flame ionization detectors, injectors for splitless and split injection for one glass column (1 m × 2 mm I.D.) packed with 10% SE-30 on Chromosorb W HP (80–100 mesh) and one WCOT fused-silica column. The fused-silica columns (50 m × 0.22 mm I.D.) were coated with CP-Sil 5 (film thickness 0.12 μm) or CP-Sil 19CB (film thickness 0.19 μm). All columns were obtained from Chrompack (Middelburg, The Netherlands). The maximum operating temperatures were: SE-30 and CP-Sil 5, 325°C (isothermal), 350°C (programmed), CP-Sil 19CB column, 300 and 325°C, respectively. The injector temperature was 325°C and of the detector 350°C. The detector sensitivity was 1 pA mV^{-1} unless stated otherwise. The recording and calculation of chromatographic data were carried out with a Shimadzu C-R2A data processor.

Alkaloid standards and chemicals

Solanidine and solanthrene were prepared at the Foundation for Agricultural Plant Breeding[19]. Demissidine, solasodine, tomatidine and solasodiene were purchased from sources described earlier[19]. 5α-Cholestane and α-solanine were obtained from Sigma (St. Louis, MO, U.S.A.), methanol, chloroform, hydrochloric acid, carbon tetrachloride, ammonium hydroxide and toluene (analytical grade) from Merck (Darmstadt, F.R.G.). Standards were dissolved in methanol–toluene (1:1, v/v).

Plant material

Tuber samples were obtained from the Potato Breeding Department of the Foundation for Agricultural Plant Breeding. They included household varieties, industrial varieties (for potato-starch production), progenies from crosses between *Solanum tuberosum* and *S. vernei* and accessions of the primitive species *S. phureja*.

Prepration of samples and standard solutions for GLC and HRGC

Tuber samples were homogenized and glycoalkaloids extracted and hydrolyzed using a two-phase technique as described before[19]. The hydrolysis conditions were:

50 ml hydrochloric acid (2 mol l^{-1}); 100 ml carbon tetrachloride; reaction time 3 h. After separation of the carbon tetrachloride phase, which contained the aglycones, the aqueous phase was washed with chloroform and then discarded. The combined organic phases were washed with 25 ml ammonium hydroxide (1 mol l^{-1}) and evaporated to near dryness. The aglycones were transferred quantitatively into crimp top vials and after evaporation of the solvent, finally redissolved in 1.0 ml methanol–toluene (1:1, v/v) or diluted further as required.

Determination of solanidine glycosides

Colorimetric determination of solanidine glycosides was done according to van Gelder as described by Morgan *et al.*[20]. A calibration curve was constructed using α-solanine as a reference substance. The determination of solanidine glycosides by HRGC was done by using 5α-cholestane as internal standard. The content of solanidine glycosides was calculated by:

$$\text{content of solanthrene} \times \frac{868.0 \text{ (mol. wt. solanine)}}{379.6 \text{ (mol. wt. solanthrene)}}$$

RESULTS AND DISCUSSION

Comparison of GLC and HRGC

In studies on the separation of potato alkaloids by GLC, attempts to separate Δ5- and 5α-aglycone pairs, like solanidine and demissidine, have been described. Although various column types and operational parameters were used, no separation was achieved[18]. The closely related chemical structures of the Δ5- and 5α-aglycone pairs, Fig. 1, result in only minute differences in boiling point. Therefore the separation of these compounds will be very difficult. Fig. 2A shows a chromatographic trace of an attempt to separate a mixture of pure alkaloid standards, representing the aglycone pair solanidine–demissidine and the spirosolanes tomatidine and solasodiene, under optimized conditions on a 1-m SE-30 column. Calculations using data from individually chromatographed compounds gave plate numbers, N, for these compounds varying from 1877 to 2656 plates m^{-1}. The plate number required for the separation of solanidine, demissidine and solasodiene with resolution $R = 1$ (98% baseline separation for solanidine–demissidine and demissidine–solasodiene) was 678 000. Thus the theoretically required minimum column length was 305 m. Consequently, no further attempts with this technique were made.

Fig. 2B shows a chromatographic trace of the same mixture of alkaloids separated by HRGC using a 50 m × 0.22 mm I.D. CP-Sil 5 capillary column. The retention time, t_R, of solanidine (peak 2) and demissidine (peak 3) were still very close, $t_{R,2} = 13.096$ min and $t_{R,3} = 13.293$ min respectively, but due to the sharp peaks a good separation was achieved, $R = 1.3$. Solasodine (22αN-spirosolenol, peak 6), which was added to the alkaloid mixture, was well separated from its 3,4 dehydration product solasodiene (peak 4) and from tomatidine (22βN-spirosolanol, peak 5). The peak symmetry appears to be quite good, although some tailing is noticeable with peaks 5 and 6. This tailing effect decreased upon optimization of the GC parameters, but never totally disappeared.

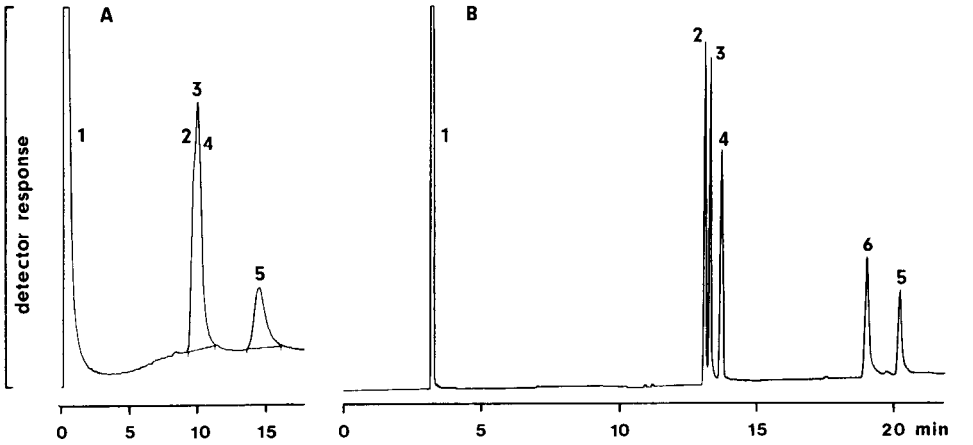


Fig. 2. Chromatograms comparing the separation of C_{27}-steroidal alkaloids by GLC using a packed column (A) and by HRGC using a capillary column (B). A, Packed column: glass, 1 m × 2 mm I.D., 10% SE-30 on Chromosorb W HP (80–100 mesh). Carrier gas: nitrogen, flow-rate 15 ml min^{-1}. Column temperature: 260–300°C at 5°C min^{-1}, finally 300°C for 10 min. Detector sensitivity: 10 pA mV^{-1}. Injection volume: 1 μl solvent containing 2 μg of each component. B, Capillary column: fused-silica WCOT, 50 m × 0.22 mm I.D., CP-Sil 5 (film thickness 0.12 μm). Carrier gas: helium, linear velocity 24.5 cm sec^{-1}. Column temperature: 290°C (isothermal). Injection volume: 1 μl solvent containing 1 μg of each component; splitting ratio 1:130. Peaks: 1 = solvent; 2 = solanidine; 3 = demissidine; 4 = solasodiene; 5 = tomatidine; 6 = solasodine. For further details see Experimental section.

Determination and optimization of chromatographic parameters

To improve the chromatographic results, operational variables were optimized using the separation of solanidine and demissidine as the main criterion. At a column temperature of 290°C the separation of this $\Delta 5$- and 5α-aglycone pair, expressed as the resolution of peaks 2 and 3, was optimum ($R_{2,3}$ = 1.7) at a carrier gas velocity, u, of 24.3 cm sec^{-1}. During further optimization, u was kept at this value.

The effect of column temperature on the resolution and peak areas was investigated at 240, 260, 280, 290, 300 and 320°C. The optimum resolution was R = 1.9 at 280°C (Fig. 3). At higher temperatures the resolution decreases because of the decrease in partition coefficient, k. At lower temperatures, k increases but this did not result in an improved resolution because even at 260°C a strong fronting behaviour (up to 52% asymmetry of leading peaks) and peak broadening set in, which is probably caused by condensation of the sample in the column. At the same time, the retention times increased exponentially. At 240°C, t_R for tomatidine was 99 min.

The peak areas of solanidine, demissidine and solasodiene showed very little variation at the different temperatures. Thus, there was hardly any decomposition of these compounds upon increasing the temperature from 260 to 320°C. Under the same conditions, solasodine and tomatidine underwent up to *ca.* 12% decomposition. This could be seen from the gradual increase of small peaks in front of the solasodine and tomatidine peaks. At 280°C, the optimum temperature for separation of the alkaloids, decomposition of solasodine and tomatidine was less then 3%.

Table I shows the relative response factor, retention time, resolution and separation number of C_{27}-steroidal alkaloids chromatographed at the optimum column

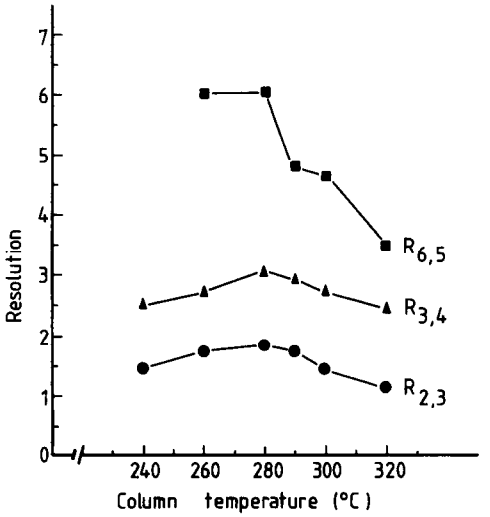


Fig. 3. Effect of temperature on the resolution, R, of solanidine–demissidine ($R_{2,3}$), demissidine–solasodiene ($R_{3,4}$) and solasodine–tomatidine ($R_{6,5}$). Because of peak asymmetry, no value for $R_{6,5}$ was obtained at 240°C. The splitting ratio and u were kept constant at 1:130 and 24.3 ± 0.2 cm sec^{-1}, respectively. For further details see Experimental section.

temperature and gas velocity. In addition, values for 5α-cholestane, which was used as a reference and to which a response factor $F = 1$ was assigned, are given. Due to a shortage of solanthrene, no response factor for this compound could be determined. Coxon *et al.*[21] reported response factors for solanthrene and solanidine of 0.73 and

TABLE I

RELATIVE RESPONSE FACTORS, F, RETENTION TIMES, t_R, RESOLUTION, R, AND SEPARATION NUMBER (SN) OF *Solanum* C_{27}-STEROIDAL ALKALOIDS AND OF 5α-CHOLESTANE

Column temperature: 280°C. Linear gas velocity: 24.3 cm sec^{-1}. Columns: CP-Sil 5 and CP-Sil 19CB (50 m × 0.22 mm). For further details see Experimental section.

Compound	*CP-Sil 5*				*CP-Sil 19CB*
	F	t_R *(min)*	*R*	*SN*	t_R *(min)*
5α-Cholestane	1.00	11.60			13.17
			6.2	4	
Solanthrene	0.73*	12.28			16.57
			35	29	
Solanidine	0.70	16.94			29.52
			1.9	0	
Demissidine	0.53	17.23			29.87
			3.1	1	
Solasodiene	0.77	17.78			31
			31	24	
Solasodine	0.59	25.22			>90
			6.1	4	
Tomatidine	0.50	27.02			>90

* Quoted from Coxon *et al.*[21].

0.72, respectively. Because their value for solanidine agrees well with mine (F = 0.70), their response factor for solanthrene was used in this study.

The retention time of the last eluted component (tomatidine) was 27 min at 280°C. When analysing *Solanum* species having unknown alkaloid compositions, the total analysis time may be longer because the retention times of alkaloids not tested in this study may exceed that of tomatidine. For example, t_R for jervine, a C_{27}-steroidal alkaloid from *Veratrum*, was 39.8 min, when chromatographed under the same conditions.

The alkaloids tested were well separated. The resolution of two consecutive peaks was always better than R = 1.5 (baseline separation). Although only one Δ5- and 5α-aglycone pair was available for this study, it can be predicted from the relatively high resolution of solanidine and demissidine ($R_{3,4}$ = 1.9) that other Δ5-5α pairs like tomatidine–tomatidenol and solasodine–soladulcidine (Fig. 1) are separated as well. Both tomatidenol (22βN-spirosolenol) and soladulcidine (22αN-spirosolanol) are expected to be eluted between solasodine (22αN-spirosolenol) and tomatidine (22βN-spirosolanol). The separation number (SN) shows that a maximum of four peaks can be separated between the last two components. Attempts further to improve the chromatographic results using a more polar liquid phase (CP-Sil 19CB) were not successful. The retention time increased drastically (Table I), and solasodine and tomatidine were not eluted after 90 min at 280°C. Solanidine and demissidine ($R_{3,4}$ = 1.0 on CP-Sil 19CB) and in particular solasodiene showed a strong tailing effect. Both phenomena are the result of adsorption of the components onto the column.

Attempts to reduce the analysis time were made by temperature programming. The best of the combinations of initial and final isothermal temperature and programming rate tested permitted the analysis time to be reduced by 9 min. However, this resulted in a 15–20% decrease in resolution and, due to the use of column temperatures up to 320°C, in an increased decomposition of solasodine and tomatidine.

Alkaloids not available for this study or novel alkaloids may be present in *Solanum* species. Therefore, in studying the alkaloid composition of *Solanum* species, the resolution must be optimum, enabling detection of such compounds. Consequently, the following temperature programme was chosen: initial temperature, 280°C; isothermal for 28 min (for optimum separation and quantitation of *Solanum* alkaloids); increased at 8°C min^{-1} to 320°C and then isothermal for at least 5 min (for testing for the presence of novel C_{27}-steroidal alkaloids which might be eluted after tomatidine).

Validation of the HRGC method

Fig. 4 shows the C_{27}-steroidal alkaloid profile determined by HRGC of a spiked potato extract. The extract was obtained from the variety Elkana, which is used for potato-starch production. Elkana contained a high content (46 mg per 100 g fresh tuber) of solanidine glycosides, determined as solanthrene. Prior to injection, 5α-cholestane, solanidine, demissidine, solasodiene, solasodine and tomatidine were added to the potato hydrolysate. The chromatogram shows that all compounds are separated and that no compounds interfering with the resolution of the alkaloids are present in the potato tuber extract.

To investigate whether the HRGC method described is useful for determining

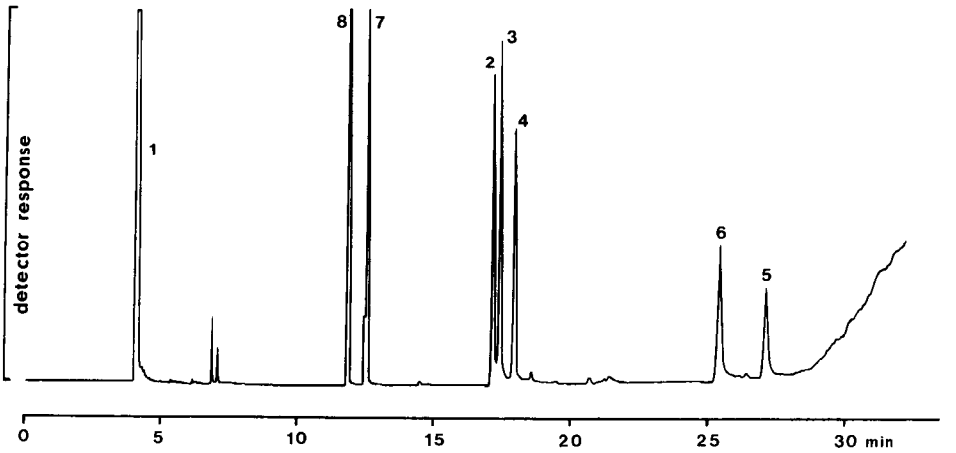


Fig. 4. Chromatogram showing the C_{27}-steroidal alkaloid profile obtained by HRGC of an extract from tubers of *S. tuberosum* cv. Elkana, spiked with 1 μg μl^{-1} of components 2, 3, 4, 5, 6 and 8. Column: fused-silica WCOT, 50 m × 0.22 mm I.D., CP-Sil 5 (film thickness 0.12 μm). Carrier gas: helium, linear velocity, u = 24.3 cm sec^{-1}. Column temperature: 280°C for 28 min, then increased at 8°C min^{-1} to a final temperature of 320°C for 5 min. Injection volume: 1 μl; splitting ratio 1:100. Attenuation: 2^6. Peaks: 1 = solvent; 2 = solanidine; 3 = demissidine; 4 = solasodiene; 5 = tomatidine; 6 = solasodine; 7 = solanthrene; 8 = 5α-cholestane; peaks not marked represent unidentified (minor) compounds. For further details see Experimental section.

the total C_{27}-steroidal alkaloid composition, the contents of solanidine glycosides determined by a colorimetric reference method were compared with those determined as solanthrene by HRGC (which were then expressed as solanidine glycosides). Ten genotypes covering a wide range in contents were used (Table II). Although differing greatly in methodology (precipitation, centrifugation, Clark reaction and colorimetry of glycosidically bound alkaloids *versus* two-phase hydrolysis, liquid–liquid extrac-

TABLE II

CONTENTS OF SOLANIDINE GLYCOSIDES (mg per 100 g FRESH TUBER) OF TEN POTATO GENOTYPES, DETERMINED BY HRGC* AND BY A COLORIMETRIC REFERENCE METHOD

Genotype	*Solanidine glycosides (mg per 100 g fresh tuber)*	
	HRGC method	*Colorimetric reference method*
1	1	2
2	1	4
3	2	6
4	15	15
5	19	21
6	22	29
7	36	31
8	38	46
9	41	40
10	47	47
Average	22.2	24.1

* Injections volume: 2 μl; splitting ratio 1:50. For other experimental conditions see Fig. 4.

tion and HRGC of aglycones, the two methods gave similar results. The correlation coefficient, r, was 0.976 and the regression equation was

$$y = 1.00\,x - 1.92$$

where y applies to the HRGC method and x to the colorimetric method.

It is concluded that this HRGC method (using the appropriate detector response factors) in combination with a hydrolysis technique, accurate and precise for the different types of C_{27}-steroidal alkaloids[19], could prove extremely useful for determining the total C_{27}-steroidal alkaloid composition of potatoes. It enables for the first time qualitative and quantitative determination of all individual compounds in one single analysis.

Various ways of improving upon the chromatographic results presented in this study are currently being investigated. A further validation study of the method is planned. This includes testing the linearity of calibration curves and comparison of determinations of the other alkaloids by HRGC and by specific reference methods. For this purpose, appropriate plant material and sufficiently large amounts of standard substances are needed.

ACKNOWLEDGEMENT

This research project is financially supported by the EEC.

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