STEROIDAL GLYCOALKALOIDS IN *SOLANUM* SPECIES: CONSEQUENCES FOR POTATO BREEDING AND FOOD SAFETY

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op woensdag 11 oktober 1989
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

STELLINGEN

1. De veelvuldig gehanteerde limiet van 200 mg steroidalkaloidglycosiden per kg verse ongeschilde aardappel, als criterium voor consumentenveiligheid, berust noch op toxicologische studies noch op kennis over het voorkomen van steroidalkaloidglycosiden in wilde Solanum-soorten en dient derhalve als arbitrair te worden beschouwd.

Dit proefschrift.

2. Voor de analyse van steroidalkaloidglycosiden in Solanum-soorten is capillaire gaschromatografie in combinatie met simultane vlamionisatiedetectie (FID) en specifieke-stikstofdetectie (NPD) minimaal noodzakelijk; bij voorkeur dient massaspectrometrie te worden toegepast.

Dit proefschrift.

3. Het vermogen van aardappelen tot het accumuleren van steroidalkaloidglycosiden onder praktijkcondities van teelt, bewaring en verwerking, dient een van de belangrijkste criteria te zijn bij het beoordelen van nieuwe rassen op hun geschiktheid voor consumptie.

Dit proefschrift.

4. Op grond van de huidige inzichten in de chemie en het voorkomen van steroidalkaloidglycosiden in Solanum-soorten kan geconcludeerd worden dat veel fytochemische en toxicologische studies tot misleidende onderzoekresultaten kunnen hebben geleid.

Bushway RJ. (1983) Am Potato J 60:793-797.

Renwick, JH, Claringbold WDB, Earthy ME, Few JD, McLean ACS. (1984) Teratology 30:371-381.

Dit proefschrift.

5. De conclusie van Osman et al. dat in microsomale fracties uit bladmateriaal van Solanum chacoense een isomeer van hydroxysolanidine gevormd zou zijn uit ^{14}C-solanidine, is niet in overeenstemming met de gepresenteerde resultaten van de massaspectrometrische analyse.

Osman S, Sinden SL, Deahl K, Moreau R. (1987) Phytochemistry 26:3163-3167.

6. Voor het bestuderen van de genetische variatie voor hoogmoleculaire gluteninesubunits in Triticum aestivum dienen naast natriumdodecylsulfaat-polyacrylamidegelelectroforese tevens andere technieken voor het analyseren van een genotype te worden toegepast.

Kolster P, Krechting CF, Van Gelder WMJ. (1988) Euphytica 39S:141-145.

7. Voor het verbeteren van de kwaliteit van tarwe als grondstof voor de broodbereiding dient te worden gestreefd naar eenvoudige methoden voor het selecteren van nieuwe tarwelijnen op de kwantitatieve samenstelling van de hoogmoleculaire gluteninesubunits.

8. Het introduceren van genen van wilde plantesoorten in voedselgewassen dient alleen te worden toegestaan, indien voldoende wetenschappelijke en experimentele ondersteuning door fytochemici en/of toxicologen is gegarandeerd.

9. Omdat tomaten tegenwoordig vaak in het "licht-kleurstadium" worden geoogst, is biochemisch onderzoek naar de degradatie van α-tomatine gedurende de doorrijping gewenst; dit geldt in het bijzonder wanneer genetisch materiaal van wilde Lycopersicon-soorten in de cultuurtomaat wordt geïntroduceerd.

Van Gelder WMJ, De Ponti OMB. (1987) Euphytica 36:555-561.

10. De gebruiksvriendelijkheid van electronisch tekstverwerken leidt niet tot een vermindering maar veeleer tot een toename van het papierverbruik en vergroot daardoor de perspectieven voor hennep als grondstof voor de papierindustrie.

11. Degenen die "natuurlijk" synoniem achten met "gezond", dienen te beseffen dat "natuurlijke toxinen" een uitzondering op die regel zijn.

12. Genetische manipulatie: van een mug een olifant maken is gemakkelijker dan het omgekeerde.

W.M.J. van Gelder.
Proefschrift: Steroidal glycoalkaloids in Solanum species: consequences for potato breeding and food safety.
Wageningen, 11 oktober 1989.

CONTENTS

VOORWOORD/ACKNOWLEDGEMENTS

Iedereen die heeft bijgedragen aan het totstandkomen van dit proefschrift wil ik bedanken, temeer omdat de wijze van samenwerking en de kontakten altijd uiterst prettig waren.
Allereerst dank ik mijn promotoren, Hans Scheffer en Jan Koeman, voor hun inspirerende begeleiding en daarnaast Albert Eenink die als directeur van de Stichting voor Plantenveredeling SVP een bijzonder stimulerende invloed heeft gehad. Voorts dank ik de co-auteurs Henk Huizing, Lidwine Dellaert, Henk Vinke, Louis Tuinstra en Jan van der Greef, voor hun waardevolle bijdragen. Dank ook aan Wim Traag, Paul Kienhuis, de heer L.G. Gramberg en Drs. C.A. Landheer voor hun bijdragen op het gebied van massaspectrometrie, aan Ben Jansen voor zijn hulp bij het hydrogeneren en aan Drs. P. Plieger en Ir. A.L.M. Sanders voor het beschikbaar stellen van standaardalkaloïden.
Thanks are due to Prof. Dr. Rodney Bushway, Dr. William Gaffield, Dr. Richard Keeler, Prof. Dr. D.N. Kirk, Dr. Stanley Osman and Dr. Steve Sinden for providing standard alkaloids.
Verder dank ik Saskia van Erkelens, Gea Jonker, Marion Janssen, Marleen de Jongh de Leeuw en Pieter de Smidt voor hun hulp o.a. bij het tekstverwerken en het verzamelen van literatuur.
Tenslotte dank ik vooral Harry Jonker en Kees Krechting voor hun vakbekwame assistentie en de bijzonder prettige werksfeer.

Het totstandkomen van dit proefschrift is gesubsidiëerd door het Directoraat Generaal voor de Landbouw van de Europese Gemeenschap en door de Stichting 'Fonds Landbouw Export Bureau 1916/1918' (LEB-Fonds).

AAN MARIE-CHARLOTTE,
JEROEN EN PHILIP

GENERAL INTRODUCTION

The potato (Solanum tuberosum L.) which is grown in 80% of all countries, is one of the world's most important crops for human consumption. In terms of global production, potato ranks fourth after wheat, maize and rice. About 300 million metric tonnes of potatoes are produced annually, of which about 70% in the developed and 30% in the developing countries (FAO, 1987).

Usually potatoes are merely seen as an energy source only. However, when potatoes are compared with other plant foods on a cooked basis, 'as eaten', it becomes clear that potatoes are also important as a source of minerals, vitamins and protein (Woolfe, 1987). The protein has a high nutritive quality because of its high content of essential amino acids, especially lysine (Van Gelder and Vonk, 1980), which makes it outstanding for complementing proteins from other plant food. The potato is superior to almost every other crop in food production per hectare per day (CIP, 1982).

In order to reduce yield losses and tuber injuries caused by parasite infestations of the potato crop, considerable amounts of fungicides, insecticides and nematocides are applied. The disadvantages, in terms of ecological damage and financial costs, of the chemical control of parasites being recognized, the attention for the genetic resistance of the potato is strongly increasing. The breeding of new potato cultivars with an enhanced resistance to parasites requires the use of wild Solanum species, as these species possess a broad spectrum of resistances (Ross, 1986). They are also a source of valuable genes for resistance to frost and drought, and for quality traits such as high contents of protein and starch and low contents of reducing sugars. A disadvantage of Solanum species, from a consumers point of view, is that they contain steroidal glycoalkaloids (SGAs).

SGAs are natural toxins generally occurring in all parts of many Solanaceous plants. The SGAs consist of a C_{27}-steroidal alkaloid (SA) and a sugar moiety, often a di-, tri- or tetrasaccharide. The tubers of the cultivated potato usually contain small quantities of a class of SGAs, the solanidine glycosides, which are believed not to present a health hazard to the consumer. However, potato tubers contain the entire enzyme system necessary for synthesis of SGAs, and many factors

during growth and post-harvest handling of potatoes can induce de novo synthesis which may lead to accumulation of undesirably high levels of SGAs. Many cases of poisoning that have occurred after consumption of potatoes, especially in the nineteenth and in the first half of this century but also more recently (see Chapter II), have been ascribed to excessive levels of solanidine glycosides, resulting from abnormal growth and storage conditions of the tubers. These cases generated much research on the potato SGAs, but the general interest in SGAs is still growing due to several other developments during this century, as is indicated below.

The discovery that SGAs from wild Solanum species are involved in the resistance of such species to certain parasites, initiated much research on this topic (for literature reviews see: Tingey, 1984; Roddick, 1987). Supply and price fluctuations between 1960 and 1975, of the steroidal sapogenin diosgenin, the starting material for the production of steroid hormones, prompted large programmes for the screening of Solanum species for the presence of the SA solasodine, that can be used as an alternative (Mann, 1978; Ripperger and Schreiber, 1981). A hypothesis, suggesting a toxin present in certain imperfect potato tubers to be involved in human birth defects (Renwick, 1972), caused great concern and led to a strong increase of the research on the toxicity and teratogenicity of the potato SGAs.

The withdrawal from commerce in the USA in 1970, of the cultivar Lenape because of excessive levels of solanidine glycosides in its tubers (Zitnak and Johnston, 1970), prompted studies on factors affecting the SGA accumulation in potato tubers. The evidence that 'Lenape' inherited the ability to synthesize large amounts of solanidine glycosides from its wild ancestor S. chacoense Bitt. (Sinden et al., 1984), forces potato breeders to realize that the introgression of genes from wild species into the cultivated potato, can result in the introduction of undesired levels or types of SGAs (Van Gelder et al., 1987 and 1988).

AIM OF THE STUDY PRESENTED IN THIS THESIS

Tuberiferous wild Solanum species are increasingly being used in potato breeding (Ross, 1986). New techniques in cell biology, such as

somatic hybridization, enable the potato breeder to recombine genes from tuberiferous and genetically strongly different, nontuberiferous Solanum species (Fish et al., 1988). The preceding facts imply that there is a growing need for knowledge on the SGAs in Solanum species which are being used or are potentially useful in potato breeding. However, the qualitative and quantitative SGA compositions of wild species used in potato breeding programmes and of their offspring, have received little or no attention until now. This can partly be ascribed to the complex methodology of the analysis of the SGAs (Van Gelder, 1989).

The aim of this study was to develop and apply a method for qualitative and quantitative analysis of the SGA compositions of Solanum species, and to put the collected information into the perspectives of potato breeding and of food safety, in order to point out the potential consequences of the introduction of undesired levels or types of SGAs into the cultivated potato. For this purpose attention has been paid to the following topics.

-Review of the literature on the distribution and accumulation of solanidine glycosides in the cultivated potato; SGAs from wild Solanum species will almost certainly accumulate similarly when introduced into the household potato, because the biosynthetic pathways of the various SGAs are closely related.

-Evaluation of the toxicity of the SGAs as the current so-called safety limits for solanidine glycosides in potatoes, reported in the literature, are conflicting and not based on such an evaluation.

-Development of an advanced procedure for the comprehensive and quantitative analysis of the SGA composition of Solanum species. Much emphasis has been given to identification and/or characterization of (novel) aglycones.

-Determination of the SGA composition of a number of wild Solanum species used in breeding programmes, and tentative study of the potential transmission of SGAs from wild Solanum species to the cultivated potato.

-Based on these studies, recommendations are made with respect to the control of the levels and the types of SGAs in potatoes to be used for human consumption.

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CHAPTER I*

OCCURRENCE OF STEROIDAL GLYCOALKALOIDS AND THEIR DISTRIBUTION AND ACCUMULATION IN THE POTATO TUBER

INTRODUCTION - HISTORY OF THE POTATO

The geographical origin of the potato is the Andes of Bolivia and Peru, where many wild tuber-bearing Solanum species occur, from one or more of which the cultivated potato can have been derived (Wright, 1977; Hawkes, 1978).

Detailed studies of starch and other cell structures enabled remains of early food plants from the Chilca Valley near Lima being identified as potatoes, which were radiocarbon dated at an age of 8000 years (Engel, 1970). At that time, potato tubers were collected from wild growing plants, as the cultivation of the potato started about 5000 years ago (Cohen, 1977). However, at the time of the Spanish conquest, the potato seems to have been an intensively cultivated crop in Bolivia and Peru.

The potato was firstly introduced into Europe in about 1570, i.e. in Spain, from where it diffused through continental Europe and parts of Asia (Hawkes, 1978). A second introduction took place in England, between 1588 and 1593, from where it spread to Ireland, Scotland, Wales, and to parts of northern Europe and to most of the British colonies. In most European countries the potato started as a botanical curiosity and, for a long time, it was hardly grown as a food crop, except for southern France, where potatoes were eaten by the poorest people, and for England and Russia, where they were esteemed as a delicacy at the royal courts. Between 1750 and 1800 the potato became increasingly popular as a field crop in many countries within and outside Europe,

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*This chapter is based on the invited review paper:
Van Gelder WMJ. (1990) Steroidal glycoalkaloids: consequences for potato breeding and food safety of utilizing wild Solanum species in breeding programmes. In Handbook of Natural Toxins, Vol. 6, RF Keeler, AT Tu (Eds). Marcel Dekker Inc., New York, in press.

and within a hundred years entire populations became dependent on potatoes as their principal source of nourishment.

DISCOVERY, OCCURRENCE AND CHEMISTRY OF SGAs

In 1820, Desfosses reported the discovery of an organic base, solanée, isolated from the berries of black nightshade (Solanum nigrum L.). A 100 mg of this compound administered orally to a dog caused considerable vomiting and unconsciousness. A compound similar to 'solanée' was found in potato and was named solanine (Baup, 1826). The discovery of this toxin in this principal food crop generated much research, which is ongoing for over 160 years now.

Important contributions to the knowledge of the chemistry and the occurrence of the SAs and SGAs have been made between 1861 and 1955. Zwenger and Kind (1861) found that solanine from potatoes was a glycoalkaloid and they named the aglycone solanidine (Fig. 1). The final establishment of the formula of solanidine as $C_{27}H_{43}NO$ (Schöpf and Herrmann, 1933) lead to the general acceptance of the formula of solanine as $C_{45}H_{73}NO_{15}$ (Henry, 1949). About 130 years after the discovery of solanine, its composition was finally revealed as a mixture of two series of glycoalkaloids, the α-, β- and γ-forms of solanine and of chaconine (Table 1), which have the same aglycone (solanidine) but different sugar moieties (Kuhn and Löw, 1954; Kuhn et al., 1955a and 1955b). About 90-95% of the solanidine in potato was found to be glycosidic-bound in α-solanine and α-chaconine (Kuhn and Löw, 1954; Kuhn et al., 1955b; Pasesnishenko and Guseva, 1956). The β- and γ-forms detected may have been artefacts resulting from the extraction procedures (Prelog and Jeger, 1960) or intermediates in SGA metabolism.

Since 1820, hundreds of SGAs have been detected in about 350 species of the families Solanaceae and Liliaceae. The chemistry and the occurrence of the SAs and SGAs in the genus Solanum have been reviewed up to 1981 (Prelog and Jeger, 1953 and 1960; Schreiber, 1968; Ripperger and Schreiber, 1981); more than 80 SAs possessing the C_{27}-skeleton of cholestane have been described. They have been divided into five groups representing different aglycone structures, namely solanidanes (e.g. solanidine), spirosolanes (e.g. tomatidine and solasodine), solanocapsines, 3-aminospirostanes (e.g. paniculidine) and epiminocholesta-

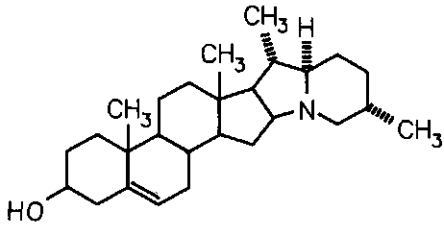
Table 1. Steroidal glycoalkaloids of tuber-bearing Solanum species.

Steroidal glycoalkaloid[1]	Sugar moiety	Glycoside structure[2]
Solanidine glycosides		
α-Solanine	Solatriose	A: R-Gal < Rham, Glu
β-Solanine[3]	Solabiose	B: R-Gal-Glu
γ-Solanine[3]	Galactose	C: R-Gal
α-Chaconine	Chacotriose	D: R-Glu < Rham-a, Rham-b
β_1-Chaconine[3]	Chacobiose	E: R-Glu-Rham-a
β_2-Chaconine[3]	Chacobiose	F: R-Glu-Rham-b
γ-Chaconine[3]	Glucose	G: R-Glu
Dehydrocommersonine	Commertetraose	H: R-Gal-Glu < Glu, Glu
Demissidine glycosides		
Demissine	Lycotetraose	I: R-Gal-Glu < Glu, Xyl
Commersonine	Commertetraose	As H
Leptinidine glycosides		
Leptinine I	Chacotriose	As D
Leptinine II	Solatriose	As A
Acetylleptinidine glycosides		
Leptine I	Chacotriose	As D
Leptine II	Solatriose	As A
Tomatidenol glycosides		
α-Solamarine	Solatriose	As A
β-Solamarine	Chacotriose	As D
Solasodine glycosides		
Solasonine	Solatriose	As A
Solamargine	Chacotriose	As D
Tomatidine glycosides		
α-Tomatine	Lycotetraose	As I
Sisunine (neotomatine)	Commertetraose	As H

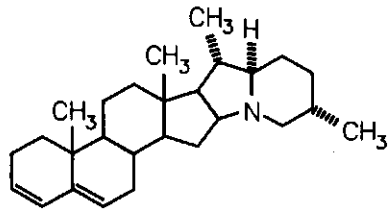
[1]Aglycone, structures are given in Fig. 1.

[2]R = aglycone; Gal = galactose; Rham = rhamnose; Glu = glucose; Xyl = xylose.

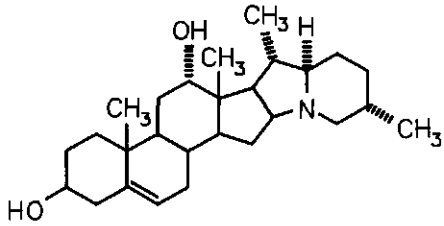
[3]Minor steroidal glycoalkaloids may be artefacts or metabolites.



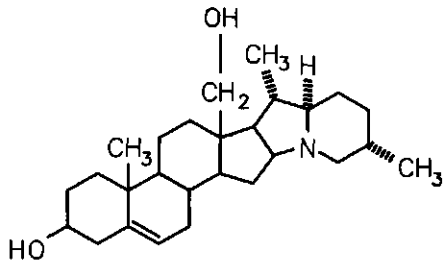
Solanidine $C_{27}H_{43}NO$



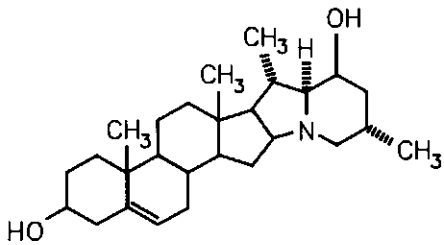
Solanthrene $C_{27}H_{41}N$



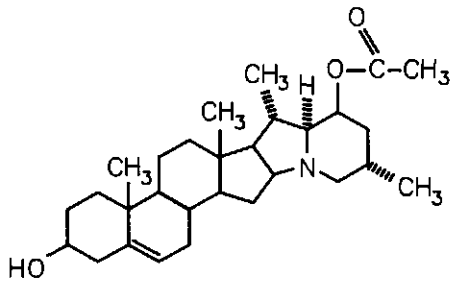
Rubijervine $C_{27}H_{43}NO_2$



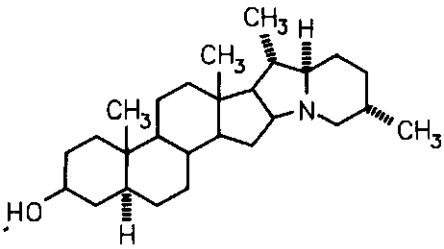
Isorubijervine $C_{27}H_{43}NO_2$



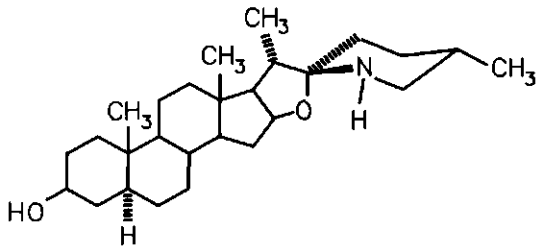
Leptinidine $C_{27}H_{43}NO_2$



Acetylleptinidine $C_{29}H_{45}NO_3$

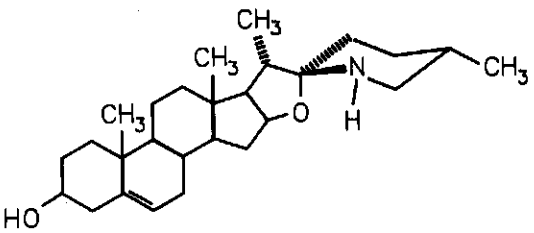


Demissidine $C_{27}H_{45}NO$

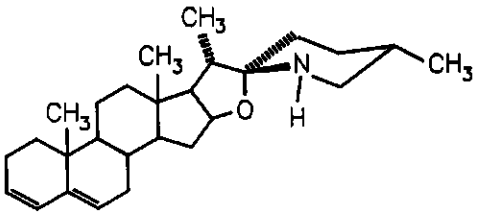


Tomatidine $C_{27}H_{45}NO_2$

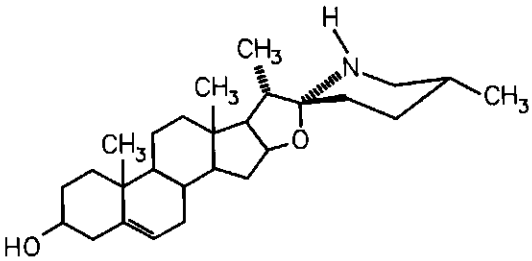
Fig. 1. Structural formulas of C_{27}-steroidal alkaloids (SAs) divided into five groups: solanidanes, spirosolanes, solanocapsines, 3-amino-spirostanes and epiminocholestanes.



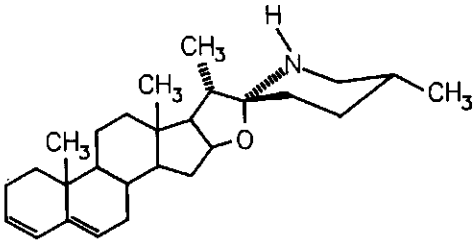
Tomatidenol $C_{27}H_{43}NO_2$



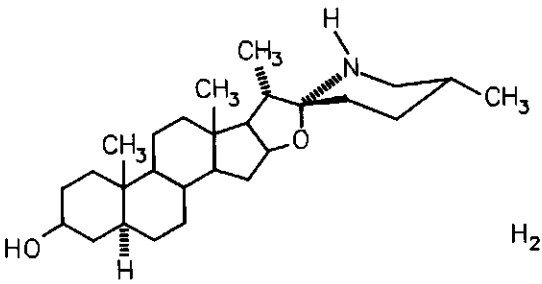
Tomatidadiene $C_{27}H_{41}NO$



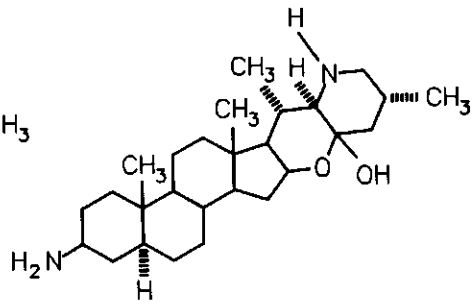
Solasodine $C_{27}H_{43}NO_2$



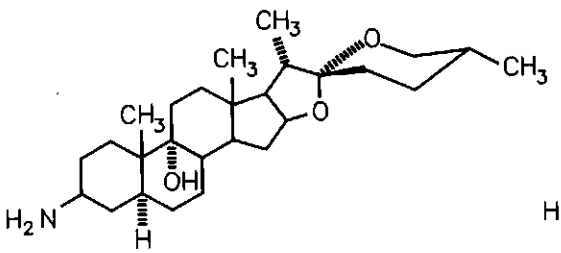
Solasodiene $C_{27}H_{41}NO$



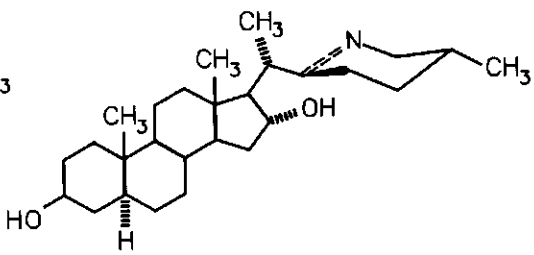
Soladulcidine $C_{27}H_{45}NO_2$



Solanocapsine $C_{27}H_{46}N_2O_2$



Paniculidine $C_{27}H_{45}NO_3$



Solafloridine $C_{27}H_{45}NO_2$

nes (e.g. solafloridine) (Fig. 1). Other structures such as that of the jervanes occur additionally in Veratrum species, family Liliaceae (Ripperger and Schreiber, 1981).

In the genus Solanum, the SAs are glycosidic-bound via the hydroxyl group at the C-3 atom. They occur in combination with different sugar moieties, e.g. solatriose, chacotriose, lycotetraose etc. As a result numerous SGAs have been reported for Solanum species, but until now only a limited number of them have been detected in the tuber-bearing species (Table 1).

New SAs and SGAs are still being reported, for example sisunine (Osman et al., 1986), 7β-hydroxy-O-methylsolanocapsine (Chakravarty and Pakrashi, 1988), and solanudine (Usubillaga, 1988). New solanidane-type SAs have recently been detected in Solanum species used in potato breeding (Van Gelder et al., 1987 and 1989; see hereafter).

BIOSYNTHESIS AND DEGRADATION OF SGAs

The biosynthetic pathways of the SAs are closely related to each other and to those of the steroidal sapogenins, which also occur in Solanum species. They all follow the general pathway of the steroid biosynthesis, starting from acetyl-coenzym A via the usual intermediates mevalonic acid, squalene, cycloartenol and cholesterol. The steps in the pathways from cholesterol to the SAs have only partially been elucidated. Fig. 2 deliniates the main steps of the (partially hypothetical) pathways for the biosynthesis of the SAs. Dormantinol and dormantinone have been isolated from a solanidine synthesizing Veratrum species (Kaneko et al., 1977), but they have not yet been proven to be intermediates by tracer experiments. The amino acid arginine was suggested as the nitrogen source for solanidine in this biosynthesis route (Kaneko et al., 1976).

The enzymatic glycosylation of the aglycones to the γ-, β- and finally the α-forms of the SGAs occurs stepwise, at least in the case of the solanidine and solasodine glycosides (Liljegren, 1971; Jadhav and Salunkhe, 1973; Lavintman et al., 1977; Osman et al., 1980). Solanidine was found to be rapidly converted into its glycosylated products, which is mentioned as a possible explanation for the fact that free solanidine does normally not occur in healthy potato tuber tissue

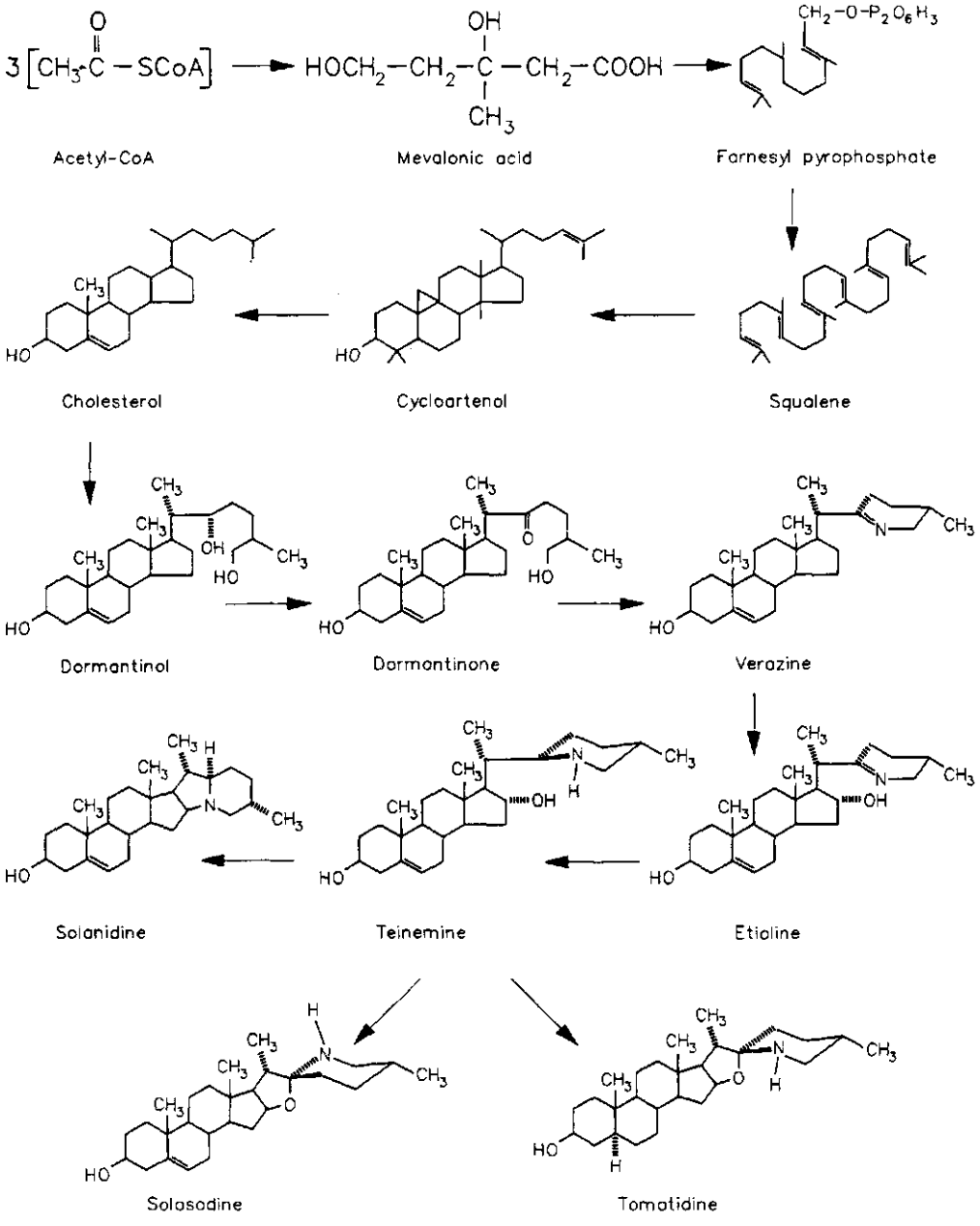


Fig. 2. Biosynthetic pathways of C_{27}-steroidal alkaloids.

(Osman et al., 1980). In Solanum species, the aglycones shown in Table 1 are the end-products of the SA biosynthesis which are glycosylated to SGAs, whereas in other genera of the Solanaceae, Solanum aglycones have been detected as precursors of other SAs. For example, in Veratrum species, solanidine was converted into jervine and veratramine (Kaneko et al., 1972) and in Fritillaria species, solanidine, solasodine and tomatidenol seemed to be metabolized to camtschatcanidine, hapepunine, anrakorinine and 27-hydroxyspirosolane (Kaneko et al., 1981).

Potato tissues contain enzymes that can degrade α-solanine and α-chaconine by cleaving one or more sugars from their sugar moieties (Swain et al., 1978). Bushway et al. (1988) characterized an enzyme from potato as a rhamnosidase; it formed mainly β_1-chaconine and a small amount of β_2-chaconine. These enzymes become active only when the tissue is disrupted, probably because the enzymes and substrates are present in different compartments within the cell. In analyses of potato tissues this enzymatic degradation of SGAs may be a source of artefacts.

Since the biosynthetic pathways of the various SAs are strongly related, it is to be expected that factors influencing the accumulation of the solanidine glycosides in household potatoes, will similarly affect alien SGAs if they would have been introduced into cultivars. It is thus necessary to know the distribution of the SGAs in the potato plant, and the factors which may affect the accumulation of the SGAs in its tubers.

DISTRIBUTION OF SGAs IN THE POTATO PLANT

SGAs are found in all organs of the potato plant, the highest concentrations in parts of high metabolic activity, such as flowers, unripe berries, young leaves and sprouts. The pure S. tuberosum cultivars contain only solanidine glycosides. The most extensive study up to now, on the distribution of the solanidine glycosides in different organs and tuber tissues of potato was carried out by Lampitt et al. (1943), who investigated different cultivars; their results are summarized in Table 2. In addition data from other reports are compiled. The variation in the concentrations of the solanidine glycosides within a certain organ or tissue can be ascribed to the different cultivars

Table 2. Contents of solanidine glycosides in various parts of the potato plant (Solanum tuberosum).

Plant part	Solanidine glycosides (mg/kg fresh weight)		References
	Lampitt et al. (1943)[1]	Other studies	
Normal tubers			
whole tuber	25- 100	11- 150	many, see text
skin, 2-3% of tuber	300- 640		
peel, 10-12% of tuber	150- 155	150- 1068	Wood and Young (1974); Zitnak (1981); Bushway et al. (1983)
flesh	12- 60	1- 100	Zitnak, (1981); Bushway et al. (1983)
Small tubers			
10-40 g		96- 448	Verbist and Monnet (1979)
0.3-17 g		92- 522	Van Gelder et al. (1988)
Bitter tubers		250- 800	Zitnak (1961)
Sprouts	1950-17700[2]	2000- 4360	Bömer and Mattis (1924); Wood and Young (1974); Fitzpatrick et al. (1977)
Stolons	150- 540		
Roots	180- 400		
Stems	23- 33		
Lateral stems		30- 71	Kozukue et al. (1987)
Leaves	550- 610	230- 1000	Wood and Young (1974); Kozukue et al. (1987)
Growing tops	300- 860		
Flowers	2150- 4160	3000- 5000	Wood and Young (1974); Kozukue et al. (1987)
Petals		3060- 4970	Kozukue et al. (1987)
Calyxes		4770- 5710	Kozukue et al. (1987)
Fruits	420	560- 1080	Bömer and Mattis (1924)

[1]Data from several varieties.
[2]Extremely high levels were due to illumination of sprouts.

studied, and to differences in the growing conditions of the plants as well as in the physiological stage of their organs.

Until now the SGA concentrations have often been expressed as mg% as well as mg/100 g plant material, but according to the rules of the International System of Units, mg/kg is to be preferred. In this thesis SGA contents are expressed as mg/kg fresh nonpeeled tuber unless stated otherwise.

Field-grown normal tubers of most of the current commercial potato cultivars show average contents of solanidine glycosides up to 100 mg/kg (Sinden and Webb, 1974; Patchett et al., 1977; Parnell et al., 1984; Van Gelder, 1985a; Lammerink, 1985; Morris and Petermann, 1985; Olsson, 1986; Uppal, 1987; De Maine et al., 1988). In The Netherlands, the average contents of solanidine glycosides of the 14 most important household cultivars which were determined in tubers grown in at least three seasons at two locations, varied from 20-85 mg/kg (Van Gelder, 1985a). Calculations based on the data reported in the just-mentioned studies, which concerned a total of 521 samples of potato tubers of many different registered cultivars (excluding 'Lenape') grown in different countries, revealed that almost 85% of the samples showed contents of solanidine glycosides below 100 mg/kg. The contents of 12% and 2% of the samples were 100-150 mg/kg and 150-200 mg/kg, respectively; another 2% of the samples showed contents of these glycosides exceeding 200 mg/kg (Fig. 3). Early and main crop potatoes purchased at sales points (supermarkets, greengrocers and farm shops) in the U.K. showed higher levels of these glycosides probably due to earliness, and post-harvest handling and storage conditions. The average contents ranged from 90-170 mg/kg and the contents of individual samples were up to 220 mg/kg (Davies and Blincow, 1984). The cultivar Lenape contained SGA levels up to 350 mg/kg in Canada (Zitnak and Johnston, 1970) and up to 650 mg/kg in the U.S.A. (Sinden and Webb, 1974).

The solanidine glycosides are usually concentrated in about a 1.5 mm layer under the skin of the potato tuber. In this case, peeling of potatoes removes 50-95% of these SGAs (Bömer and Mattis, 1924; Lepper, 1949; Verbist and Monnet, 1979; Bushway et al., 1983; Uppal, 1987). However, when potatoes contain concentrations above 250 mg/kg, the solanidine glycosides can be present throughout the flesh. Peeling may

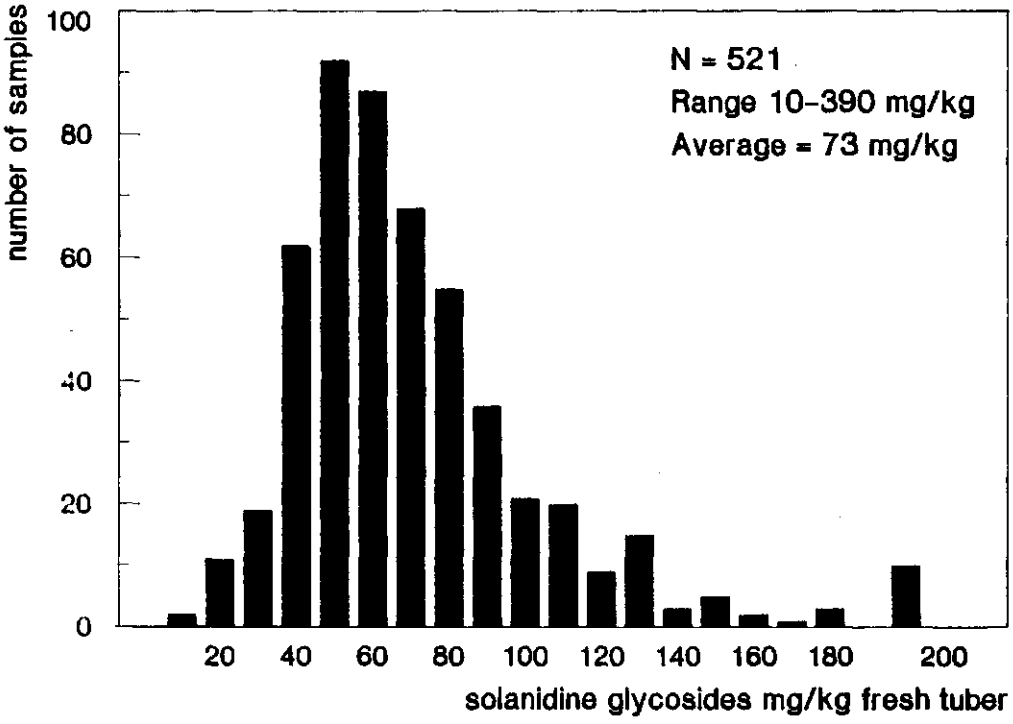


Fig. 3. Frequency distribution of the contents of solanidine glycosides (mg/kg fresh tuber) in 521 samples of potato tubers. These contents were reported in the literature and include those of many registered household potato cultivars grown in Australia, England, India, The Netherlands, New Zealand, Scotland, Sweden and the U.S.A. (the cultivar Lenape and breeding clones were excluded from this frequency distribution).

then remove only 30-35% of the total amount of SGAs present (Zitnak, 1961; Wood and Young, 1974; Verbist and Monnet, 1979).

Morris and Petermann (1985) found that the amount of α-solanine varied from 28% to 57% of the total amount of the solanidine glycosides in potato tubers. Free solanidine up to 33% of the total amount (free and glycosidic-bound solanidine) present, has been reported to occur in bitter potato tubers (Zitnak, 1961), but Lampitt et al. (1943) could not detect free solanidine in tubers of nine cultivars, not even when the tubers contained large amounts of solanidine glycosides due to light exposure for 18 weeks.

FACTORS AFFECTING THE ACCUMULATION OF SOLANIDINE GLYCOSIDES IN POTATO TUBERS

Genetic variation and growth conditions

The contents of solanidine glycosides in field-grown tubers of commercial potato cultivars can vary widely for individual samples. Values between 10 mg/kg and 650 mg/kg have been reported (Baerug, 1962; Cronk et al., 1974; Fitzpatrick et al., 1978; Verbist and Monnet, 1979; Davies and Blincow, 1984; see also the studies of which the values have been summarized in Fig. 3). The genetic variation is the largest source of variation of the contents of solanidine glycosides, but these contents may also vary among crops of a single cultivar grown at different locations or in different years, due to the varying environmental conditions (Sanford and Sinden, 1972; Sinden and Webb, 1972 and 1974; Parnell et al., 1984; Van Gelder and Dellaert, 1988).

Although interactions between genotype and environment have been demonstrated, the ranking order of genotypes as to their SGA contents remains quite constant under different environmental conditions (Lepper, 1949; Sinden and Webb, 1974; Patchett et al., 1977; Lammerink, 1985; Olsson, 1986). In a recent study on 27 genotypes grown in four seasons at two locations, a strong correlation ($r = 0.86$) was found between the genotype-averages of the contents of solanidine glycosides (10-650 mg/kg; measured over the years and locations) and the coefficients of variation calculated for each genotype (Van Gelder and Dellaert, 1988). This may explain why cultivars with genetically determined 'high' contents of solanidine glycosides in the tubers (above 100 mg/kg) accumulated easily excessive amounts (above 200 mg/kg) compared with cultivars with low contents.

Most of the environmental variation seems to result from climatic influences (Maga, 1980; Jadhav et al., 1981). Especially an unusually cool growing season accompanied with an abnormally high number of overcast days, probably resulting in an immature potato crop, has been suggested to be the cause of excessive levels of solanidine glycosides which were held responsible for cases of acute poisoning (Bömer and Mattis, 1924; Norberg, 1987).

Immaturity has been associated with high or even excessive levels of solanidine glycosides (Bömer and Mattis, 1924; Wolf and Duggar, 1946;

Patchett et al., 1977; Ahmed and Müller, 1979; Verbist and Monnet, 1979). Tuber size as well as maturity, are inversely related with the content of solanidine glycosides within a cultivar, as these SGAs are formed early during tuber development and as they are "diluted" during the process of enlarging and maturation of the tubers. Moreover, the SGAs are concentrated in the peel, which is a relatively larger part of the tubers when they are smaller. Verbist and Monnet (1979) found in 20 tuber samples of eight current cultivars contents of solanidine glycosides varying from 96-448 mg/kg; the average tuber weights varied for the different samples from 9-40 g. They supposed that such small potatoes can be responsible for cases of gastroenteritis. Other factors that have been investigated in relation to SGA accumulation during the growth of potato tubers in the field, are planting date, fertilization with major elements and soil type. These factors exerted only little effect on the accumulation of solanidine glycosides and are not associated with potentially hazardous levels of these compounds (Maga, 1980; Jadhav et al., 1981; Sinden et al., 1984).

Light exposure and storage conditions

When harvested potatoes are exposed to light, their contents of solanidine glycosides may increase considerably. The increase depends on the cultivar and is positively related to the light intensity and the duration of the exposure (Jadhav and Salunkhe, 1975; Maga, 1980; Zitnak 1981; Jadhav et al., 1981; Sinden et al., 1984; Uppal, 1987). Upon exposure to bright sunlight (35000-50000 lx) for 6 h, the solanidine glycoside content of freshly harvested tubers increased from 50 mg/kg to above 200 mg/kg (Baerug, 1962). Levels as high as 450 mg/kg have been reported for tubers exposed to intense solar irradiation when left in the field for 72 h (Zitnak, 1961 and 1977). During light exposure, the solanidine glycoside contents of immature and small tubers increased more strongly (up to 480 mg/kg) than those of mature and large tubers (up to 200 mg/kg) (Bömer and Mattis, 1924; Wolf and Dugar, 1946; Lepper, 1949; Sinden and Webb, 1974; Patchett et al., 1977). Artificial light during storage or marketing also induces de novo synthesis of solanidine glycosides. The increases are usually not as dramatic as those resulting from sunlight, and tubers of cultivars normally showing contents below 100 mg/kg, do usually not exceed 150 mg/kg upon exposure

to artificial light (Ahmed and Müller, 1981; Sinden et al., 1984; Lammerink, 1985; De Maine et al., 1988).

Light-induced synthesis of SGAs is often accompanied with greening of the tubers as a result of the synthesis of chlorophyll in the potato peel. Greened potatoes are often associated with bitterness and high SGA levels. Although the spectral responses to light of the mechanisms synthesizing the chlorophylls a and b and the SGAs α-solanine and α-chaconine were similar (Petermann and Morris, 1985), these biochemical processes seem to be able to proceed independently of each other (Grison, 1987; De Maine et al., 1988). Potatoes with levels of solanidine glycosides as high as 600-1000 mg/kg, did not show any greening (Zitnak, 1977). These data clearly show that greening is not a reliable indicator of too high SGA levels in light-exposed potatoes.

Storage of potato tubers can increase their contents of solanidine glycosides (Cronk et al., 1974; Ahmed and Müller, 1981), but the litterature is contradictory with respect to the influence of the temperature during, and the duration of the storage period. Storage in the dark at various temperatures between 0°C and 28°C for 12 weeks (Nair et al., 1981; Linneman et al., 1985) or between 10°C and 15°C for up to 7 months (Zitnak, 1953, quoted from Jadhav et al., 1981; Lammerink, 1985) hardly affected the accumulation of solanidine glycosides. Storage at 4°C resulted in a slight increase after 3 months in a study by Wu and Salunkhe (1976), but in non-elevated levels after 3-8 months in other studies (Wolf and Duggar, 1946; Fitzpatrick et al., 1977; Bostock et al., 1983). Storage at 4°C under humid conditions resulted in high contents of solanidine glycosides after 6 weeks (Zitnak, 1953).

A large number of physical and chemical treatments to control post-harvest accumulation of SGAs have been described. Among these are vacuum packaging and packaging in coloured polyethylene bags, ionizing radiation, submerging in water to which often chemicals were added, heating of tubers and treating them with waxes, chemicals or oils. Some treatments simultaneously inhibited SGA accumulation in, and greening and/or sprouting of the tubers. For information on the efficacy and limitations of these treatments reviews by Salunkhe and Wu (1979), Maga (1980) and Jadhav et al. (1981) are referred to.

Tuber injury

Damage to tubers resulting from dropping, puncturing, cutting, hammering, brushing, or caused by diseases or by animals, stimulated the SGA synthesis (Sinden and Webb, 1974; Wu and Salunkhe, 1976; Ahmed and Müller, 1978; Olsson, 1986; Mondy et al., 1987). This effect is thought to be due to a physiological defense mechanism in the tuber. In slightly injured tubers the contents of solanidine glycosides can increase 200-300% (Sinden and Webb, 1974). Severely damaged tubers contained levels of these glycosides sometimes exceeding 200 mg/kg, with extreme values of 319 and 530 mg/kg (Olsson, 1986). The accumulation of the glycosides occurred in the peel as well as in the flesh, mainly in the first weeks of storage after the induction of the synthesis by mechanical injury (Wu and Salunkhe, 1976). The extent of the SGA accumulation depends strongly on the type of injury, on the cultivar and on the storage conditions after the injury. Damaged tubers stored at ca 5°C in the dark accumulated less solanidine glycosides than tubers stored at ca 20°C (Wu and Salunkhe, 1976; Ahmed and Müller, 1978; Mondy et al., 1987). Probably, the tuber defense mechanism is more active at higher temperatures. According to Fitzpatrick et al. (1978), damaged potatoes purchased on the retail market contained levels of SGAs up to 193 mg/kg. Olsson (1986) observed a high correlation between the initial solanidine glycoside contents of cultivars and breeding clones and their elevated contents after damage, and recommended potato breeders to select for low SGA contents to prevent accumulation of SGAs due to damage.

Potato processing

SGAs are fairly heat-stable compounds, their melting points, at which some SGAs may start decomposing, vary in general from 230-280°C (Prelog and Jeger, 1960; Schreiber, 1968; Ripperger and Schreiber, 1981). At these temperatures their aglycones do not show decomposition, and even during gas chromatography at 280-320°C they hardly decompose (Van Gelder, 1985b). It is therefore not surprising that steaming, boiling, baking, cooking, frying and microwaving of potatoes did not affect their contents of solanidine glycosides (Baker et al., 1955; Zitnak and Johnston, 1970; Bushway and Ponnampalam, 1981).

When during the manufacturing of potato chips (Eng. = crisps) and

French fries (Eng. = chips), half products such as slices or strips are left for some time (3 h to 3 days) before frying or cooking, the SGA contents can increase with 100% (Ahmed and Müller, 1978; Maga, 1981). In aged potato slices of the cultivar Kennebec, which contains germplasm of S. demissum Lindl., the tomatidenol glycosides α- and β-solamarine, not originating from S. tuberosum, were detected in addition to α-solanine and α-chaconine (Shih and Kuc, 1974; Fitzpatrick et al., 1977). The above results are especially important in the light of the results of Sizer et al. (1980), who showed that during the frying of potato chips water was lost, which resulted in a three- to fourfold concentration of SGAs. They found strongly varying SGA contents, 95-720 mg/kg, in commercially purchased chips containing most of the potato skin. The excessive content did not cause a noticeable bitterness, but the authors suggested that the bitterness could have been masked by the salt on, or the high oil content of the chips. Bitterness can possibly also be masked by other flavour additives. As especially infants and adolescents sometimes consume relatively large amounts of chips, it is advisable that potatoes are peeled well and that delays in processing of slices or otherwise nonintact potatoes are avoided.

CONCLUSIONS

It can be stated that in view of the data given in the literature many factors influencing the accumulation of solanidine glycosides in potatoes should be kept in mind.

In order to control the accumulation of these SGAs resulting from the growth conditions, it is important that potato tubers are well-covered with soil during growth, and that they are harvested when they are mature. When the crop has been exposed to physiological stress, especially unusual climatic conditions, it is advisable to check the SGA content of the tubers after harvest. Exposure of potatoes to bright light, especially sunlight, must be minimized.

Potato tubers can be stored for a considerable period of time, probably up to eight months or even longer, without a marked increase in the contents of solanidine glycosides. However, in such cases the storage conditions must be optimum. Indications that storage in the dark at ca 9-15°C and at a low air humidity is to be preferred, can be found in

the literature, but further study of the effects of storage conditions on the accumulation of SGAs is needed. This is the more so, in view of the currently increasing interest for cold storage (2-4°C) of potatoes as an alternative to chemical sprouting inhibitors.

Growers of potatoes and those involved in marketing usually avoid injury that deteriorates the outward appearance of potatoes, but it is important also from the viewpoint of food safety, that any kind of injury to potatoes should be avoided.

Potatoes must be peeled well before they are prepared and delays in processing of slices or otherwise non-intact potatoes should also be minimized.

Data in the literature suggest that, as a result of unusual post-harvest conditions, SGAs from wild Solanum species may be synthesized in household potatoes containing germplasm of such species. Knowledge on the occurrence and the toxicity of SGAs in wild Solanum species and their offspring is therefore required.

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CHAPTER II

EVALUATION OF THE TOXICITY OF STEROIDAL GLYCOALKALOIDS

INTRODUCTION

The objective of this chapter is to evaluate the toxicological data on SGAs in order to assess possible consumer hazards and to derive safe levels for household potatoes. Also an assessment will be made of the acceptable levels or comparable standards proposed in the literature so far.

TOXICOLOGY OF SGAs

Toxicokinetics

Human data

Harvey et al. (1985a, 1985b and 1986) demonstrated that solanidine glycosides as well as free aglycone were present in all sera of the several hundreds of human beings (women and men) tested in the course of a number of separate experiments. Calculations based on the data presented in these studies showed that the concentrations varied from about 2-110 ng expressed as solanidine glycosides per ml serum. Harvey et al. (1985a and 1985b) found that the concentrations of these SGAs in the sera were proportionally related to the SGA contents of the potatoes, and to the amount of potatoes (which had been prepared in various ways), consumed by the individuals. The relationship between the SGA concentration in the serum and the monthly potato intake found in the male subjects was different from that found in the female subjects (Fig. 1), which was ascribed to a greater day to day variation in the size of potato portions eaten by the female subjects (Harvey et al., 1985a). Upon abstention of potatoes and potato products by two subjects, their serum SGA concentrations decreased with 35-55% after the first week and 'became minimal' after 2-3 weeks (Harvey et al., 1985a).

Claringbold et al. (1982) administered tritiated solanidine to human volunteers (two men, one woman) by intravenous (iv) injections. Such

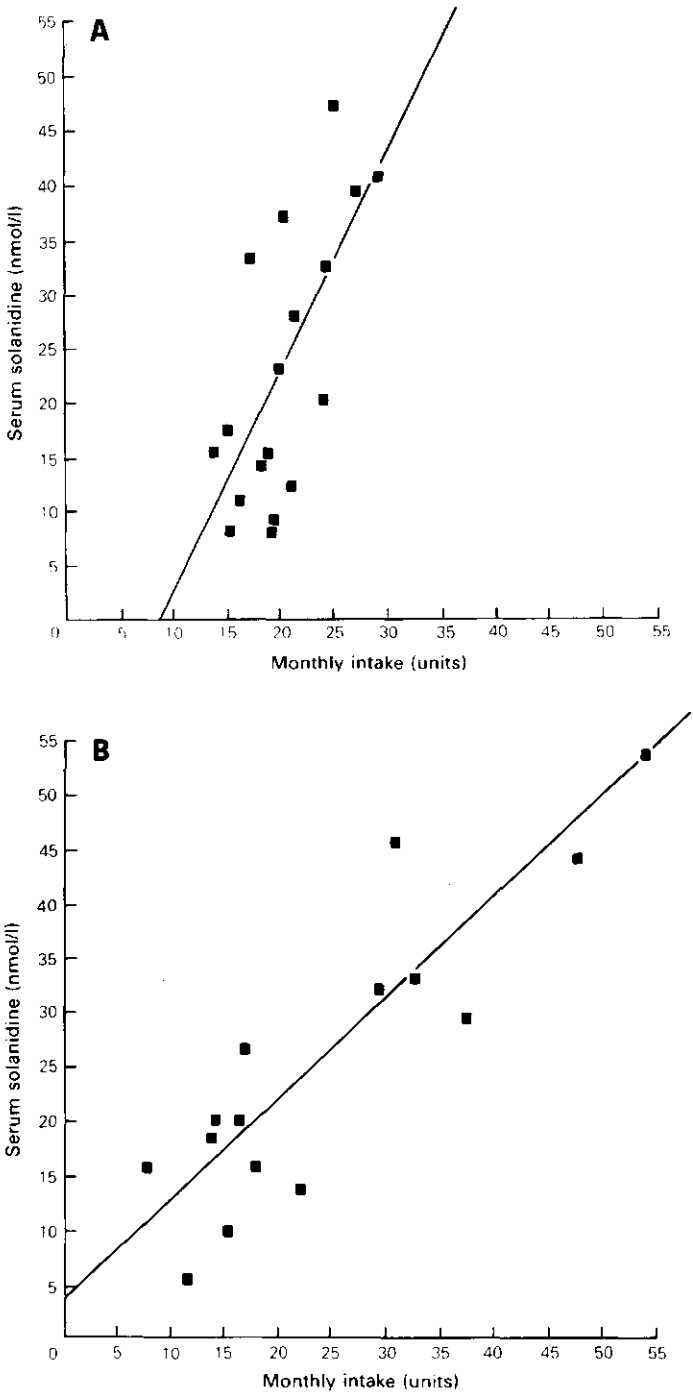

Fig. 1. Relationship between dietary potato intake and concentration of SGAs in serum of female (A) and male (B) subjects. A: n = 18; y = 2.11x - 18.36; r = 0.70. B: n = 15; y = 0.91x + 3.11; r = 0.88. After Harvey et al. (1985a).

experiments are relevant from a consumers point of view, in that they provide valuable information on the elimination of the SGAs. The excretion rates of the tritiated solanidine were low; during the first 24 h, 3-7% of label was excreted and during the following week about 2% of the dose daily. A half-time of excretion of 34-68 days was calculated. The authors also examined human post-mortem livers which were of normal histological appearance and originated from subjects who died suddenly from cardiovascular disease. They detected free solanidine as well as solanidine glycosides in total concentrations up to 106 mg solanidine (230 mg α-solanine equivalents) per kg liver (calculated from the data reported).

Claringbold et al. (1980) studied the kinetics of tritiated solasodine after iv administration to two men. The men had excreted about 16% of the labeled solasodine after 24 h and about 25% after 3 days. After 8 days, labeled compound was still present in the erythrocytes and the plasma of the humans.

These studies show that after consumption of potatoes or potato products, human beings absorb the potato SGAs from the gastrointestinal tract in amounts proportionally related to the amounts ingested. After the SGAs have entered the blood stream they are eliminated only slowly. This implies that a certain level of accumulation of SGAs may occur in the human body, which is confirmed by the relatively high SGA levels that were found in post-mortem livers. The experiments also showed that α-solanine and α-chaconine are partly hydrolysed liberating the aglycone solanidine. The long retention times of the SGAs in the human body concur with the finding of Rühl (1951) who detected 'solanine' in post-mortem urine 14 days after it had been ingested.

Experimental-animal data

Nishie et al. (1971) studied the kinetics of α-solanine in the rat. After oral administration of 5 mg of tritiated α-solanine per kg body weight (bw), this SGA was poorly absorbed from the gastrointestinal tract; 78% was excreted after 24 h and 94%, (of which 84% with the faeces and 10% with the urine) after 4 days. The highest concentrations of α-solanine were found after 12 h in spleen, kidney, liver, lung, fat, heart, brain and blood, in descending order. After intraperitoneal (ip) administration of tritiated α-solanine the excretion rate was much

lower: about 34% after 24 h at a dose range of 5-15 mg/kg bw. At a high dose of 25 mg/kg bw the excretion almost stopped, resulting in accumulation of labeled compound in liver, spleen, kidney and intestine. After oral as well as ip administration, solanidine was formed upon hydrolysis. Comparable results were obtained by Norred et al. (1976) who studied the kinetics using tritiated α-chaconine. Thus both potato SGAs, α-solanine and α-chaconine, are poorly absorbed from the gastrointestinal tract in the rat, and the patterns of distribution and excretion of these compounds are similar. Solanidine was formed also upon hydrolysis of α-chaconine.

Alozie et al. (1978) studied the kinetics of tritiated α-chaconine in hamsters. Contrasting to the rat, the hamster absorbed the labeled compound readily from the gastrointestinal tract after oral administration of 10 mg/kg bw. After 7 days only 24% was excreted, much of the labeled compound persisted in spleen, liver, lung, testes, kidney and brain, in concentrations, in descending order, which were 3-6 times as high as that in the blood. After ip administration, only 3% of the label was excreted after 7 days.

Claringbold et al. (1980) found tritiated solasodine to be still present in the livers of hamsters 22 days after (intracardial) administration.

The animal studies are generally in agreement with the findings in humans. The solanidine glycosides are (partially) absorbed from the gastrointestinal tract and once present in the blood stream these compounds are absorbed by many organs and are then only slowly eliminated. The amount of solanidine glycosides found in the blood represents only a relatively small fraction of the total amount present in the body. The SGAs and the aglycones, solanidine as well as solasodine (which result from hydrolysis of the glycosides in the body), are retained in the body for several weeks.

The experiments with rats and hamsters show that considerable differences may exist between species, in absorption and elimination of the solanidine glycosides. They further suggest that the hamster resembles man more closely with respect to these kinetics than the rat, and thus the hamster may possibly be a more adequate test model for studying the kinetics.

Toxicodynamics

Acute and subacute toxicity

Human data

Potato SGAs. In the literature case studies as well as experimental data on the acute and subacute toxicity of potato SGAs in man are available. The two sources of information will be dealt with subsequently.

In the epidemiological literature a large number of cases of potato poisoning have been described. The most extensive and careful examination of an epidemic of potato poisoning is found in McMillan and Thompson (1979). The epidemic involved 78 boys between 11 years and 13 years old that became ill after eating a lunch at school, which included potatoes. Seventeen of the boys required hospital admission; three being dangerously ill. The authors present detailed clinical findings, including a description of the symptoms of the suspected potato poisoning, which started 4-14 h after the meal. The common symptoms were gastrointestinal, such as vomiting (for 1-3 days), diarrhoea (for 2-6 days) and abdominal pain; and neurological, such as restlessness, confusion, delirium, stuporose, drowsiness or hallucination. Most of the boys had fever sometimes as high as 40-40.5°C. Other symptoms recorded were nausea, malaise, a rapid but very weak pulse (160/min), difficult but accelerated respiration (48/min), a low or unrecordable blood pressure, unconsiousness, skin lesions and disturbance of vision.

After 6-11 days of admission, the boys were fit to be discharged. Some still complained of visual disturbance, dizziness or pain on walking, but within the next few weeks all of them recovered.

Elaborate microbiological, bacteriological and virological examinations, and chemical tests for numerous organic and anorganic compounds, were carried out by experts on blood, serum, vomit, urine and faeces, as well as on kitchen equipment and samples of food recovered from the meal. It was concluded that the illnesses were due to the consumption of a relatively small number of toxic potatoes, which had been in the foodstores of the school for some months. The unpeeled potatoes contained 330 mg solanidine glycosides per kg whilst toxic concentrations of many other substances for which the potatoes were analysed were not detected. Six days after the meal, plasma cholinesterase levels of the boys were on average about 25% below normal. Ten boys had levels below

the lower limit of the normal range of children up to 14 years old, but one month later the values of all but one boy were within the normal range. As potato SGAs are inhibitors of cholinesterase (Orgell et al., 1958; Orgell, 1963; Patil et al., 1972; Bushway et al., 1987), extracts of the toxic potatoes were tested for their inhibitor activity, which appeared three times higher than the anticholinesterase activity of an extract of potatoes which did not cause symptoms of illness. From a comparison of the anticholinesterase activity of extracts of prepared but uneaten batches of the toxic potatoes with the activity of the pure SGAs, the content of solanidine glycosides in the prepared potatoes was estimated to be about 250-300 mg/kg. Finally the conclusion was drawn that "the gastrointestinal, circulatory, neurological and dermatological findings and the results of laboratory investigations were in keeping with solanine poisoning".

A fatal case of poisoning of a two year old girl that ingested (at least) one potato fruit (berry) was described in detail by Rühl (1951). Potato fruits have been shown to contain amounts of solanidine glycosides varying from 420-1080 mg/kg fresh weight (Chapter I, Table 2). Considering all evidence (the nature of the plant material ingested, the clinical symptoms observed and the course of the illness, post-mortem and histological examinations, pathological information from a similar case (Terbruggen, 1936), toxicological information in the literature, and the presence of 'solanine' in the post-mortem urine 14 days after the potato fruit was ingested), Rühl concluded that death was caused by "solanine poisoning".

A massive outbreak of poisoning in Scotland in 1917 involved 61 persons; one subject, a five year old boy, died (Harris and Cockburn, 1918). Symptoms reported were headache, vomiting, diarrhoea and debility. The onset of symptoms varied from almost immediately after the meal to 2 days after the meal; the duration of the illnesses varied from a few hours to 3 days. Investigations showed conclusively that all the persons that became ill had consumed potatoes obtained from one source, whilst those persons (often in the same households) who did not partake of the potatoes were unaffected. The toxic potatoes were normal in appearance, although quite a number of them showed sprouts about 0.6 cm in length. Analyses showed that the toxic potatoes contained 410 mg solanidine glycosides per kg, whilst normal potatoes

'considered nontoxic' showed contents of about 80 mg/kg. The authors concluded: "the outbreak of poisoning was due to the eating of potatoes containing an excessively large quantity of solanine".

Table 1 presents a historical overview of cases of poisoning in humans reported in the literature and ascribed to the consumption of tubers or other parts of the potato plant. In all the cases the symptoms of poisoning were comparable to those reported by McMillan and Thompson (1979). From these cases, it can be concluded that in general, the onset of symptoms varies from 1-2 h to about 2 days after consumption of the potatoes; the duration of the illness varies from a few hours to about 2 weeks. In most cases, circumstantial evidence justified the conclusion that the potato material was the cause of the intoxication. The epidemic ascribed to the rotten potatoes (Reelah and Keem, 1958) can have been caused by a variety of substances due to the microbial contamination and cannot be ascribed with certainty to the SGAs. In the cases of poisoning due to consumption of potato fruits, foliage and greened tubers, the SGA contents were not reported, but it is known that such tissues and also small tubers usually contain high levels of solanidine glycosides (see Chapter I, Table 2).

It should be noted in general that in many cases described in this chapter the potatoes involved in poisoning showed a normal outward appearance.

In addition to the case studies referred to above, there are some data from experiments with human volunteers (summarized in Table 2). A recent experiment involved six male volunteers who consumed potato tubers containing 320 mg solanidine glycosides per kg fresh weight. The tubers were baked and consumed including the skin. The amounts of SGAs ingested varied for different volunteers from 1.75-2.58 mg/kg bw. All persons showed symptoms of poisoning such as nauseating and severe distress, which started after about 2 h; one person showed no other symptoms; one vomited after 4 h; and four suffered from diarrhoea which started after 4 h. Symptoms tapered off after 8-10 h (Bushway, 1987).

The cases described above, clearly indicate that potato tubers, may cause poisoning in humans, due to their high levels of solanidine glycosides.

Table 1. Historical overview of reports on human poisoning according to the authors due to consumption of potato material or potato steroidal glycoalkaloids (SGAs)[1].

Reference	Location	Number of people affected	Number of fatal cases	Potato material consumed	SGA content reported[2]	Remarks[3]
Norberg (1987)	Sweden	A number	None	Normal tubers[4]	200 and >	Only few details reported.
McMillan and Thompson (1979)	England	78 Boys	None	Old tubers	330	Age between 11 and 13 years. Detailed report on clinical and laboratory findings; see text.
Wilson (1959)	England	4	None	Non-peeled baked tubers	500	Less severe case, gastrointestinal symptoms only.
Reelah and Keem (1958)	North Korea	382	22	Rotten tubers	-	Death occured either within 24 h often from cardiac arrest, or between the fifth and tenth day preceded by respiratory failure.
Rühl (1951)	Germany	1 Child	1	Potato fruit	-	Two years old girl died after 13 d; see text.
Lepper (1949)	Germany	Several	None	Tubers	325	No remarks on quality of potatoes.
Viollier (1941)	Schweiz	-	-	Tubers	230-350	
Zettel (1937)	Germany	5	None	Light exposed, sprouted tubers	-	Tubers of the same lot which were not exposed to light caused no illness.
Terbruggen (1936)	Germany	1 Child	1	Potato fruits	-	Three years old child died after 2 d.
Willimott (1933)	Cyprus	60	1	Potato foliage	-	Foliage boiled as vegetables.
Hansen (1925)	U.S.A.	7	2	Greened tubers	-	Two women 45 and 16 years old died after 6 and 8 d respectively.
Bömer and Mattis (1924)	Germany	A large number	None	Normal tubers[4]	257-583	See text.
Griebel (1923)	Germany	A large number	None reported	Normal tubers[4]	380-790	See text.
Alfa and Heyl (1923)	Germany	A large number	None reported	Normal tubers[4]	173-468	Peeled tubers.
Harris and Cockburn (1918)	Scotland	61	1	Tubers with small sprouts	410	Five years old boy died after 31 h; see text.
Rothe (1919)	Germany	14	None	Tubers with black spots	260-435	Consumption of about 500 g potatoes.

Pfuhl (1899)	Germany	56 Soldiers	None	Sprouted tubers	240-380	Peeled tubers, sprout eyes removed, doses calculated max. 300 mg SGA per person; illnessess for at least 5 d.
Schmiedeberg (1892)	Germany	357 Soldiers	None	Early tubers	-	Although fresh, the tubers were soft. Illnessess up to 10 d.
Cortial (1888)	France	101 Soldiers	None reported	Small tubers	-	Illnessess for 4-8 d.
Morris (1859)	England	1 Woman	1	Small green tubers	-	Symptoms started after 1 d; death after another 2 d.
Muncke (1845)	Germany	1 Woman	None	Early tubers	-	Early potatoes consumed for 14 d. Severe illness 2 d, diarrhoea 10 d.

[1]All the persons affected showed symptoms diagnosed as 'solanine poisoning' or 'potato poisoning' as described in the text. The conclusions of potatoes or potato SGAs being responsible for the cases of poisoning were based on circumstantial evidence.

[2]In all cases potato SGAs can be considered to be solanidine glycosides. Contents as mg/kg fresh non-peeled tuber unless otherwise stated under Remarks.

[3]Periods of time mentioned under Remarks are from onset of symptoms.

[4]Potato tubers of normal appearance. Some authors ascribed the high SGA contents to unusual climatic conditions during growth.

Table 2. Results from experiments with volunteers, showing acute oral toxicity in man of potato steroidal glycoalkaloids (SGAs) consumed with potatoes, and of SGAs isolated from potatoes.

SGA	Dose	Subject(s)	Onset/Duration of symptoms	Symptoms	Reference
Potato SGAs	1.75-2.58 mg/kg bw[1]	6 Adult males	2 h/8-10 h	Nausea, vomiting diarrhoea	Bushway (1987)
Potato SGAs	200 mg/person[1]	1 Adult male	2 h/1 d	Stomach pain, tachycarida, thin stools	Rothe (1919)
Solanine[2]	200 mg/person	- Adults	-	Headache, very weak interrupted pulse, stuporose, abdominal pain	Schroff (1873)
Solanine	370 mg/person	1 Adult male	3 h/12 h	Vomiting, dilated pupils	Fronmüller (1865)
Solanine	400 mg/person	1 Adult male	8 h/1 d	Headache, vomiting, apathy, increased but weak pulse, dyspnoea	Clarus (1857)

[1]Dose of SGAs ingested was calculated from the amount and the SGA content of the potatoes consumed.

[2]Solanine means the natural mixture of solanidine glycosides isolated from potato material.

Experimental-animal data

Potato SGAs. Symptoms of acute toxicity caused by feeding potato material or potato SGAs to animals are described in detail for the cow, sheep, horse and pig by Rühl (1951) and by König (1953) and for the rabbit, mouse, rhesus monkey and rat by Patil et al. (1972) and by Swinyard and Chaube (1973). The symptom complex is essentially the same in all species studied and can be summarized as follows. Neurological effects were prominent in all species and included apathy, drowsiness, weakness or paralysis, tachycardia, dyspnoea, asphyxia, cyanosis and unconsiousness. Dilated pupils or tachypnoea may occur. Gastrointestinal effects such as vomiting were observed in the cow, sheep, horse and pig and diarrhoea in the cow, sheep, horse and mice. In some cases (cow) exanthematic symptoms such as skin eruptions were reported. Autopsy findings included mild to severe haemorrhagic gastroenteritis, haemorrhagic damage in mouth, nose, lungs, liver, spleen, kidneys, lymph nods, adrenals and salivary glands. Other effects observed were hepatic leucocytic infiltration in the mouse (Sharma et al., 1979), accumulation of pleural and peritoneal sanguineous fluids in the rat and the rhesus monkey (Swinyard and Chaube, 1973; Chaube and Swinyard, 1976), cholinesterase inhibition in vivo in the rabbit and the dog (Patil et al., 1972) and increased body temperature up to 40.3°C in sheep (König, 1953). An extensive gross and microscopic examination of the pathologic effects of gavaging potato sprouts and potato SGAs to the hamster has been reported by Baker et al. (1987). In this experiment, 9 out of 10 hamsters receiving dried potato sprout material and 3 out of 5 hamsters receiving potato SGAs died within 72 h after dosing. At autopsy the most important effects were severe gastric and intestinal mucosal necrosis and heamorrhage. The lesions observed were indicative for exposure of the gastrointestinal epithelium to an irritant poison. The hamsters also showed congestion of the kidneys and lungs and gross and microscopic lesions of a variety of tissues. All lesions in the hamsters that were fed sprout material were similar to those in the hamsters that received extracted SGAs. The 3 hamsters that survived to the time of euthanasia showed similar but milder symptoms. Gavaging sprout material from which the SGAs had been eliminated by extraction (5 hamsters), or gavaging water (3 control animals), did not produce abnormalities. These experiments showed that the SGAs were responsible

Table 3. Acute toxicity of steroidal glycoalkaloids (SGAs) after intraperitoneal (ip) or intravenous (iv) administration to mammals.

Species	SGA	Administration	Dose (mg/kg)	Number of test animals	Effect (on)	Reference
Mice	α-Solanine	Ip	10	10	Decreased spontaneous motor activity	A: Nishie et al. (1971)
			10	10	Toxic; symptoms not specified	B: Patil et al. (1972)
			12	6	Hepatoleukocytic infiltration	C: Sharma et al. (1979)
			20	10	Increase PST[1]	As A
			30	Nr[2]	LD50 (S.E. ± 2.0 mg)	D: Nishie et al. (1975)
			32	10/dose	LD50	As B
			32	6/dose	LD50	As C
			50	10	Death after 1-3 h	As B
	a-Chaconine	ip	12	6	Heamorrhage liver, kidney	As C
			19	6-10/dose	LD50	As C
			28	Nr	LD50 (S.E. ± 1.8 mg)	As D
	a-Tomatine	Ip	34	Nr	LD50 (S.E. ± 1.3 mg)	As D
		Iv	18	54	LD50, death within 2 min	E: Wilson et al. (1961)
Rat	α-Solanine	Ip	10	3	Large increase PST[1]	F: Dalvi and Peeples (1981)
	α-Chaconine	Ip	8	12	Respiratory impairment, tensing of abdomen, sedation	G: Aldous et al. (1980)
			10	3	EEG, tachycardia	As G
			30	3	Death 1/3 within 4 h	H: Alozie et al. (1978)
			60	3	Death 2/3 within 4 h	As H
			90	3	Death 3/3 within 4 h	As H
	α-Tomatine	Iv	0.5-2	20	Respiration, blood pressure	As E

Rabbit	α-Solanine	Ip	10	1	Tachypnoea, tachycardia	As A
			15	1	Bradypnoea	As A
			20	3	Death 2/3 after 2-24 h	As B
			20	1	Death 1/1 within 24 h	As A
			30	1	Death 1/1 within 7 h	As A
			30	1	Death 1/1 within 1 h	As B
			40	1	Death 1/1 within 24 h	As D
			50	1	Death 1/1 within 24 h	As D
		Iv	1	Nr	Heart rate, blood pressure	As D
			2-3	Nr	Heart rate, blood pressure, respiration	As D
			10	1	Death within 2 min	As A
	α-Chaconine	Ip	50	3	Death 1/3 after 8-24 h	As D
			60	1	Death 1/1 after 8-24 h	As D
		Iv	1-2	Nr	Heart rate, blood pressure	As D
	α-Tomatine	Ip	50	3	No death after 24 h	As D
			100	3	Respiration, heart rate	As D
		Iv	1-2	Nr	Heart rate, blood pressure	As D
		Iv	0.5-2	7	Respiration, blood pressure	As E
Sheep	'Solanine'	Iv	17	1	Severe neurological syptoms for 2 d	I: König (1953)
			50	1	Death after 1 h	As I

[1]PST = pentobarbital sleeping time; inhibition hepatic drug-metabolizing enzyme system.
[2]Nr = not reported.

Table 4. Acute toxicity of steroidal glycoalkaloids (SGAs) after oral administration to mamals.

Species	SGA	Dose (mg/kg)	Number of test animals	Effect (on)	Reference
Mice	α-Solanine	1000	Nr[1]	No mortality after 7 d	Nishie et al. (1971)
Rat	α-Tomatine	900	5	Death 5/5 within 24 h	Wilson et al. (1961)
Sheep	'Solanine'	100	1	Tachycardia, tachypnoea, apathy, paralyzed peristalsis for 2-3 d (adult)	König (1953)
		225	1	Idem but very severe, diarrhoea (adult)	Idem
	Potato SGAs[2]	50	1	Death within 24 h (lamb 16.5 kg)	Idem
Hamster	α-Solanine	200	55	Death 3/55 (pregnant)	Renwick et al. (1984)
	α-Chaconine	165	60	Death 4/60 (pregnant)	Idem
		185	60	Death 6/60 (pregnant)	Idem
	Potato SGAs[2]	240	55	Death 28/55 (pregnant)	Idem
		330	15	Death 12/15 within 72 h	Baker et al. (1987)

[1]Nr = not reported.

[2]Solanidine glycosides isolated from, or ingested with potato material.

for the toxic effects. In sheep, rats, rabbits and rhesus monkeys, feeding of potato foliage, sprouts, greened or sprouted tubers, produced the same effects as administration of purified potato SGAs (Rühl, 1951; König, 1953; Swinyard and Chaube, 1973).

From these findings it can be concluded that the SGAs were responsible for the toxic effects caused by the potato material. The clinical and autopsy findings reported for potato SGA poisoning in animals are comparable to those reported for poisoning in human beings caused by potatoes or potato SGAs.

Acute toxic effects reported in the above-mentioned studies are included in Table 3 (parental administration) and Table 4 (oral administration).

An interesting study on the acute and subacute toxicity of potato SGAs is that of Chaube and Swinyard (1976) who studied the lethality of α-solanine and α-chaconine after ip administration to female Wistar rats (Table 5). The authors reported that the signs of toxicity were externally: nasal, oral and periorbital haemorrhage; and internally: sanguineous ascitic and pleuritic fluid. A clear dose-response (lethality) relationship for α-solanine and α-chaconine was found. The authors also reported a study on the subacute toxicity of α-solanine and α-chaconine in pregnant and nonpregnant female Wistar rats. The animals received daily 5, 10 or 20 mg α-solanine or α-chaconine per kg bw ip for 8 days, or 40 mg/kg bw ip for 2 days. The results are shown in Table 6. External symptoms were similar to those described for acute toxicity. Sanguineous ascitic fluid was recovered from all animals that died and from about half of those that survived the dosages of 20-40 mg SGA per kg bw. Histological changes associated with the treatment were limited to moderate congestion of lung, spleen, and liver. The results indicate that in the rat the subacute lethality of the SGAs is quantitatively related to the daily dose and not to the total dose; for instance two daily injections of 40 mg/kg bw (80 mg/kg bw total) were at least as effective as 8 daily doses of 20 mg/kg bw (160 mg/kg bw total). The authors also concluded that pregnant rats are more sensitive to α-chaconine than nonpregnant female rats.

Table 5. Acute toxicity of α-solanine and α-chaconine in the rat after intraperitoneal administration[a].

α-Solanine				α-Chaconine			
Dose (mg/kg)	Lethality		LD50 (mg/kg)	Dose (mg/kg)	Lethality		LD50 (mg/kg)
	Dead/total	%			Dead/total	%	
20	0/22	0	84.0	20	0/15	0	67.0
50	3/11	27[b]		40	1/9	11[b]	
75	2/6	33[c]		60	3/8	38[c]	
85	5/10	50[c]		85	6/6	100[c]	
100	4/4	100[c]					

[a]Female rats received single ip injections.

[b]Rats died 3-7 days after injection.

[c]Rats died within 24 h after injection.

After Chaube and Swinyard, (1976)

Table 6. Subacute toxicity of α-solanine and α-chaconine in the rat after intraperitoneal administration to nonpregnant-female and to pregnant animals.

Dose (mg/kg)		Lethality			
Daily	Total	α-Solanine		α-Chaconine	
		Nonpregnant Dead/total	Pregnant Dead/total	Nonpregnant Dead/total	Pregnant Dead/total
5[a]	40	0/5	0/7	0/6	0/6
10[a]	80	0/10	0/8	0/11	3/7[c]
20[a]	160	0/5	0/6	3/7[d]	2/4[c]
40[b]	80	1/6[d]	0/6	2/5[d]	4/6[c]

[a,b]Rats received 8 (a) or 2 (b) consecutive ip injections.

Although the data on the oral toxicity in experimental animals are scarce, they suggest that the sensitivity to potato SGAs of human beings corresponds to a larger extent with that of the hamster than with that of the rat or the mouse. Therefore for future experiments one should consider the hamster as an adequate model.

SGAs from wild Solanum species. Wilson et al. (1961) studied the acute toxicity of α-tomatine. A single oral dose of 900-1000 mg/kg bw to rats (5 test animals) was fatal within 24 h. Histologic examination showed an acute gastric mucosal erosion with a mild acute inflammation in the underlying submucosa. This inflammation had produced spasm of the pylorus, which in turn was considered the cause of the stomach to be distended with much fluid. After iv administration of a lethal dose in mice, death occurred within 0.5-2 min, due to a severe disturbance of the central nervous system. There was often bloody nasal discharge. Doses (iv) of 15 mg/kg bw or below were always nonfatal; a dose of 17.5 and 20 mg/kg bw produced mortality in 11 out of 17 and 7 out of 11 mice, respectively, whereas doses above 22.5 mg/kg bw were always fatal. Thus, in this experiment the maximum nonfatal dose differed only slightly from the always fatal dose.

Nishie et al. (1975) compared the acute toxicity of α-tomatine with that of α-solanine and α-chaconine (Table 3). The authors concluded that these three SGAs were similarly toxic except for the lethal doses in rabbits; that of α-tomatine was more than twice as high than those of the potato SGAs. The data on the acute toxicity of α-tomatine are tabulated in the Tables 3 and 4.

Reproduction and developmental toxicity

Human data

Potato SGAs. Studies that demonstrate effects of SGAs on reproduction in human beings have not been reported. However, potato SGAs have been associated indirectly with teratogenicity. In 1972, Renwick reported a correlation between seasonal, geographical and year-to-year variation in the incidence of anencephaly and spina bifida (ASB) in humans and the severity of blight-infestation (Phytophthora infestans) in potatoes. He guessed that a teratogenic substance in the blight-infected potatoes might be responsible for these severe birth defects, which

occur relatively frequently in Britain; the incidence varying for different regions from 2-10 per 1000 births. He suggested that the teratogen could be a potato SGA. On the basis of this rather weak evidence Renwick (1972a; 1972b) even made the suggestion that avoidance of potatoes by pregnant women would virtually eliminate these severe malformations.

A subsequent short-term avoidance trial of potatoes and potato-containing food products showed that ASB occurred in 2 out of 23 pregnancies in a potato-free group, whereas in a potato-consumers group 2 out of 56 pregnancies resulted in an ASB infant (Nevin and Merret, 1975). This report is regularly referred to in the literature as being evidence against Renwick's hypothesis, but it can only be concluded that this very limited study does not provide any evidence which can be used to throw light on this problem.

It is concluded that not any evidence of a causal relationship existing between ingestion of blighted potatoes or potato SGAs and ASB in human beings has ever been presented. The literature reports on this subject are suggestive and do not allow conclusions to be drawn.

Experimental-animal data

Potato SGAs. Renwick's hypothesis initiated much research to test whether blighted or otherwise imperfect potatoes or potato SGAs, can induce ASB in experimental animals. Many studies provided negative evidence; those that were of a good standard are summarized hereafter. Neither tuber material infested with Phytophtora infestance or with other potato pathogens, nor aged tubers showing high SGA contents, were teratogenic upon gavaging in rats, marmosets, hamsters, mice or rabbits (Poswillo et al., 1973; Chaube et al., 1973; Ruddick et al., 1974; Keeler et al., 1975). Administration of α-solanine or α-chaconine, either in pure form or in potato extracts, did not result in birth defects in the following experimental animals: rats, given daily doses up to 25 mg/kg bw orally during day 8-11 or day 6-15 of gestation (Ruddick et al., 1974) or of 40 mg/kg bw ip on day 5 and 6 or 5-20 mg/kg bw ip during day 5-12 (Chaube and Swinyard, 1976); hamsters, given single doses varying from 20-40 mg/kg bw orally on day 7 or day 8 (Keeler et al., 1978); mice, given 20 mg/kg bw ip daily during day 7-11 (Bell et al., 1976); and chick embryos, after injection into the yolk

sack of amounts up to 30 mg/kg egg (LD50 19 mg/kg egg) (Nishie et al., 1971 and 1975).

In several other papers however, it was reported that α-solanine, α-chaconine or potato preparations, some of which were derived from blighted potatoes or from potato sprouts, can produce teratogenic effects. These positive studies must be considered carefully. The few referred to hereafter do not provoke major critical comments with respect to the experimental design.

Mun et al. (1975) injected into the yolk sack of fertile chicken eggs an amount of 0.26 mg SGA/egg. The SGAs were isolated from potatoes infected with blight (Phytophthora infestance). This preparation produced 26% embryonal mortality (out of 92 embryos) and 29% abnormalities, mainly tail, trunk and rump defects. Control groups showed 1% (out of 88) and 8% (out of 125) mortality and 9% and 10% abnormalities, respectively. For an assessment of the hazard of the SGAs in potatoes, the information on the teratogenicity of the blight infected potato preparations cannot be used, because other compounds formed in the infected potatoes can be responsible for the effect as well. A 'solanine' preparation isolated from potato flowers produced 0-27% mortality and 15-25% abnormalities when doses of 0.015-0.26 mg/egg were injected into the yolk sack. These effect levels were very low compared with those reported by Nishie et al. (1971 and 1975). However, it cannot be excluded that this preparation from flowers contained SGAs not found in potato tubers. Mun et al. (1975) did not find a dose-effect relationship. In summary it can be stated that these authors showed that certain SGA preparations from potato material can induce malformations in chicken embryos, but it is not clear whether their results are of any significance for human beings.

Renwick et al. (1984) reported cranial defects and exencephaly in hamsters given orally on day 7 or 8 of gestation, either α-solanine or α-chaconine in single doses varying from 165-200 mg/kg bw, or SGAs isolated from potato sprouts in a single dose of 240 mg/kg bw. The authors also reported a dose-related teratogenicity of potato sprouts in hamsters, but upon a closer look this relationship appeared to be based on the highest dose level only. This level, which was equivalent to about 225 mg SGA per kg bw, was not a realistic dose as it produced 31% maternal lethality.

Keeler et al. (1976 and 1978) found potato sprouts, from a number of cultivars, which contained 220-400 times as much SGAs as tubers to be teratogenic in hamsters. However, the doses administered caused 11-48% maternal mortality. The terata included spina bifida and exencephaly. Potato tubers or peels were not teratogenic at doses up to four times as high as the sprout dosages (5-21% maternal lethality) (see also Keeler, 1979).

With respect to the birth defects in hamsters, it should be mentioned that a particular strain of hamsters (Simonsen) was chosen as test animal to study teratogenicity of SGAs and SAs, because this strain had proven to be sensitive to low doses of SA teratogens and to produce a high incidence of deformities (Keeler et al., 1976). The oral doses of isolated SGAs or of sprouts, which produced teratogenic effects in the hamsters in the above studies, were quite high, as was apparent from maternal mortality (see also Table 4 Renwick et al., 1984). In this respect it should be noted that recently, the WHO Expert Task Group on Updating the Principles for the Safety Assessment of Food Additives and Contaminants in Food (WHO, 1987) recommended that: "for assessing, whether the organism is more sensitive to the agent under test during its reproductive and developmental stages than during its adult phase, the highest dose of food chemical that is administered is generally the amount that would be expected to cause slight maternal toxicity". The group further stated that: "If the test substance injures reproduction or development at levels comparable with levels that cause toxicity in adults, then no special concern should be attached to the results of the reproduction/developmental toxicity studies" (WHO, 1987). Consequently, one can not conclude from the hamster trials that potato material or potato SGAs should be considered as teratogens.

Considering all evidence, it can not be excluded that very high doses of potato SGAs may produce birth defects. However, there is fair evidence from experience with known teratogens (e.g. thalidomide, vitamin A) that a 'no effect level' concept applies to these chemicals. Therefore, it does not seem likely that the potato SGAs are teratogenic at levels present in the current potato cultivars.

Most of the above studies, regardless of whether or not birth defects were induced, showed that potato SGAs and potato preparations con-

taining the SGAs adversely affected the reproduction rate in experimental animals. Especially foetal mortality and resorption, but also abortion and sometimes decreased fertility of proven breeders, were reported.

The lowest ip administered doses reported in the literature to produce effects on the reproduction of mammals are 5-20 mg/kg bw (Chaube and Swinyard, 1976; Bell et al., 1976). The relevant data from these literature reports are tabulated in Table 7.

Kline et al. (1961) reported a low oral dose of 'solanine' to produce effects on the reproduction of the rat. In this study, five groups of pregnant rats were fed ad libitum throughout pregnancy a commercial laboratory diet or this diet mixed either with ground frozen potato sprouts or with 'solanine' in concentrations of 30 or 40 mg/kg diet. Unfortunately, the food consumption was not reported. The results of this experiment (Table 8) showed that the number of pups per litter did not differ between the treatments, but that both the potato sprouts and 'solanine' caused a high incidence of neonatal mortality. Death occurred within 3 days of birth due to starvation. As the pups that died did not have milk in their stomachs, the authors presumed that the potato SGAs "may be toxic for certain functions such as lactation, possibly by virtue of an anti-hormone effect." Further information to support this presumption was not given. As the amount of 'solanine' ingested by the animals is not known, this report can not be used for an appropriate hazard assessment, but anyhow the oral dose level of 'solanine', 30 mg/kg in the diet, was low. The relatively low levels (oral and ip) of SGAs that produced effects on the reproduction in rats and mice, show that this aspect deserves further attention.

Table 7. Lowest doses of solanidine glycosides reported to produce effects on reproduction in mammals after intraperitoneal administration.

Daily doses (mg/kg)	Days adminis-tered	Solanidine glycoside	Test animal (Mother)			Effect on embryo (Number affected/total)	Level of signifi-cance	Reference
			Species	Number	Mortality			
5	8	α-Chaconine	Rat	6	0	Resorption 16%	P<0.001	Chaube and
10	8	α-Chaconine	Rat	7	3	Resorption 65%	P<0.001	Swinyard
10	8	α-Solanine	Rat	8	0	Resorption 18%	P<0.001	(1976)
20	5	Solanine[1]	Mice	20	0	Not pregnant 12/20[2]; resorption of 26-100% of foetuses in 5/8 pregnant	P<0.01	Bell et al. (1986)
20	4	Solanine	Mice	16	0	Not pregnant 7/16[2]; resorption of 26-100% of foetuses in 4/9 pregnant	P<0.05	

[1]Solanine means that either α-solanine, or the natural mixture of α-solanine and α-chaconine isolated from potato (plant) material was used.

[2]According to the authors due to abortion.

Table 8. Effect of potato sprouts and of 'solanine' on neonatal mortality in rat pups.

Test diet	Number of litters	Number of pups			Number of litters with 100% mortality
		Born	Weaned (%)		
Control (C)	11	115	95	(82.6)	1
C+10% potato sprouts	9	83	42	(50.6)	5
C+30 mg/kg solanine[1]	4	41	8	(19.5)	2
C+30 mg/kg solanine[2]	10	100	31	(31.0)	6
C+40 mg/kg solanine[2]	10	106	33	(31.1)	5

[1]Isolated from potato sprouts.

[2]Commercially obtained solanine standard.

After Kline et al. (1961).

SGAs from wild Solanum species. With respect to teratogenicity of the SGAs from wild Solanum species, only few studies have been reported. Tomatine injected into the yolk sack of chicken eggs in concentrations varying from 0.5-25 mg/kg egg weight produced a significant ($P = 0.05$) embryo mortality in chicken eggs, but it was not teratogenic (Nishie et al., 1975).

As part of a study for revealing structural requirements for teratogenicity of Veratrum and Solanum SAs, the aglycones solasodine and tomatidine were tested (Keeler et al., 1976). A single high dose of solasodine between 1180 and 1630 mg/kg bw, gavaged to hamsters at day 7 or 8 of gestation, resulted in a large increase in foetal resorption (total resorption in 21/115 pregnant dams; in two control groups 1/135 and 2/57) and a high incidence of malformations (gross birth defects in 33% of litters involving 50/727 foetuses; in two control groups 3/1400 and 2/548 foetuses), amongst which neural tube defects. Tomatidine was not teratogenic nor did it produce resorptions at a single dose of 1200 or 2700 mg/kg bw. Both these apolar aglycones produced little or no

maternal mortality, probably due to their poor absorption from the gastro-intestinal tract[1].

It can be concluded that tomatidine does probably not present a teratogenic hazard to humans. However, it can not be excluded that solasodine at high dosage levels is teratogenic. As there are uncertainties towards the occurrence of solasodine in current potato cultivars (Chapter IX), one remark should be made. The aglycone solasodine is probably poorly absorbed from the gastrointestinal tract compared to its naturally occurring (glycosidic) form, which will be much better solubilized in the stomach. Therefore, it can not be totally excluded that the solasodine glycosides are teratogenic in humans.

EVALUATION OF THE TOXICOLOGICAL LITERATURE DATA

Evidence for toxicity of solanidine glycosides in humans

The studies on the toxicokinetics showed that SGAs are absorbed by the human body in amounts proportionally related to the amounts of potatoes (or potato products) consumed. Upon regular consumption of potatoes, the solanidine glycosides as well as free aglycone may accumulate to a certain level in various organs. In many countries potatoes are an important component of the diet and they are consumed almost daily. This means that chronic exposure may occur.

Two sources of information on the toxicity of SGAs are available in the literature: firstly, case studies of poisoning in man; secondly,

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[1]In a similar study (Brown and Keeler, 1978) four synthetic solanidanes, the 22S,25R epimer of solanidine and the 22R,25S, the 22S,25S and the 22S,25R dihydro-5α forms of solanidine were tested, in doses varying from 39-343 mg/kg bw. All four solanidanes produced dose-related foetal resorption. The 22R,25S and 22S,25S solanidanes were not, but the two 22S,25R solanidanes were highly teratogenic, producing malformations at doses above 80 mg/kg bw. This is a low effect-level, considering that probably also these aglycones are poorly absorbed from the gastrointestinal tract. These data show that SAs possessing the 22S,25R configuration, including solasodine, are more likely to produce birth defects than 22R,25S SAs, such as solanidine and tomatidine. However, the structural and configurational specificity for teratogenicity among SAs and SGAs is still not clear (Keeler, 1986; Gaffield, 1986). Especially the reported teratogenicity of the potato SGAs (solanidine glycosides) cannot be explained by the current hypothesis based on the stereochemical configuration.

data from experiments, a few of which carried out with human volunteers and those involving experimental animals.

In the first source of information, many cases of acute and subacute poisoning of human beings due to consumption of sprouted, greened, small, or otherwise imperfect potatoes or of other parts of the potato plant have been described. The statement often made in the literature for over 60 years now, that qualitatively imperfect potatoes are poisonous due to contents of solanidine glycosides exceeding 200 mg/kg, is obviously based on this information. It can not be refuted that potatoes of inferior quality can cause poisoning in human beings. The literature data showed that many cases of acute poisoning have occurred after consumption of potatoes containing solanidine glycosides in amounts of 230 mg/kg or above. In several reports, the facts clearly pointed towards these SGAs as being responsible for the cases of poisoning, although the evidence was circumstancial; causal relationships have not been demonstrated. The data arguing on the possible teratogenicity of potatoes in man are suggestive and no other data on chronic adverse effects in human beings have been reported. Therefore, from the first source of information it can only be concluded that sprouted, greened, small or otherwise imperfect potatoes can cause poisoning due to excessive levels of solanidine glycosides.

The second source of information, the literature describing the experiments with human volunteers and with experimental animals, showed that purified solanidine glycosides and potatoes containing high levels of these SGAs can exert acute toxic effects. In various animal species, a causal relationship between administration of purified potato SGAs, α-solanine as well as α-chaconine, and the production of toxic effects was demonstrated. The symptom complex, the clinical as well as the autopsy findings, in human beings of so-called 'potato poisoning' or 'solanine poisoning' is essentially the same as the symptom complex produced in test animals that received purified potato SGAs or potato material containing high SGA levels. These facts support the hypothesis that the SGAs are responsible for cases of potato poisoning in man.

Acceptable level for SGAs in potatoes

Many scientists who carried out studies on contents of solanidine glycosides in potatoes (see Chapter I) generally accepted the idea that

excessive levels of these SGAs cause poisoning in human beings. Most of them refer in their papers to a so-called upper safety limit (acceptable level) for contents of solanidine glycosides in potatoes.

Different safety limits have been proposed in the literature, but the one most often referred to is that of Bömer and Mattis (1924). These authors analysed batches of potatoes involved in cases of acute and/or subacute poisoning of human beings in different parts of Germany, and batches which did not cause symptoms of illness. The toxic tubers contained levels of solanidine glycosides varying from 257-583 mg/kg (Tabel 1), whilst the nontoxic potatoes contained levels of 20-150 mg/kg. At that time, Griebel (1923) had reported that potatoes containing 380-790 mg 'solanine'/kg (Tabel 1) caused nauseating, vomiting and a strong irritation in the neck region in a large number of adults. Potatoes, which had been grown on the same site as the toxic potatoes, but which did not cause noticeable symptoms of illness contained 196 mg 'solanine'/kg. From Griebel's and their own results, Bömer and Mattis (1924) concluded: "potatoes with a solanine content exceeding 200 mg/kg seem to cause adverse effects on human health".

Since then, many authors have assumed without further evidence that levels below 200 mg/kg are safe. They ignore the fact that the 200 mg/kg level only relates to acute and/or subacute effects and not to possible chronic effects. It should also be mentioned in this context that most of the researchers concerned are not toxicologists, but chemists, geneticists and plant physiologists.

Other authors have proposed lower acceptable levels because they took additional relevant facts into consideration. Ross et al. (1978) stated that as the contents of solanidine glycosides in potatoes vary with the year and location of growth, an upper limit of 60-70 mg/kg is to be applied for cultivars to be selected for human consumption. Recently, the National Institute of Agricultural Botany (NIAB) at Cambridge (UK) recommended, that the overall amount of SGAs ingested by the public should not be allowed to rise. NIAB therefore suggested 'the average SGA content over all eight maincrop recommended potato varieties', which was calculated (over years and locations) to be 60 mg/kg, as a guideline for potato breeders to bear in mind when submitting new potato cultivars for National List testing (Parnell et al., 1984).

More recommendations on safety limits have been made in the literature, but none of them is based on an appropriate toxicological evaluation.

Morris and Lee (1984) calculated the toxic doses for potato SGAs from the cases of potato poisoning reported in the literature. According to these authors toxic doses amounted to about 2-5 mg/kg bw. Their estimate was based on the upper SGA levels reported for the toxic potatoes (cf. Table 1). They assumed the amount of potatoes consumed to be 500 g (except for one epidemic for which 250 g was taken, as the serving had been specified being small) and the body weights of the persons affected to be 80 kg for adults, 60 kg for children and 40 kg for small children. The authors stated that the estimates were designed to provide conservative values for the toxic doses, and that the SGAs could be twice as toxic as calculated.

Lepper (1949), Viollier (1941) and Alfa and Heyl (1923) reported SGA levels of toxic potatoes (Table 1) not included in the study of Morris and Lee (1984). In these cases the toxic doses, estimated by the method of Morris and Lee (1984), range from 2-3 mg/kg bw.

Pfuhl (1899), who examined a severe epidemic of 'solanine' poisoning in the German army, stated that 200-400 mg of pure 'solanine' caused symptoms of poisoning in human subjects. These amounts corresponded with his calculations of the amounts of potato SGAs ingested by the poisoned soldiers, which were at maximum 300 mg, corresponding to about 4 mg/kg bw.

In the experiments with human volunteers, amounts of 200 mg pure potato SGAs or more were reported to cause symptoms of illness in adults, which means that about 3 mg/kg bw is an acute toxic dose. The acute toxic doses of potato SGAs consumed in their natural matrix, calculated from experiments with volunteers and from cases of potato poisoning, were between 1.75 mg/kg bw and 5 mg/kg bw. The lowest dose reported to cause acute toxic effects in human beings was thus 1.75 mg/kg bw. This means that an amount of 105 mg potato SGAs ingested by an average person (60 kg) may potentially cause poisoning. This dose is ingested when 525 g of potatoes containing 200 mg solanidine glycosides per kg is consumed. This is a fairly large amount of potatoes but consumption of such amounts is not exceptional.

Although an acceptable level for solanidine glycosides in potatoes

can not be derived from data on acute toxicity, these data appear useful in showing that the so-called safety limit of 200 mg/kg, often referred to in the literature, is inadequate as virtually no margin exists between amounts of potatoes that may cause acute toxic effects and amounts that are being consumed. It must therefore be concluded that the levels of solanidine glycosides in household potatoes should remain considerably below 200 mg/kg; this applies of course also for new cultivars containing germplasm from wild Solanum species.

Literature on chronic effects in human beings is not available thus the no-adverse effect level is unknown. Consequently, an acceptable level for solanidine glycosides in household potatoes can not be derived from the data on human toxicity.

Much literature on the toxicity of the potato SGAs in experimental animals is available. However, many studies show shortcomings from a toxicological point of view and/or with respect to essential information on or the quality of the potato material or the SGA preparations that were tested. The results of such studies can therefore not be used for an adequate toxicological evaluation either.

The animal studies on the acute and subacute toxicity do not provide information on the no-adverse effect level. The data presented in the (sub)chronic study by Kline et al. suggesting that relatively low oral doses of 'solanine' may produce neonatal mortality in the rat, can not be used either because data on the 'solanine' intake are lacking. Therefore, it must unfortunately be concluded that, because of the virtual absence of chronic toxicity data, an adequate no-adverse effect level for potato SGAs can not be assessed. Thus there is no appropriate starting point for the establishment of a so-called acceptable daily intake (ADI) figure for man, and consequently, the acceptable level for solanidine glycosides in potatoes can not be derived.

Hardly any information on the toxicity of the SGAs from wild Solanum species used in potato breeding programmes is available in the literature. Consequently, appropriate ADI figures for man can not be established for these SGAs, and they must therefore not be introduced into the household potato.

In conclusion, it can be stated that the 'safety limit' of 200 mg solanidine glycosides per kg, proposed in the literature for household

potatoes as well as with respect to the registration of new cultivars, must be considered potentially acute toxic. The ingestion by the public of the amount of solanidine glycosides should not be allowed to rise but should, on the contrary, preferably be reduced. Therefore, the SGA contents of new household potato cultivars should not be higher than the average level of the current cultivars; preferably their contents should be lower. SGAs alien to S. tuberosum must not be introduced into the household potato. As certain conditions during the growth and post-harvest period of potatoes can result in a considerable accumulation of these SGAs (Chapter I), it is advisable that for the selection of new cultivars the guideline of about 60-70 mg SGA per kg potatoes -recommended by Ross et al. (1978) and by NIAB (Parnell et al., 1984)- is used in potato breeding, until an appropriate acceptable level has been assessed.

Quantitative data reported in the following literature have been consulted for the present study but were not included for reasons such as: improper experimental design of the study, lack of information on essential experimental details, questionable quality of the potato material or SGA preparations tested, recantation of the results by the authors, or insignificance of results for human beings; Azim et al. (1982), Clarke et al. (1973), El Shabrawy and Negm (1980), Fontaine et al. (1947), Gull et al. (1970), Harvey et al. (1986), König and Staffe (1953), Lorber et al. (1973), Pierro et al. (1977), Poswillo et al. (1972), Sackmann et al. (1959) and Swinyard and Chaube (1973).

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CHAPTER III*

TWO-PHASE HYDROLYSIS OF STEROIDAL GLYCOALKALOIDS FROM SOLANUM SPECIES

INTRODUCTION

Wild and cultivated primitive Solanum species are being used in potato breeding to introduce a variety of desirable characteristics into the cultivated potato, for instance: disease and insect resistances; tolerances to frost, heat and other physiological stresses; improved yield, starch content and processing quality.

The cultivar Lenape inherited from its wild ancestor S. chacoense several of the mentioned useful characteristics but in addition the potency to accumulate large amounts of SGAs (Sinden et al., 1984). This was a serious warning that the utilization of germplasm from wild Solanum species must be approached with caution (see also Chapter I). Other examples that this utilization may lead to new cultivars with high contents of SGAs have also been reported. In The Netherlands, genotypes containing germplasm from various wild Solanum species, which had been developed for industrial starch production only, showed contents of solanidine glycosides up to 630 mg/kg (Van Gelder, 1982). In Scotland many advanced breeding clones derived from S. vernei Bitt. et Wittm., which is a source of resistance against the potato cyst nematode, appeared useless due to contents of solanidine glycosides up to 1570 mg/kg (Coxon et al., 1983). As a result, potato breeders are now becoming more and more aware that introduction of alien germplasm into potato cultivars must be supported by SGA analyses. Gregory the present Scientific Director of the International Potato Center at Lima (Peru) stated: "Absence of glycoalkaloid assessment from a breeding programme can result in wasted effort, time and money" (Gregory, 1984).

Chapter II showed that due to the scarce information on the toxicity

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*This chapter is based on the paper:
Van Gelder WMJ. (1984) A new hydrolysis technique for steroid glycoalkaloids with unstable aglycones from Solanum spp. J Sci Food Agric 35:487-494.

of the SGAs from wild Solanum species, their acceptable levels for food products can not be established, and that consequently these SGAs must not be introduced into the cultivated potato. It is therefore necessary to analyse wild species for their SGA composition before they are used in a breeding programme.

A common step in the methods for SGA analysis is acid hydrolysis of the SGAs in order to cleave the sugar moieties from the aglycones. The aglycones are subsequently extracted with an organic solvent and usually quantified as 'total glycoalkaloids' by a colorimetric or titrimetric procedure (Coxon, 1984). For determining SGAs in (wild) tuber-bearing Solanum species these quantitative techniques have been employed in combination with separate qualitative analyses of the main aglycones by chromatographic techniques (Osman et al., 1978; Gregory et al., 1981). The methodology for the analysis of SGAs has recently been reviewed (Van Gelder, 1989).

However, the conventional acid hydrolysis of SGAs, applied in the studies reported until now, yields low and irreproducible recoveries, varying from 35-90%, at least for the solanidine glycosides and α-tomatine, even under optimized hydrolysis conditions (Coxon et al., 1979; Mackenzie and Gregory, 1979; Blincow et al., 1982; Davies and Blincow, 1984).

The initial aim of the present study was to investigate the hydrolysis of glycosides of solanidine, demissidine, tomatidine and solasodine in order to select a procedure giving optimum recoveries, equal for the various aglycones. As solasodine glycosides were not available, only their aglycone could be tested for its stability under the hydrolysis conditions.

The results showed that the conventional acid hydrolysis methods were not satisfactory because of degradation of aglycones. Alkaline hydrolysis was not promising either, because alkalis do not hydrolyse solanine (Henry, 1949). Therefore a new technique was developed, allowing a simultaneous quantitative recovery of the aglycones from different types of SGAs. It exploits the differences in polarity between the SGAs and their aglycones. The hydrolysis medium consists of an aqueous acid and a nonpolar organic solvent, resulting in two immiscible phases. The polar SGAs are hydrolysed in the acid phase and the nonpolar aglycones formed are continuously withdrawn from the acid

into the organic phase, in which they are protected from further disintegration. Thus the hydrolysis can proceed until the various SGAs are quantitatively cleaved. Afterwards, the aglycones can easily be recovered from the organic phase. The significance of this procedure for analytical and preparative applications is discussed.

EXPERIMENTAL

Standard alkaloids and other chemicals

Solanidine glycosides were kindly supplied by Mr P. Plieger (Proefstation voor Aardappel Verwerking-TNO, Groningen, The Netherlands), solasodine and solasodiene by Mr A.L.M. Sanders (Diosynth, Oss, The Netherlands). α-Tomatine was obtained from Roth GmbH & Co (Karlsruhe, F.R.G.), α-solanine from Sigma (St. Louis, MO, U.S.A.) and tomatidine from Fluka AG (Buchs, Switzerland). Solanidine and solanthrene were prepared by hydrolysing the solanidine glycoside mixture, and separated and purified as described in the Thin-layer chromatography paragraph. Demissine was extracted from leaves of S. demissum Lindl. by the method of Wang et al. (1972). After concentrating the extract, the pH was adjusted to 10 by adding ammonia (5 mol/l) at 70°C. Then demissine was allowed to precipitate overnight at 2°C. The precipitate was collected by centrifugation for 20 min at 11000 g, washed with 10 ml of aqueous ammonia (1 mmol/l)/methanol (1:1 v/v), and centrifuged again. The demissine precipitate was recrystallized twice from hot ethanol (96%). The purity of the preparation was checked by thin-layer chromatography (TLC), as described hereafter for SGAs. The identity of demissine was checked by comparison of its aglycone with demissidine (Roth) using TLC and mass spectrometry (MS) (Mr C.A. Landheer, Department of Organic Chemistry, Agricultural University, Wageningen, The Netherlands). Also the identity of solanidine and solanthrene was confirmed by MS. All other chemicals were analytical grade (E. Merck, Darmstadt, F.R.G.).

Plant material

Tuber samples of the potato household cultivar Bintje and of the cultivar Astarte, which is used only for industrial starch production, were obtained from the Potato Breeding Department of the Foundation for Agricultural Plant Breeding SVP (Wageningen, The Netherlands).

Hydrolysis of pure SGAs

Standard solutions were made up of, accurately weighed, 4 mg SGA or 2 mg SA per ml solvent. SGAs were dissolved in methanol/aqueous hydrochloric acid (0.25 mol/l) (1:1 v/v), and SAs in methanol/chloroform (2:1 v/v).

For conventional acid hydrolysis, 2.0 ml samples of the SGA and SA standard solutions were added to 100 ml of aqueous hydrochloric acid (Table 2). During hydrolysis, the temperature of the acid was 97°C.

For hydrolysis in a two-phase system, 1.0 ml samples of the SGA and SA solutions were pipetted into flasks, to which were added 50 ml of hydrochloric acid in the concentrations given in Table 2, and 100 ml of carbon tetrachloride. During hydrolysis, the temperature of the lower (organic) layer was 80°C and that of the upper (aqueous) layer 85°C. Hydrolyses were carried out in duplicate, using reflux condensers, for the reaction periods given in the Tables 2 and 3.

Estimation of recovery of total aglycones

For the estimation of the recovery after conventional acid hydrolysis, 4.0 ml of the hydrolysate were pipetted into a 10 ml volumetric flask. The acid was neutralized with aqueous sodium hydroxide (12 mol/l), then 2.0 ml of glacial acetic acid were added, and the flask was filled to the mark with demineralized water. To estimate total aglycones, 5.0 ml of this solution were transferred to a separatory funnel for complex formation with methyl orange in acetate buffer, as described by Birner (1969).

After hydrolysis in the two-phase system, 10.0 ml of the hydrochloric acid phase and 20.0 ml of the carbon tetrachloride phase were used to estimate the aglycone recovery. The former phase was adjusted to pH 10 with aqueous sodium hydroxide (12 mol/l) and extracted with 10 ml of chloroform. The extract was combined with the 20 ml carbon tetrachloride phase and the whole washed with 20 ml of aqueous ammonia (1 mmol/l). The organic liquid was evaporated to dryness and the aglycones were dissolved in 10.0 ml of aqueous acetic acid (50% v/v). Then 2.0 ml of this solution and 3.0 ml of demineralized water were transferred to a separatory funnel to estimate the aglycones by complex formation as above.

Table 1. Colour development of steroidal alkaloids after TLC on Whatman LK5D plates and spraying with a saturated solution of cerium(IV) sulphate in concentrated sulphuric acid. Colours were observed before and after heating at 120°C, in daylight and under UV light of 366 nm.

Steroidal alkaloid	R_F	Colour development					
		Room temperature		After 3 min 120°C		After 3 + 10 min 120°C	
		Daylight	UV light	Daylight	UV light	Daylight	UV light
Solanthrene	0.73	Brown	Dark brown	Blue/grey	Dark blue	Grey	Grey
Solanidine	0.51	Brown/red	Pink/brown	Purple	Blue	Grey	Grey
Demissidine	0.51	Colourless	Colourless	Light yellow	Light blue (fluorescent)	-	Light blue (fluorescent)
Solasodiene	0.64	Brown	Brown	Dark blue/ grey	White (fluorescent)	Grey/green	White (fluorescent)
Solasodine	0.36	Brown	Brown	Blue	Grey (slightly fluorescent)	Grey	Grey
Tomatidine	0.40	Blue/green	Violet (slightly fluorescent)	Blue/brown	Blue/grey (slightly fluorescent)	Grey	Grey/green

Thin-layer chromatography

For TLC, the aglycones were recovered from the hydrochloric acid phase and the carbon tetrachloride phase as described above. After evaporation of the organic solvent, they were dissolved in 100 μl methanol/chloroform (2:1 v/v) and 2 μl of this solution were applied to a Whatman TLC plate LK5D 20 x 20 cm, together with 10 μg of standard alkaloids. After equilibration of the TLC chamber for 30 min with 100 ml cyclohexane/ethyl acetate (55:45 v/v), the plate was developed to a height of 14 cm. The SAs were detected by spraying the chromatogram with a solution of iodine in carbon tetrachloride (1 g/100 ml). For preparative TLC, 1 mg samples of the SAs were applied to Whatman PLK plates. After the brown/yellow colour of the iodine-sprayed SAs had disappeared, the SAs were recovered unchanged (Schreiber et al., 1963) by elution with methanol/chloroform (2:1 v/v). Identification of the SAs was performed by comparison of their R_F values and their colour development with those of standard compounds. For this colour development the TLC plates were sprayed with a saturated solution of cerium(IV) sulphate in concentrated sulphuric acid. Subsequently, observations of the coloured SAs were made at room temperature, after heating the plates in an oven at 120°C for 3 min, and after heating the plates a second time for 10 min. The R_F values and the colour development of the SAs are presented in Table 1.

TLC was also used in preliminary experiments carried out to study the partitioning of α-solanine and its hydrolysis products over the two-phase systems hydrochloric acid and carbon tetrachloride, hydrochloric acid and chloroform, and hydrochloric acid and n-hexane. The aglycones were recovered as described above and the SGA was recovered from both separated phases of each of the systems as follows. After washing with aqueous ammonia (1 mmol/l), the organic phase was evaporated to dryness and the residue dissolved in 50 μl of pyridine. A 10 μl sample was applied to a Whatman LK5D plate for TLC. The hydrochloric acid phase was made alkaline (pH 10), kept at 70°C for 30 min and stored overnight at 2°C. α-Solanine was precipitated by centrifugation for 20 min at 11000 g, washed with 10 ml of aqueous ammonia (1 mmol/l) and collected by centrifugation again. Subsequently it was dissolved in a minimum of pyridine, and samples containing 10-20 μg of the SGA were then applied to a LK5D plate which was developed to a height of 14 cm

using methanol/chloroform/water (5:5:1 v/v) as eluent (Sachse and Bachmann, 1969). Visualization of the spots was as described for the aglycones.

Hydrolysis of SGAs in potato tuber extracts

Fresh potato tubers of 'Bintje' and 'Astarte', with a low and high content of solanidine glycosides respectively, were used. From samples of ten tubers of each, longitudinal sections were cut, to obtain subsamples of 300 g. The subsamples, which were representative of each tuber in the sample as was tested earlier, among others in an interlaboratory study (Morgan et al., 1985), were homogenized with 75 ml of water in a Waring Blendor. While homogenizing, nine samples each of 25 g of brei (corresponding to 20 g of fresh tubers) were taken for analysis, and to each sample 100 ml methanol/chloroform (2:1 v/v) were added (immediately) in order to prevent enzymic action. For extraction of the glycosides, a modification of the extraction procedure of Wang et al. (1972) was used. The suspension of tuber brei in methanol/chloroform was transferred to a Waring Blendor using 25 ml of the methanol/chloroform for rinsing the glasswork. The suspension was homogenized for 2 min and then filtered through a Buchner funnel fitted with filter paper (Type 589/5, Schleicher and Schüll, Dassel, F.R.G.). The remaining pulp and the filter were re-extracted for 2 min using 75 ml of the methanol/chloroform mixture. The combined filtrates were transferred to a separatory funnel, and 80 ml of a sodium sulphate solution (55 mmol Na_2SO_4 per l) were added. The mixture was gently shaken and then left overnight to allow complete separation of the layers. The chloroform (lower) layer was discarded and 15 ml of the sodium sulphate solution were added to the remaining liquid. After gently shaking, this mixture was left for 4 h and then the newly formed chloroform layer was discarded. The remaining (aqueous) methanolic layer was used for analysis. Allowing the solvent layers to separate for a sufficiently long period of time is essential.

To study the influence of tuber extracts on the recovery of the aglycone solanidine after the two-phase hydrolysis, 2 mg accurately weighed solanidine glycosides dissolved in 1.0 ml methanol/chloroform (2:1 v/v) were added as a spike to the methanolic layers of three of the nine brei samples of each cultivar.

The methanolic layer was concentrated to near dryness in a water bath at 50°C and the residue transferred to an Erlenmeyer flask using 50 ml of aqueous hydrochloric acid. The conditions for conventional hydrolysis were: hydrochloric acid 1 mol/l, reaction period 2 h; and for the two-phase hydrolysis: hydrochloric acid 2 mol/l to which 100 ml of carbon tetrachloride were added, reaction period 2.5 h.

After conventional acid hydrolysis, 5.0 ml of the hydrolysate were transferred to a 10 ml volumetric flask and processed further as described for estimation of the recovery of total aglycones.

After the two-phase hydrolysis, the system was allowed to cool and the pH of the aqueous phase was adjusted to 10 with ammonia (7 mol/l). After separation of the layers, the aqueous phase was washed with 20 ml of carbon tetrachloride and this carbon tetrachloride layer was added to the 100 ml carbon tetrachloride phase; then this total amount was washed with 25 ml of aqueous ammonia (1 mmol/l). From the total volume of carbon tetrachloride, amounts of 12.0 ml and 6.0 ml, for the 'Bintje' and the 'Astarte' samples respectively, were evaporated to dryness. The aglycones were redissolved in 5.0 ml of aqueous acetic acid (20% v/v), and these solutions were transferred to a separatory funnel to estimate the SAs as described above.

RESULTS AND DISCUSSION

Hydrolysis of pure SGAs

Table 2 shows that the acid hydrolysis of solanidine glycosides resulted in varying losses up to 59%. Cleavage of these SGAs yields the aglycone solanidine and the sugar moieties solatriose and chacotriose (Chapter I, Fig. 1 and Table 1). Monitoring by TLC of a mild and a vigorous hydrolysis of α-solanine showed that solanidine was converted to solanthrene. This dehydration product was very unstable and disappeared with concomitant formation of 2-12 degradation products. When solanidine was subjected to the hydrolysis conditions, the solanthrene formed was disintegrated even more than during the hydrolysis of α-solanine. Probably, the presence of the sugar moiety retards the formation and through that the disintegration of solanthrene, and thus influences the recovery of aglycones.

Table 2. Recovery of aglycones, including dehydrated derivatives, upon conventional acid hydrolysis of steroidal glycoalkaloids, and recovery of steroidal alkaloids subjected to the same conditions[1].

Steroidal (glyco)alkaloid	Reaction period (h)	Concentration of HCL (mol/l)	Recovery (%)
Solanidine	2	0.5	77
glycosides	1	1	83
	1	2	78
	2	2	78
	4	2	73
	1	4	64
	2	6	41
Solanidine	2	2	55
	3	2	37
	4	2	16
Solasodine	2	2	97
	3	2	98
	4	2	92
Demissine	2	2	102
	3	2	98
	4	2	99
	2	6	90
	4	6	93
α-Tomatine	2	2	82
	3	2	90
	4	2	86
	2	6	0
	4	6	0

[1]SGAs, 8 mg; SAs, 4 mg; aqueous hydrochloric acid, 100 ml at 97°C.

As solasodine glycosides (solamargine, solasonine) were not available, experiments were carried out with the aglycone solasodine. Solasodine plus its dehydration product solasodiene could be recovered almost quantitatively after a period of 2-3 h under rather mild hydrolysis conditions. These data suggest that the hydrolysis of the solasodine glycosides may proceed without significant losses, because both aglycones can be quantified together.

Hydrolysis of demissine yields the aglycone demissidine; this SA was stable even after a prolonged hydrolysis period under vigorous conditions. About 90% of tomatidine, the aglycone resulting from the cleavage of α-tomatine, could be recovered after employing rather mild hydrolysis conditions, but this SA was totally disintegrated when a higher acid concentration was used. Preliminary tests using TLC indicated that differences existed between demissine and α-tomatine, as to reaction rate of the glycosidic cleavage. However, these SGAs possess the same tetrasaccharide moiety, lycotetraose (Chapter I, Table 1). Thus the difference between these SGAs with respect to hydrolysis seems to be due to differences in the chemical structures of the aglycones. This, together with the demonstrated influence of the sugar moiety on the stability of solanidine, suggests that hydrolysis of the SGAs is influenced by the chemical structures on both sides of the glycosidic bond.

After the studies described here were carried out, Davies and Blincow (1984) reported that losses of α-solanine upon acid hydrolysis varied largely, when in different laboratories the same method was applied for assessing the content of solanidine glycosides in potatoes. The losses depended on the concentration of solanidine glycosides and on the presence or absence of potato tuber extract in the hydrolysis medium. Those results and the results reported in this chapter, show that conventional acid hydrolysis is not suitable for the hydrolysis of mixtures of SGAs.

Table 3 shows the aglycone recoveries after hydrolysis of glycosidic-bound solanidine and tomatidine in a two-phase system of carbon tetrachloride and aqueous hydrochloric acid as well as the recovery of solasodine after being subjected to these hydrolysis conditions. Compared to conventional hydrolysis, recovery of the unstable aglycones was little affected by the reaction period and the acid concentration.

Table 3. Recovery of aglycones, including dehydrated derivatives, upon hydrolysis of steroidal glycoalkaloids using a two-phase system consisting of carbon tetrachloride and hydrochloric acid, and recovery of solasodine subjected to the same conditions[1].

Steroidal (glyco)alkaloid	Reaction period (h)	Concentration of HCL (mol/l)	Recovery (%)
Solanidine	1.5	2	70
glycosides	2	2	95
	2.5	2	96
	4	2	93
	1.5	4	95
	2	4	90
	2.5	4	92
α-Tomatine	2	2	94
	3	2	96
	4	2	92
Solasodine	1.5	4	103
	2	4	97
	2.5	4	98

[1]SGAs, 4 mg; solasodine, 2 mg; hydrochloric acid, 50 ml at 85°C; carbon tetrachloride, 100 ml.

As was to be expected, recovery of the stable SA solasodine was also very high. The low recovery of solanidine after 90 min in the two-phase system (hydrochloric acid concentration 2 mol/l) was due to incomplete hydrolysis and not to aglycone degradation. The optimum conditions for the two-phase hydrolysis of the solanidine glycosides and α-tomatine were a reaction period of 2.5-3 h and an acid concentration of 2 mol/l, giving a recovery of about 96%. The lower reaction temperature (85°C) compared to the conventional procedure (97°C) may also have had some favourable influence on the recoveries.

The high recoveries were largely explained using TLC experiments. α-

Solanine still yielded (via solanidine) solanthrene, but this compound did not disintegrate. α-Solanine and the major part of the solanidine remained in the aqueous acid phase, but the unstable solanthrene all remained in the protective organic phase. In experiments with chloroform instead of carbon tetrachloride, TLC showed that α-solanine still remained in the acid phase, but that solanidine almost quantitatively remained in the protective chloroform phase, resulting in much less dehydration to solanthrene. Using a two-phase system of n-hexane and aqueous hydrochloric acid (2 mol/l), α-solanine, solanidine and the major part of the solanthrene were found in the acid phase. This resulted in a very low recovery because of disintegration of the aglycones.

Hydrolysis of SGAs in potato tuber extracts

Table 4 shows that the precision (coefficient of variation) and the yield in the determination of solanidine glycosides in tubers of the household potato cultivar Bintje and the industrial starch cultivar Astarte, improved considerably by using the two-phase hydrolysis technique. Again low coefficients of variation were obtained; the average recoveries of the theoretical amounts of solanidine glycosides were about 95% and 101% for 'Bintje' and 'Astarte', respectively. These values are higher than those reported by other researchers who accepted recoveries of 70% and coefficients of variation of 15% (Coxon et al., 1979; Coxon, 1984; Davies and Blincow, 1984). In many other papers on this topic, data on accuracy and precision of the methods applied are worse (see Coxon, 1984) or not even presented.

The recoveries in Table 5 compare well with the optimum values in Table 3. This means that the solanidine glycosides can be converted to their aglycones reproducibly and sufficiently quantitatively, by two-phase hydrolysis of pure SGAs as well as of SGAs in potato tuber extracts. A probable explanation is that the nonpolar compounds in the potato tuber extract that can potentially interfere with the aglycones during hydrolysis are discarded with the chloroform layer after the extraction step with the methanol/chloroform mixture. The polar compounds which can potentially interfere with the aglycones are separated from the aglycones liberated during two-phase hydrolysis, as a result of the differences in polarity.

Table 4. Comparison of conventional acid hydrolysis[1] (CAH) and two-phase hydrolysis[2] (TPH) in the determination of solanidine glycosides in tubers of two potato cultivars.

Cultivar	Hydrolysis technique	Solanidine glycosides in fresh tuber			Yield CAH / Yield TPH (%)
		Absolute (mg/20 g)	Average (mg/20 g)	Coefficient of variation (%)	
Bintje	CAH	0.56	0.61	25.61	71.7
		0.48			
		0.78			
	TPH	0.88	0.85	11.16	
		0.74			
		0.92			
Astarte	CAH	3.66	3.83	10.38	83.7
		4.28			
		3.54			
	TPH	4.48	4.57	2.49	
		4.70			
		4.54			

[1]CAH conditions: 50 ml hydrochloric acid 1 mol/l, reaction period 2 h, reaction temperature 97°C.

[2]TPH conditions: 50 ml hydrochloric acid 2 mol/l, 100 ml carbon tetrachloride as protective phase, reaction period 2.5 h, reaction temperature 85°C.

The procedure used for extraction of SGAs from potato tubers as introduced by Wang et al. (1972) has been criticized by some authors (Mackenzie and Gregory, 1979; Speroni and Pell, 1980), but proved to be most satisfactory when applied in the present as well as in other studies (Clement and Verbist, 1980; Morgan et al., 1985). The present study showed that reproducible and quantitative results can be obtained by this extraction procedure, provided that the tuber homogenate is extracted twice and the solvent layers of the chloroform/methanol/sodium

Table 5. Recovery of solanidine, expressed as glycosides upon two-phase hydrolysis[1] of tuber extracts of two potato cultivars spiked with solanidine glycosides.

Cultivar	Solanidine glycosides in sample			Solanidine glycosides determined			
	Determined in fresh tuber (mg/20 g)[2]	Added (mg)	Theoretical amount (mg)	Absolute (mg)	Relative (%)	Average (%)	Coefficient of variation (%)
Bintje	0.85	2.0	2.85	2.73	95.8	95.4	4.25
	0.85	2.0	2.85	2.83	99.3		
	0.85	2.0	2.85	2.60	91.2		
Astarte	4.57	2.0	6.57	6.67	101.5	100.6	2.79
	4.57	2.0	6.57	6.39	97.5		
	4.57	2.0	6.57	6.76	102.9		

[1]Conditions of two-phase hydrolysis as in Table 4.

[2]See Table 4.

sulphate mixture are allowed to separate for the periods of time described in the Experimental section.

Although the data presented in the Tables 4 and 5 can have been affected by a number of analytical errors, such as inhomogeneity of the tuber brei, weighing errors, losses due to extraction, hydrolysis, clean-up and quantification of the solanidine glycosides, the overall error of the analytical procedure now presented is rather small, especially in comparison with those of the reports mentioned before.

To obtain optimum results with the two-phase system, the unstable aglycones must almost totally remain in the organic phase, and to limit the duration of hydrolysis, the substrate has to remain largely in the acid phase. Variation in liquid phases, especially by combining different organic solvents, enables one to obtain an optimum partition coefficient for almost any compound. So this technique may be applicable to other chemical procedures where a hydrolysis step leads to side reactions or degradation. For instance, the estimation of cardiac glycosides, which is disturbed by side reactions during the hydrolysis necessary for genin formation (Meilink, 1980), may be improved by application of a two-phase system. The production of solasodine from Solanum spp. for the pharmaceutical industry is attended by a considerable loss, resulting from the formation of solasodiene, which can not be used for steroid hormone synthesis (Mann, 1978). This dehydration is analogous to the solanthrene formation, which was shown in this study to be controllable. Therefore, solasodine dehydration may be prevented by using a two-phase hydrolysis with a carefully selected organic phase.

In conclusion it can be stated that the two-phase hydrolysis technique can succesfully be applied to different SGAs, and that it offers prospects for developing a comprehensive and quantitative method for determining the SGA compositions of Solanum species and their hybrid offspring. The procedures for sample preparation and clean-up seem useful too, but quantitation, separation and identification of SGAs are rather complicated and time-consuming. Application of more advanced chromatographic techniques will perhaps be more efficient.

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CHAPTER IV*

CAPILLARY GAS CHROMATOGRAPHY OF STEROIDAL ALKALOIDS

INTRODUCTION

The methodology for SGA analysis is very complex, which is illustrated by the hundreds of publications describing the development or modification of methods for SGA analysis, of which more than 60 appeared during the past decade only, and which may also be apparent from the structures of these compounds (Chapter I, Fig 1 and Table 1). Many of these methods have been developed for determining the contents of solanidine glycosides in potatoes. Such methods, which do not apply to SGAs alien to S. tuberosum, have been used for determining the total SGA contents of wild Solanum species and their offspring (Coxon et al., 1983; Grassert and Lellbach, 1987). This may lead to underestimation of these contents, and possibly the introduction of undesired types of SGAs into new potato cultivars will be overlooked. The aim of the study presented in this chapter was to develop a method for the separation and individual quantification of the SGAs of wild Solanum species. Several authors recently stated that there is an urgent need for such a method which must be at the same time comprehensive, quantitative, and efficient enough, for routine incorporation into breeding programmes (Gregory, 1984; Sinden et al., 1984; Tingey, 1984).

SELECTION OF CHROMATOGRAPHIC TECHNIQUE

Techniques that can be applied for analysis of SGA compositions are TLC, high-pressure liquid chromatography (HPLC), gas chromatography

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*This chapter is based on the papers:
Van Gelder WMJ. (1985) Determination of the total C_{27}-steroidal alkaloid composition of Solanum species by high-resolution gas chromatography. J Chromatogr 331:285-293.
Van Gelder WMJ, Scheffer JJC. (199*) Strategy for analysis of steroidal glycoalkaloids in potato and tomato genotypes containing wild Solanum and Lycopersicon germplasm, in preparation.

(GC) using packed columns, and capillary GC. In this section the applicability of these techniques for analysis of the SGAs is evaluated in order to select the most promising technique(s) for further study.

TLC is especially valuable for qualitative analyses of SAs and SGAs. Many TLC systems for the separation and detection of these compounds have been developed, and are reviewed by Baerheim Svendsen and Verpoorte (1983). Sensitive reagents for visualization of SAs and SGAs are also available (Jellema et al., 1980) and quantification by densitometry, although generally quite difficult, is feasible (Ahmed and Müller, 1978; Caddle et al., 1978; Jellema et al., 1981 and 1982). A disadvantage of TLC is that for the separation of complex mixtures of SAs or SGAs, different systems have to be applied in succession.

In the past decade, TLC analysis of SAs and SGAs has been superseded by HPLC and GC, because the resolution of these instrumental techniques improved greatly and they can be automated for sample injection and quantification. Moreover, microprocessor-controlled programming allows variables such as column temperature, flow rate, mobile phase, or detector sensitivity, to be changed automatically during analyses. HPLC and GC are complementary techniques. For separation of thermolabile, strongly polar, high-boiling or non volatile compounds, HPLC is often preferable. An advantage of GC is the availability of highly sensitive and generally applicable detectors.

HPLC and GC on packed columns can be applied for the separation and individual quantification of SGAs and their aglycones when only a single SGA is present in plant material, or when only a single aglycone is present glycosidically bound to different sugar moieties such as solanidine in α-solanine and α-chaconine. HPLC methods for quantification of α-solanine and of α-chaconine in extracts of potato tubers, have been described (Morris and Lee, 1981; Bushway et al., 1986), and such methods specifically developed for the solasodine glycosides in Solanum species of potential use to the pharmaceutical industry, are available too (Crabbe and Fryer, 1980; Eldridge and Hockridge, 1983; Cham and Wilson, 1987). However, in many Solanum species, several SAs may occur simultaneously, and each of them may be bound to various sugar moieties (Chapter I); then many SGAs occur in a single species and the separation efficiency of HPLC will be insufficient. A GC method for the separation of SGAs on a 3% OV-1 column programmed up to 350°C

has been described (Herb et al., 1975), which could potentially be applied to mixtures of SGAs such as those occurring in wild Solanum species (Osman et al., 1979). However, this method suffered from several disadvantages. The samples had to be derivatized by permethylation, which is difficult and laborious, and a chromatographic run lasted for almost two hours. Only relative quantification was permitted, thus a separate analysis for the total SGA content was required. Moreover, due to the high GC operation temperature, column deterioration occurred already after 100 analyses (Herb et al., 1975; Osman et al., 1978; Gregory et al., 1981).

An alternative approach consists of extraction and two-phase hydrolysis of the SGAs, separation and individual quantification of the aglycones, and expression of their contents as glycosidic-bound SAs.

For the separation of aglycones, HPLC is not preferable as two time-consuming analyses, one for the more polar and one for the less polar SAs were required (Hunter et al., 1980). Moreover, although HPLC was not applied quantitatively by Hunter et al. (1980), their data revealed that the ultraviolet-detector responses varied largely for the different SAs. This limits the possibilities for quantification of the SAs by this technique. In general, the ultraviolet and refractive-index detectors used in HPLC are insensitive to saturated SAs, whereas flame ionisation detection (FID) used in GC is very sensitive and generally applicable to SAs and SGAs.

Quantification by FID of solanidine injected directly, i.e. underivatized, into the gas chromatograph has been reported (Osman and Sinden, 1977; Coxon et al., 1979; King, 1980). However, the Δ5- and 5α-aglycone pair solanidine and demissidine could not be separated. For the quantification of these aglycones, a separate analysis had to be carried out in which solanidine was converted into solanthrene and demissidine remained unchanged.

A recently developed GC technique which is potentially useful to analysis of SAs, is capillary GC, also called high-resolution GC (HRGC). This technique enables efficient separations of complex mixtures in relatively short periods of time, because open tubes with an internal diameter (I.D.) of 0.1-0.3 mm are used in which the stationary phase is directly coated as a thin film, usually 0.1-0.4 μm, onto the wall of the column; as a result a low carrier gas pressure can be

applied enabling the use of very long columns (10-100 m). With increasing length and decreasing I.D. of the column, the separation efficiency increases proportionally. However, these improvements are attended by a proportionate increase in the complexity of the technique and of the number of problems which can be encountered. Therefore, capillary GC demands a much more careful approach than GC on packed columns.

The literature referred to above as well as preliminary experiments showed that with respect to detection and quantification, GC of aglycones liberated during two-phase hydrolysis of SGAs was the most promising approach, and that with respect to the separation efficiency, capillary GC offered prospects for developing a method for determination of the SGA composition of Solanum species. Therefore GC analyses of SAs using packed as well as capillary columns were compared and the most promising technique was further optimized.

EXPERIMENTAL

Apparatus

A Packard 439 microprocessor-controlled gas chromatograph was used, equipped with two flame ionization detectors and two injectors for splitless and split sampling techniques. One injector was used for a glass column (1 m x 2 mm I.D.) packed with 10% SE-30 on Chromosorb W HP (80-100 mesh) and the second one for fused-silica columns. The fused-silica columns of 50 m x 0.22 mm I.D. were coated with CP-Sil 5, film thickness 0.12 μm, or CP-Sil 19 CB, film thickness 0.19 μm. All columns were obtained from Chrompack Nederland (Middelburg, The Netherlands). The maximum operating temperatures of the SE-30 and CP-Sil 5 columns were 325°C (isothermal) and 350°C (programmed), and of the CP-Sil 19 CB column, 300°C and 325°C, respectively. Other conditions were: injector, 325°C; detector, 350°C; flow-rates for FID, hydrogen 25 ml/min, air 250 ml/min; detector sensitivity, 1 pA/mV, unless stated otherwise. The injection volumes and splitting ratios are given either in the text or in the captions of tables and figures. Chromatographic data were recorded and calculated using a Shimadzu C-R2A data processor.

Standard compounds and other chemicals

5α-Cholestane was obtained from Sigma and toluene (analytical grade)

from E. Merck. All other chemicals were as described in Chapter III. Standards were dissolved in methanol/toluene (1:1 v/v).

Plant material

Tuber samples from household potato cultivars, cultivars for industrial starch production, progenies from crosses between Solanum tuberosum L. and S. vernei Bitt. et Wittm. and accessions of the primitive cultivated species S. phureja Juz. et Buk. were studied. They were obtained from the Potato Breeding Department of the Foundation for Agricultural Plant Breeding SVP.

Quantification of solanidine glycosides

Potato tubers were sampled and homogenized, and the SGAs extracted as described in Chapter III. The aqueous methanolic layer obtained was evaporated to near dryness.

For the quantifications by capillary GC the concentrated extract was hydrolysed using the two-phase technique. The hydrolysis conditions were: 50 ml of hydrochloric acid (2 mol/l) and 100 ml of carbon tetrachloride; reaction period 3 h. After hydrolysis, the phases were alkalinized and the carbon tetrachloride phase containing the aglycones was separated, and the aqueous phase washed with 25 ml of chloroform and then discarded. The combined organic phases were washed with 25 ml of ammonia (1 mmol/l) and evaporated to near dryness. Subsequently, the aglycones were transferred quantitatively to crimp top vials and after evaporation of the solvent, they were redissolved in 1.0 ml methanol/toluene (1:1 v/v) or diluted further as required. The additional capillary GC conditions were as follows: carrier gas, helium, linear velocity 24.3 cm/s; oven temperature, 280°C; injection volume, 2 μl; splitting ratio, 1:100. For quantification of solanidine and its dehydration product solanthrene, their (combined) peak areas were compared with those of a calibration line that was constructed using amounts of 0.1, 0.5, 1.0, 2.0 and 3.0 mg solanidine in 1.0 ml methanol/toluene (1:1 v/v). 5α-Cholestane was used as internal standard. The concentration of an SGA was calculated from the concentration of the corresponding aglycone (including its dehydration product) by multiplying the latter concentration by the ratio of the molecular masses of the SGA and its aglycone.

For quantification of solanidine glycosides by a colorimetric method, the concentrated aqueous methanolic layer was transferred to a 30 ml centrifuge tube and the beaker was rinsed twice with 5 ml of acetic acid (0.8 mol/l). The solution was brought to pH 10 with about 3 ml of ammonia (7 mol/l) and heated to 70°C. The solanidine glycosides were left overnight at 2°C and were then collected by centrifugation for 15 min at 11000 g. The dried precipitates were redissolved in 1 ml of phosphoric acid (0.5 mol/l), and subsequently 10 ml freshly prepared Clarke reagent, containing 300 mg paraformaldehyde in phosphoric acid (8.5 mol/l), were added. After 30 min, the absorbances were measured at 600 nm. The concentrations of the solanidine glycosides were calculated using a calibration line that was constructed using amounts of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg of α-solanine in 1.0 ml of phosphoric acid (0.5 mol/l). Depending on the colour intensities of the solutions, absorbances were measured using spectrophotometer cells of 5.0 mm or 10.0 mm path length. An inter-laboratory study (Morgan et al., 1985), in which this colorimetric method was compared with two other methods for quantification of solanidine glycosides in potato tubers, showed that the colorimetric method used here was sufficiently accurate and precise in order to be used as a reference method.

RESULTS AND DISCUSSION

GC analysis of SAs using packed and capillary columns

In studies on the GC separation of SAs using packed columns, attempts to separate the Δ5- and 5α-aglycone pair solanidine and demissidine were not successful, although various column types and GC conditions were applied (King, 1980). The closely related chemical structures of Δ5- and 5α-aglycone pairs (Chapter I, Fig. 1) result in only minute differences between their boiling points. Therefore, separation of these compounds will be very difficult. Fig. 1A shows a gas chromatogram obtained for a mixture of SA standards, the solanidanes solanidine and demissidine, and the spirosolanes tomatidine and solasodiene, using a 1 m SE-30 column. Calculations using data from the individually chromatographed SAs gave plate numbers, N, for these compounds varying from 1877 to 2656 plates/m. The plate number required for the separation of solanidine, demissidine and solasodiene with resolution

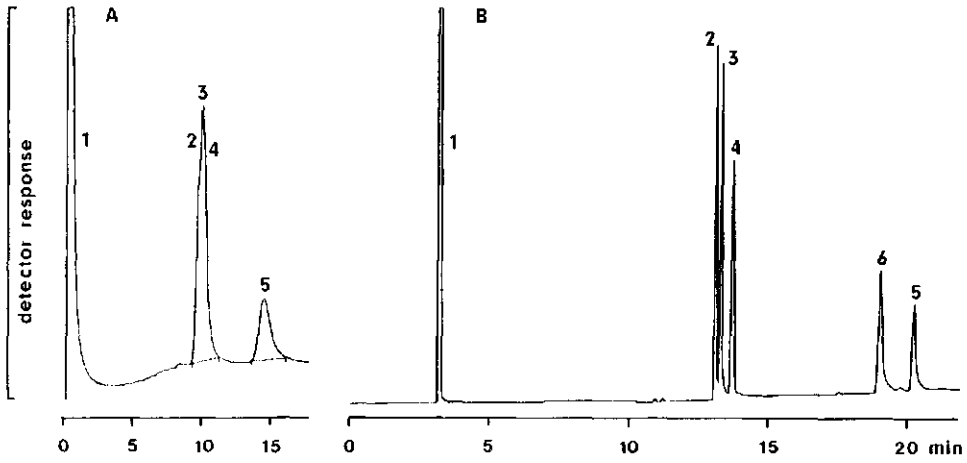

Fig. 1. Gas chromatograms obtained for steroidal alkaloids using packed (A) and capillary (B) columns. Packed column: glass, 1 m x 2 mm I.D., 10% SE-30 on Chromosorb W HP (80-100 mesh). Carrier gas: nitrogen, flow-rate 15 ml/min. Oven temperature: 260-300°C at 5°C/min, then 300°C for 10 min. Detector sensitivity: 10 pA/mV. Injection volume: 1 μl containing 2 μg of each component. Capillary column: fused silica, 50 m x 0.22 mm I.D., CP-Sil 5, film thickness 0.12 μm. Carrier gas: helium, linear velocity 24.5 cm/s. Oven temperature: 290°C. Injection volume: 1 μl 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.

R = 1 (98% baseline separation for solanidine-demissidine and demissidine-solasodiene) was 678000. Thus the theoretically required minimum column length was 305 m. Consequently, no further attempts with this technique were made.

Fig. 1B shows a chromatogram of the same mixture of SAs obtained by capillary GC using a 50 m CP-Sil 5 column. The retention times, t_R, of solanidine (peak 2) and demissidine (peak 3) were still very close, $t_{R,2}$ = 13.10 min and $t_{R,3}$ = 13.29 min respectively, but due to the small peak widths a good separation, R = 1.3, was achieved. Solasodine (22αN-spirosol-5-en-3-ol, peak 6), which was added to the alkaloid mixture, was well separated from its dehydration product solasodiene (peak 4) and from tomatidine (22βN-spirosolan-3-ol, peak 5). The peak

symmetry appeared to be quite good, although some tailing of the peaks 5 and 6 was noticeable. This tailing effect decreased upon optimization of the GC parameters, but it never totally disappeared.

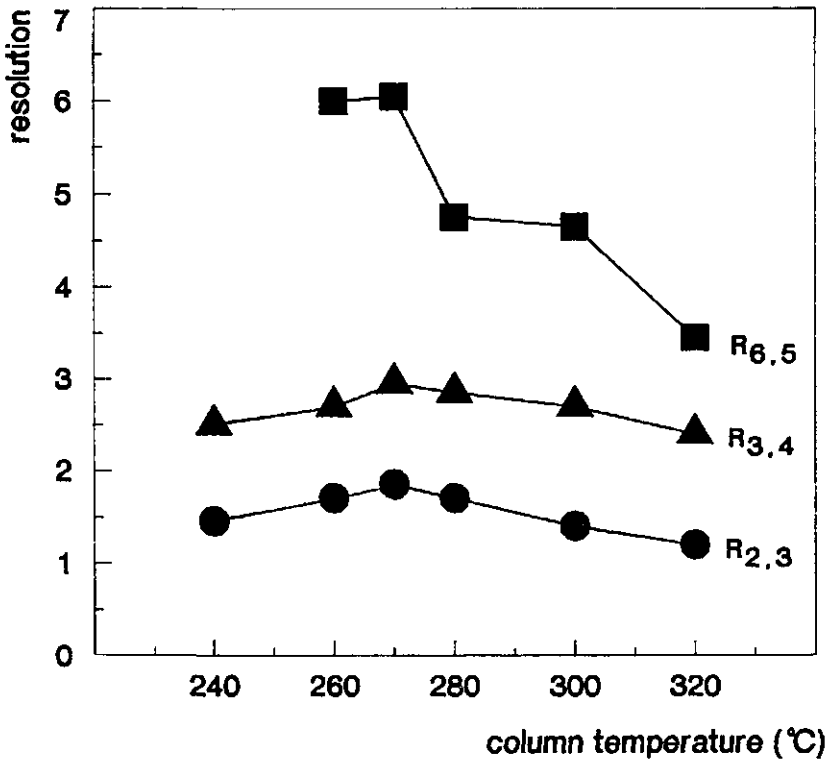

Fig. 2. Effect of GC-oven temperature on the resolution, R, of solanidine-demissidine ($R_{2,3}$), demissidine-solasodiene ($R_{3,4}$) and solasodine-tomatidine ($R_{5,6}$). Because of peak asymmetry, no value for $R_{6,5}$ was obtained at 240°C. The splitting ratio and the carrier gas velocity were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

Optimization of GC conditions

To improve the chromatographic results, the GC conditions were studied using the separation of solanidine and demissidine as the main criterion. At an oven temperature of 290°C, the separation of this Δ5- and 5α-aglycone pair, expressed as the resolution of the peaks 2 and 3, was optimum, $R_{2,3}$ = 1.7, at a carrier gas velocity, u, of 24.3 cm/s. During further optimization, u was kept at this value.

The effect of the oven temperature on the resolution and peak areas was investigated at 240, 260, 280, 290, 300 and 320°C. The optimum resolution, $R_{2,3}$ = 1.9, was observed at 280°C (Fig. 2). At higher temperatures the resolution decreased 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 was 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 oven temperatures. Thus, there was hardly any decomposition of these compounds upon increasing the temperature from 260°C to 320°C. Under the same conditions, solasodine and tomatidine underwent up to 12% decomposition, which 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 SAs, decomposition of solasodine and tomatidine was less then 3%.

Table 1 shows the retention time, resolution and separation number for six SAs chromatographed at the optimum oven temperature and carrier gas velocity. Values for the internal standard are also given.

The retention time of the last eluted component (tomatidine) was 27 min at 280°C. When analysing Solanum species having unknown SGA compositions, the total analysis may last longer because the retention times of SAs not tested in this study may exceed that of tomatidine. For example, t_R of jervine, an SA from Veratrum species, was 39.8 min, when chromatographed under the same conditions.

The SAs 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 is likely from the relatively high resolution of solanidine and demissidine, $R_{2,3}$ = 1.9, that other Δ5-5α pairs like tomatidine-tomatidenol and solasodine-soladulcidine (Chapter I, Fig. 1) will be separated as well. Both tomatidenol (22βN-spirosol-5-en-3-ol) and soladulcidine (22αN-spirosolan-3-ol) are expected to be eluted between solasodine (22αN-spirosol-5-en-3-ol) and tomatidine (22βN-spirosolan-3-ol). The separation number, SN, shows that a maximum of four peaks can

Table 1. Retention time, t_R, resolution, R, and separation number, SN, of steroidal alkaloids and of 5α-cholestane obtained by capillary GC using a fused-silica column (50 m x 0.22 mm I.D.) coated with CP-Sil 5 (film thickness 0.12 μm); the t_Rs on a similar column coated with CP-Sil 19 CB are also given.

Compound	CP-Sil 5			CP-Sil 19 CB
	t_R (min)	R	SN	t_R (min)
5α-Cholestane	11.60			13.17
		6.2	4	
Solanthrene	12.28			16.57
		35	29	
Solanidine	16.94			29.52
		1.9	0	
Demissidine	17.23			29.87
		3.1	1	
Solasodiene	17.78			31
		31	24	
Solasodine	25.22			>90
		6.1	4	

Oven temperature: 280°C. Linear carrier gas (helium) velocity: 24.3 cm/s. Injection volume: 1 μm; splitting ratio 1:100.

be separated between the last two components. Attempts to improve the GC results using a more polar liquid phase (CP-Sil 19 CB) were not successful. The retention times increased drastically (Table 1), and solasodine and tomatidine were still not eluted after 90 min at 280°C.

Attempts to reduce the analysis time were made by oven temperature programming. The best of the programmes 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.

SAs not available for this study or novel SAs may also be present in Solanum species. Therefore, in studying the SA composition of Solanum

species, the resolution must be optimum, enabling detection of such compounds. Consequently, the following temperature programme was chosen: 280°C for 28 min (for optimum separation and quantification of the known SAs from tuber-bearing Solanum species); temperature increase, 8°C/min to 320°C, and then isothermal for at least 5 min (for detection of SAs which might elute after tomatidine).

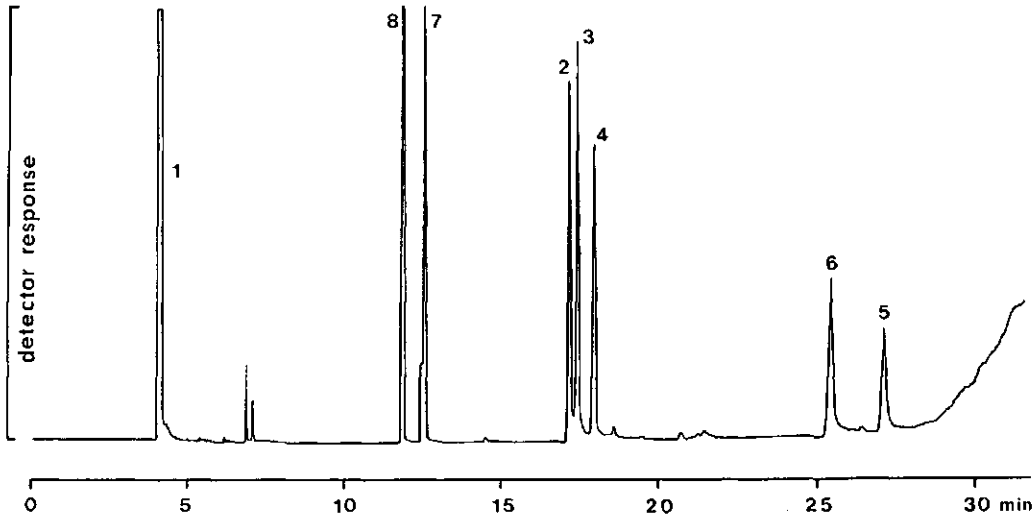

Fig. 3. Capillary gas chromatogram showing the steroidal alkaloid profile of an extract from tubers of S. tuberosum cv Elkana, spiked with 1 μg/μl of components 2, 3, 4, 5, 6 and 8. Column: fused silica, 50 m x 0.22 mm I.D., CP-Sil 5, film thickness 0.12 μm. Carrier gas: helium, linear velocity 24.3 cm/s. Oven temperature: 280°C for 28 min, then 280-320°C at 8°C/min, finally 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.

Validation of the capillary GC method

Fig. 3 shows a chromatogram obtained by capillary GC of a spiked potato extract. The extract was obtained from tubers of the cultivar Elkana, which is used for potato-starch production. 'Elkana' contained a high concentration (460 mg/kg) of solanidine glycosides, determined as solanthrene. Prior to injection, 5α-cholestane, solanidine, demissi-

dine, solasodiene, solasodine and tomatidine were added to the potato hydrolysate. The chromatogram shows that all compounds were separated and that compounds interfering with the resolution of the SAs were not present in the potato tuber extract.

To investigate the prospects of the capillary GC method for qualitative and quantitative analysis of the SGAs of various Solanum species, the contents of solanidine glycosides determined by a colorimetric reference method were compared with those determined by capillary GC of the aglycone of which the concentration was expressed as solanidine glycosides. Ten genotypes covering a wide range in these contents were used (Table 2). Although the methods differ strongly (precipitation, centrifugation, Clarke reaction and colorimetry of SGAs versus two-phase hydrolysis of SGAs, liquid-liquid extraction and capillary GC of the aglycone), they yielded similar results. The correlation coefficient, r, was 0.976 and the regression equation was

$$y = 0.95x + 2.98$$

where y applies to the colorimetric method and x to the capillary GC method.

Calculation of the SGA content

In methods used for determining the total SGA content, which have been reported in the literature (Fitzpatrick and Osman, 1974; Coxon et al., 1979; Bushway et al., 1980), the aglycones liberated after acid hydrolysis are determined, and then they are expressed as SGAs by multiplying the concentration of the aglycones by the ratio of the molecular masses of the corresponding SGAs and aglycones. In this way, only average conversion factors, which neglect the variation in chain length of the sugar moieties, can be applied. However, when the aglycones are separated by chromatography and identified and quantified individually, accurate conversion factors can be used. For this purpose, it must be taken into account that in tuber-bearing Solanum species, solanidine, solasodine, tomatidenol, leptinidine and acetylleptinidine occur bound to trisaccharides, and demissidine and tomatidine to tetrasaccharides (Chapter I, Table 1), although exceptions may exist. Such an exception is dehydrocommersonine, composed of solanidine and the tetrasaccharide commertetraose, which has been detected in the foliage of S. commersonii and S. toralapanum (Gregory et al., 1981; Johns and Osman, 1986).

Table 2. Contents of solanidine glycosides of ten potato genotypes, determined by capillary GC and by a colorimetric reference method.

Genotype	Solanidine glycosides (mg/kg fresh weight)[1]	
	Capillary GC	Colorimetry
1	10	20
2	10	40
3	20	60
4	150	150
5	190	210
6	220	290
7	360	310
8	380	460
9	410	400
10	470	470
Average	222	241

Injection volume: 2 µl; splitting ratio: 1:50. For other experimental conditions see Fig. 3.

[1]The contents have originally been determined and reported as mg/100 g fresh weight and have now been multiplied by a factor 10 because of standardization.

In that case, the results would have been calculated 14.6% too low. Even more accurate data can be obtained when the concentrations are expressed as moles/kg. Then the values will be independent of the molecular mass of the aglycone, and of the presence of a sugar moiety and its number of sugars.

In conclusion, the capillary GC method described here, in combination with the extraction and two-phase hydrolysis described in Chapter III, offers prospects for quantitative analysis of the SGA composition of Solanum species. Further sample clean-up and derivatization of the SAs is not required. For optimum accuracy, a calibration line should be constructed for each SA and the contents expressed as moles/kg.

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CHAPTER V*

CAPILLARY GAS CHROMATOGRAPHY OF STEROIDAL ALKALOIDS: RETENTION INDICES AND SIMULTANEOUS FLAME IONIZATION/NITROGEN-SPECIFIC DETECTION

INTRODUCTION

Capillary GC analyses using the method described in Chapter IV, showed that SGAs, such as solanidine, demissidine, solasodine or tomatidine glycosides, occur in tubers and leaves of wild Solanum species used in potato breeding and in leaves of progeny derived from such species (Van Gelder and Jonker, 1986; Van Gelder et al., 1987). In addition to peaks of the aglycones of these SGAs, the chromatograms showed peaks of other compounds with retention times within the range of those of the SAs. These peaks could not be identified by comparison of their retention times with those of the standard SAs available. In the framework of a tomato breeding programme on resistance to the glasshouse whitefly (Trialeurodes vaporariorum Westw.), a study on the SGA composition of the wild tomato species Lycopersicon hirsutum glabratum C.H. Muller, and of tomato breeding lines derived from this species, was carried out (Van Gelder and De Ponti, 1987). Also in this study, some unidentified (minor) compounds were detected, which eluted somewhat before and after tomatidine. Preliminary experiments in which chromatograms were recorded by nitrogen-specific detection (NPD) using a thermoionic nitrogen-phosphorus detector, indicated that some of the unidentified compounds were probably SAs. In a screening of breeding material for SAs, the characterization of each unknown peak in a chromatogram by costly spectrometric techniques is hardly feasible. Therefore a method was developed that enables differentiation between peaks of unknown SAs and peaks of nonnitrogen-containing (non-N) compounds. The method in-

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*This chapter is based on the paper:
Van Gelder WMJ, Jonker HH, Huizing HJ, Scheffer JJC. (1988) Capillary gas chromatography of steroidal alkaloids from Solanaceae. Retention indices and simultaneous flame ionization/nitrogen-specific detection. J Chromatogr 442:133-145.

volves a capillary column connected by a splitting device to a dual detector system for FID and NPD, which is connected to a two-channel system for interactive processing of the two sets of data. As a further aid for identification of the SAs, a retention index system was introduced. The chromatographic conditions were optimized and hydrogen was used as carrier gas in order to decrease the duration of the analyses. Spectrometric techniques will have to be employed for characterization only when new SAs are detected.

EXPERIMENTAL

Apparatus

The gas chromatograph described in Chapter IV was equipped with detectors for FID and NPD and with two split/splitless-type injectors. One injector was in the split sampling mode, splitting ratio 1:50 unless otherwise stated; the vaporization tube was a modified Jennings tube (Freeman, 1981) provided with a fritted glass filter and partly filled with deactivated quartz-wool. The other injector was used for controlled supply of nitrogen, 20 ml/min, as make-up gas for a four-way effluent-splitting device, which consisted of a quartz tube (9 mm x 1.3 mm I.D.) and two two-hole vespel ferrules in a stainless-steel housing. The effluent splitter connected the column with both detectors via two deactivated fused-silica restriction tubes (20 cm x 0.22 mm I.D.). The effluent-splitting ratio for FID and NPD which depends on the length ratio of the restriction tubes was kept at 1:1 in order to obtain identical retention times for the FID and the NPD traces. Other GC conditions were as follows. Column: fused silica, 50 m x 0.22 mm I.D., CP-Sil 5 CB, film thickness 0.12 μm (Chrompack Nederland); oven temperature, 270°C or 280°C; detector, 325°C; injector, initially 300°C but after the optimization experiment 350°C; carrier gas, hydrogen, linear velocity 49 cm/s, unless stated otherwise; flow-rates for FID, hydrogen 25 ml/min, air 250 ml/min; and for NPD, hydrogen 5.0 ml/min, air 50 ml/min. The detectors were supplied with nitrogen, 30 ml/min each, as make-up gas (unless stated otherwise), including 10 ml/min each of the make-up gas from the effluent splitter. For the injector, Chromsep Red, high-temperature (up to 450°C) septa (Chrompack) were employed. Two Shimadzu C-R3A data processors were used for recording

the FID and NPD signals and for interactive processing of the data. The processors were interconnected by a PC14N current loop interface, an RS232C and a MIC-loop.

Software

For communication between the data processors, a programme was written partly in BASIC and partly in machine language. After the introduction of the identification system using NPD/FID response ratios and retention indices, a BASIC programme was written for automated calculation of these values. Integrated into the BASIC programme, concentrations were measured with the ROM-software of the data processors.

Standard alkaloids and other chemicals

Solanidine, solanthrene, demissidine, solasodiene, solasodine, tomatidine, 5α-cholestane, the solvents and other chemicals, were as described in the Chapters III and IV. For comparison of SAs and test compounds with respect to the relationship between injection volumes and peak areas, a commercially available test mixture (Chrompack, Cat.No. 6606) was used. This contained n-octanal, n-octanol-1, n-undecane, 2,6-dimethylphenol, methyl n-octanoate, 2,6-dimethylaniline, naphthalene, n-dodecane, n-tridecane and methyl n-decanoate, each in a concentration of 1% in cyclohexane. Cholesterol and stigmasterol were obtained from Roth, diosgenin, n-octacosane and n-tetratriacontane from Sigma and jervine and tigogenin from ICN Pharmaceuticals, (Plainview, NY, U.S.A.).

Calculation of retention indices

Retention indices, I, on CP-Sil 5 CB at 270°C were calculated using the formula:

$$I_{270}^{\text{CP-Sil 5 CB}} = 100 \cdot C_1 + 100 \cdot (C_2 - C_1) \cdot \frac{\log(t_{R,i} - t_{R,0}) - \log(t_{R,1} - t_{R,0})}{\log(t_{R,2} - t_{R,0}) - \log(t_{R,1} - t_{R,0})}$$

where C_1 = the carbon number of the first reference compound (n-octacosane, $C_{28}H_{58}$), C_2 = the carbon number of the second reference compound (n-tetratriacontane, $C_{34}H_{70}$), $t_{R,0}$ = the retention time of an unretarded component (methane), $t_{R,i}$ and $t_{R,1}$ and $t_{R,2}$ = the retention

Table 1. Retention times, t_R, and resolution, R, of steroidal alkaloids (SAs) using nitrogen, helium and hydrogen as carrier gases.

SA	Oven temperature (OT) 280°C						OT 270°C	
	Nitrogen u 13.5 cm/s		Helium u 24.3 cm/s		Hydrogen u 48.8 cm/s		Hydrogen u 49.0 cm/s	
	t_R (min)	R	t_R (min)	R	t_R (min)	R	t_R (min)	R
Solanidine	30.74		16.98		8.60		10.96	
		2.00		1.84		1.76		1.92
Demissidine	31.28		17.27		8.74		11.15	
		3.64		3.04		3.01		3.19
Solasodiene	32.28		17.83		9.02		11.57	
		35.8		30.3		33.2		33.2
Solasodine	46.56		25.30		13.03		17.16	
		6.71		6.13		6.29		6.23
Tomatidine	49.85		27.10		13.94		18.43	

Column: fused silica, 50 m x 0.22 mm I.D., CP-SIL 5 CB, film thickness 0.12 μm.
Optimized average linear velocities of carrier gas (u). Injector temperature: 350°C.

times of the component of which I has to be determined, of the first reference compound and of the second reference compound, respectively.

RESULTS AND DISCUSSION

Separation of SAs

The effect of the injector temperature on the retention time, relative response, chromatographic stability and resolution was investigated by increasing this temperature from 300°C to 450°C by increments of 25°C. The temperature increase gradually resulted in shorter retention times of the SAs but the effect was negligible (< 2%) even at 450°C. The compound ratios, for instance that of solanidine and tomatidine, did not change at the various injector temperatures, which meant that all the SAs were vaporized to the same extent at the various injector temperatures. Thus, the injector temperature did not influence the possible discrimination of the SAs. However, increasing the injector temperature deteriorated the baseline stability and peak symmetry, and caused ghost peaks, especially at temperatures above 375°C. These phenomena probably resulted from septum bleeding and/or sample decomposition. The resolution of the early eluting compounds solanidine and demissidine was optimum at an injector temperature of 350°C, whereas for the late eluting compounds such as solasodine and tomatidine it was optimum at 375-400°C. Because the chromatographic stability deteriorated at the higher temperatures and an optimum resolution of the Δ5- and 5α-aglycone pair solanidine and demissidine was important, 350°C was chosen as the injector temperature.

Table 1 shows the retention times and the resolution of the SAs chromatographed on the CP-Sil 5 CB column at an oven temperature of 280°C using helium, nitrogen and hydrogen as the carrier gases at optimized average linear velocities. The highest resolution was obtained with nitrogen, but this was attended with long retention times. The separation efficiencies obtained with helium and hydrogen were similar, but using hydrogen the analysis duration was half as long. Comparison of the separation efficiencies with hydrogen at oven temperatures of 260, 270, 280 and 290°C, showed that the optimum temperature for the separation of solanidine, demissidine and solasodiene was 270°C. Compared with helium as the carrier gas and the oven temperature of 280°C

used before, 270°C was favourable with regard to the column durability (maximum isothermal operating temperature 300°C), and a 30% shorter analysis duration was obtained (Table 1).

Split injection of SAs

In isothermal capillary GC, the split injection technique is preferable, especially when automated injection is applied. However, when quantititive results are required, many problems may arise (Grob Jr. and Neukom, 1982). Therefore, the splitting process was investigated in order to study whether quantitative results could be obtained for the individual SAs.

For quantitative analyses it is necessary that the splitting is linear both for various concentrations and sample sizes. This was checked by constructing FID calibration curves for the internal standard 5α-cholestane, using two injection volumes, 0.9 μl and 1.9 μl. For both curves, the peak areas were found to be directly proportional to the concentrations. So, splitting was independent of concentration. However, the normalized FID responses of 5α-cholestane found for the 0.9 μl injection volumes were only 80% of those found for 1.9 μl. This suggested that the splitting ratio varied depending on the sample size. For quantitative analyses, the normalized peak areas must be similar for the various sample sizes and for each SA. Therefore the concentrations of the compounds were kept constant, whilst the sample sizes were varied. Table 2 gives the average of the normalized peak areas of solanidine, demissidine, solasodiene, solasodine and tomatidine for each sample size. The normalized peak areas were expressed as percentages of the peak areas, averaged for the SAs, at the injection volume of 2.4 μl. For each sample size the variation (range and % S.D.) in normalized peak areas between the individual SAs is also given. The experiments showed that the normalized peak areas decreased with decreasing sample sizes. The linearity of the calibration plots (see hereafter) showed that this was not due to irreversible adsorption of the components onto the column. Therefore, it is most probable that the pressure pulse caused by flash vaporization of the sample decreases at smaller sample sizes, and as a consequence, the splitting ratio is lowered, which in turn results in a reduction of the peak areas. Concomitantly with this reduction in normalized peak areas, the variation

Table 2. Variation in normalized peak areas for steroidal alkaloids (SAs) and for test compounds dependent on the injection volume.

Injection volume (μl)	Relative normalized peak areas[1] (%)					
	SAs			Test compounds		
	Average	Range	% S.D.	Average	Range	% S.D.
2.4	100	-	-			
1.9	98	95-103	4.3	100	-	-
1.4	94	91- 98	3.1	100	99-107	2.4
1.0	67	59- 80	9.9	85	78- 86	2.7
0.8	53	46- 57	10.7	80	77- 81	1.5
0.4	30	17- 39	31.6	76	72- 79	3.1
0.3				69	64- 72	4.1

SAs: solanidine, demissidine, solasodiene, solasodine and tomatidine; for the composition of the test mixture see Experimental section.
[1]Normalized peak areas in percentages of the area at 2.4 μl for SAs and at 1.9 μl for the test compounds, respectively.

in these areas between the individual SAs increased, as is shown by the range and % S.D. From replicate analyses it was clear that this variation was not due to a lack in precision of operation or detection (n = 5; % S.D. for the peak areas of solanidine 2.22; demissidine 2.06; solasodiene 2.15; solasodine 4.31 and tomatidine 3.08). Comparison of the peak areas of the individual SAs showed that at injection volumes $\leq$ 1 μl the relative peak areas of the compounds decreased with their order of elution. This can be ascribed to discrimination of the compounds according to their boiling points. Discrimination is due to variation of splitting ratio for the different compounds present in the splitter (Grob Jr. and Neukom, 1982). This nonlinearity of the splitting process apparently increased at smaller sample sizes. So, discrimination occurred in spite of the precautions taken with respect to the configuration of the vaporization tube, to ensure that the sample was heated sufficiently rapidly for a complete vaporization during injec-

tion (see Experimental section).

A similar experiment was done using a commercially available mixture of test compounds of well defined chromatographic behaviour. Also in this experiment, although to a lesser extent, the normalized peak areas decreased (relative to 1.9 μl) with decreasing sample sizes (Table 2). This shows the characteristics of the splitter configuration used. However, the decrease in the peak areas was similar for all compounds of the test mixture. The results obtained for the SAs and for the test compounds show that the SAs are difficult compounds for capillary GC. For quantitative results, extra care is needed. A constant injection volume is essential, which disallows correction of peak areas by changing the injection volume when the column is overloaded, or when the peaks are too small to be detected. Also an assessment of the smallest volume that gives uniform results for the different SAs is required. In our study 1.4 μl was found to be appropriate, even more so because at this volume the results for 5α-cholestane agreed well with those for the SAs, allowing this compound to be used as internal standard.

Nitrogen-specific detection of SAs

The selectivity and sensitivity of a N-specific detector depend on the position of its alkali metal source, on the supply of thermal energy for emission of the alkali metal ions and on the composition and flow of the gas surrounding the alkali source (Sevcik, 1976; Verga, 1983; Shmidel et al., 1986). Each of these factors is the resultant both of detector characteristics determined by the instrumental design and of adjustments of chromatographic conditions and detector variables. The selectivity and sensitivity of the detector used in this study were mainly determined by the distance between the rubidium bead and the detector jet, by the flow of hydrogen used for combustion, by the flow of the nitrogen make-up gas and by the current of the electrically heated bead for adjustment of the background ionization current (BIC). As an instrumental prerequisite, the position of the rubidium bead was carefully tuned and the hydrogen flow adjusted to 5.0 ml/min. Then the influence of the flow-rate of the make-up gas and of the BIC on the selectivity and sensitivity were studied. In studying the SA composition of Solanaceae, the former is more important than the latter,

Table 3. Influence of make-up gas flow and background ionization current (BIC) on the selectivity and sensitivity of NPD for solasodine and diosgenin in comparison to the FID response.

BIC (pA)	Nitrogen make-up gas flow (ml/min)	Relative sensitivity NPD/FID		Selectivity[1] Solasodine/Diosgenin	
		Solasodine	Diosgenin	NPD	FID
80	20	3.2	0.119	25	0.91
80	25	3.1	0.019	139	0.87
80	30	2.9	0.006	418	0.88
80	35	2.4	0.005	462	0.86
40	20	1.5	0.082	16	0.90
40	25	1.5	0.011	117	0.88
40	30	1.1	0.003	389	0.90
40	35	0.9	0.002	409	0.91

Injection volume: 3 μl.

[1]Normalized for concentration of diosgenin = 7.11 • concentration of solasodine.

because the selectivity allows differentiation between SAs and non-nitrogen-containing compounds (non-N compounds), whilst for quantification of SAs the sensitivity of FID is usually sufficient. A number of experiments were carried out, using SAs and several more or less related non-N compounds, to compare different BICs and make-up gas flows. An example is given in Table 3. The relative sensitivity of NPD (NPD/FID) was defined as the ratio of the NPD and FID responses to the same amount of a compound. The selectivity was defined as the ratio of the response to a compound under study, i.e. solasodine and the normalized response to a reference compound, i.e. diosgenin. At nitrogen flow-rates of 20, 25 and 30 ml ml/min and a BIC of 80 pA, the relative sensitivity of NPD to solasodine (22αN-spirosol-5-en-3-ol) remained similar, whilst the relative sensitivity to diosgenin, the non-N (22αO) analogue, strongly decreased when the flow-rate was increased. As a result the selectivity of NPD for the N-containing compound greatly

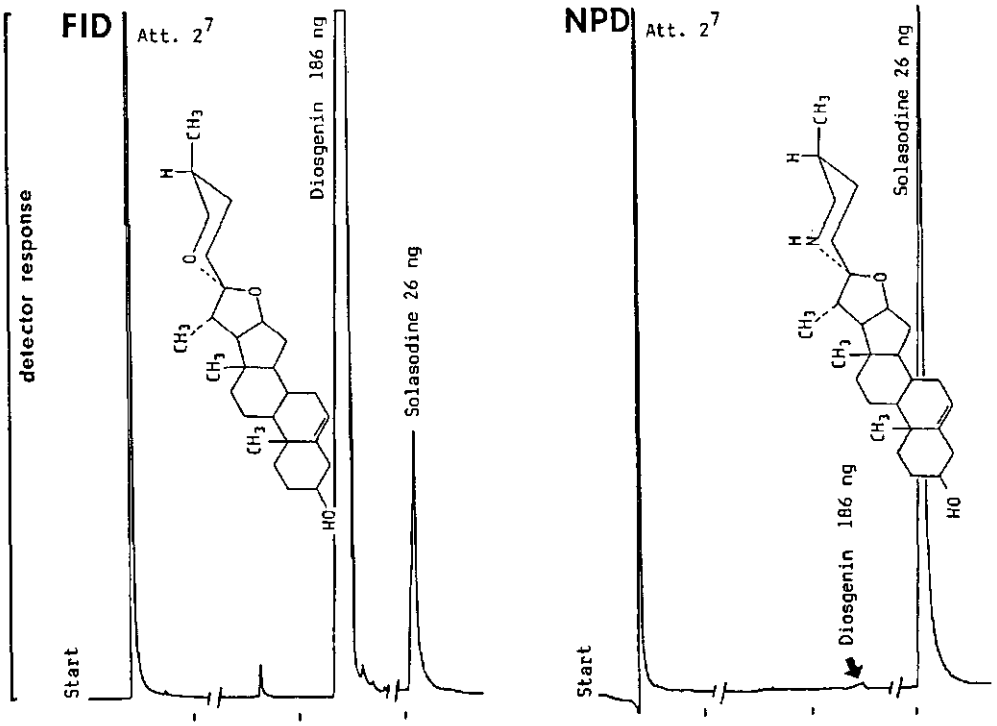

Fig. 1. Gas chromatograms obtained simultaneously by FID and NPD, showing the selectivity of NPD for solasodine, 22αN, compared to the 22αO analogue diosgenin. Column: fused silica, 50 m x 0.22 mm I.D., CP-Sil 5 CB, film thickness 0.12 μm. Carrier gas: hydrogen, linear velocity 53 cm/s. Oven temperature: 270°C. Injection volume: 3 μl; splitting ratio: 1:50; effluent splitting ratio: 1:1 (FID:NPD). Detector sensitivity: 1pA/mV. Attenuation: 2^7. For further details see Experimental section.

improved. At 35 ml/min and 80 pA the selectivity was further improved, but the sensitivity to solasodine became rather low for subtraction of FID from NPD traces (see hereafter). Lowering the BIC to 40 pA reduced the sensitivity of NPD to solasodine more than to diosgenin, and thus the selectivity slightly decreased. Increasing the BIC above 80 pA negatively affected the baseline stability and the noise level.

In summary, the make-up gas flow especially influenced the NPD sensitivity to non-N compounds and so it had a strong effect on the selectivity for SAs, whereas the BIC mainly influenced the sensitivity of NPD to the SAs. After these experiments a make-up gas flow of nitro-

gen of 30 ml/min and a BIC of 80 pA were chosen as optimum for the present study.

The relative sensitivity and the selectivity of NPD for the SAs were rather low compared with the corresponding values for pyridine: NPD/FID response ratio 22; selectivity pyridine/cyclohexane 2900. This might be explained by the low N/C ratio of the SAs, i.e. 1:27.

Fig. 1 shows the gas chromatograms of a mixture of solasodine and diosgenin recorded simultaneously by FID and NPD. Only high levels of the non-N compound, far above the theoretical sample capacity of the column, were able to interfere with the N-specific detection. This means that simultaneous detection by FID and NPD unambiguously differentiates between SAs and non-N compounds.

Quantification of SAs

Calibration lines were constructed for FID and NPD. As samples, solutions of solanidine, of demissidine plus tomatidine and of solasodiene plus solasodine were used. Each compound was used in 11 concentrations, varying from 0.05-10 mg/ml. Although concentrations between 4 mg/ml and 10 mg/ml may cause overloading of the column, they were used because preliminary experiments showed that tubers of wild Solanum species may contain levels of solanidine glycosides above 6000 mg/kg.

Table 4. Slope, m, intercept, c, and coefficient of correlation, r, of the calibrations $y = mx - c$ for steroidal alkaloids (SAs) with FID and NPD, where y = response (counts $\cdot 10^3$) and x = concentration (mg SA/ml).

SA	FID			NPD		
	m	c	$r \cdot 10^4$	m	c	$r \cdot 10^4$
Solanidine	70	4.4	9997	190	15.5	9997
Demissidine	70	5.3	9996	186	1.4	9984
Solasodiene	70	6.7	9996	183	8.0	9983
Solasodine	58	4.0	9999	149	11.7	9994
Tomatidine	62	13.6	9980	153	9.5	9994

The calibration lines for FID and for NPD were linear over the entire concentration range, for all SAs tested (Table 4). Under the conditions described for sample preparation (Chapter IV) and chromatography, 50- and 150-fold concentrations for the individual SAs could be quantified by FID and NPD respectively, without exceeding the column capacity. Under these conditions and using the NPD calibrations of Table 4, 2.7 mg/kg was the minimum content for accurate quantification. The actual detection limit that can be achieved using modified procedures for sample preparation is lower, but it was not relevant to determine this limit. The concentrations between 4 mg/ml and 10 mg/ml, which exceeded the column capacity, had no effect on the peak symmetry. Thus, when closely eluting SAs are not present, extremely high levels of SAs can be quantified without dilution of the samples.

Identification of SAs

The reproducibility of the retention times expressed as % S.D. (n = 6) was 0.045 for solanthrene and 0.037 for solanidine; for demissidine, solasodiene, solasodine and tomatidine it varied from 0.040-0.068. These values agreed with those reported for accurate analyses (Rooney and Freeman, 1981). However, such determinations are usually made under ideal conditions. This means that injections are made successively within one day and using one concentration. In studying the SA composition of Solanaceae, samples with widely different concentrations of SAs must be analysed in the course of weeks or months. Progressive phase stripping of the column due to the high oven temperature, and especially a large variation in peak heights due to variation in the concentrations of the SAs, might result in less reproducible retention times. Therefore, we also determined routinely the reproducibility of the retention times of the SAs. Nine different tuber samples of six Solanum species, obtained from the Potato Breeding Department of the Foundation for Agricultural Plant Breeding SVP were analysed for their SA composition using the extraction and hydrolysis procedures described in the Chapters III and IV. The analyses were carried out between routine analyses in the course of a five-day period. Most of the samples contained only solanidine glycosides -their contents varying widely: 600-6200 mg/kg- so calculations were made only for the corre-

Table 5. Retention times, t_R, retention indices, I, and NPD/FID response ratios of steroidal alkaloids, 5α-cholestane, sterols and steroidal sapogenins.

Peak number[1]	Compound	t_R	I	NPD/FID response ratio
1	n-Octacosane ($C_{28}H_{58}$)[2]	5.87	2800.0	-
2	5α-Cholestane	6.61	2866.5	-
3	Solanthrene	7.61	2941.8	3.04
4	Cholesterol	10.13	3068.3	-
5	Solanidine	11.07	3129.3	2.96
6	Demissidine	11.25	3136.8	3.01
7	Solasodiene	11.59	3151.1	3.06
8	Stigmasterol	13.22	3212.9	-
9	Diosgenin	13.43	3220.4	-
10	Tigogenin	13.78	3232.4	-
11	Solasodine	17.36	3337.6	3.02
12	Tomatidine	18.65	3369.8	2.88
13	n-Tetratriacontane ($C_{34}H_{70}$)[2]	19.96	3400.0	-
14	Jervine	28.73	3559.4	3.43

Column: fused silica, 50 m x 0.22 mm I.D., CP-SIL 5 CB, film thickness 0.12 μm. Oven temperature: 270°C. Carrier gas: hydrogen, linear velocity 49 cm/s.

[1]Peak numbers correspond with those of Fig. 2.

[2]Reference compound.

sponding aglycones solanthrene and solanidine. The reproducibility of the retention times deteriorated strongly; the % S.D. was now 0.864 and 0.690, respectively. Due to the large variations in retention time, it was not possible to set a time window for solanidine along with one for demissidine, in order to identify these peaks by retention times.

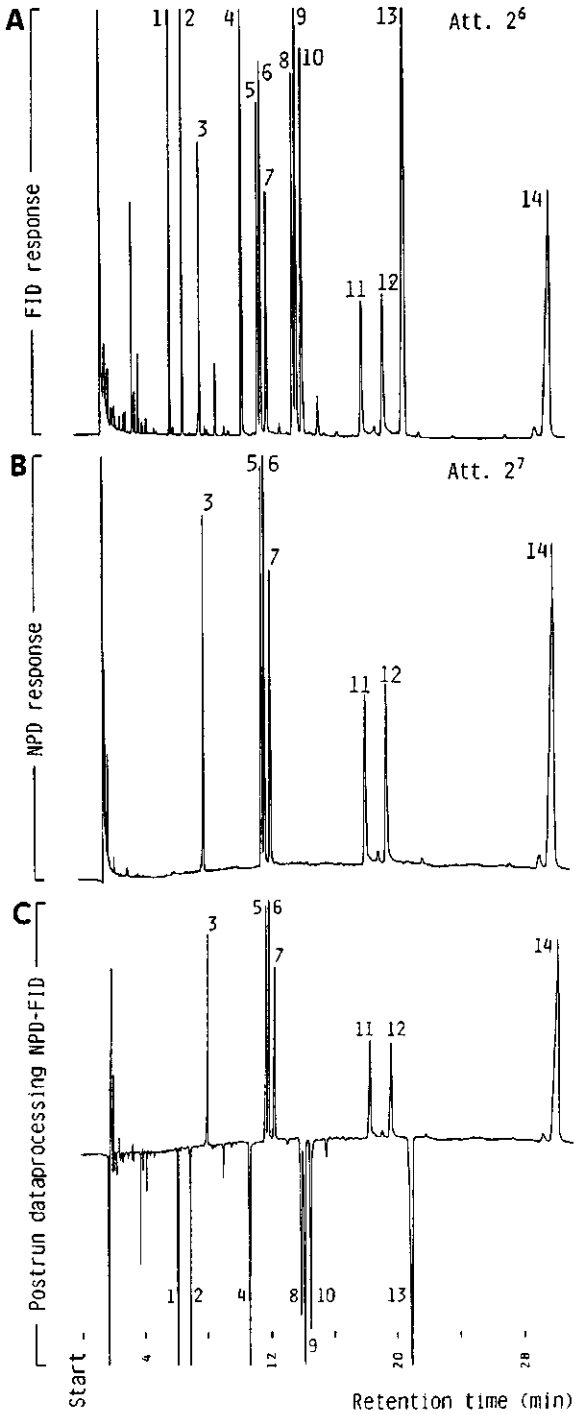
A
FID response
Att. 2^6
1
2
3
4
5
6
7
8
9
10
11
12
13
14
B
NPD response
Att. 2^7
C
Postrun dataprocessing NPD-FID
Start
Retention time (min)

Fig. 2. Gas chromatograms obtained simultaneously by FID (A) and NPD (B) for a potato extract spiked with steroidal alkaloids, and with 5α-cholestane, sterols and steroidal sapogenins. Post-analysis reprocessing of the raw data from A and B (subtraction of the FID trace from the NPD trace) is shown in C. Carrier gas: hydrogen, linear velocity 49 cm/s. Injection volume: 1.4 μl. Attenuation: FID 2^6, NPD 2^7. Further details as in Fig. 1. Amounts (ng) of compounds in the column: 1 = n-octacosane, 19.0; 2 = 5α-cholestane, 13.7; 3 = solanthrene, 12.9; 4 = cholesterol, 14.4; 5 = solanidine, 15.2; 6 = demissidine, 16.0; 7 = solasodiene, 13.6; 8 = stigmasterol, 14.1; 9 = diosgenin, 20.9; 10 = tigogenin, 14.2; 11 = solasodine, 15.7; 12 = tomatidine, 17.4; 13 = n-tetratriacontane, 17.9; 14 = jervine, 31.3.

For certain Solanum species, complex chromatograms showing large numbers of SAs may be obtained. As potato breeding may require many SA analyses, the characterization and identification of compounds by costly spectrometric techniques is hardly feasible. Therefore a cheap and relatively simple system for characterization and identification using retention indices and NPD/FID response ratios was developed.

The retention indices shown in Table 5 were sufficiently reproducible. Under ideal conditions, the % S.D. of the indices for the SAs varied from 0.006-0.010. In the above mentioned routine experiment the % S.D. of the retention index was 0.046 for solanthrene and 0.028 for solanidine. Tests with widely different concentrations of solanidine and demissidine showed that typical identification windows for the retention indices of these closely eluting compounds would be 3129.3 ± 1.7 and 3136.8 ± 1.7 units, respectively. This meant that the retention index system could be used as an aid in the identification of the SAs. For automated identification in our laboratory, the indices are compared, using the BASIC programme, with the values of stored retention index windows. These values have been determined for individual SAs, using samples of plant material or standard solutions, with a wide concentration range. The accuracy and precision of the retention indices may be further improved by using for each SA two reference compounds differing by only one in their number of carbon atoms (Schomburg

and Dielman, 1973). For practical reasons this was not done. Future studies on the SA composition of Solanaceae will reveal whether improvement of the retention index system is required.

The NPD/FID response ratios of the SAs were quite similar (Table 5) and they will thus be useful in the characterization of unknown compounds. As the NPD/FID response ratios of the SAs differed markedly from that of pyridine, which was 22, it should be worthwhile to investigate whether these ratios are sufficiently specific for different types of N-containing compounds to discriminate between SAs and other N-containing compounds which may be present in plant extracts. If so, this could allow a considerable simplification of the complex procedure for sample preparation currently in use. Fig. 2 shows gas chromatograms of a potato extract spiked with SAs and related non-N compounds (5α-cholestane, sterols and steroidal sapogenins) obtained by FID (A) and NPD (B). The peak numbers correspond with those of the compounds listed in Table 5. The FID chromatogram shows that, under the conditions applied, all compounds were well separated. The Solanum SAs tested were eluted within 19 min. The Veratrum SA jervine ($C_{27}H_{39}NO_3$) had a retention time of almost 29 min. This shows the necessity of a temperature rise after elution of tomatidine (or the reference compound tetratriacontane) to facilitate the detection of other, late eluting, SAs as stated in Chapter IV. Comparison of the FID chromatogram with the NPD chromatogram reveals the peak vacancies in the latter, which correspond with the non-N compounds of Table 5. Fig. 2C shows the result of post-analysis reprocessing of the chromatograms, which was realized by the software mentioned in the Experimental section. By subtraction of the FID chromatogram from the NPD chromatogram, the peak vacancies in the NPD trace are converted into negative peaks. So, the differentiation between SAs and non-N compounds is unambiguously achieved.

In conclusion, quantification of individual SAs using capillary GC can be done routinely either by FID or by NPD, provided extra care is taken over the injection of the samples. Usually FID is more stable than NPD, but the latter will be more sensitive. The NPD/FID response ratios can be used together with retention indices in the identification and characterization of SAs. GC-MS analysis can probably be restricted to compounds with NPD/FID response ratios which correspond

to those of the SAs and with retention indices which do not agree with those of the known SAs.

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CHAPTER VI*

ANALYSIS OF STEROIDAL GLYCOALKALOIDS IN POTATO GENOTYPES CONTAINING GERMPLASM FROM WILD SOLANUM SPECIES

INTRODUCTION

The procedures for bisolvent extraction, two-phase hydrolysis and capillary GC were developed using pure SAs and SGAs, and tubers of commercial potato cultivars. Preliminary experiments carried out for studies on tomato fruits (Van Gelder & De Ponti, 1987) and potato leaves (Van Gelder et al., 1987), showed that the hydrolysis conditions may require optimization for analyses of different plant organs. This can be explained by the differences in the chemical composition of diverse plant tissues which may vary considerably in their concentrations of starch, proteins, organic acids, sugars, fats and other constituents. Such variation also exists between tubers from different Solanum species and from their hybrid offspring.

SGAs containing solanidine, solasodine and tomatidine as aglycones have been detected in tubers of wild Solanum species (Van Gelder and Jonker, 1986) and in the plant material analysed in the studies mentioned above. However, not all of these glycosides could be used during the development of the various analytical procedures. Recent preparation of sufficient amounts of standard SGAs and of plant materials containing them, enabled these procedures to be optimized for application to largely diverse samples, among others including different plant organs. A large number of standard SAs became available recently, and enabled their potential separation to be studied and their retention indices and NPD/FID response ratios to be determined. The results of these experiments are presented in this chapter.

Procedural difficulties of literature methods for SGA analysis,

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*This chapter is based on a part of the paper:
Van Gelder WMJ, Scheffer JJC. (199*) Strategy for analysis of steroidal glycoalkaloids in potato and tomato genotypes containing wild Solanum and Lycopersicon germplasm, in preparation.

which may easily introduce experimental errors when applied to diverse breeding material, are discussed in relation to the method developed in the studies described in this thesis.

EXPERIMENTAL

Plant material

For determining the number of bisolvent extractions required, tuber samples were obtained from the Potato Breeding Department of the Foundation for Agricultural Plant Breeding SVP. These samples included field-grown tubers of the household potato cultivar Bintje and of the potato cyst nematode resistant SVP clone AM 78-3778 which has S. vernei Bitt. et Wittm. as progenitor, and glasshouse-grown tubers of S.spegazzinii Bitt. and of two clones, hybrids 1 and 2, from a cross between a diploid genotype of S. tuberosum L. and S. leptophyes Bitt.

The samples for determining the recoveries of SGAs, after two-phase hydrolysis and capillary GC were tubers and/or leaves of S. tuberosum ssp. andigena (Juz. et Buk.) Hawkes, S. brevicaule Bitt. and S. venturii Hawkes et Hjerting collected from plants grown in 1986 under controlled short-day conditions (see Chapter VII), field-grown tubers of the household potato cultivar Pimpernel, green berries (fruits) of S. nigrum L. collected in the field, and fruits of the tomato cultivar Allround (Lycopersicon esculentum L.) and of the wild species L. hirsutum glabratum C.H. Muller.

For comparing the capillary GC method and a colorimetric method, selected as reference, tuber samples of S. gourlayi Hawkes, S. leptophyes, S. spegazzinii and S. sucrense Hawkes, of 'Bintje' and 'Pimpernel', of the SVP clones AM 78-3778 and AM 78-3787 ('Arabesque') and of clones from crosses between diploid genotypes of S. tuberosum and accessions of S. spegazzinii, S. vernei, S. sparsipilum (Bitt.) Juz. et Buk. and S. leptophyes were used. The samples were from different locations, including experimental field, glasshouse and growth cabinet. For the comparison of the GC method, tuber samples containing only solanidine glycosides were selected.

Standard alkaloids and other chemicals

Solasodine, solasodiene, solanthrene, α-tomatine, tomatidine and demis-

sidine and α-solanine were as described in Chapter III, 5α-cholestane was as in Chapter IV, and diosgenin and jervine were as in Chapter V. Solanidine was from Roth. Tomatidenol, solanocapsine and solanid-4-en-3-one were obtained from Prof. Dr. D.N. Kirk, Steroid Reference Collection (University of London, U.K.), veratramine, rubijervine, isorubijervine and cyclopamine from Dr. R.F. Keeler, Poisonous Plant Research Laboratory (Logan, UT, U.S.A.), N-acetyltomatidine from Dr. S.F. Osman, Eastern Regional Research Center (Philadelphia, PA, U.S.A.) and deacetylmuldamine (teinimine) from Dr. W. Gaffield, Western Regional Research Center (Albany, CA, U.S.A.). Soladulcidine was prepared by hydrogenation, at room temperature for 24 h, of 100 mg solasodine in 20 ml of glacial acetic acid, using hydrogen at 200 kPa, and 25 mg palladium 10% on carbon as catalyst. Tomatidan-3-one and demissidan-3-one (5α-solanidanone) were prepared by dehydration of tomatidine and demissidine according to Schreiber et al. (1963). Tomatidadiene was obtained by hydrolysis of tomatidenol using the two-phase system described in Chapter III. Solasodine glycosides were isolated from 300 g of green berries of S. nigrum by bisolvent extraction, using adjusted volumes of solvents, and alkali precipitation as described for the colorimetric method (Chapters III and IV). The SGA precipitate was washed with ammonia (1 mmol/l) and recentrifuged, then dissolved in 100 ml of hot ethanol (70%) and left for crystallization at -20°C. The crystals were collected by centrifugation at 20000 g for 15 min, and washed with n-pentane, after which the procedure for crystallization and centrifugation was repeated twice. About 160 mg of dried glycoalkaloids were obtained. TLC on HPTLC plates Silica gel 60 (E. Merck) using ethyl acetate/pyridine/water (30:10:30 v/v) (Boll, 1962) and visualization by Clarke reagent containing 300 mg paraformaldehyde in 10 ml of phosphoric acid (8.5 mol/l), showed two spots (R_F = 0.30 and 0.48), which corresponded with those of the standard compounds α-solasonine and α-solamargine obtained from Dr. S.L. Sinden, Agricultural Research Service (Beltsville, MD, U.S.A.). Upon two-phase hydrolysis, the glycoalkaloids produced solasodiene when carbon tetrachloride was used, and solasodiene and solasodine when chloroform was used as organic phase. The identity of these SAs was confirmed by their retention indices, by their NPD/FID response ratios determined by capillary GC (Chapter V) and by GC-MS, using standard compounds. The extract of S. nigrum was

then regarded a mixture of α-solasonine and α-solamargine; this mixture of solasodine glycosides was used as such.

Solvents and other chemicals were analytical grade.

Sample preparation

For the analysis of tubers, at least 15 tubers were taken from each genotype. Small tubers of wild species and hybrids were brought into a Waring Blendor and homogenized using an amount of water equal to 1/4 of the sample weight. Large tubers, such as those of commercial cultivars, were cut into longitudinal sections representative to each tuber in order to obtain subsamples of 240 g, which were then homogenized in 60 ml of water.

While homogenizing, 6.25 g aliquots of the tuber brei were taken for analysis by the GC method, and 12.5 g aliquots for analysis by the colorimetric method. These aliquots were transferred to a Waring Blendor with 75 ml of methanol/chloroform (2:1 v/v) as described in Chapter III. When the tubers of the wild Solanum species were very small -their weight may vary from 0.2-10 g under the conditions used for their propagation- and the weight of a sample of 15 tubers was 25 g or less, the samples were directly brought into the blender together with adjusted volumes of the methanol/chloroform mixture.

For the analysis of leaves, samples of 10 g were brought into the blender together with 75 ml of the methanol/chloroform mixture.

The tomato samples consisted of ten fruits which were homogenized until a fine homogeneous slurry was obtained. While spinning this slurry, 25 g samples were taken for 'Allround' and 10 g samples for L. hirsutum glabratum, which were each transferred to a Waring Blendor with 100 ml of the methanol/chloroform mixture.

The samples of S. nigrum consisted of 10 g of green berries which were brought into the blender with 75 ml of the methanol/chloroform mixture.

All samples were prepared in duplicate, unless otherwise stated.

The plant material was extracted with methanol/chloroform during homogenization (bisolvent method; Chapter III). After concentrating the methanolic layer obtained by this extraction, the residue was either worked up for quantification of the solanidine glycosides by colorimetry as described in Chapter IV, or analysed by capillary GC

after two-phase hydrolysis.

Optimization of two-phase hydrolysis

For optimization of the two-phase hydrolysis procedure described in Chapter III, the following organic phases were tested: benzene; n-butanol; n-butanol/chloroform (1:1, 1:3, 1:5, 1:7 and 1:9 v/v); n-butanol/chloroform/acetic acid (2:4:1 v/v); carbon tetrachloride; chloroform; dichloromethane; dichloromethane/chloroform (1:1 v/v); diethyl ether and 2-propanol. Various hydrochloric acid concentrations (0.5, 2, 4, and 6 mol/l) and hydrolysis durations varying from 1-18 h, depending on the hydrochloric acid concentrations, were applied.

The residue from the bisolvent extraction was transferred quantitatively to a 500 ml flask using 100 ml of the acid phase applied; then 100 ml of the organic phase tested were added and the hydrolysis was carried out on a hot plate. Subsequently, the two phases were allowed to cool and the pH was adjusted to 10 with ammonia (7 mol/l). The aqueous phase was separated off in a separatory funnel and washed with 20 ml of chloroform, which were then added to the organic phase. The combined organic phases were washed with 25 ml of ammonia (1 mmol/l), and evaporated to dryness, and the residue was transferred quantitatively to a crimp top vial and finally dissolved in 1.5 ml of methanol/toluene (2:1 v/v) containing 1 mg/ml of 5α-cholestane and diosgenin as reference compounds.

In the recovery experiments carried out for selection of the optimum two-phase system, 1.6 mg α-solanine, 1.7 mg solasodine glycosides and/or 2.2 mg α-tomatine, in 2 ml of methanol/chloroform (2:1 v/v), were added to the methanolic layers after their separation from the bisolvent extraction mixtures.

For determining the recoveries for a variety of Solanaceous plant samples, using the bisolvent extraction and the optimized two-phase hydrolysis system consisting of 100 ml of chloroform and 100 ml of hydrochloric acid (4 mol/l), and applying a hydrolysis duration of 5 h, 2.3 mg α-solanine, 2.3 mg solasodine glycosides and/or 3.2 mg α-tomatine in 2 ml of methanol/chloroform (2:1 v/v), were added to the homogenates after addition of the 75 ml of methanol/chloroform used for the bisolvent extraction.

GC, determination of retention indices and NPD/FID response ratios,

and quantification of SAs were carried out as described in Chapter V. The content of a specific SGA was calculated by multiplying the content of its aglycone by the ratio of the molecular masses of the SGA and the aglycone moiety.

RESULTS AND DISCUSSION

Quantitative determination of SAs and SGAs

For determining the number of repetitive extractions required for quantitative analyses, homogenates were prepared in triplicate from tubers of five markedly different potato genotypes. Each homogenate was extracted four times using the bisolvent extraction method and after each extraction the amount of solanidine glycosides extracted was determined. The contents of solanidine glycosides of the five genotypes, calculated from the combined results after four repetitive extractions, were on average 26, 168, 235, 391 and 1180 mg/kg fresh weight for 'Bintje', the hybrids 1 and 2, AM 78-3778 and S. spegazzinii respectively. On average, 84.3 ± 3.1%, 96.5 ± 0.8% and > 99.5% of the total amounts of solanidine glycosides were extracted from these diverse tuber samples after the first, second and third extraction, respectively. It was concluded that two repetitive extractions will usually be sufficient, provided the solvent layers are allowed to separate for sufficiently long periods of time as described in Chapter III.

Most of the organic solvents and mixtures of solvents, tested as organic phases in combination with hydrochloric acid for the hydrolysis of extracts from tubers, leaves and fruits spiked with α-solanine, solasodine glycosides and/or α-tomatine, resulted in low recoveries. This was due either to improper partition coefficients, which often caused degradation of SAs, or to the formation of an SA absorbing emulsion between the organic and the acid phases, or to problems with respect to the solubility of the glycoalkaloids or aglycones. Only the chlorinated hydrocarbons yielded acceptable or good recoveries. Therefore, these phases were further tested using various hydrochloric acid concentrations and various hydrolysis durations. The optimum two-phase system for the hydrolysis of SGAs in extracts from different types of plant material consisted of chloroform and hydrochloric acid (4 mol/l) and a hydrolysis duration of 5 h. Subsequently, this system was used

Table 1. Average recoveries (n = 2 or 3) of steroidal glycoalkaloids (SGAs) from spiked homogenates of samples of Solanum and Lycopersicon species, determined by capillary GC after bisolvent extraction and two-phase hydrolysis for 5 h in chloroform/hydrochloric acid 4 mol/l (1:1 v/v).

Sample	Average tuber/fruit weight (g)	Content of SGA[1] (mg/kg fresh wt)	Recovery of SGA added (%)		
			α-Solanine	Solasodine	α-Tomatine
S. tuberosum ssp. andigena tubers	26	SNG 940	93	88	82
S. brevicaule tubers	6	SNG 1422	93		92
S. tuberosum 'Pimpernel' tubers	105	SNG 102	89		92
S. venturii tubers	6	SNG 1837	94	89	84
S. brevicaule leaves		SNG 955	97	95	90
S. venturii leaves		SNG 2500	99		94
L. esculentum 'Allround' fruits	112	TMG 7	98		91
L. hirsutum glabratum fruits	12	TMG 3140	93		93
S. nigrum green fruits (berries)	1	SSG 1239			96
Control, standard SGAs			91	97	89

[1]SNG = solanidine glycosides, TMG = tomatidine glycosides, SSG = solasodine glycosides.

for determining the recoveries of α-solanine, solasodine glycosides and α-tomatine, added to homogenates of a variety of Solanaceous samples. A minor disadvantage of using chloroform as organic phase was that the unsaturated SGAs yielded two SA peaks, one representing the corresponding aglycone and a second one representing the dehydrated aglycone (ratio 1:3). The contents and the recoveries of the unsaturated SGAs of the samples listed in Table 1 were therefore assessed by measuring the areas of both peaks. Although the recoveries (Table 1) were lower than those obtained in the study using tubers of potato cultivars only (Chapter III) they were still good, especially when one considers the diversity of the samples used in the present study, and the low recoveries which had to be accepted by other authors using acid hydrolysis in studies of household potato cultivars (see Chapter III). The results also indicated that the precision of the method was better than that of the methods applying conventional acid hydrolysis, as the differences between duplicates or triplicates were always within 6% of the averages.

In the presently developed procedure for sample preparation, certain steps for the purification of SGA extracts reported in the literature, which may cause considerable losses of SGAs, were circumvented. Precipitation of proteins, in order to prevent their interference with quantification of SGAs, as carried out by Rooke et al. (1943), Schwarze (1962 and 1963), Ross et al. (1978), Blincow et al. (1982) and by Deahl and Sinden (1987) should be avoided, because it results in losses due to coprecipitation of SGAs. Baker et al. (1955) reported such losses of solanidine glycosides up to 20%, and Olsson (1986) of about 10%, of the total SGA content of potatoes. Slijm and Weinans (1973) found 25-80% of the solanidine glycosides in the heat-precipitated tuber proteins of six potato cultivars that varied considerably in their contents of proteins and of SGAs. During an earlier evaluation of literature methods for determining the contents of solanidine glycosides in potato tubers, losses of 15-35% were found to be due to coprecipitation of SGAs and proteins.

A considerable experimental error will occur when SGAs with different aglycones are present in a sample and the purification and concentration of the SGAs is performed by alkaline precipitation at pH 9.4-10 and at 70°C. Under these conditions the alkali soluble leptines

are totally and demissine is partly lost (Kuhn and Löw, 1961; Gregory et al., 1981; Sinden et al., 1986), whilst the solanidine glycosides are quantitatively precipitated (Sachse and Bachmann, 1969).

When high concentrations of SGAs are present, such as in wild Solanaceous species, losses may occur due to interactions between SGAs and other compounds present (Osman, 1983); especially complex formation between SGAs and sterols must be taken into consideration (Heftmann, 1967; Roddick, 1974, 1979 and 1980). The solubility of such complexes, for instance those of a tomatine-sterol and a solanine-sterol complex, varied considerably. However, aglycones do not form complexes with sterols (Arneson and Durbin, 1968; Roddick, 1979 and 1980).

In the present procedure, bisolvent extraction is applied so that proteins are not extracted and sterols are eliminated, preventing these compounds to interfere with the SGA clean-up, and instead of precipitating the SGAs at pH 10, they are hydrolysed in a two-phase system after which the aglycones are collected in the organic phase; as a result the above-mentioned losses are prevented.

The precision of the present method was further tested using samples of a leaf homogenate of S. brevicaule and of tuber homogenates of S. brevicaule and S. venturii. The average contents of solanidine glycosides of the samples were 955, 1374 and 1831 mg/kg fresh weight, respectively, and the standard deviations were 5.2% (n = 7), 3.1% (n = 8) and 3.0% (n = 8), respectively.

From a number of tuber samples, the contents of solanidine glycosides were determined by the present method as well as by a colorimetric reference method; they ranged from 35-1310 mg/kg. Fig. 1 shows the correlation between both methods; the coefficient of correlation, r, was 0.98 (n = 30) and the linear regression equation was

$$y = 0.80x - 1.14$$

where y = the colorimetric reference method and x = the capillary GC method. These results agreed with those of a preliminary comparative study using ten genotypes with SGA contents ranging from 20-470 mg/kg (Chapter IV).

Comparison of the capillary GC method with reference methods with respect to quantification of solasodine and tomatidine were not made, because these SAs can be quantified equally well as solanidine, as appeared from the GC calibration plots, and because the recoveries of

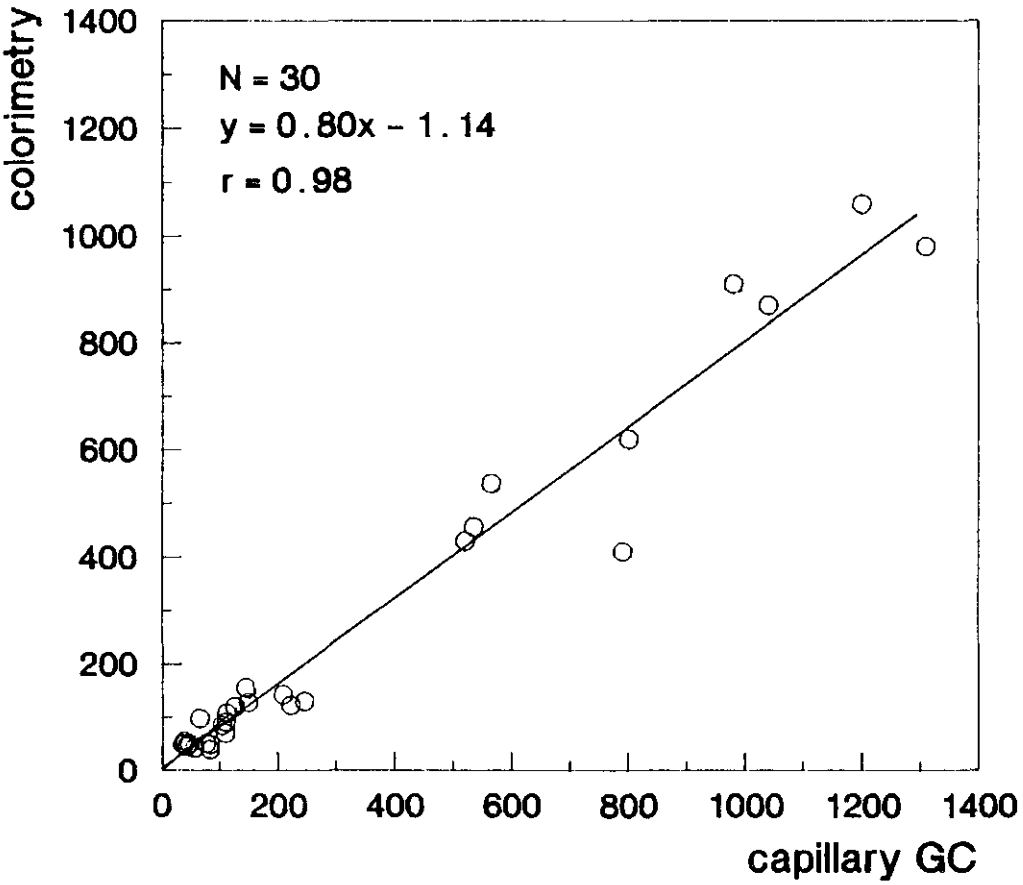


Fig. 1. Contents of solanidine glycosides (mg/kg fresh weight) in tubers of potato cultivars, wild Solanum species and hybrids determined by capillary GC and a colorimetric reference method.

the corresponding SGAs were all similar. Besides, adequate sets of samples containing these SGAs, which were needed for such a comparison, were not available.

The contents of solanidine glycosides of individual potato tubers can vary considerably (Chapter VII), showing coefficients of variation up to 60% (Ross et al., 1978). Analysis of individual tubers of two potato cultivars indicated that for obtaining a representative value for a batch of a given genotype, samples should preferably consist of at least 15 tubers (Van Gelder, 1987). However, in many studies referred to in Chapter I, samples consisted of 4-6 tubers only. It is thus advisable that before the capillary GC method is applied for

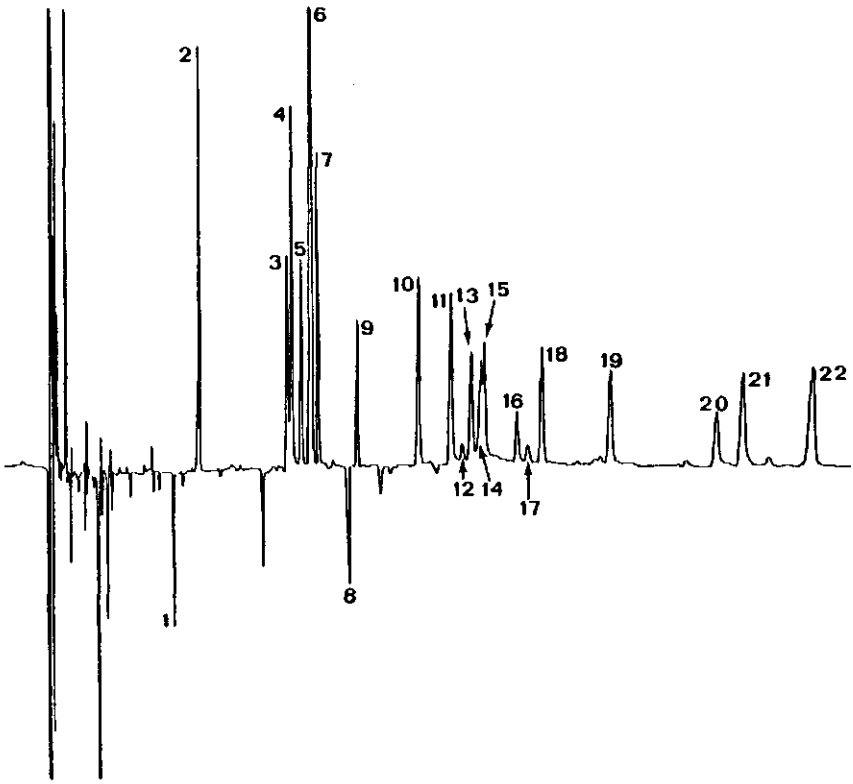


Fig. 2. Chromatogram of a potato tuber extract spiked with steroidal alkaloids, obtained by post-analysis reprocessing of FID and NPD data from capillary GC. Peak numbers and GC conditions as in Table 2.

studying genetic variation or the inheritance of individual SGAs, or for selection purposes, the minimum sample size (number of tubers) is assessed. The same applies to analysis of other plant parts.

Separation and identification of SAs

Fig. 2 shows the result of post-analysis reprocessing of the simultaneously obtained NPD and FID data after GC of a potato tuber extract spiked with SAs on a CP-Sil 5 CB column, 50 m x 0.22 mm I.D., with a film thickness of 0.12 μm. The separation efficiency towards this complex SA mixture was very good; a similar separation has not been reported before. Separation of the mixture using the same column type with a film thickness of 0.4 μm, further improved the resolution, but then the retention times were longer: t_R's for the last-eluting peak

Table 2. Retention indices, I, and NPD/FID response ratios of steroidal alkaloids from Solanaceae and Liliaceae determined by capillary GC[1].

Peak number[2]	Compound	I	NPD/FID response ratio
1	5α-Cholestane[3]	2867	-
2	Solanthrene	2943	2.98
3	Solanidine	3130	2.96
4	Demissidine	3137	3.04
5	Solasodiene	3152	3.01
6	Demissidan-3-one	3167	3.05
7	Tomatidadiene	3172	3.02
8	Diosgenin[3]	3221	-
9	Solanid-4-en-3-one	3232	2.96
10	Rubijervine	3305	3.01
11	Solasodine	3338	3.00
12	Soladulcidine	3349	3.03
13	Tomatidenol	3361	2.93
14	Tomatidine	3370	2.88
15	Isorubijervine	3374	3.00
16	Tomatidan-3-one	3400	2.99
17	Solanocapsine	3409	3.80
18	Cyclopamine	3422	3.20
19	Veratramine	3475	3.13
20	Deacetylmuldamine	3544	3.26
21	Jervine	3560	3.35
22	N-acetyltomatidine	3598	2.60

[1]Column: fused silica, 50 m x 0.22 mm I.D., CP-Sil 5 CB, film thickness 0.12 μm. Oven temperature: 270°C.

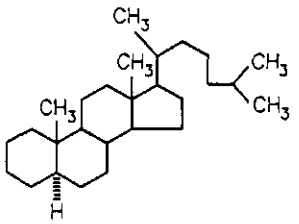
[2]Peak numbers correspond with those of Fig. 2; structural formulas are given in Fig. 3 and in Chapter I, Fig. 1.

[3]Reference compound.

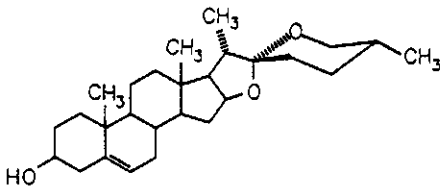
(N-acetyltomatidine) were 29.85 min on 0.12 μm and 84.5 min on 0.4 μm, respectively. Such a column can be useful when an extremely high resolution is required. This resolution is about the ultimate which can presently be achieved, as application of the same column type with a film thickness of 1.2 μm did not result in a further increase but in a decrease of the resolution and, in adddition, in extremely long retention times.

Retention indices and NPD/FID response ratios are useful for the identification and characterization of SAs (Chapter V and VII). Table 2 shows these indices and ratios determined for a large number of SAs. Because the structural relationship between diosgenin and 5α-cholestane and the SAs is favourable with respect to the accuracy of the identification and quantification, these steroidal compounds were used as reference compounds instead of n-octacosane and n-tetratriacontane used before. All the solanidane and spirosolane type SAs, which are commonly found in tuber-bearing Solanum species (see Chapter I, Fig 1 and Table 1), showed normalized NPD/FID response ratios of about 3. SAs which are structurally different with respect to the E-ring or to the N-containing F-ring, especially N-acetyltomatidine, jervine and cyclopamine (Fig. 3), showed somewhat deviating NPD/FID response ratios. The latter two compounds and also the Veratrum SAs veratramine and deacetylmuldamine have not (yet) been found in Solanum and Lycopersicon species. The high value of solanocapsine is due to the presence of two nitrogen atoms.

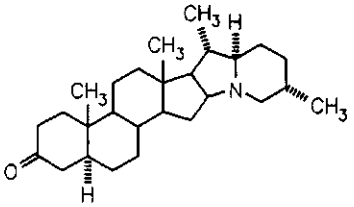
There are a number of N-containing plant compounds which potentially could be coextracted with the SAs and hence could interfere with their identification. However, compounds of this nature have not been detected so far. This is due to the current procedure for sample preparation, which is rather specific for the SAs because the bisolvent extraction and two-phase hydrolysis exploit the difference in polarity between the glycoalkaloids and their aglycones. Moreover, the SAs require high temperatures for their elution during capillary GC, so most compounds present together with the SAs were found to pre-elute, and simultaneous NPD and FID unambiguously differentiate between SAs and steroidal sapogenins, which might have been (partly) isolated together with the SAs and which may have similar retention times (Fig. 2).



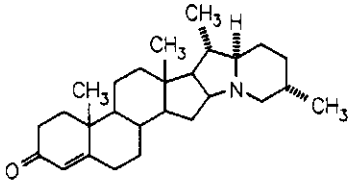
5α-cholestane $C_{27}H_{48}$



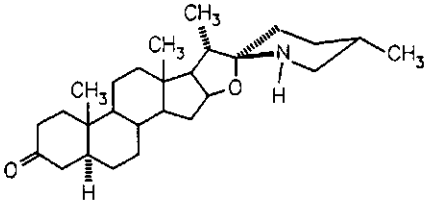
Diosgenin $C_{27}H_{42}O_3$



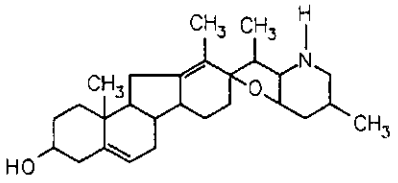
Demissidan-3-one $C_{27}H_{43}NO$
(Solanidan-3-one)



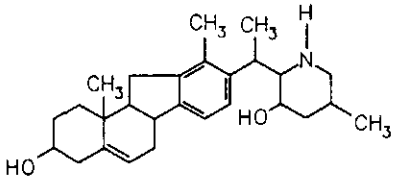
Solanid-4- en-3-one $C_{27}H_{41}NO$



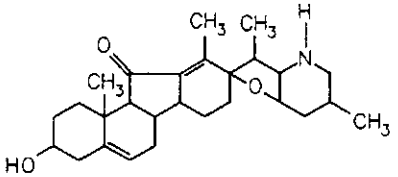
Tomatidan-3-one $C_{27}H_{43}NO_2$



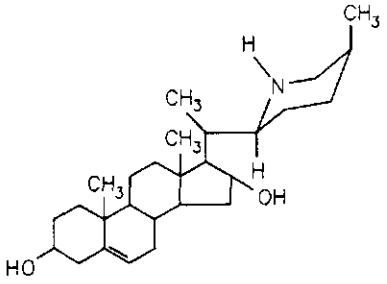
Cyclopamine $C_{27}H_{41}NO_2$



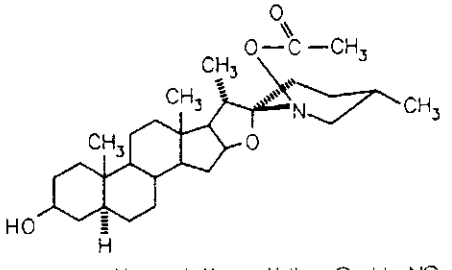
Veratramine $C_{27}H_{38}NO_2$



Jervine $C_{27}H_{39}NO_3$



Deacetylmuldamine $C_{27}H_{45}NO_2$



N-acetyltomatidine $C_{29}H_{47}NO_4$

Fig. 3. Structural formulas of 5α-cholestane, diosgenin and steroidal alkaloids.

In conlusion, the above results showed that the method described in this thesis, is useful for qualitative and quantitative analysis of the SAs in diverse samples of Solanaceous plant material.

When wild Solanum species are utilised in potato breeding programmes, it is advisable to make a qualitative and quantitative inventory of the SAs present in the potential crossing parents. For this analysis, a carefully developed procedure for sample preparation followed by capillary GC should be applied, because other procedures for sample preparation, reported in the literature, can lead to losses of various SGAs or SAs, and other analytical techniques do not offer the separation efficiency which can be achieved to date by capillary GC.

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CHAPTER VII*

GLYCOSIDIC-BOUND STEROIDAL ALKALOIDS IN TUBERS AND LEAVES OF SOLANUM SPECIES USED IN POTATO BREEDING

INTRODUCTION

In most studies on SAs or SGAs present in Solanum species, aerial plant parts have been analysed, usually for compounds of pharmaceutical interest (Petroshenko, 1956; Schreiber et al., 1961; Schreiber, 1963; Bognár and Makleit, 1965; Schreiber, 1968; Bradley et al., 1978; Weiler et al., 1980; Ripperger and Schreiber, 1981). Most attention has been paid to solasodine, which is a raw material for the industrial production of steroid hormones. Only few and limited surveys on the SA or SGA compositions of tubers of Solanum species have been presented (Osman et al., 1978; Van Gelder and Jonker, 1986; Johns and Osman, 1986). In these studies, only the main SAs or SGAs have been detected and/or quantified.

In this chapter, the entire composition of glycosidic-bound SAs, including the minor SAs, of tubers and in some cases of leaves of a number of Solanum species which are being used in potato breeding, are reported.

EXPERIMENTAL

Plant material

The species studied were: S. acaule Bitt., S. berthaultii Hawkes, S. brevicaule Bitt., S. bukasovii Juz., S. canasense Hawkes, S. gourlayi Hawkes, S. leptophyes Bitt., S. multidissectum Hawkes, S. oplocense Hawkes, S. sparsipilum (Bitt.) Juz. et Buk., S. spegazzinii Bitt., S. sucrense Hawkes, S. venturii Hawkes et Hjerting and S. vernei Bitt. et

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*This chapter is based on the paper:
Van Gelder WMJ, Vinke JH, Scheffer JJC. (1988) Steroidal glycoalkaloids in tubers and leaves of Solanum species used in potato breeding. Euphytica 39S:147-158.

Wittm. The taxonomic nomenclature is according to Hawkes (1978). Clones of these species were derived from accessions of the Dutch-German potato collection at Braunschweig Genetic Resources Centre (BGRC, Braunschweig, F.R.G.), except for S. berthaultii of which the provenance is not known. The first number of the accession numbers mentioned in this study, corresponds to the BGRC accession number (Hoekstra and Seidewitz, 1987), the second number refers to the clone number. The plant material to be analysed was produced in a glasshouse under controlled conditions, which are routinely used at the Foundation for Agricultural Plant Breeding SVP to induce tuber formation in wild species. The growing period was from mid March to mid August. The day-length was 12 h from 6.00 h to 18.00 h; it was controlled by an automated PVC awning, which covered and darkened the growing area of the plants completely. In 1985, the sums of solar radiation (350-3000 nm) were for March (two weeks), April, May, June, July and August (two weeks): 11, 36, 48, 46, 51 and 23 kJ/cm^2, respectively, and for the same period in 1986: 11, 26, 59, 56, 59 and 24 kJ/cm^2, respectively. The temperature was kept at ca 18°C from 7.30 h to 18.00 h and at ca 13°C from 18.00 h to 7.30 h. The relative air humidity was kept at ca 60-70%. If necessary for keeping the temperature at 18°C, the glasshouse was somewhat shaded on hot bright days. In 1985, the plants were grown in earthenware pots of 11 cm diameter for propagation of tubers as part of a breeding programme; in 1986 the plants were especially grown for SA analysis, for which plastic pots of 18 cm diameter were used. To enable a comparison of the contents of solanidine glycosides in the small tubers of the wild species with those of cultivars, plants of 'Bintje', 'Pimpernel' and of the SVP progenitors AM 78-3778 and AM 78-3787 ('Arabesque') were grown under the same conditions as those of the wild species in the glasshouse in 1986. After harvesting, the tubers were stored in the dark at 5-7°C for six weeks, before they were analysed.

Chemicals and procedures

Standard SAs, solvents and solutions for extraction and hydrolysis, sample preparation, capillary GC, and identification and characterization of SAs by retention indices and simultaneous NPD and FID were as described in the Chapters V and VI. The compounds 5α-cholestane and

diosgenin were used as reference compounds. The NPD/FID response ratios were calculated from the peak areas, but when integration of small overlapping peaks was inaccurate, the peak heights were measured. Contents of the individual SGAs were calculated from the GC peak areas of the corresponding aglycones (and also of their Δ3,Δ5-dehydration products in the case of Δ5-unsaturated aglycones) and were expressed on the basis of solatriose in the case of solanidine, solasodine and unidentified SAs, and of lycotetraose in the case of demissidine and tomatidine. The contents of the solanidine glycosides of the field-grown tubers of the cultivars were determined using the colorimetric method described in Chapter IV.

RESULTS AND DISCUSSION

The SGA content of small tubers

The wild species produced tubers varying in fresh weight from 0.2-15 g. Such small sizes might influence the SGA contents of the tubers. In order to get an impression of the magnitude of this effect, tubers corresponding in small size to those of the wild species were selected from the glasshouse-grown tubers of the four cultivars. For each cultivar, the contents of the solanidine glycosides of seven individual tubers were determined. The results are shown in Table 1. The "normal" contents of the cultivars are given as well. These figures are average values determined for batches of 20 field-grown mature tubers collected over at least three years at two locations. The contents of solanidine glycosides in the glasshouse-grown small tubers were two to three times higher than those of the field-grown normal tubers. Within a cultivar, the smallest tubers showed, in general, the highest contents. The ranking order of the cultivars as to their contents was equal for both environments. These data might be useful for placing the SGA contents of the exotic tubers in a more realistic perspective. However, as small tubers of wild species can be mature and such tubers of cultivars are usually immature, wild species may show a different (less strong) correlation between tuber size and SGA content compared to the cultivated potato. Therefore, further studies on the relation between the SGA content and the size and maturity of tubers should be carried out.

Table 1. Fresh weight (g) and content of solanidine glycosides (mg/kg fresh weight) of seven individually analysed small tubers of the cultivars Bintje, AM 78-3778, AM 78-3787 ('Arabesque'), and Pimpernel, grown in pots in a glasshouse. In comparison, the contents of field-grown mature-harvested tubers are given[1].

Cultivar		Tuber fresh weight		Solanidine glycosides	
		Range	Average	Range	Average
Bintje	glasshouse	1.0-15	8.2	73- 188	126
	field		139		40
AM 78-3778	glasshouse	0.2-15	5.7	321-1484	721
	field		140		360
AM 78-3787	glasshouse	3.9-12	7.1	95- 265	155
	field		152		58
Pimpernel	glasshouse	0.3-17	8.6	132-1287	522
	field		122		146

[1]Average values determined for samples of 20 tubers collected over at least three years at two locations.

The SGA composition of Solanum species

Table 2 shows the SA compositions, expressed as glycoalkaloids, of the tubers (T) and in some cases of the leaves (L) of a number of Solanum species used in potato breeding. Until now, such compositions have not been reported for the species S. brevicaule, S. bukasovii, S. canasense, S. gourlayi, S. leptophyes, S. multidissectum, S. oplocense, S. sparsipilum, S. spegazzinii and S. venturii.

Schreiber et al. (1961) reported for the foliage of S. berthaultii a content of 12000 mg solanidine glycosides per kg dry weight. Gregory et al. (1981) found solasodine glycosides in a concentration of 1000 mg/kg dry foliage in this species. The S. berthaultii accession we analysed

contained 1090 mg solasodine glycosides per kg fresh weight, which corresponded to about 8000 mg/kg dry foliage (Fig. 1A). We could not detect any solanidine, not even when the detection limit was as low as 0.2 mg/kg. In the tubers of S. berthaultii, for which the SA composition has not been reported before, we found solanidine but no solasodine glycosides (Fig. 1B). Small amounts of other compounds were present in the foliage as well as in the tubers. Several of these are most probably SAs as is indicated by the NPD/FID response ratios which are shown at the peak tops in the figures. Superimposition of the chromatograms of the leaf and tuber samples and comparison of the NPD/FID response ratios, showed that none of the leaf SAs corresponded to any of the tuber SAs, possibly except for the compound which eluted after 11.00 min. MS may show whether these two peaks correspond to the same compound.

Grafting experiments using combinations of species from Solanum and other genera of Solanaceae, have suggested that the SAs in the leaves are synthesized independently from the SAs in the root system (Petroshenko, 1956), and that no translocation of SAs from the roots to the leaves occurs. Our data obtained for S. berthaultii indicated that the SAs in the leaves are synthesized also independently from the SA metabolism in the tubers (stem system) and vice versa, which means that the SA synthesis in S. berthaultii is organ specific or regulated by light.

The literature on the SA composition of S. acaule is contradictory as to the presence of demissidine and tomatidine glycosides (for details see Gregory, 1984). Both our present and earlier analyses (Van Gelder and Jonker, 1986) showed that in the accessions studied, demissidine glycosides as well as tomatidine glycosides were always present, but we also found that in addition solanidine glycosides can be present.

The discrepancies between the compositions described for Solanum species by different authors may be due to intraspecific heterogeneity and/or differences in the growing conditions applied, but probably, imperfection of the analytical techniques, with respect to the identification accuracy and detection limits, has played an important role.

Table 2. Composition of glycosidic-bound steroidal alkaloids of tubers (T) and leaves (L) of Solanum species used in potato breeding.

Solanum species	Accession	Year	Plant organ	Solanidine glycosides[1]	Demissidine glycosides[1]	Solasodine glycosides[1]	Tomatidine glycosides[1]	Unidentified compounds	
								Number[2]	Total amount[1]
S. acaule	1028	1985	T	15	152	-	75	-	-
	15466-1	1985	T	-	115	-	8	-	-
	15466-2	1985	T	-	503	-	350	-	-
S. berthaultii	VS2A	1985	T	160	-	-	-	9	140
	VS2A	1985	L[4]	-	-	1090	-	9	55
S. brevicaule	18291-4	1986	T	1758	-	-	-	9	150
	18291-27	1986	T	1374	-	-	-	2	Tr[3]
	18291-27	1986	L[5]	955	-	-	-	4	Tr
S. bukasovii	1033-5	1985	T	840	-	-	-	-	-
	1033-6	1985	T	920	-	-	-	-	-
	1033-20	1985	T	700	-	-	-	-	-
S. canasense	1038-5	1985	T	322	-	-	-	4	Tr
	1038-M	1985	T	363	-	-	-	3	Tr
S. gourlayi	25257-3	1986	T	1130	-	-	-	-	-
	25257-6	1986	T	1634	-	-	-	-	-
S. leptophyes	1029-17	1985	T	718	-	-	-	6	Tr
	1029-39	1985	T	778	-	-	-	5	Tr
	27208-12	1986	T	1210	-	-	-	-	-
	27208-27	1986	T	1355	-	-	-	-	-
S. multidissectum	1031-2	1985	T	360	-	-	-	12	55
S. oplocense	5507-290	1986	T	599	-	-	-	-	-
	24661-29	1986	T	400	-	-	-	-	-
S. sparsipilum	1040-1	1985	T	824	-	-	-	8	75
	1040-3	1985	T	482	-	-	-	6	60
	8206-7	1985	T	255	-	-	-	16	950

S. sparsipilum	8206-11	1985	T	325	-	-	-	17	365
	8206-29	1985	T	442	-	-	-	15	850
	27060-14	1985	T	332	-	-	-	15	270
	27060-34	1985	T	258	-	-	-	16	300
	8206-15	1986	T	2780	-	-	-	5	Tr
S. spegazzinii	8218-16	1985	T	171	-	-	-	16	700
	8218-21	1985	T	316	-	-	-	15	660
	8218-25	1985	T	169	-	-	-	9	480
	8219-8	1985	T	849	-	-	-	12	230
	15458-23	1985	T	407	-	-	-	6	30
	15458-26	1985	T	319	-	-	-	9	350
	8218-4	1986	T	1758	-	-	-	4	Tr
	15456-15	1986	T	1672	-	-	-	4	Tr
	15456-25	1986	T	2207	-	-	-	4	Tr
	15458-17	1986	T	919	-	-	-	4	Tr
	15458-21	1986	T	952	-	-	-	4	Tr
S. sucrense	8153-23	1986	T	772	-	-	-	2	Tr
	27275-42	1986	T	1562	-	-	-	3	Tr
S. venturii	24720	1985	T	1380	-	-	-	-	-
	24720	1986	T	1618	-	-	-	-	-
	24720	1986	L[6]	2501	-	-	-	14	Tr
S. vernei	8240-10	1986	T	6093	-	675	-	4	580
	15451-12	1986	T	4711	-	1590	-	6	410
	15451-28	1986	T	2742	-	3483	-	6	800
	15451-12	1986	L[7]	-	-	2452	-	1	Tr

[1]Contents in mg/kg fresh weight.
[2]Number of unidentified compounds with NPD/FID response ratios corresponding to steroidal alkaloids and expressed as glycoalkaloids on a trisaccharide basis.
[3]Tr = traces (< 2.5 mg/kg).
[4-7]Dry matter contents of the leave samples 13.6 %, 14.1 %, 14.0 %, 17.6 %, respectively.

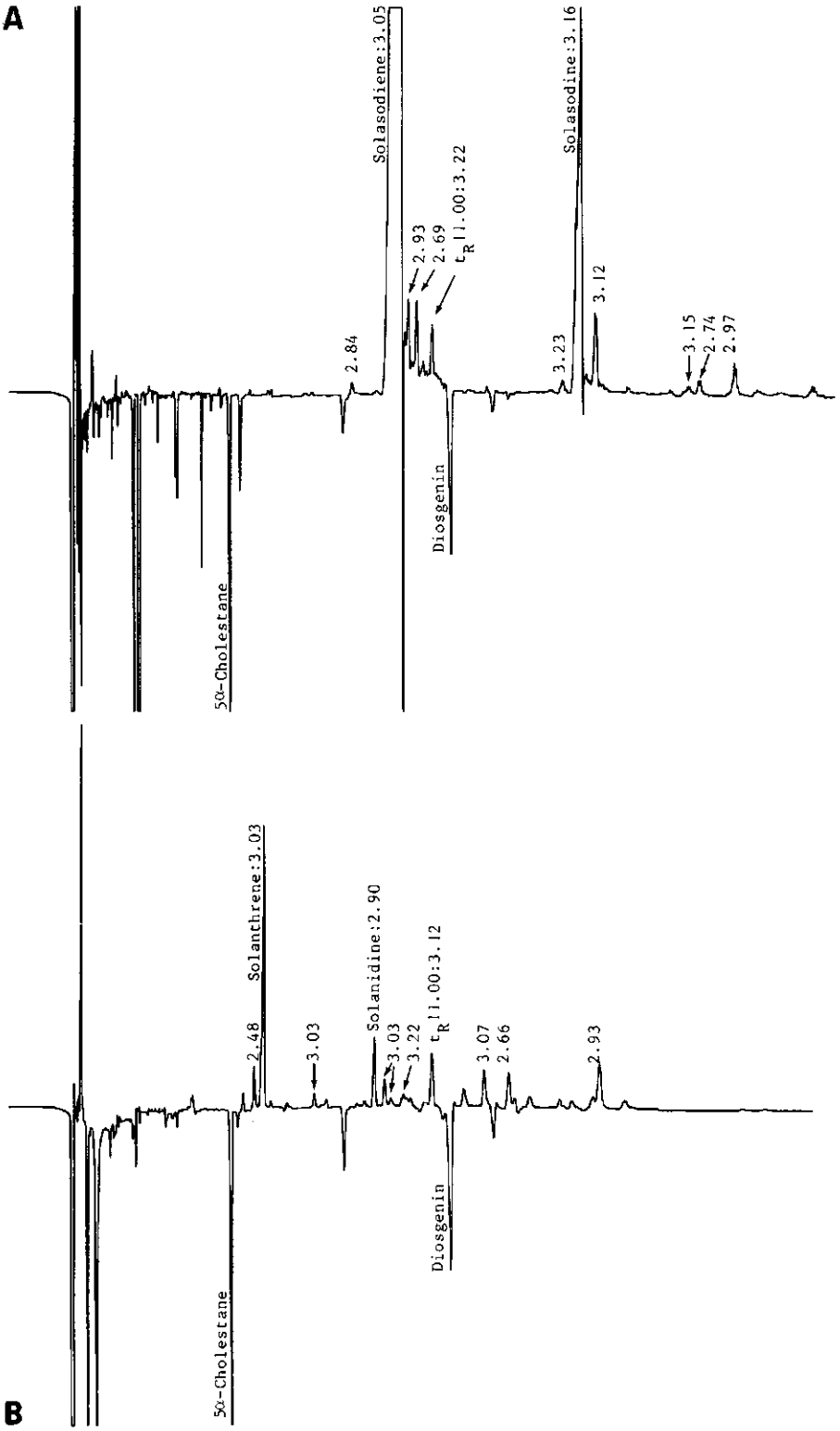
A
Solasodiene:3.05
Solasodine:3.16
2.93
2.69
t_R 1.00:3.22
2.84
3.23
3.12
3.15
2.74
2.97
Diosgenin
5α-Cholestane
B
Solanthrene:3.03
2.48
3.03
Solanidine:2.90
3.03
3.22
t_R 1.00:3.12
3.07
2.66
2.93
Diosgenin
5α-Cholestane

Fig. 1. Steroidal alkaloid composition of leaves (A) and tubers (B) of S. berthaultii shown by post-analysis reprocessing of NPD and FID data simultaneously obtained by capillary GC. Column: fused silica, 50 m x 0.22 mm I.D., CP-Sil 5 CB, film thickness 0.12 μm. Carrier gas: hydrogen, linear velocity 50 cm/s. Oven temperature: 270°C. Injection volume: 1.4 μl. Reference compounds: α-cholestane and diosgenin. Positive peaks represent nitrogen-containing compounds with NPD/FID response ratios as shown at the peak tops, negative peaks represent nonnitrogen-containing compounds.

For S. vernei, 2000-5000 mg solanidine glycosides per kg dry weight have been reported for foliage samples (Schreiber, 1963). Although more data on the SAs of S. vernei have not been reported since then, this species has been widely used in potato breeding (Ross, 1986), for example as a source of resistance to the potato cyst nematode. Probably, it was assumed that the data reported by Schreiber would be valid in general for foliage as well as tubers of all S. vernei accessions used in breeding programmes (cf. Holden, 1981). Our analyses showed that the leaves and tubers of clones from S. vernei accession 15451, contained high levels of solasodine glycosides (Fig. 2A and B). This is the first time solasodine is reported to occur in tubers of wild Solanum species. Solasodine has been reported to possess a weak teratogenic activity in hamsters, but the activity of the solasodine glycosides is not known (see Chapter II). It is therefore advisable to study whether these SGAs are synthesized in tubers of hybrid offspring.

In addition to the solasodine glycosides, only one other SA was present in trace amounts in the leaves of S. vernei, whereas the tubers contained high levels of solanidine glycosides and considerable amounts of unidentified compounds. The NPD/FID response ratios indicated that the unidentified peaks represented SAs. The tubers of the accessions 15451 (Fig. 2B) and 8240 (Fig. 3A) showed different SA compositions, especially with respect to some of the major unidentified compounds. This indicates that intraspecific variation exists.

S. sparsipilum accession 8206 showed remarkable differences between the SA composition of clone 7, grown in 1985, and that of clone 15, grown in 1986. (Table 2 and Fig. 4A and B). The origin of these differences is not clear. It is not likely from the viewpoint of the

Fig. 2. Steroidal alkaloid composition of leaves (A) and tubers (B) of S. vernei 15451-12. Experimental conditions as in Fig. 1.

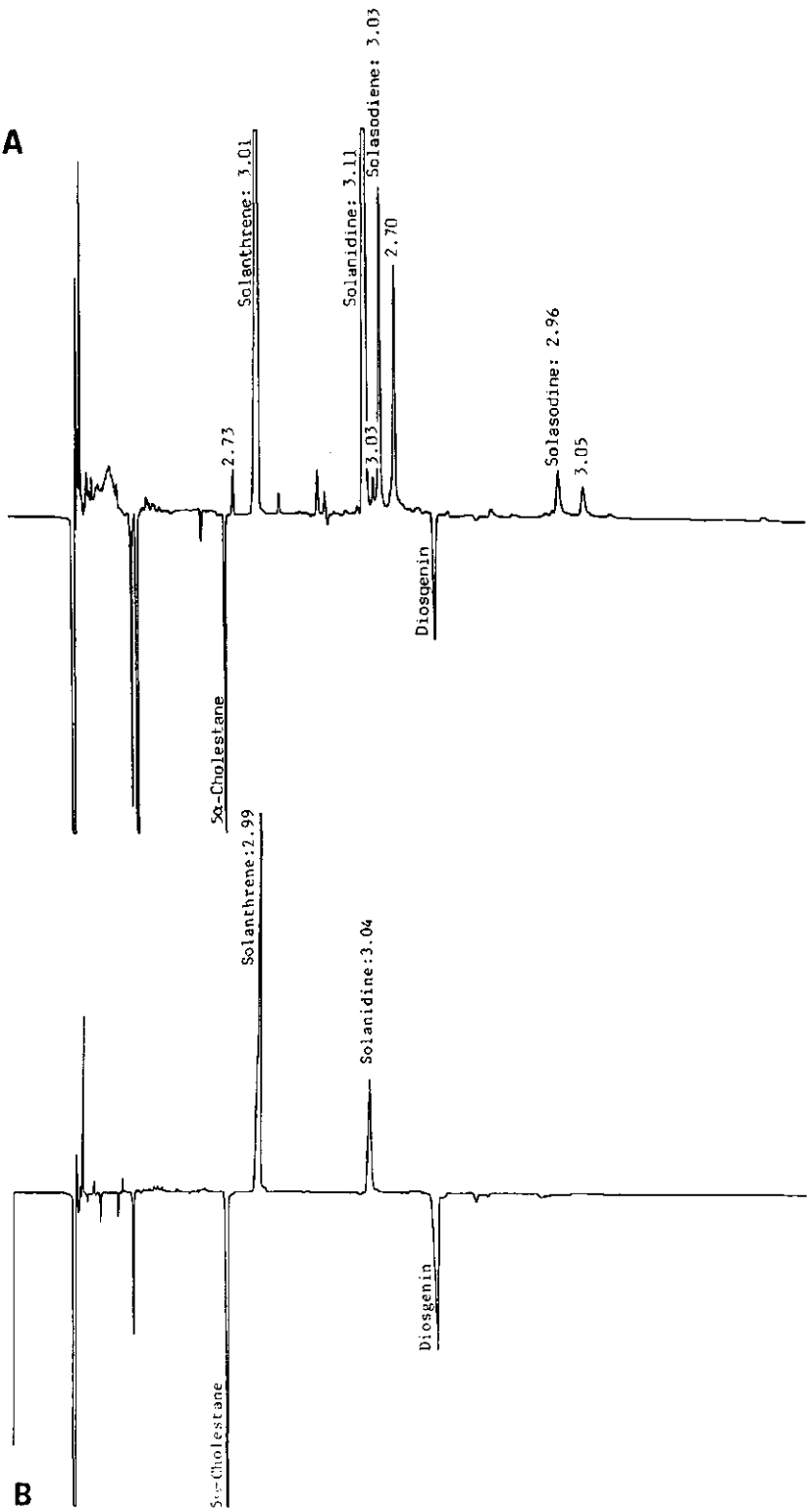

Fig. 3. Steroidal alkaloid composition of tubers of S. vernei 8240-10 (A) and S. bukasovii 1033-20 (B). Experimental conditions as in Fig. 1.

Fig. 4. Steroidal alkaloid composition of tubers of S. sparsipilum 8206-7 grown in 1985 (A) and 8206-15 grown in 1986 (B).

biogenesis of SAs, that such differences within accessions are genetically determined. On the other hand, it is not unlikely that the growing conditions may influence the SA composition. In 1985, a low solanidine content was attended by the presence of a relatively large number of unidentified SAs. The small earthenware pots used for growing the plants in 1985 might have induced some stress which could have been (partly) responsible for this. The same was true for S. spegazzinii, but the phenomenon was not observed for tubers of other species such as S. bukasovii (Fig. 3B) and S. venturii, grown in 1985 under the same conditions. Anyhow, the occurrence of markedly different SA compositions within accessions is most interesting. Further studies on the structure of the unidentified compounds may possibly yield information on their nature.

Except for S. acaule, the tubers of all species studied contained solanidine glycosides as main components. S. acaule belongs to the series Acaulia, whilst the other species belong to the series Tuberosa. In most tuber samples, unidentified compounds were detected, which most likely were SAs as was shown by their NPD/FID response ratios. Such compounds, which were later characterized as SAs using GC-MS, had been found earlier in foliage of S. vernei offspring (Van Gelder et al., 1987) and in fruits of Lycopersicon species (Van Gelder and De Ponti, 1987). Based on the procedure for their isolation, it can be concluded that such SAs occurred in the leaves and tubers as glycosides (see Chapter VI). In the presently analysed tubers, the individual concentrations of the unidentified SAs, expressed as glycoalkaloids, varied from less than 2.5 mg/kg (traces) to more than 200 mg/kg, and the total amounts varied from about 2.5-950 mg/kg.

Most of the species showed high total SGA contents. Under the growing conditions applied, all species produced small tubers, which might have been partly responsible for their high contents, as shown above. However, even when this is taken into consideration, several species showed total contents which were extremely high. Thus, analysis of the hybrid progeny of such species is necessary.

The SVP progenitors AM 78-3778 and AM 78-3787 ('Arabesque') were both derived from S. vernei accessions by backcrossing with S. tuberosum. Both genotypes showed a high level of resistance to potato cyst nematodes (Dellaert and Vinke, 1987). The clone AM 78-3787 which com-

bines a high level of nematode resistance with a low content of SGAs, had been selected in spite of the extremely high SGA contents normally found in S. vernei. However, in the course of the breeding programme, many hybrids were discarded because of their high SGA contents. In the tubers of 'AM 78-3787' which were analysed for their SA compositions, glycosidic-bound solanidine was found. In the leaves, solasodine and an unidentified compound, which was shown by GC-MS to be possibly an isomer of solanidine, were also present (Van Gelder et al., 1987). We did not yet investigate whether these foliar SAs are organ specific, or whether they may be synthesized in the tubers, dependent on the environmental conditions.

Until now studies on the composition of glycosidic-bound SAs of Solanum species have been carried out from a phytochemical viewpoint, in which genetic and environmental aspects have been disregarded. Our results show that studies on the inheritance of the various SAs, on the inter- and intraspecific variation in SA compositions, and on the influence of the environment on the SA composition, will be useful for efficiently utilizing wild germplasm. Intraspecific variation may be used profitably by selecting crossing parents which combine a desired trait with the least unfavourable SA composition.

In conclusion it can be stated that high concentrations of solanidine glycosides and a number of other SGAs, can be present in wild Solanum species. As in some cases the SGA composition varied within a species or between different organs of one plant, the limited data available in the literature can not be used as a general guide in potato breeding. It is therefore recommended to analyse the SAs of wild crossing parents, and to identify or characterize the unknown SAs, before they are used in a breeding programme. Those genotypes, that combine a desired trait with the least unfavourable SA composition, can then be selected as crossing parents. When species showing extremely high levels of SGAs or containing SAs which should not be allowed in potato tubers, are used in breeding programmes, the hybrid progeny must also be analysed. Studies on the effect of tuber size and maturity on the SGA contents of wild species, cultivars and their hybrids, will show whether it is possible to select for a low SGA content in an early stage of a breeding programme.

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CHAPTER VIII*

CHARACTERIZATION OF NOVEL STEROIDAL ALKALOIDS FROM TUBERS OF SOLANUM SPECIES BY COMBINED GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

INTRODUCTION

Tubers of Solanum species contained, in addition to known glycosidic-bound SAs, unidentified compounds which were tentatively characterized as SAs by their NPD/FID response ratios determined by capillary GC. Based on the procedure for their isolation it was concluded that these SAs originated also from glycosides (Chapter VII).

This chapter describes the characterization of such unidentified compounds using retention indices, GC-MS, high-resolution MS (HRMS) and hydrolysis in two-phase systems. By combining the data obtained by these techniques, a number of novel SAs were characterized. The SA composition (including main and minor SAs) of tubers of three wild Solanum species and one primitive cultivated subspecies, widely used in potato breeding, is presented. The implications of the results for potato breeding are discussed.

EXPERIMENTAL

Plant material

Tubers of Solanum chacoense Bitt. accession 8054, S. leptophyes Bitt. accession 27208, and S. tuberosum ssp. andigena (Juz. et Buk.) Hawkes accession 1024, were produced by cultural techniques described in Chapter VII for 1986, as were the taxonomic nomenclature and the provenance of the accession numbers. The analyses of S. sparsipilum (Bitt.) Juz. et Buk. were carried out on the extract from the tubers of acces-

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*This chapter is based on the paper:
Van Gelder WMJ, Tuinstra LGMTh, Van der Greef J, Scheffer JJC. (1989) Characterization of novel steroidal alkaloids from tubers of Solanum species by combined gas chromatography and mass spectrometry: implications for potato breeding. J Chromatogr, in press.

sion 8206 genotype number 7 grown in 1985, as described in Chapter VII.

Chemicals and sample preparation

Standard SAs, solvents, extraction of the tubers, two-phase hydrolysis, sample clean-up, and capillary GC using retention indices and NPD/FID response ratios, were as described in the Chapters V and VI.

GC-MS and HRMS

A Finnigan 4500 gas chromatograph-mass spectrometer coupled to an Incos data system was used to record the mass spectra of the SAs. The SAs were gas chromatographed using a fused-silica column, 25m x 0.22 mm I.D., CP-Sil 5 CB, film thickness 0.12 μm (Chrompack Nederland). The temperature programme of the oven was: 280°C (20 min), increase 8°C/min for 5 min, then 320°C for 5 min. Other GC conditions were: injector, 300°C; interface, 290°C; carrier gas, helium, linear velocity 30 cm/s; injection volume, 2 μl, splitting ratio 1:50. The conditions of electron impact ionization (EI) were: ion source temperature 230°C, emission current 0.25 mA, ion source energy 70 eV; those of chemical ionization (CI): ionization gas methane, ion source temperature 180°C, emission current 0.25 mA, ion source energy 100 eV, ionizer pressure 67 Pa. The mass range was monitored from 50 to 500 mass units in the EI mode and from 90 to 450 mass units in the CI mode. The scan rate was 1 scan/0.5 s.

The peaks of the total ion current (TIC) profile obtained by GC-MS were compared with the peaks of the mass chromatograms recorded at m/z 150 and m/z 204, which are specific for the solanidanes, and at m/z 114 and m/z 138, which are specific for the spirosolanes (Ripperger and Schreiber, 1981). For each tuber extract, the TIC profile and mass chromatograms were compared with the chromatogram of the N-containing compounds, obtained by capillary GC using simultaneous detection by NPD and FID, in order to investigate whether other types of SAs were present. Coelution of components was checked by comparing the TIC profile and mass chromatograms recorded at m/z 150 and m/z 204, with mass chromatograms recorded at specific m/z values.

HRMS was used for confirmation of the masses obtained by GC-MS, for determination of the exact masses of individual components and for calculation of their elemental compositions. A Finnigan MAT-711 mass spec-

trometer coupled to a Tracor TN-1750 multichannel analyser was used. The EI conditions were: ion source temperature 240°C, emission current 800 μA, ion source energy 100 eV, and acceleration voltage 8 kV. The resolution was 20000 (10% valley definition), the probe temperature was increased from 50°C to 350°C in 500 s, and the mass range was fixed. An amount of 1-5 μl of tuber extract was introduced into the mass spectrometer via a direct insertion probe. Only the tuber extracts of S. chacoense and S. sparsipilum were analysed by HRMS.

RESULTS AND DISCUSSION

Identification and characterization of SAs

HRMS of the tuber extracts of S. chacoense and S. sparsipilum showed abundant ions with the masses 150.1283 and 204.1752, for which the formulas $C_{10}H_{16}N$ and $C_{14}H_{22}N$ were calculated, respectively. These formulas agree with those of the diagnostic fragments at m/z 150 (base peak) and m/z 204 of the solanidanes (Ripperger and Schreiber, 1981). Fragments with exact masses corresponding to the base peaks of other groups of SAs, such as spirosolanes, epiminocholestanes, aminospirostanes and solanocapsines, were not present.

All compounds in the tuber extracts of the four Solanum (sub)species that were characterized as SA by their NPD/FID response ratios, showed main ions at m/z 150 (base peak) and m/z 204 in the spectra obtained by GC-MS (EI mode). This means that they all were solanidanes with unsubstituted E and F rings (Ripperger and Schreiber, 1981; Kaneko et al., 1981). Fig. 1 shows the TIC profile (D) and the mass chromatograms at the m/z values of the diagnostic ions of the solanidanes (B and C). Base peaks corresponding to other groups of SAs were not detected, as is illustrated for the spirosolanes by the mass chromatogram recorded at m/z 114 (Fig. 1A).

Substitutions in the steroid skeleton (rings A-D) do not markedly influence the fragmentation patterns (Budzikiewics, 1964). However, a comparison of the EI mass spectra of nine standard alkaloids showed that differentiation between the saturated SAs (demissidine, soladulcidine and tomatidine), the Δ5-unsaturated SAs (solanidine, solasodine and tomatidenol) and their 3,5-diene dehydration products (solanthrene, solasodiene and tomatidadiene) was possible by using the ratios of the

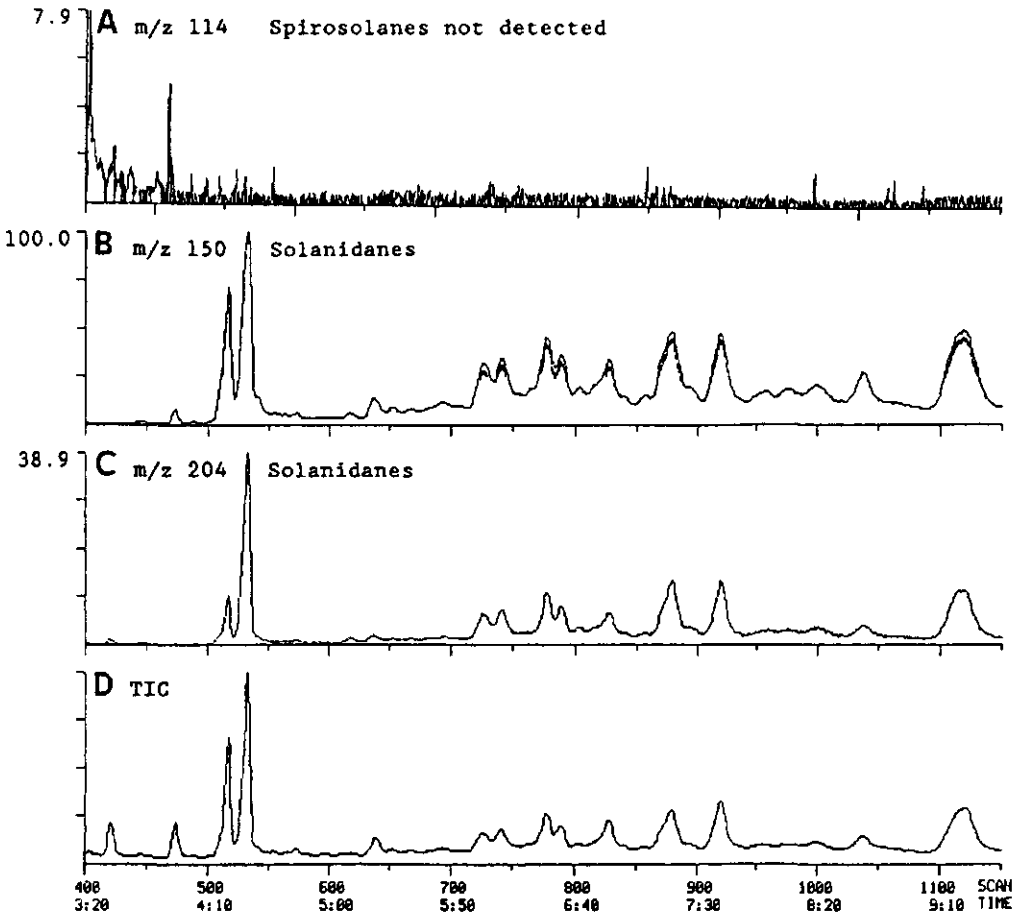

Fig. 1. Mass chromatograms recorded at the diagnostic m/z values of spirosolanes (A: m/z 114, base peak) and solanidanes (B: m/z 150, base peak; C: m/z 204) and the total ion current (TIC) profile, obtained by GC-MS (EI mode) analysis of a steroidal alkaloid extract from S. sparsipilum tubers.

abundances of the ions at m/z 91 and m/z 93, which were about 0.8 for the saturated SAs, 1.3 for the Δ5-unsaturated compounds and 2.0 for the 3,5-dienes, respectively. Similar ratios for the three respective SA groups tested were found for the abundances at m/z 105 and m/z 107.

GC-MS (CI mode) of the SAs showed $(M+1)^+$ ions with a relative abundance of 30-100%. SAs containing a hydroxyl group showed in addition $(M+1-18)^+$ ions with a relative abundance of 20-50%, due to the loss of water from the protonated molecules (Fig. 2).

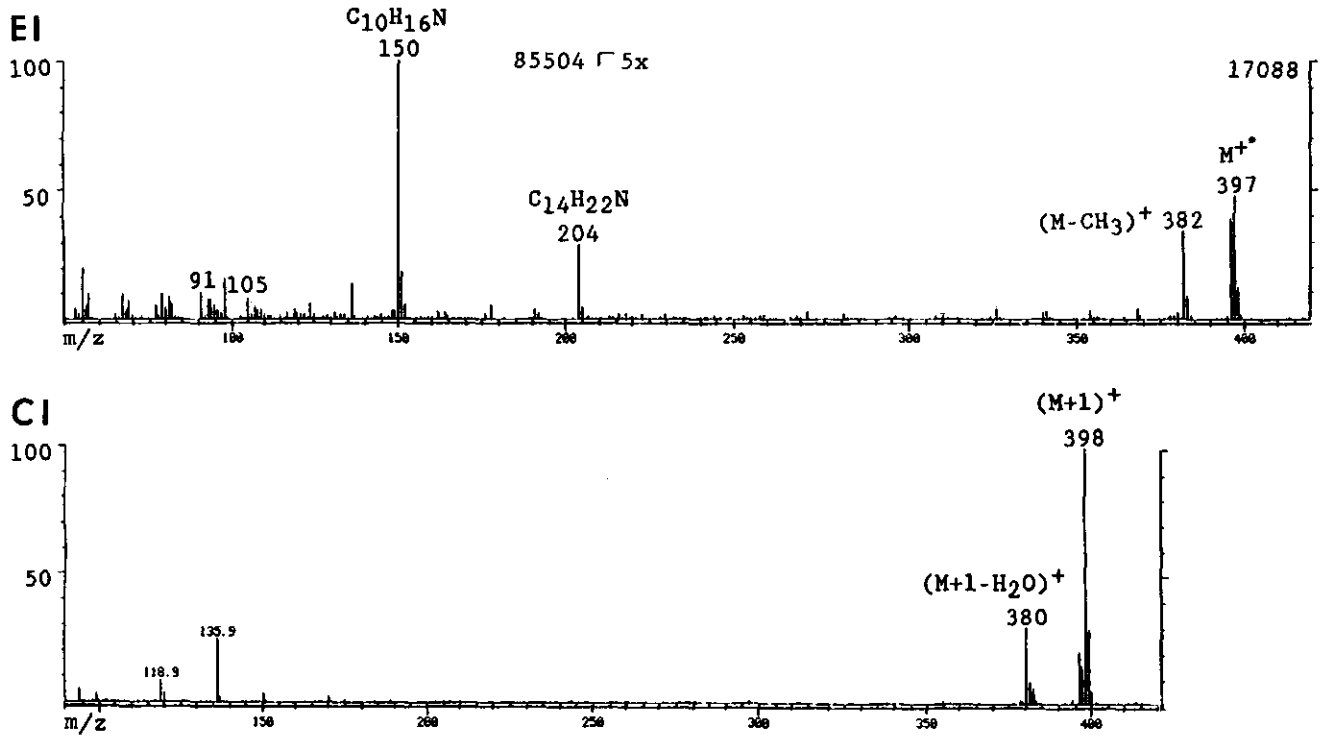

Fig. 2. EI and CI mass spectra of solanidine. Peaks in the CI mass spectrum at m/z 118.9 and 135.9 resulted from column bleeding.

Table 1. Steroidal alkaloids from tubers of Solanum (sub)species used in potato breeding, characterized by retention indices, I, high-resolution mass spectrometry (HRMS), gas chromatography-mass spectrometry (GC-MS) and by two-phase hydrolysis.

Steroidal alkaloid[1]	$I^{CP\text{-}Sil\ 5\ CB}_{270}$	HRMS		GC-MS diagnostic ions (% abundance)	Loss of H_2O	
		Mass	Formula		CI[2]	Hydrolysis[3]
1 Solanida-dien-ol	3040	395.3188	$C_{27}H_{41}NO$	150(100), 204(14), 380(2), 395(5)	1	1
2 Solanidine[4]	3130	397.3345	$C_{27}H_{43}NO$	150(100), 204(20), 382(4), 397(5)	1	1
3 Solanidane-diol	3143	415.3450	$C_{27}H_{45}NO_2$	150(100), 204(22), 400(2), 415(3)	2	0
4 Solanida-dien-ol	3169	395.3188	$C_{27}H_{41}NO$	150(100), 204(23), 380(5), 395(9)	1	1
5 Solanida-dien-ol	3176	395.3188	$C_{27}H_{41}NO$	150(100), 204(22), 380(6), 395(7)	1	1
6 Substituted solanid-en-ol	3195	427.3086	$C_{27}H_{41}NO_3$	150(100), 204(18), 380(2), 395(2), 396(2), 412(2), 427(3)	Nr[5]	1
7 Hydroxymethyl or methoxysolanid-en-ol	3236	427.3450	$C_{28}H_{45}NO_2$	150(100), 204(28), 380(3), 395(7), 396(4), 412(1), 427(2)	1	1
8 Solanidane-diol	3293	415.3450	$C_{27}H_{45}NO_2$	150(100), 204(22), 400(2), 415(5)	2	0
9 Substituted solanid-en-ol	3319	425.3303	$C_{28}H_{43}NO_2$	150(100), 204(14), 410(5), 425(4)	Nr	1
10 Methylsolanid-en-ol	3353	411.3501	$C_{28}H_{45}NO$	150(100), 204(20), 396(16), 411(14)	Nr	1
11 Substituted solanidan-ol	3357	423.2772 423.3136	$C_{27}H_{37}NO_3$ $C_{28}H_{41}NO_2$	150(100), 204(19), 394(2), 408(4), 409(4), 423(1)	Nr	0

[1]Nomenclature according to Ripperger and Schreiber (1981).

[2]$(M+1-18)^+$ resulting from chemical ionization.

[3]Formation of dehydration product upon hydrolysis, which corresponds to mass difference of 18.0105.

[4]Solanid-5-en-3β-ol.

[5]Nr = CI mass spectrum not recorded.

Upon two-phase hydrolysis using chloroform as organic phase, the Δ5-unsaturated SGAs produce 5-ene-3β-ol aglycones as well as the corresponding 3,5-diene dehydration products, but using carbon tetrachloride, they produce almost entirely 3,5-dienes (Chapter III). This phenomenon (shift from the concentration of the 5-ene-3β-ol to the concentration of the 3,5-diene) was used for the detection of the enols and the dehydration products by capillary GC. The final identification of each 5-ene-3β-ol and 3,5-diene pair was achieved using the data from EI mode GC-MS (ratios of the abundances at m/z 91/93 and m/z 105/107), from CI mode GC-MS (presence of $(M+1-18)^+$ ions), and from the HRMS (absence of oxygen atoms in the dehydration products, and a difference of 18.0105 between the masses of the SAs and their dehydration products).

Table 1 shows the main physical and chemical data which enabled a characterization of the SAs. Only the naturally occurring SAs are given and not their dehydration products. Compound 1, a solanida-dien-ol, may be dehydrosolanidine according to its retention time and that of its dehydration product $C_{27}H_{39}N$ (mass 377.3082), which can be dehydrosolanthrene. Compound 3 is a saturated SA, which contains two hydroxyl groups and is probably an isomer of compound 8. Compounds 4 and 5 are isomers of compound 1. Compound 7 with $M^{+\bullet}$ at m/z 427, showed the loss of one molecule of water upon hydrolysis (difference in mass of 18.0105 with its dehydration product), an $(M+1-18)^+$ peak in CI mode GC-MS, and an $(M-31)^+$ peak in the EI mode. Therefore compound 7 may be methoxysolanidine or hydroxymethylsolanidine. The data from the HRMS revealed the presence of an SA showing the mass 427.3450 which corresponds with the formula $C_{28}H_{45}NO_2$; this supported this hypothesis. A second compound with $M^{+\bullet}$ at m/z 427 was detected by GC-MS, namely compound 6, of which the mass spectrum differed from that of compound 7. HRMS also revealed the presence of a compound with a mass of 427.3086, and consequently, it was assumed that this mass corresponded to compound 6, for which the formula $C_{27}H_{41}NO_3$ was calculated. Although the compound contained three oxygen atoms, it lost only one molecule of water upon hydrolysis (mass difference 18.0105); it is therefore supposed to be a solanid-en-ol containing no second enol group but more likely keto and/or hydroxy groups. Compound 10 may be methylsolanidine as it produced a dehydration product $C_{28}H_{43}N$ (mass 393.3395), which is probably

a methylsolanidadiene (methylsolanthrene). One solanidane-type SA with a molecular mass of 423 was detected by the EI mode GC-MS. This compound (11) could either be $C_{27}H_{37}NO_3$ or $C_{28}H_{41}NO_2$, because exact masses corresponding to both formulas were detected by the HRMS. Low intensities of the molecular ions 411.3137 ($C_{27}H_{41}NO_2$) and 425.2930 ($C_{27}H_{39}NO_3$) were also measured by the HRMS, but corresponding peaks were not detected by GC-MS; these masses probably correspond to trace compounds.

Biological implications

A large number of naturally occurring steroidal sapogenins, a well-studied class of compounds closely related to the SAs, have been described in a recent review (Patel et al., 1987). Among these were methyl-, di- and trihydroxy-, and mono- and diketo-sapogenins as well as ene- and diene-forms. Also, though to a lesser extent, a variety of SAs has been described; until 1981, more than 80 aglycones have been reported for the genus Solanum only (Ripperger and Schreiber, 1981). Regularly, novel SAs and SGAs are being detected in Solanum species, mostly in aerial plant parts, as a result of qualitative studies on pharmacologically interesting compounds (Chakravarty and Pakrashi, 1987; Chun-Nan Lin et al., 1987; Chakravarty and Pakrashi, 1988; see also Chapter I). Only few studies on the SA composition of tubers have been carried out, and consequently only occasionally novel SAs or SGAs have been found since 1981 (Osman et al., 1986; Van Gelder et al., 1987; see also Chapter VII). Until now, six SAs of the solanidane group have been described for Solanum species, of which only two, solanidine and demissidine, have been detected in tubers.

Table 2 shows that a variety of (minor) SAs occurred in the tubers of the Solanum (sub)species used in this study. The detection of these SAs was achieved by the application of capillary GC using simultaneous FID and NPD. In addition to solanidine, at least four of these SAs may be regarded as (minor) compounds common to the genus Solanum, as they occurred in species which belong to different series of this genus, namely the series Commersoniana (S. chacoense) and Tuberosa.

The nature of the novel SAs of the Solanum (sub)species studied could be revealed, as was described above. They all belonged to the solanidane group and were most probably substituted, dehydrogenated,

Table 2. Contents[1] of steroidal alkaloids (SAs) of tubers of Solanum (sub)species. All SAs possessed a solanidane skeleton.

SA (glycosidically bound)	Solanum chacoense	Solanum leptophyes	Solanum sparsipilum	Solanum tuberosum ssp. andigena
1 $C_{27}H_{41}NO$	30	61	200	19
2 Solanidine	2121	199	255	450
3 $C_{27}H_{45}NO_2$	40	12	35	14
4 $C_{27}H_{41}NO$		30	125	
5 $C_{27}H_{41}NO$			100	
6 $C_{27}H_{41}NO_3$			25	
7 $C_{28}H_{45}NO_2$	5	18	100	12
8 $C_{27}H_{45}NO_2$	15			
9 $C_{28}H_{43}NO_2$		5	40	
10 $C_{28}H_{45}NO$	17	78	200	31
11 $C_{27}H_{37}NO_3$ or $C_{28}H_{41}NO_2$	75			
Unidentified	Tr[2]	Tr	140	Tr
Total	2228	403	1300	526

[1]SAs expressed as glycosides on a trisaccharide basis in mg/kg fresh weight.

[2]Tr = traces.

or substituted saturated forms of solanidine, in which for instance a hydrogen has been replaced by a hydroxyl or methyl group. However, their origin is not yet clear. In the S. sparsipilum sample studied, elevated levels of novel SAs were present. This may have been (partly) the result of the different growing conditions of these tubers (Chapter VII), as it has been shown that environmental conditions may affect the biosynthesis of SAs in Solanum species in many ways (Kuc, 1984). In Veratrum species, solanidine accumulated, or was converted to jervine and veratramine, depending on the absence or presence of illumination, respectively (Kaneko et al., 1978). Solanidine, solasodine and tomatidenol, common end-products in the SA biogenesis in Solanum species, seemed to be precursors in the biogenesis of camtschatcanidine, hapepu-

nine, anrakorinine and 27-hydroxyspirosolane, which are SAs of Fritillaria species (Kaneko et al., 1981). Thus the SAs found in the tubers of the wild and primitive species studied, might be intermediates in SA metabolism and/or stress metabolites.

The specific biological activities of SGAs are often associated with structural characteristics of their aglycones. For instance, the anticholinesterase activity of SGAs (Bushway et al., 1987) depended much more on the type of SA than on the composition of the sugar moiety. Furthermore, a comparison between the solanidine-type glycoalkaloids leptine I, leptinine I and α-chaconine, which possess the same sugar moiety and differ only with respect to the substituent (acetyl, hydroxyl and hydrogen, respectively) at C_{23} of the solanidane skeleton (Chapter I, Fig. 1 and Table 1), showed that the C_{23}-acetyl group is responsible for the high antifeedant activity of leptine I against the Colorado potato beetle (Kuhn & Löw, 1961). Thus, unknown and/or minor SAs as detected in this study must not be overlooked. However, this can easily happen when imperfect analytical techniques are used, or when insufficient attention is paid to them since their biological significance is not clear.

For a complete structure elucidation, the SAs and their glycosides need to be isolated and costly and time-consuming studies by MS, infrared and nuclear magnetic resonance spectroscopy must be carried out. Once the entire structure of the individual compounds is known, information on their toxicity will still be lacking. It is therefore more efficient to investigate whether these (minor) compounds occur in cultivars, either normally or induced by environmental (stress) conditions during cultivation and storage of the tubers. If these compounds do not occur in the current household potato, it is advisable to prevent their introduction into the future cultivars.

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CHAPTER IX*

TRANSMISSION OF STEROIDAL GLYCOALKALOIDS FROM SOLANUM VERNEI TO THE CULTIVATED POTATO (S. TUBEROSUM)

INTRODUCTION

S. vernei is widely being used in potato breeding programmes in many countries, mainly as a source of resistance to potato cyst nematodes (Globodera species) and because of its high starch content. Commercial household cultivars containing S. vernei germplasm have already been released in several countries (Ross, 1986) and an increasing number will be released in the near future. Until now it was believed that this wild species contains only solanidine glycosides (see Chapter VII) and in some reports it has been mentioned that its offspring was analysed for these SGAs (Coxon et al., 1983; Grassert and Lellbach, 1987). As a result, advanced breeding clones were discarded because of the high levels of solanidine glycosides - up to 1570 mg/kg (see Chapters III and VII). However, tubers of S. vernei which we analysed contained in addition to the solanidine glycosides, solasodine glycosides and several unidentified SAs (Chapter VII). Compared with the potential acute toxic level of 200 mg/kg of the solanidine glycosides (see Chapter II), the contents of these compounds in tubers of S. vernei were extremely high (2742-6093 mg/kg). The contents of solasodine glycosides were also high (675-3483 mg/kg). Toxicological information on the solasodine glycosides as well as on other SGAs from wild Solanum species is lacking and therefore it has been stated that alien SGAs must not be introduced into the household potato (Chapter II).

The aim of the study described in this chapter was to identify and quantify the SAs in tubers of S. vernei and in offspring of S. vernei and S. tuberosum in order to investigate whether the ability to syn-

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*This chapter is based on the paper:
Van Gelder WMJ, Scheffer JJC. (199*) Transmission of steroidal glycoalkaloids from Solanum vernei to the cultivated potato (S. tuberosum), submitted for publication.

thesize solasodine or other alien aglycones can be transmitted from this wild species to potato cultivars. Some of the data presented here are part of a study on the inheritance of leaf and tuber SGAs and the relationships between the contents of these SGAs and important agricultural characteristics, using various Solanum species. The results of that study will be published in a separate paper.

EXPERIMENTAL

Plant material

The provenance of S. vernei Bitt. et Wittm. accession 8240, clone number 19, and accession 15451, clone number 12, and of the SVP potato clones AM 78-3778 and AM 78-3787 ('Arabesque') has been described in Chapter VII. The hybrids 24 and 32 were part of the offspring from a cross between S. vernei 15451-12 and the diploid S. tuberosum L. clone SH 78-202-3265 grown in a study on the inheritance of the SGAs. The tubers of S. vernei grown under a short-day (12 h light-12 h dark) photoperiod, were produced in a glasshouse under the controlled conditions as described in Chapter VII for the plants grown in 1986. The sums of the solar radiation (350-3000 nm) were in the present study for April, May, June, July and August (2 weeks): 34, 54, 34, 55 and 17 kJ/cm^2, respectively. The plants were grown in a randomized block design with six replicates.

The tubers of the genotypes grown under a long-day photoperiod were also produced in a glasshouse. The growing period was from April till the third week of August 1987; the average monthly temperature in the glasshouse was 17, 18, 19, 24 and 33°C, respectively, and the minimum night temperature 15°C. The photoperiod was the natural daylength. The relative air humidity was kept at 60-70%. The sums of the solar radiation were as above. The plants were grown in a randomized block design with ten replicates.

Chemicals and procedures

The tuber batches of each replicate were analysed separately. Standard SAs, and extraction and determination of the SAs were as described in the Chapters V and VI. Columns used for capillary GC were fused silica, 50 m x 0.22 mm I.D., CP-Sil 5 CB, film thickness 0.12 μm and 0.4 μm,

and CP-Sil 19 CB, film thickness 0.19 μm (Chrompack Nederland). SA concentrations were calculated from the peak areas of the 5-ene-3β-ol and 3,5-diene pairs, and expressed as SGAs on a trisaccharide basis. Solanidine epimers synthesized according to Brown and Keeler (1978) were kindly supplied by Dr. W. Gaffield. Identification of SAs by comparison of retention indices, NPD/FID response ratios, hydrolysis behaviour and mass spectra of the SAs and their corresponding 3,5-diene dehydration products with those of reference compounds was as described before (Chapter VIII).

For TLC comparison of the synthetic 22R,25R solanidine and the supposed natural 22R,25R solanidine isolated from S. vernei tubers and from leaves of 'AM 78-3787', high-performance TLC plates, 5 x 10 cm, silica gel 60 (E. Merck) were used. Volumes of 1 μl of the appropriately diluted tuber extracts prepared for capillary GC and of a methanol/toluene (2:1 v/v) solution containing 5 μg of the synthetic epimer were applied to a TLC plate. Plates were developed using t-butyl methyl ether/methanol (95:5 v/v) as eluent (Gaffield, 1988). A saturated solution of cerium(IV) sulphate in sulphuric acid (65%) was used as visualization reagent. The spots became brown, and turned to grey after heating the plates for 5 min at 120°C.

Identification of the diagnostic ions of GC-MS (EI mode, 70 eV) was as described in Chapter VIII and by fragmentation mapping (Barber et al., 1968). GC-MS diagnostic ions: solanthrene (I = 2943) and solanthrene isomer (I = 2889) ($C_{27}H_{41}N$) m/z 379 $M^{+\bullet}$, 364 $[M\text{-}CH_3]^+$, 204 $[C_{14}H_{22}N]^+$, 150 base peak $[C_{10}H_{16}N]^+$; solanidine (I = 3130), 22R,25R epimer of solanidine (I = 3121) and solanidine isomer (I = 3058) ($C_{27}H_{43}NO$) m/z 397 $M^{+\bullet}$, 382 $[M\text{-}CH_3]^+$, 204, 150 base peak; solasodiene and tomatidadiene ($C_{27}H_{41}NO$) m/z 395 $M^{+\bullet}$, 380 $[M\text{-}CH_3]^+$, 367 $[M\text{-}C_2H_4]^+$, 138 $[C_9H_{16}N]^+$, 114 base peak $[C_6H_{12}NO]^+$, 113 $[C_6H_{11}NO]^+$; solanid-5-ene-3β-ol derivative ($C_{27}H_{41}NO_3$) m/z 427 $M^{+\bullet}$, 412 $[M\text{-}CH_3]^+$, 204, 150 base peak; solasodine and tomatidenol ($C_{27}H_{43}NO_2$) m/z 413 $M^{+\bullet}$, 398 $[M\text{-}CH_3]^+$, 385 $[M\text{-}C_2H_4]^+$, 271 $[C_9H_{27}NO]^+$, 138, 114 base peak.

RESULTS AND DISCUSSION

Table 1 shows the contents of the various SAs, expressed as SGAs on a trisaccharide basis, of tubers of the S. vernei clones 8240-19 and

Table 1. Contents[1] (± standard errors) of steroidal alkaloids (SAs) of tubers of Solanum vernei grown under short-day (SD) and long-day (LD) photoperiods. Plants were grown in six replicates which were analysed separately.

Steroidal alkaloid	Retention index	S. vernei 8240-19	S. vernei 15451-12	
		SD	SD	LD
Solanidine isomer	3058	63 ± 10.4	25 ± 11.7	Tr[2]
22R,25R Solanidine	3121	Nd[3]	45 ± 9.9	Tr
Solanidine[4]	3130	4961 ± 484	2769 ± 553	4595 ± 879
$C_{27}H_{41}NO_3$[5]	3195	12 ± 6.2	Nd	Nd
Solasodine	3338	495 ± 71	1301 ± 366	105 ± 62
Tomatidenol	3360	455 ± 256	291 ± 184	Nd
Total		5986 ± 713	4431 ± 1088	4700 ± 931

[1]SAs expressed as glycosides on a trisaccharide basis in mg/kg fresh weight.

[2]Tr = trace (< 2.5 mg/kg).

[3]Nd = not detected.

[4]22R,25S configuration.

[5]Substituted solanidenol (see Chapter VIII).

15451-12, obtained by capillary GC on a CP-Sil 5 CB fused-silica column, 50 m x 0.22 mm I.D., film thickness 0.12 μm. These contents are averages of six separately analysed tuber batches obtained from six plants which had been grown as replicates, as the tuber SGA contents of different plants of a single genotype grown in the same compartment may vary considerably.

High concentrations of solanidine, solasodine, and tomatidenol were found. In clone 8240-19, low concentrations of a solanid-5-ene-3β-ol derivative with a molecular weight of 427 and a retention index ,I, of 3195 were found. This compound was identical to the SA which had previously been detected in other Solanum species and was identified as $C_{27}H_{41}NO_3$ (Chapter VIII).

Two solanidanes with molecular ion peaks, $M^{+\bullet}$, at m/z 379 (I = 2889)

Table 2. Retention indices, I, obtained by capillary GC and R_F values obtained by TLC of solanidine (22R,25S), of its supposed natural epimer (22R,25R) isolated from tubers of S. vernei 15451-12 and from leaves of the potato progenitor AM 78-3787, and of its synthetic epimers.

Configuration of solanidine	I (Capillary GC)			R_F (TLC)
	CP-Sil 5 CB 0.12 μm	CP-Sil 5 CB 0.4 μm	CP-Sil 19 CB 0.19 μm	Silica gel 60
22R,25R; Natural	3120.9	3122.2	3130.0	0.80
22R,25R; Synthetic	3120.6	3122.8	3129.9	0.81
22R,25S; Solanidine	3129.8	3131.1	3134.3	0.66
22S,25R; Synthetic	3246.3	3248.0	3227.3	-
22S,25S; Synthetic	3250.1	3253.3	3228.0	-

and m/z 397 (I = 3058), respectively, were identified as a 3,5-diene and 5-ene-3β-ol pair. These compounds appeared to be isomers of solanthrene (solanida-3,5-diene) and solanidine, respectively. The dienes are produced by dehydration of the 5-ene-3β-ols upon hydrolysis and are an aid in the identification of SAs (Chapter VIII). In the accession 15451, another isomer of solanidine (I = 3121) which had earlier been detected in leaves of the potato clone AM 78-3787 ('Arabesque') was present (Van Gelder et al., 1987; cf. also Chapter VII). The mass spectra and the retention indices of the two just-mentioned natural solanidine isomers were compared with those of the synthetic 22R,25R, 22S,25R, and 22S,25S epimers. The solanidine isomer with I = 3058 did not correspond to any of the epimers, but the results strongly suggested that the isomer with I = 3121 that was found in the tubers of the S. vernei clone 15451-12 and in the leaves of the clone AM 78-3787, was the 22R,25R epimer of solanidine (solanidine shows the 22R,25S configuration). Further comparison of these compounds by capillary GC using the same column type with a film thickness of 0.4 μm, which increased the resolution by almost 40%, and using a more polar column (CP-Sil 19 CB) provided additional evidence that this natural isomer was identical with the synthetic 22R,25R epimer (Table 2).

The results were confirmed by TLC on silica gel 60 using t-butyl methyl ether/methanol (95:5 v/v) as eluent (Gaffield, 1988). To our knowledge this is the first report on a naturally occurring epimer of solanidine. The 22S,25R and 22S,25S epimers were not detected in S. vernei nor in any other genotype investigated so far.

A short-day regime was applied in this study because it is favourable for the tuberization of S. vernei. Earlier studies strongly suggested that the environment may influence the SA composition, both qualitatively and quantitatively (Van Gelder et al., 1987; Chapter VII, see also Chapter VIII). Therefore the individual SA contents (expressed on a glycoside basis) of tubers of the S. vernei clone 15451-12 grown under a long-day photoperiod, which was applied in the inheritance study mentioned above, are given for comparison (Table 1). The total contents of the SGAs in the tubers did not differ between the two regimes ($P = 0.05$), and they were as (extremely) high as those found before in tubers of S. vernei (Chapter VII). Under long-day conditions, the content of solanidine glycosides was much higher, whereas the contents of the other SA glycosides were significantly lower ($P = 0.05$). Such concentration shifts resulting from differences in photoperiods have also been observed for various sterols in leaves of S. tuberosum ssp. andigena (Bae and Mercer, 1970).

The aglycones in S. vernei are all Δ5-unsaturated SAs and they have teinemine as common precursor (Chapter I). It seems therefore most likely that the environment changes the expression of the genes regulating the conversion of teinemine to the various aglycones. Under the long-day conditions, the precursor, the synthesis rate of which apparently did not change, was almost entirely converted into solanidine, whereas under the short-day conditions considerable amounts of other teinemine-derived SAs were synthesized. The influence of environmental conditions on the biosynthetic pathways of the SAs has been reported earlier. For instance, conversion of solanidine to jervine and veratramine in a Veratrum species was regulated by light (Kaneko et al., 1978), and the ability of tubers of the potato cultivar Kennebec to synthesize tomatidenol glycosides, became specifically expressed when sliced tubers were stored (Shih and Kuc, 1974; Chapter I). Storage of slices is not uncommon in the potato processing industry.

Table 3. Contents[1] (± standard errors) of the main steroidal alkaloids (SAs) of tubers of S. vernei 15451-12, of S. tuberosum SH 78-202-3265, of two of their hybrids, and of three cultivars, grown under a long-day photoperiod.

Genotype	N[2]	Solanidine glycosides	Solasodine glycosides
S. vernei	6	4595 ± 879	105 ± 62
Hybrid 24	5	897 ± 157	20 ± 5
Hybrid 32	9	1370 ± 141	22 ± 5
S. tuberosum	8	92 ± 20	Nd[3]
AM 78-3778	10	825 ± 86	7 ± 1
AM 78-3787	7	149 ± 22	3 ± 0.4
Bintje	9	153 ± 12	Nd

[1]SAs expressed as glycosides on a trisaccharide basis in mg/kg fresh weight.

[2]Number of replicates which produced tubers and were analysed.

[3]Nd = not detected.

Although in tubers of Solanum species, solanidine as well as solasodine and tomatidenol have been found so far linked to solatriose and chacotriose (in α-solanine and α-chaconine, in solasonine and solamargine, and in α-solamarine and β-solamarine, respectively), these aglycones can occur bound to other sugars (Chapters I and IV). Therefore a study on the sugar moieties of the SGAs of S. vernei is in progress.

S. vernei belongs to the Solanum series Tuberosa. However, the SGA composition of the tubers of S. vernei was different from those of all other species of this series which we investigated (Van Gelder and Jonker, 1986; Chapters VII and VIII). This is quite interesting from a chemotaxonomic point of view. Further study of this series, for which 68 wild species were described by Hawkes (1978), will reveal its diversity with respect to the SGAs.

Table 3 shows the contents of the main SGAs of tubers of the parents and of two hybrids of a cross between S. vernei and a diploid S. tuberosum. For comparison, the contents of the cultivar Bintje and of the

clones AM 78-3778 and AM 78-3787, which both have a S. vernei clone as progenitor, are given. The tubers were produced in a glasshouse under a natural long-day photoperiod.

The contents of the solanidine glycosides of 'Bintje', 'AM 78-3778' and 'AM 78-3787' agreed fairly with those of an earlier study which showed that under the growing conditions applied, the contents of the solanidine glycosides were two to three times higher than in normal field-grown tubers (Chapter VII). For instance, field-grown tubers of 'Bintje' contain usually about 40 mg solanidine glycosides per kg. However, even when this aspect is taken into consideration, the contents of the solanidine glycosides of the hybrids are above the potential acute toxic level.

Also solasodine glycosides were found in the tubers of the hybrids; fortunately the concentrations were low. Solasodine has been reported to cause birth defects when a single high dose of 1180 mg/kg bw was gavaged to hamsters of a strain that is considered sensitive to SA teratogens (see Chapter II). To administer an equivalent amount of this aglycone, about 10 kg of tubers of the hybrids would have to be gavaged to a single hamster, and thus the level of solasodine in the hybrids seems irrelevant from the viewpoint of teratogenicity. However, in its natural matrix, solasodine is ingested glycosidically bound, and toxicological information on the solasodine glycosides, administered either as pure compounds or in the natural matrix, is not available. Most probably the glycosides are absorbed to a larger extent from the gastrointestinal tract than the aglycone and thus they may perhaps show a higher teratogenic potency. As, unfortunately, the necessary toxicological data are lacking, it can not be concluded unequivocally that solasodine glycoside levels as found in the hybrids do not present a hazard to human beings.

Also in the tubers of 'AM 78-3778' and 'AM 78-3787', low contents of solasodine glycosides were detected. These SVP-progenitors have been derived from S. vernei interspecific hybrids which had been backcrossed three times using S. tuberosum cultivars and species in which only solanidane-type SGAs have been detected in the tubers. Therefore it was not expected that solasodine would be still synthesized in these genotypes.

Toxicological information on the other SAs detected in S. vernei is

not available. With respect to the relationship between the structure of SAs or SGAs and their teratogenic potency, it should be noted that induction of congenital malformations by solanidanes and spirosolanes has been associated with the 22S,25R configuration occurring for instance in solasodine (see Chapter II). Demissidine (22R,25S) and tomatidine (22R,25S) did not induce malformations. Thus if this stereochemical concept appears to be valid, tomatidenol (22R,25S) would be beyond suspicion.

Studies on the teratogenicity of SAs showing the 22R,25R configuration have not been reported. However, these SGAs were found in low concentrations in the tubers of the S. vernei clones, and they were not even detected in the offspring.

In conclusion it can be stated that the considerably reduced contents of the solanidine and solasodine glycosides in the hybrids compared with the S. vernei parent and the absence of other SGAs found in S. vernei, is reassuring for the potato breeder, as it is likely that genotypes that are safe for consumption can be produced by (repeated) backcrossing. However, it must not be overlooked that S. vernei offspring can possibly accumulate larger amounts of solasodine glycosides or synthesize alien SGAs, inherited from the wild species, under cultivation conditions differing from those applied here. Furthermore, to our knowledge, in not one country where S. vernei has been utilized in breeding programmes, its offspring or new cultivars containing its germplasm, have been analysed for their total SGA composition. It is therefore advisable to analyse the SGAs of such new cultivars yet, and to carry out the analysis on tubers produced under various environmental conditions.

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SUMMARY AND CONCLUDING REMARKS

Tuberiferous and nontuberiferous wild Solanum species are increasingly being used in potato breeding as a source of genes for disease and pest resistances and for other valuable characteristics. A disadvantage of Solanum species, from a consumers point of view, is that they contain steroidal glycoalkaloids (SGAs), which are natural toxins occurring in all parts of these plants. The SGAs consist of a C_{27}-steroidal alkaloid (SA) and a sugar moiety, often a tri- or tetrasaccharide. The tubers of the cultivated potato usually contain small quantities of one type of SGAs, the solanidine glycosides.

Evidence has been presented that utilization of wild Solanum species can result in the introduction of hazardous levels of solanidine glycosides into the cultivated potato. However, little is known of the qualitative and quantitative SGA composition of wild Solanum species used in potato breeding. Such information on hybrid offspring or on cultivars containing germplasm from wild species is entirely lacking.

The aim of the studies described in this thesis was to evaluate the possible health hazards of the SGAs from the genus Solanum and to develop and apply methods for analysis of the SGA compositions of Solanum species. The collected information was placed into the perspectives of potato breeding and of food safety, in order to point out possible consequences of introducing undesired levels or types of SGAs into the cultivated potato.

In Chapter I, the literature on the distribution and accumulation of solanidine glycosides in the cultivated potato and on the biosynthesis of the SGAs is reviewed. It was shown that many factors during the growth and post-harvest period of potatoes can lead to levels of solanidine glycosides exceeding 200 mg/kg fresh weight. This information is important also with respect to introduction of SGAs from wild Solanum species into the household potato, as alien SGAs will almost certainly accumulate similarly, because the biosynthetic pathways of the various SGAs are closely related.

The objective of Chapter II was to evaluate the toxicity of the SGAs, based on the data available in the literature, in order to assess possible consumer hazards and to derive safe levels for household pota-

toes. The data showed that acute poisoning in man may occur due to consumption of potatoes with a solanidine glycoside content above 200 mg/kg fresh weight. This level is only two to three times higher than levels that are considered normal for tubers of current cultivars. Because of the virtual absence of chronic toxicity data, an adequate 'no-adverse effect level' for potato SGAs could not be assessed. Consequently an acceptable daily intake (ADI) figure for man or an acceptable level for potatoes could not be derived. It was concluded that the SGA content of new household potato cultivars should not be allowed to rise above the average level of the current cultivars; preferably it should be lower. SGAs alien to S. tuberosum must not be introduced into the household potato as acceptable levels have not been established.

The methods for SGA analysis described in the literature are not adequate for determining -qualitatively and quantitatively- the SGA composition of breeding material. Therefore, a comprehensive, quantitative and efficient method applicable to diverse plant material, was developed.

In Chapter III, a new hydrolysis technique employing a two-phase system is described. The technique was developed in order to prevent the losses of aglycones which do usually occur during conventional hydrolysis of different SGAs. The two-phase system consists of an aqueous acid phase, in which the glycosides are hydrolysed, and an immiscible nonpolar organic phase, which serves as a protective phase for the unstable nonpolar aglycones. Using the technique quantitative recoveries of various SAs after simultaneous hydrolysis of different SGAs were obtained.

In Chapter IV a capillary gas chromatography (GC) method is described, which enabled -for the first time- the separation of the Δ5- and 5α-aglycone pair solanidine and demissidine as well as separation of the other SAs studied. Derivatization of the SAs was not required. The capillary GC method in combination with the bisolvent extraction and two-phase hydrolysis described in Chapter III offered prospects for quantitative analysis of complex SGA compositions of Solanum species in a single run.

Chapter V deals with the development of a method that enabled an unambiguous differentiation during capillary GC between peaks of unknown SAs and peaks of closely related nonnitrogen-containing (non-N)

compounds such as sterols and steroidal sapogenins. The method involves a splitting device that connects a capillary column to a dual detector system, for flame ionization detection (FID) and N-specific detection (NPD), respectively. This system is connected to a two-channel system for interactive processing of the two sets of data. The application of hydrogen as carrier gas was introduced to achieve a short analysis duration. As tubers of wild Solanum species appeared to vary strongly in SGA contents, identification by retention times was not possible. Therefore a retention index system for the SAs was introduced. The retention indices were sufficiently reproducible, even for identification of the closely eluting Δ5- and 5α-aglycone pair solanidine and demissidine. Application of NPD/FID response ratios together with retention indices proved to be useful in the identification and characterization of SAs. Costly spectrometric techniques will have to be employed for characterization only when new SAs are detected.

Chapter VI deals with an evaluation of the developed methods using diverse plant tissues of wild Solanum and Lycopersicon species, cultivars and hybrid progeny. The results showed that the methods were useful for qualitative and quantitative analysis of the SAs in the diverse samples. A potato extract spiked with 20 SAs, including three Δ5- and 5α-aglycone pairs, could be analysed in a single GC run. A comparison with procedures described in the literature showed that the procedure developed for sample preparation followed by capillary GC should be applied, because literature procedures for sample preparation can lead to losses of various SGAs or SAs, and other chromatographic techniques do not offer the efficiency required for separation of the various SAs.

In Chapter VII, the SGA compositions of a large number of wild Solanum species used in potato breeding, are presented. The tubers of most of the species contained high concentrations of solanidine glycosides. Some species contained glycosidic-bound demissidine, solasodine and/or tomatidine. Unidentified compounds were also detected, which were most probably SAs as was shown by their NPD/FID response ratios. The total SGA contents of the tubers of the wild species varied from 123 to 7348 mg/kg. In order to place these contents in a realistic perspective, tubers of cultivars, corresponding in small size with and grown under the same conditions as the tubers of the wild species, were analysed. The contents of solanidine glycosides of these tubers were

two to three times higher than those of field-grown normal tubers. Thus, even when this factor was taken into consideration, the SGA contents of the tubers of several wild species were extremely high. This study also revealed that samples of some wild species contained high or low levels of one or many other glycosidic-bound SAs, and that the SGA composition can vary within species and between different organs of one plant. The results further suggested that the SGA synthesis can be organ specific or regulated by light and that translocation of SGAs from leaves to tubers and vice versa did not occur. It was concluded that wild crossing parents should be analysed as to their SA composition, before they are used in a breeding programme.

A procedure developed for the characterization of unidentified SAs present in plant extracts with complex SA compositions, is described in Chapter VIII. The combined results of GC-mass spectrometry (MS) applying electron impact and chemical ionization, and high-resolution MS, of the application of retention indices and NPD/FID response ratios, and of a comparison of the degree of dehydration of SAs in different two-phase hydrolysis systems, were used. This study revealed that the SAs all possessed a solanidane skeleton; they were characterized as substituted, e.g. hydroxylated or methylated, forms; dehydrogenated forms; or substituted saturated forms of solanidine. Most of these SAs had not been reported before. The complete SA composition, including the minor new SAs, of Solanum (sub)species used in potato breeding, is presented in this chapter and the importance of analysis of the minor SAs is indicated.

S. vernei is widely being used in potato breeding. Although the SGA composition of its tubers had not been reported, cultivars containing S. vernei germplasm have been released and an increasing number will be released in the future.

Chapter IX describes the identification and characterization of the SAs of S. vernei tubers produced under various growth conditions, and deals with a tentative study on the transmission of these SAs to hybrid offspring. High levels of solanidine and solasodine glycosides were found together with tomatidenol and novel glycosidic-bound SAs, amongst which the 22R,25R epimer of solanidine, a structural configuration not reported before for naturally occurring solanidanes. It was revealed that the SA composition can vary significantly in tubers grown under

different cultivation conditions.

In the tubers of S. vernei offspring, high levels of solanidine glycosides were found but the newly identified and characterized SAs of S. vernei were not detected. Solasodine was also found in the offspring, even in tubers of cultivars obtained after several times backcrossing, but fortunately, the levels were low. However, it can not be totally excluded that S. vernei offspring may synthesize hazardous levels of solasodine glycosides under particular growth or post-harvest conditions, and in this respect the possible teratogenic potency of solasodine should be kept in mind.

In conclusion it can be stated that the ingestion by the public of the amount of solanidine glycosides should not be allowed to rise but should preferably be reduced. SGAs alien to S. tuberosum should not be introduced into the household potato. The basis for the production of a potato crop safe for consumption is, to grow current cultivars and to breed new ones which accumulate low levels of only solanidine glycosides under various growth and post-harvest conditions. Utilization of germplasm from wild Solanum species must be approached with caution. Therefore it is recommended to analyse the SGAs of potential wild crossing parents before they are used in a breeding programme, in order to select the genotypes that combine a desired trait with the least unfavourable SGA composition. Depending on the SGA composition of the parents, the offspring should be monitored too.

For analysis of SGA compositions, the newly developed procedures for separation, quantification and identification/characterization, described in this thesis, must be applied, as the conventional methods described in the literature do not meet the required standards.

The accumulation of SGAs in new cultivars containing wild-species germplasm should be studied under various environmental conditions before such cultivars are registered. The guideline of 60-70 mg solanidine glycosides per kg fresh tuber recommended in the literature from the viewpoint of consumer safety (see Chapter II), should be used in potato breeding until an adequate acceptable level for these compounds has been established.

SAMENVATTING EN SLOTOPMERKINGEN

In de aardappelveredeling wordt in toenemende mate gebruik gemaakt van knoldragende en niet-knoldragende wilde Solanum-soorten, om belangrijke eigenschappen zoals resistentie tegen ziekten en plagen te introduceren in de gecultiveerde aardappel. Vanuit het oogpunt van voedselveiligheid hebben Solanum soorten als nadeel dat zij steroidalkaloidglycosiden bevatten (SGAs). Dit zijn toxinen die van nature in alle delen van planten van het geslacht Solanum voorkomen. SGAs zijn opgebouwd uit een C_{27}-steroidalkaloide (SA) en een suikergroep, meestal een tri- of tetrasaccharide. De knollen van de gecultiveerde aardappel bevatten slechts één type SGAs, de solanidineglycosiden, gewoonlijk in kleine hoeveelheden.

Het benutten van wilde Solanum-soorten kan in bepaalde gevallen leiden tot nieuwe aardappelrassen met ongewenst hoge gehalten aan solanidineglycosiden. Er is echter weinig bekend over de kwalitatieve en kwantitatieve SGA-samenstelling van de wilde Solanum-soorten die in de aardappelveredeling gebruikt worden. Informatie over deze samenstelling van nakomelingen van hybriden en van rassen die erfelijk materiaal van wilde soorten bevatten, ontbreekt geheel.

Het onderzoek dat in dit proefschrift beschreven wordt, had tot doel de potentiële gezondheidsrisico's van de SGAs te schatten en methoden te ontwikkelen, ten einde de SGA-samenstelling van Solanum-soorten te kunnen analyseren. De verzamelde informatie werd geëvalueerd, vanuit het oogpunt van zowel de aardappelveredeling als van de voedselveiligheid, om de mogelijke gevolgen aan te geven van het introduceren van ongewenste gehalten of typen SGAs in de gecultiveerde aardappel.

In Hoofdstuk I wordt beschreven waar en via welke biosynthese-route de SGAs in de aardappel worden gevormd. Het blijkt dat velerlei factoren tijdens de groei van de aardappel en tijdens opslag, verwerking en distributie van aardappelen, kunnen leiden tot toename van het gehalte aan solanidineglycosiden tot boven 200 mg/kg vers gewicht. Omdat de biosynthese van de verschillende SGAs uit wilde Solanum-soorten verloopt via routes die nauw verwant zijn aan die van de solanidineglycosiden uit aardappelen, is het aannemelijk dat deze soortvreemde SGAs,

indien geïntroduceerd in de aardappel, op een zelfde wijze zullen accumuleren.

Het doel van Hoofdstuk II is de toxiciteit van de SGAs te evalueren op basis van de beschikbare literatuurgegevens, teneinde de risico's voor de consument vast te stellen en SGA-gehalten voor de aardappel te kunnen afleiden, die voor de consument als veilig beschouwd kunnen worden. Uit de gegevens blijkt dat acute vergiftiging bij de mens kan optreden als gevolg van de consumptie van aardappelen met gehalten aan solanidineglycosiden boven 200 mg/kg vers gewicht. Dit gehalte is slechts twee tot drie keer zo hoog als de gehalten die voor aardappelen als normaal worden beschouwd. Omdat gegevens over chronische toxiciteit nagenoeg ontbreken, kon een geen-nadelig-effect niveau (no-adverse effect level) niet worden vastgesteld. Als gevolg daarvan kon noch de aanvaardbare dagelijkse opname (acceptable daily intake, ADI), noch een geschikt maximum aanvaardbaar gehalte (acceptable level) van solanidineglycosiden in aardappelen worden berekend. Op grond van de toxiciteitsgegevens werd gesteld, dat het gehalte aan solanidineglycosiden van nieuwe consumptierassen niet hoger dient te zijn dan het gemiddelde gehalte van de huidige rassen -bij voorkeur zou het lager moeten zijn- en dat voorkomen dient te worden dat soortvreemde SGAs in de consumptie-aardappel worden geïntroduceerd.

De methoden die in de literatuur beschreven zijn voor de analyse van SGAs zijn niet geschikt voor het bepalen van de kwalitatieve en kwantitatieve SGA-samenstelling van veredelingsmateriaal. Daarom werd een efficiënte analysemethode ontwikkeld, die ook bij ander plantmateriaal dan aardappelen kan worden toegepast.

In Hoofdstuk III wordt een nieuwe hydrolysetechniek beschreven, waarbij gebruik wordt gemaakt van een twee-fasensysteem: een zure waterfase waarin de glycosiden worden gehydrolyseerd, en een daarmee niet mengbare apolaire organische fase, die dient als beschermende fase voor de instabiele apolaire aglyconen. Deze techniek werd ontwikkeld om de gebruikelijke doch ongewenste verliezen aan SGAs die bij conventionele zure hydrolyse optreden, te vermijden.

In Hoofdstuk IV wordt beschreven hoe met behulp van capillaire gaschromatografie (GC) het $\Delta 5$- en 5α-aglyconen-paar solanidine en demissidine alsmede andere SAs kon worden gescheiden. Een dergelijke scheiding was niet eerder gerapporteerd. Derivatiseren van de SAs was

niet nodig. De capillaire GC methode, in combinatie met de bisolvent-extractie en het twee-fasensysteem voor hydrolyse beschreven in Hoofdstuk III, bood goede perspectieven voor het efficiënt en kwantitatief analyseren van complexe SGA-samenstellingen van Solanum-soorten.

Hoofdstuk V handelt over het ontwikkelen van een methode met behulp waarvan tijdens capillaire GC, pieken van onbekende SAs onderscheiden kunnen worden van pieken van nauwverwante niet-stikstofhoudende (non-N) verbindingen zoals sterolen en steroidsapogeninen. Bij deze methode werden simultaan vlamionisatie-detectie (FID) en N-specifieke detectie (NPD) toegepast, alsmede twee-kanaals gegevensverwerking. Voor het verkorten van de analyseduur werd waterstof gebruikt als dragergas. Omdat in wilde Solanum-soorten de SA-concentraties aanzienlijk bleken te variëren, kon de retentietijd niet voor identificatie worden gebruikt. Daarom werd een retentie-indexsysteem geïntroduceerd dat voldoende reproduceerbare waarden gaf, zelfs voor het identificeren van direct na elkaar eluerende verbindingen, zoals het Δ5- en 5α-aglyconen-paar solanidine en demissidine. Het toepassen van kostbare spectrometrische technieken kan daardoor beperkt blijven tot het identificeren en/of karakteriseren van nieuwe SAs.

De evaluatie van de ontwikkelde methoden wordt beschreven in Hoofdstuk VI. Uit de resultaten bleek dat de methoden toepasbaar waren bij verschillend materiaal van Solanum- en Lycopersicon-soorten, van rassen, en van hybriden. Een aardappelextract waaraan 20 SAs waren toegevoegd, kon in een enkele GC-run worden geanalyseerd.

In Hoofdstuk VII wordt de SGA-samenstelling van een groot aantal wilde Solanum-soorten weergegeven. De knollen van de meeste wilde soorten bevatten hoge gehalten aan solanidineglycosiden in vergelijking met de huidige consumptie-aardappelrassen. Daarnaast kunnen wilde soorten uiteenlopende concentraties aan diverse andere glycosidisch gebonden SAs bevatten. De SGA-samenstelling bleek binnen een en dezelfde Solanum-soort te kunnen variëren maar ook tussen verschillende organen van dezelfde plant. Het is daarom raadzaam wilde kruisingsouders te analyseren voordat zij in een veredelingsprogramma worden gebruikt.

Omdat in wilde Solanum-soorten onbekende SAs bleken voor te komen, werd een procedure ontwikkeld om dergelijke SAs indien aanwezig in een extract, te karakteriseren en/of te identificeren. Deze procedure wordt

beschreven in Hoofdstuk VIII. De gecombineerde resultaten van GC-massaspectrometrie (GC-MS), waarvoor 'electron impact' en chemische ionisatie werden toegepast, en van hoge-resolutie MS, van het toepassen van retentie-indices en NPD/FID responsverhoudingen, en van een vergelijking van de graad van dehydratie van SAs in verschillende twee-fasensystemen voor hydrolyse toonden het volgende aan. Alle onbekende SAs bezaten een solanidaanskelet en waren dus verwant aan solanidine uit de aardappel; zij bevatten bijvoorbeeld een extra hydroxyl- of methylgroep. De meeste van deze verbindingen waren niet eerder gerapporteerd. De totale SA-samenstelling, inclusief de afzonderlijke concentraties van de nieuwe SAs, van enkele Solanum-(onder)soorten die in de veredeling worden gebruikt, wordt in dit hoofdstuk gepresenteerd.

S. vernei wordt veelvuldig in de aardappelveredeling gebruikt. Hoewel de SGA-samenstelling van de knollen van deze soort niet bekend is, zijn er rassen ontwikkeld die erfelijk materiaal van deze soort bevatten. Een toenemend aantal van dergelijke rassen zal in de toekomst in vele landen op de rassenlijsten verschijnen.

Hoofdstuk IX beschrijft de identificatie en karakterisering van de SAs uit knollen van S. vernei, en van enkele hybride nakomelingen en rassen die erfelijk materiaal van deze soort bevatten. In de wilde soort werden hoge gehalten aan solanidine- en solasodineglycosiden gevonden. Daarnaast kwamen, glycosidisch gebonden, tomatidenol en nieuwe SAs voor, waaronder de 22R,25R-epimeer van solanidine, een verbinding met een ruimtelijke structuur die nog niet eerder is vermeld voor natuurlijke solanidanen. In dit onderzoek bleek ook dat de SA-samenstelling van knollen aanzienlijk kan variëren afhankelijk van de teeltcondities van de plant.

Knollen van hybride nakomelingen en van rassen die erfelijk materiaal van S. vernei bevatten, kunnen lage of hoge gehalten aan solanidineglycosiden bevatten. Daarnaast werden solasodineglycosiden gevonden, zelfs in rassen die meerdere malen waren teruggekruist, maar de concentraties waren gelukkig laag. Het kan echter niet worden uitgesloten, dat nakomelingen van S. vernei onder bepaalde groei-omstandigheden of tijdens na-oogstbehandelingen potentiëel schadelijke hoeveelheden aan solasodineglycosiden in de knol synthetiseren. In dit verband verdient de mogelijke teratogene activiteit van solasodine de nodige aandacht.

Samenvattend kan worden gesteld dat de opname van de hoeveelheid solanidineglycosiden door de consument niet dient toe te nemen, maar bij voorkeur gereduceerd dient te worden. SGAs die van oorsprong niet in S. tuberosum voorkomen dienen niet in de consumptie-aardappel te worden geïntroduceerd. Het introduceren van erfelijk materiaal van wilde Solanum-soorten in de cultuuraardappel dient behoedzaam te geschieden. Het is daarom aan te bevelen de SGAs van potentiële wilde kruisingsouders te analyseren, voordat zij in een veredelingsprogramma worden gebruikt, om zodoende die genotypen te kunnen selecteren die een gewenste eigenschap bezitten in combinatie met de minst ongunstige SGA-samenstelling. Afhankelijk van de SGA-samenstelling van de ouders dienen vervolgens de nakomelingen geanalyseerd te worden. Voor deze analyses zijn de in dit proefschrift beschreven nieuwe methoden geschikt; de methoden die in de literatuur zijn beschreven voldoen niet aan de te stellen eisen.

Rassen die erfelijk materiaal van wilde Solanum-soorten bevatten, dienen voordat zij als consumptieras worden toegelaten, onderzocht te worden op een eventuele accumulatie van SGAs onder diverse teelt-, opslag- en verwerkingsomstandigheden. De richtlijn van 60-70 mg solanidineglycosiden per kg verse aardappel, die uit het oogpunt van consumentenveiligheid in de literatuur wordt aanbevolen (zie Hoofdstuk II), dient in de aardappelveredeling te worden aangehouden totdat een geschikt maximaal toelaatbaar gehalte voor deze stoffen is vastgesteld.

CURRICULUM VITAE

W.M.J. van Gelder werd geboren te 's-Hertogenbosch op 10 maart 1947. Na zijn HBS-B opleiding en het vervullen van zijn militaire dienstplicht, volgde hij aan de Hogere Laboratoriumschool te Oss de avondopleidingen HBO-I analytische chemie en HBO-B biochemie; einddiploma 1972. Van 1971 tot 1985 was hij werkzaam bij de Stichting voor Plantenveredeling SVP te Wageningen, als hoofd van de Sectie Chemie. Tijdens deze periode volgde hij colleges (kandidaats-B en doctoraal) en (PAO)cursussen, onder andere in de moleculaire biologie, toxicologie, en instrumentele analysemethoden, aan de Landbouwuniversiteit en aan de Technische Universiteit te Eindhoven. Hij verrichtte onderzoek naar de relatie tussen mitochondrion-activiteit en heterosis bij mais, en naar de samenstelling van eiwit in aardappelen. Van 1985 tot 1989 was hij bij de SVP werkzaam als hoofd van de Afdeling Biochemie. Tijdens deze periode verrichtte/leidde hij onderzoek onder andere naar de biochemisch-genetische achtergrond van de bakkwaliteit van tarwe, onderzoek gericht op de ontwikkeling van snelle en gevoelige immunochemische diagnostische technieken en onderzoek naar kwaliteitsaspecten bij verschillende gewassen. Het onderzoek dat in dit proefschrift wordt beschreven heeft hij tussen 1983 en 1988 bij de SVP verricht. Met ingang van 1 september 1989 is hij aangesteld bij het Agrotechnologisch Onderzoekinstituut ATO te Wageningen, als hoofd van de Hoofdafdeling Agrificatie, Nieuwe Gewassen en Produkten.