to another in untreated rats. Problems arise, however, when the method is used to detect changes in pharmacokinetics by some experimental factors. Although the tail method detected changes in antipyrine kinetics by short-term, low-dose treatment of phenobarbital (11) and long-term treatment with ethanol (9), interpretation of data is obscured by possible influences of the treatment on tail blood flow and tail antipyrine distribution. Further investigations of such effects on drug kinetics, therefore, should include *in vitro* studies (9) or kinetic studies with the use of arterial blood samples.

REFERENCES

(1) H. C. Grice, *Lab. Anim. Care*, **14**, 488 (1964).
(2) B. H. Migdalof, *Drug Metab. Rev.* **5**, 295 (1976).
(3) W. Johannessen, G. Gadeholt, and J. Aarbakke, *J. Pharm. Pharmacol.*, **33**, 365 (1981).
(4) R. P. Rand, A. C. Burton, and T. Ing, *Can. J. Phys. Pharmacol.*, **43**, 257 (1965).
(5) S. Kety, *Pharmacol. Rev.*, **3**, 1 (1951).
(6) S. Riegelman, J. C. K. Loo, and M. Rowland, *J. Pharm. Sci.*, **57**, 117 (1968).
(7) M. Gibaldi and D. Perrier, "Pharmacokinetics," Marcel Dekker, New York, N.Y. 1975, p. 45.
(8) J. R. Gillette, "Concepts in Biochemical Pharmacology," Bind 28, Part 3, Springer Verlag, Berlin, Heidelberg, New York, 1975, p. 51.
(9) G. Gadeholt, J. Aarbakke, E. Dybing, M. Sjöblom, and J. Mørland, *J. Pharmcol. Exp. Ther.*, **213**, 196 (1980).
(10) T. Enta, S. D. Lockey, Jr., and C. E. Reed, *Proc. Soc. Exp. Biol. Med.*, **127**, 136 (1968).
(11) O. M. Bakke, M. Bending, J. Aarbakke, and D. S. Davies, *Acta Pharmacol. Toxicol.*, **35**, 91 (1974).
(12) G. M. Cohen, O. M. Bakke, and D. S. Davies, *J. Pharm. Pharmacol.*, **26**, 348 (1974).
(13) A. M. Rudolph and M. A. Heymann, *Circ. Res.*, **21**, 163 (1967).
(14) M. A. Heymann, B. D. Payne, J. E. Hoffman, and A. M. Rudolph, *Prog. Cardiovasc. Dis.*, **XX**, 55 (1977).
(15) S. Kety, *Met. Med. Res.*, **8**, 228 (1960).
(16) M. Reivich, J. Jehle, L. Sokoloff, and S. Kety, *J. Appl. Physiol.*, **27**, 296 (1969).
(17) A. Hope, G. Clausen, and K. Aukland, *Circ. Res.*, **39**, 362 (1976).
(18) A. H. Conney *et al.*, *Ann. N.Y. Acad. Sci.*, **179**, 155 (1971).
(19) M. Danhof, D. P. Krom, and D. D. Breimer, *Xenobiotica*, **9**, 695 (1979).
(20) A. C. Guyton, "Basic Human Physiology: Normal Function and Mechanisms of Disease", W. B. Saunder's, 2nd ed., 1977, p. 798.
(21) T. R. Tephly and G. J. Mannering, *Mol. Pharmacol.*, **4**, 10 (1968).
(22) W. F. Bousquet, B. D. Rupe, and T. S. Miya, *J. Pharmacol. Exp. Ther.*, **147**, 376 (1965).
(23) R. E. Stitzel and R. L. Furner. *Biochem. Pharmacol.*, **16**, 1489 (1967).
(24) G. D. Buckberg, J. C. Luck, D. B. Payne, J. E. Hoffman, J. P. Archie, and D. E. Fixler, *J. Appl. Physiol.*, **31**, 598 (1971).
(25) Y. Sasaki and H. N. Wagner, *ibid.*, **30**, 879 (1971).
(26) S. Kaihara, P. D. van Heerden, T. Migita, and H. Wagner Jr., *ibid.*, **25**, 696 (1968).
(27) D. J. Warren and J. G. Ledingham, *Cardiovasc. Res.*, **8**, 570 (1974).
(28) J. Onarheim and I. Tyssebotn, *Undersea Biomed. Res.*, **7**, 47 (1980).
(29) T. Høyem-Johansen, L. Slørdal, A. Høylandskjaer, and J. Aarbakke, *Acta Pharmacol. Toxicol.*, **47**, 279 (1980).

ACKNOWLEDGMENTS

This work was supported by grants from Norsk Medisinaldepot, Oslo, Norway.

Evaluation of Chemical Analysis for the Determination of Solasodine in *Solanum Laciniatum*

P. G. CRABBE [‡] and C. FRYER [*x]

Received September 29, 1981, from the *Department of Chemical Engineering, Monash University, Clayton, Victoria, Australia, 3168.* Accepted for publication February 5, 1982. *Present address: Fluor Australia Pty. Ltd., Melbourne, 3001, Australia. ‡Present address: Department of Chemical and Materials Engineering, University of Auckland, New Zealand.

Abstract □ A detailed study of a method for solasodine analysis has been carried out and the suitability of chemical analysis for solasodine determination in plant material evaluated. A number of problems with the analytical isolation of solasodine and its subsequent colorimetric determination have been highlighted: oven drying of plant material >100° leads to solasodine loss; cell disruption of the dry plant material is required if complete and rapid extraction of solasodine is to take place; hydrolysis of plant extract residues in >1 *N* acid leads to solasodine loss; the colorimetric procedure is more temperamental than past methods have indicated, especially with regard to the specificity of the reaction and the instability of the complex.

Keyphrases □ Solasodine—steroidal alkaloid from *Solanum laciniatum*, colorimetric determination by evaluation of chemical analysis □ Steroids—colorimetric determination of the alkaloid, solasodine, in *Solanum laciniatum* □ Colorimetry—determination of solasodine in *Solanum laciniatum*

Since problems arose 6 or 7 years ago with the supply, cost, and steroid content of *Dioscorea*, the source of diosgenin for steroid drug production, there has been renewed interest in alternative raw materials, including solasodine from plants of the genus *Solanum*. This steroidal alkaloid occurs in *S. aviculare* and *S. laciniatum* as the glycosides, solasonine and solamargine.

As part of a study into the production of solasodine from *Solanum* plant material, chemical analysis was considered for the determination of solasodine and its related species. Several important observations were made in the course of this investigation concerning both the isolation of solasodine from the plant (sample preparation, extraction, and hydrolysis) and the subsequent determination of solasodine using colorimetry. Chemical analysis was found not to be suitable for this study, and subsequently, a procedure using high-pressure liquid chromatography was developed (1). However, chemical analysis is suitable for certain purposes and has the advantage of not requiring expensive equipment. Matters concerning the isolation of solasodine from the plant material are important in solasodine analysis in general (including instrumental analysis) and in commercial solasodine production.

Previous methods for chemical analysis of solasodine

0022-3549/82/1200-1356$01.00/0

© *1982, American Pharmaceutical Association*

have been reviewed. In particular, a colorimetric procedure (2) has been studied; analytical scale sample preparation, extraction of solasodine glycosides, hydrolysis, and determination of solasodine by methyl orange complexing also have been evaluated.

BACKGROUND

In *Solanum* species, steroid alkaloids constitute only 0–5% of the dried plant, making it necessary to determine these components in the presence of a large amount of inert material. In most procedures for determination of solasodine in *Solanum*, solasodine is separated from the plant matrix by selective extraction, then purified before analysis.

Solasodine must be isolated in a relatively pure form before its determination. There is only one reported method in which plant material is analyzed directly for solasodine without extraction or purification steps (3). In all other reported methods, a fairly standard procedure is followed involving sample preparation, extraction of glycosides, and hydrolysis to the aglycon. Because drying stabilizes the solasodine content of the plant material, most procedures start with dry material, although fruits have often been analyzed fresh. Refrigeration has also been used and gives comparable solasodine contents to oven drying of samples at temperatures <100° (4). Oven drying of samples >100° may lead to loss of solasodine (4).

It is normal to crush the dried plant material before extraction. There are reports which suggest that as the average particle size is decreased, the amount of solasodine extracted increases (5, 6). Also, defatting the dry powdered plant material with light petroleum prior to extraction has been shown to reduce significantly the error in the analysis (7).

Solvents used for extraction include dilute aqueous acids, alcohols, and acidified alcohols. Dilute aqueous acid is more specific than alcohols and extracts fewer inert materials. However, if solasodine is present in the plant in partial glycoside or aglycon form because of poor drying and storage, it may not be extracted by dilute aqueous acid, and an alcohol solvent will be more suitable. It has been shown (8) that due to this breakdown of glycosides, previous procedures (9–11) all gave incomplete extraction, and this modified method (8), avoiding acid extraction, gave 30–50% greater solasodine contents for the same material.

Normally solasodine is analyzed as the aglycon, since hydrolysis is a useful purification step, especially if aqueous extraction is used. Increased acid concentration gives increased hydrolysis rates but losses of solasodine have been reported when plant extracts are hydrolyzed with strong acid (4, 7). Many methods combine the extraction and hydrolysis step into a single, direct hydrolysis. Completeness of extraction is ensured due to the more rigorous extraction conditions, and because the less soluble aglycon is produced, mother liquor losses during precipitation, filtration, and washing will be lower. However, such a procedure will extract more inerts, and because the hydrolysis is carried out in the presence of the plant extract, losses of solasodine may occur.

Isolation of solasodine as discussed so far aims only at determining the total steroid base content. To make the isolation procedure more specific, a chromatographic separation (paper, thin-layer, or ion-exchange chromatography) can be introduced at some stage before the final determination is made. If glycoalkaloids are to be distinguished, then separation should follow hydrolysis. In some cases *in situ* colorimetric analytical procedures have been developed using densiometry (12–19). Such methods are rapid but require continual calibration and are subject to large errors.

Three major methods have been used for determination of the amount of solasodine recovered: gravimetric, titrimetric, and colorimetric. Colorimetric determination is the method most widely used. Although there are many dyes that may be used (20, 21), bromthymol blue and methyl orange have been the usual dyes employed.

None of the reported procedures for solasodine determination has been systematically investigated to check for errors arising at each stage in the procedure. Some overall tests have been applied using repeated analysis of plant material to determine reproducibility. This approach identifies random but not systematic errors. A few investigators have used addition of pure solasodine to determine the percentage recovery of solasodine, but this technique will not identify losses during sample preparation and extraction.

In the present investigation, the Birner method (2) for solasodine analysis was subjected to systematic evaluation. The basic procedure of this method was followed, but in a scaled-up form so that larger initial plant samples could be used (1.0 instead of 0.1 g). Three considerations of the Birner method were made: to ensure that the procedure was at optimum conditions, to verify that the solasodine determined was the total solasodine content of the plant material, and to estimate the reproducibility of the method. To achieve these objectives it was necessary to study each step of the method separately and to work backwards through the method starting at the colorimetric assay.

EXPERIMENTAL

The reagents specified previously (2) were used. Standard solutions of solasodine (20 and 100 μg/ml in 20% acetic acid), solasonine (100 and 500 μg/ml in water), and solasodiene (100 μg/ml in ethanol) were prepared from purified compounds. In all cases *S. laciniatum*[1] plant material was used.

Analytical Procedure—Leaf material was oven dried at 70–80° to constant weight, then ground to <75-μm sieve size. This material was divided into six 1.000-g portions, and each portion was analyzed. For the analysis, each portion was refluxed with ethanol (70 ml) for 40 min and then filtered. The filtrate and residue washings were made up to 100 ml with ethanol. An aliquot (20 ml) was evaporated to dryness and 1 *N* HCl (7.5 ml) was added. The mixture was heated on a steam bath (temperature 95–98°) for 5 hr to effect hydrolysis. The solution was cooled and neutralized with 1 *N* NaOH. Glacial acetic acid (5 ml) was added and the solution filtered and made up to 25 ml with water. Aliquots of this hydrolysate (2 ml) were then taken and the solasodine content determined colorimetrically. For the standard solution, an aliquot of hydrolysate plus sodium acetate–acetic acid buffer (5 ml, pH 4.7) and aqueous methyl orange (1.0 ml) were added to chloroform (5 ml) in a separating funnel (100 ml) and the resulting mixture shaken for 4 min. The chloroform layer was separated and dried over anhydrous sodium sulfate and the absorbance read in a spectrophotometer at 425 nm. The same procedure was carried out for the reference solution as for the standard solution, but without added methyl orange.

Instability of the Complex—The solasodine–methyl orange complex formed from 1.0 and 2.0 ml of the 100-μg/ml standard solasodine solution was kept in a capped cuvet and either exposed to room light or left in the spectrophotometer. The absorbance at 425 nm was measured in the spectrophotometer after various time periods.

Hydrolysis of Plant Extract—A standard ethanol extract solution was prepared by refluxing dry leaf material (10 g) in ethanol (1000 ml) for 45 min. Portions of this solution (20 ml) were refluxed with aqueous acid (7.5 ml) for the given period; the solution was cooled and neutralized with equal normality base (7.5 ml) and the solasodine content was determined.

Hydrolysis of Standard Solasonine Solution—For 2 *N* HCl hydrolysis, solasonine (3.2 ml of 500-μg/ml solution) was refluxed on a steam bath with 3.48 *N* HCl (4.3 ml) for the specified period. For 1 *N* HCl hydrolysis, solasonine (3.2 ml of 500-μg/ml solution) with 1.74 *N* HCl (4.3 ml) was used.

Hydrolysis of Plant Extract and Standard Solasodiene—Standard plant extract solution (20 ml) plus solasodiene (5 ml of 100 μg/ml) were evaporated to dryness and 2 *N* HCl added (7.5 ml). The solution was refluxed for the given period then cooled and neutralized with 2 *N* NaOH. The absorbance of the conjugated double bonds of solasodiene at 240 nm was found to be linear and was used to determine the solasodiene content of the final chloroform extract. Reference solution was pure chloroform. For the zero hydrolysis time case, the residue was shaken with the acid for 2 min at room temperature before being neutralized.

Effect of Extraction Time on Solasodine Yield from Crushed Leaf Material—A uniform sample of dry leaf material in the sieve range of 0.5 to 1.0 mm was prepared. Samples (1 g) were refluxed with 70 ml of ethanol for set periods.

Effect of Solid/Solvent Ratio on Extracted Solasodine—The leaf material was oven dried at 70–80° and ground to pass a 1-mm sieve. Samples of 0.25, 0.5, 1.0, and 2.0 g were taken and repeatedly extracted. For each extraction the sample was refluxed in 70 ml of 95% ethanol for 40 min, after which the solution was allowed to cool and settle and the extract solution decanted. The residue leaf material was washed with 95% ethanol and the washings decanted to make the extract solution up to 100 ml. An additional 70 ml of 95% ethanol was then added immediately

[1] The plant material was obtained from plants grown from seeds collected from plants identified as *S. laciniatum* Ait. by Dr. D. E. Symons (Waite Agricultural Research Institute, South Australia, Voucher No. ADW 47361). Seed samples JMS 75/176 are held by Dr. Fryer.

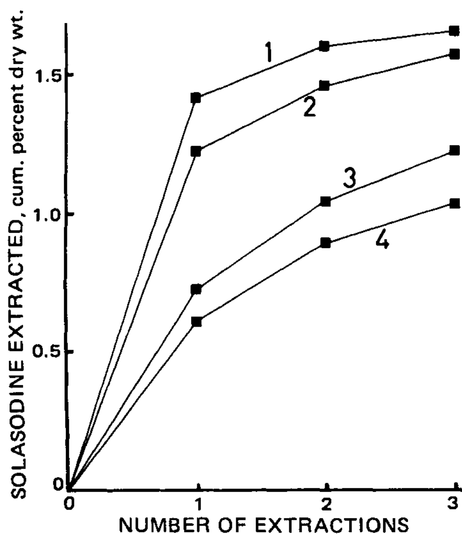

Figure 1—*Combined effect of added base and size reduction on solasodine extracted from dry leaf material. Key: (1) ethanol, ground < 0.25 mm; (2) ethanol, 2% triethylamine, ground < 0.25 mm; (3) ethanol, 2% triethylamine, crushed < 2.812 mm; (4) ethanol, crushed < 2.812 mm.*

to the damp leaf material in the flask and the extraction repeated. Between extractions 4 and 5 of the 1.0-g and 2.0-g samples, the residue leaf material was allowed to stand for 1 week while the analysis of the previous extractions was obtained. During this time the residue dried out completely.

Effect of Particle Size on Extracted Solasodine—A sample of leaf material dried at 70–80° was taken and broken into pieces by crushing. Material in the screen size range of 2.812–1.204 mm was retained, with as much of the leaf stalk material as possible being removed. A portion of this material was put aside and the remainder divided into four quantities. These were either crushed or ground in a mortar and pestle to pass 1.004-, 0.599-, 0.250-, and 0.075-mm screen sizes. This gave five samples of different average particle size, as indicated in Fig. 1. The averge sizes reported are volume average diameters based on sieve analysis, the particle volume estimation allowing for the platelet shape of the leaf particles as described previously (22). Portions of each sample were repeatedly extracted and analyzed as described in the *Analytical Procedure* section. The results were corrected to allow for the contribution of fines (<75 μm) in the samples.

Microscopic observation of the samples, numbered 1–5 as in Fig. 1, revealed: sample 1, large pieces of leaf with no fines; samples 2 and 3, large pieces of leaf plus material of various sizes including cell-size particles; sample 4, mostly fine particles of cell size but also small leaf particles consisting of several cells together (<50 cells); and sample 5, completely ruptured cell material.

The cell structure could be seen clearly on the flat surface regions of the leaf material. Ruptured cells only occurred along the thin edges of the leaf material and not on the flat surface regions. By counting cells along a linear dimension (*e.g.*, 1-mm length), it was found that the average cell size was 40 ± 5 μm for the dried leaf material.

Effect of Added Acid-Base on Extracted Solasodine—The leaf material used was the same as that in the section, Effect of Solid/Solvent Ratio on Extracted Solasodine. Three 1-g portions of this material were taken for repeated extraction and analysis as described in the previous two sections. One portion was repeatedly extracted with 70 ml of ethanol and 1.4 ml of glacial acetic acid (2% v/v acetic acid in ethanol); the second with 70 ml of ethanol and 3.5 ml of glacial acetic acid (5% v/v acetic acid in ethanol); and the third with 70 ml of ethanol and 5 ml of concentrated ammonia solution (2% v/v ammonia/ethanol). Each extraction was carried out under reflux for 40 min.

Combined Effect of Particle Size and Added Base on Extracted Solasodine—Leaf material dried at 70–80° was divided into two portions. One portion was crushed to pass a 2.812-mm sieve size, the other ground to pass a 0.25-mm sieve size. Portions (1 g) of each of these size fractions were repeatedly extracted and analyzed using both 70 ml of ethanol and 1.37 ml of triethylamine. Each extraction was carried out for 40 min under reflux. When the solvent was removed from the portion of the extract solution taken for hydrolysis, care was taken to remove all the triethylamine (bp < 90°) so that it would not interfere in the subsequent colorimetric analysis.

Effect of Extraction Time on Solasodine Yield from Ground Leaf Material—A uniform sample of dry leaf material was ground to pass a 90-μm sieve size. Samples (0.8 g) were refluxed with 60 ml of methanol for set periods. For the zero-extraction time case, the sample was shaken with the methanol for 30 sec at room temperature before filtering.

Effect of Oven Drying Temperature on Solasodine Yield—Leaf material from the same plant and of a similar size and age was divided into six samples of 100–150 g each and dried at various temperatures in an air-circulated oven. Once dry, each leaf sample was ground to pass a 90-μm sieve before extraction. Extraction was a 40-min reflux of 1.0 g/70 ml of ethanol.

RESULTS AND DISCUSSION

Colorimetric Analysis—Results of the colorimetric analysis procedure outlined above showed that the absorbance of the solasodine–methyl orange complex obeys Beer's law over the range of 2–50 μg/ml.

The original method (2) involves no reference for the absorbance readings. Normal plant colored matter, soluble in chloroform, is carried through the procedure and gives absorbance in the <450-nm region. It was found that the use of a reference solution, similar to the standard solution but without the methyl orange, eliminated this effect. The mass transfer of the solasodine–methyl orange complex from the aqueous to the chloroform layer is slow and was found to take up to 5 min.

The methyl orange–solasodine complex was found to be relatively unstable and several precautions must be taken. Contact with rubber, metal, or plastics gave breakdown of the complex and led to erroneous results. Because of this, the complex should be contained in clean, smooth glassware rinsed with an acetone solution of 0.5% *m*-cresol before use (4).

The complex was found to be stable in the dark for up to 3 days after formation but was unstable when exposed to room light or to light of wavelength 425 nm in the spectrophotometer. Losses ranging from 20 to 50% were observed within 20 min of exposure to these conditions, with initial rates of loss from 1 to 5%/min. To obtain reproducible results, it is essential that the time between formation of the complex and measurement of its absorbance be the same in all analysis and calibration tests.

Methyl orange was found to form complexes with amines other than solasodine, including most simple organic amines and even ammonia. Such compounds may be formed during degradation of plant material in storage. These complexes had similar absorption characteristics to the solasodine–methyl orange complex.

Hydrolysis—Only the effects of acid concentration and time on the hydrolysis were considered. Temperatures and solvent were left unchanged from the original method. It was found that, using 0.5 and 1 *N* HCl, hydrolysis was not complete until after 5-hr digestion on a steam bath. Using 1 *N* HCl and a hydrolysis time of 2 hr as in the original procedure (2), the hydrolysis is only 55–60% complete (Fig. 2). At higher acid

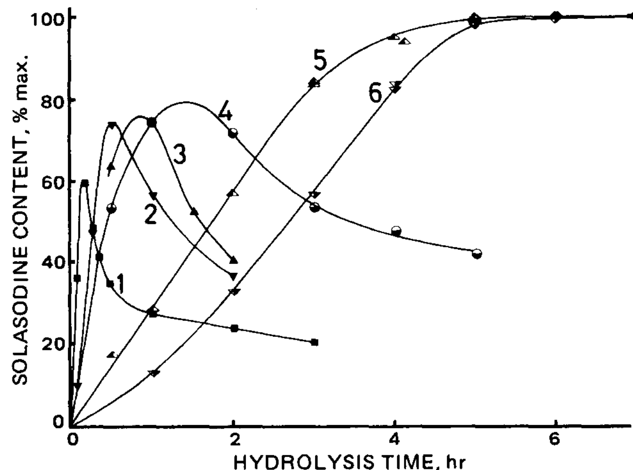

Figure 2—*Effect of acid concentration on the hydrolysis of plant extracts (solasodine contents are expressed as a percentage of the maximum solasodine content, i.e., that found after stable hydrolysis: 0.5 or 1.0* N *HCl for 5 hr at 100°). Key: (1) 2.0* N *HCl; (2) 1.5* N *HCl; (3) 1.5* N *H_2SO_4; (4) 1.25* N *HCl; (5) 1.0* N *HCl; (6) 0.5* N *HCl.*

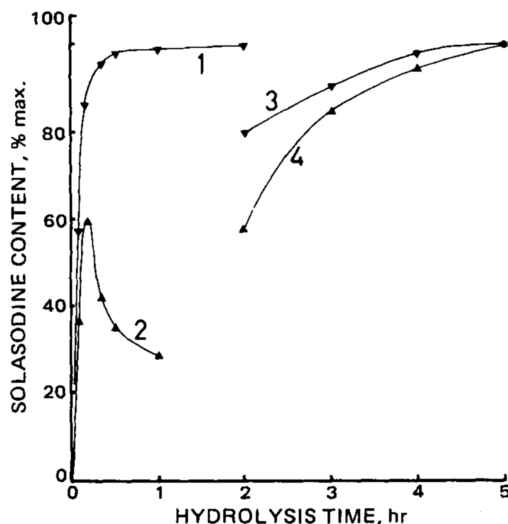

Figure 3—*Hydrolysis of pure solasodine with aqueous hydrochloric acid at 100°, compared with the similar hydrolysis of leaf extracts. Key: (1) 2 N HCl, pure solasonine; (2) 2 N HCl, plant extract; (3) 1 N HCl, pure solasonine; (4) 1 N HCl, plant extract.*

concentrations, the apparent solasodine contents did not stabilize and dropped away after reaching a maximum value. Unstable hydrolysis, as shown in Fig. 2, has only been found by two workers. Unstable hydrolysis with 2 *N* HCl but stable hydrolysis with 0.5 *N* HCl was found (4), while another report (7) indicated that apparently unstable hydrolysis with each of 4, 2, and 1 *N* HCl was found. For the 1 *N* HCl hydrolysis case in the latter study (7), the decrease of solasodine content after the maximum is reached is based on only one experimental point, and represents a <5% change in the apparent solasodine content.

To investigate further the losses of solasodine during hydrolysis, two separate trials were made. In the first experiment, hydrolysis of pure glycoalkaloid (solasonine) was carried out using 1 and 2 *N* HCl levels. The results are given in Fig. 3 and show stable hydrolysis, producing the required amount of solasodine in both cases. The comparable curves for the hydrolysis of the plant extract are reproduced from Fig. 2 for comparison. In the second experiment, 2 *N* HCl hydrolysis of the leaf extract, to which a known amount of pure solasodine was added, gave a reduction of solasodiene with hydrolysis time as shown in Fig. 4. The solasodiene added amounted to 20 μg/ml. The initial increase of solasodiene concentration to this value may be due to the leaching of the solasodiene into aqueous solution from the plant extract residue—a thin, tarry residue on the walls of the flask. The need for such leaching explains why, as

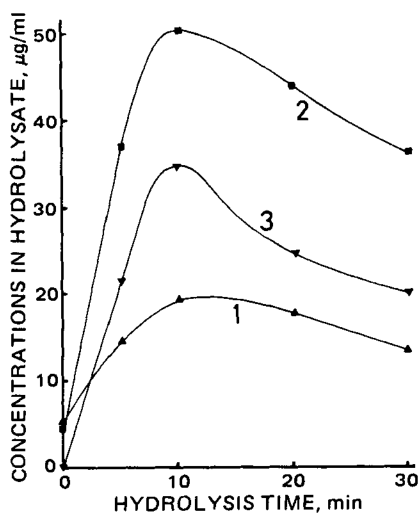

Figure 4—*Hydrolysis with 2 N HCl at 100° of a leaf extract containing added pure solasodiene compared with the similar hydrolysis of leaf extract. Key: (1) solasodiene from extract with added solasodiene; (2) total steroidal base from extract with added solasodiene; (3) solasodine from leaf extract (from Fig. 3).*

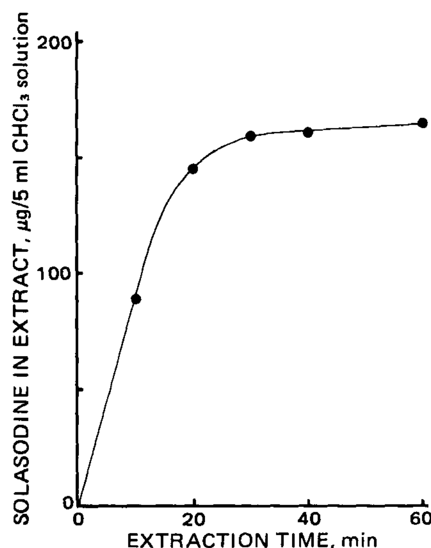

Figure 5—*Effect of extraction time on extraction of solasodine with ethanol from crushed leaf material.*

shown in Fig. 3, hydrolysis of pure solasonine occurred faster than the corresponding hydrolysis of the plant extract: The pure solasonine was being hydrolyzed in solution and no leaching was necessary. The change in total steroidal base is also shown in Fig. 4. This was determined by the normal colorimetric assay procedure and represents the total solasodine plus solasodiene content. The 2 *N* HCl hydrolysis curve from Fig. 2 is also reproduced in Fig. 4 for comparison. The sum of the lower curves approximates the upper (solasodine + solasodiene) curve.

From these two experiments, it appears that it is the formation and the subsequent further reaction of solasodiene which is causing the overall loss of solasodine (determined as total steroidal base). The further reaction of solasodiene is linked to other compounds in the plant extract and is important only >1 *N* acid. Sulfuric acid reportedly produces less solasodiene than hydrochloric acid for a given acid strength (23) but, as shown in Fig. 2, at the 1.5 *N* level, the use of sulfuric acid still does not give stable hydrolysis.

Extraction—Figure 5 shows the effect of time on extraction for uniform dry leaf material of a sieve size range of 0.5–1.0 mm. A 40-min extraction time appears adequate for this material as the curve tends to approach an upper limit after this period. For the extraction of 1 g of plant material with 70 ml of ethanol, the solubility limit of solasodine (in either glycoside or aglycon form) in ethanol is not exceeded. Because of this and the leveling-off of the curve in Fig. 5, it was initially assumed that complete extraction had taken place. However, when the residue of the plant material from the first extraction was re-extracted with ethanol as before, more solasodine was extracted, amounting to as much as 30–40% of that of the first extraction. Further extractions of this same residue yielded more solasodine; complete extraction was not taking place in a single 40-min ethanolic extraction.

To characterize further this problem of incomplete extraction, a series of extractions of the same material but with different solid/solvent ratios was carried out. The results are shown in Fig. 6. The greater the solid/solvent ratio, the less solasodine per weight of sample extracted. Even so, not all the solasodine is extracted after repeated extractions. For example, even after six repeated extractions of the 2.0-g sample, the solasodine extracted in the seventh extraction represents 4% of the total solasodine extracted. There is a discontinuity in the curves for 1.0- and 2.0-g samples between extractions 4 and 5. This corresponded to the extracted residue being allowed to stand for several days before the fifth extraction. As a result, more solasodine was extracted in the fifth extraction, but this has not altered the shape of the curve between the sixth and seventh extractions from the shape prior to the fourth extraction.

Figure 7 shows the results of Fig. 6 plotted as cumulative solasodine extracted against sample size; the total solasodine extracted per weight of sample decreases with an increase in sample size, but the total solasodine extracted increases with sample size. Thus, as the solid/solvent ratio is increased, more solasodine is extracted (per volume of solvent), but the extraction process is less efficient (per weight of solid). These trends are more important <1 g/70 ml than above this ratio.

The effect of decreasing the particle size on solasodine extraction was investigated. The results are shown in Fig. 8. Lower particle size gives

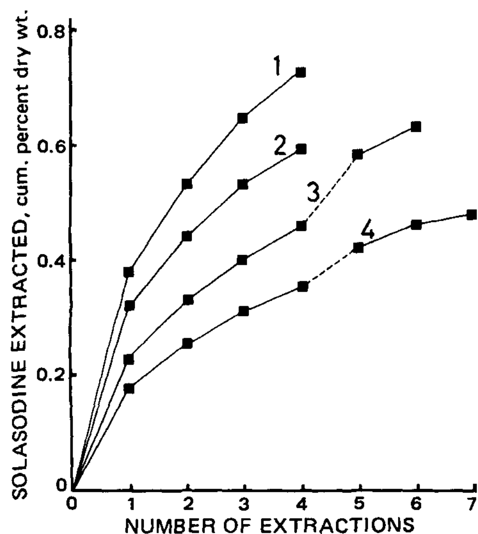

Figure 6—*Effect of altering the proportion of dry leaf material to extractant on solasodine extracted. Key: (1) 0.25 g/70 ml; (2) 0.5 g/70 ml; (3) 1.0 g/70 ml; (4) 2.0 g/70 ml.*

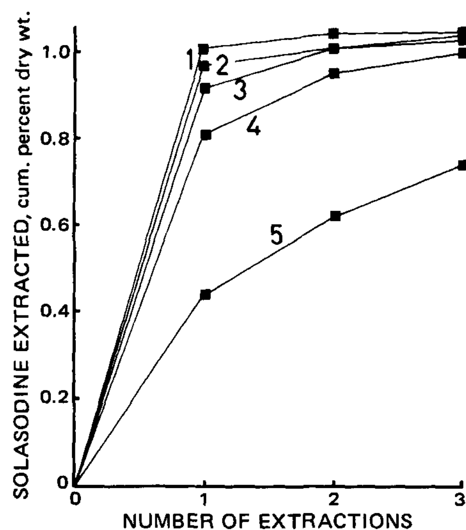

Figure 8—*Effect of average particle size,* D, *on solasodine extracted from dry leaf material. Key: (1)* D < *0.075 mm; (2)* D = *0.16 mm; (3)* D = *0.42 mm; (4)* D = *0.60 mm; (5)* D = *1.93 mm.*

increased extraction, and for the sample of the finest material, 97% of the total solasodine was obtained after a single 40-min extraction. The cell size of the dry leaf material used in this experiment was calculated at 40 ± 5 μm by viewing the material through a microscope. This means that for the material ground to pass the 75-μm sieve size, the majority of the cells are ruptured, while for the largest material [particle size (D) = 1.93 mm] only a small proportion of the cells, those along the edges of the leaf pieces, are ruptured.

It is suggested from these observations that ruptured cells have readily extractable solasodine, while the solasodine within intact cells can only be removed by much slower diffusional transport through cell walls. This would explain the shape of Fig. 5. The curve represents not a single exponential type diffusional process but the combination of two diffusional processes, one much more rapid than the other. The apparent leveling-off seen in Fig. 5, therefore, is misleading and represents the much slower diffusional process becoming dominant in the latter stages of the extraction. This also explains why in Fig. 6 the sudden jump between extractions 4 and 5 of the 1.0- and 2.0-g samples occurred. The greater time permitted to elapse before the next extraction has allowed diffusional processes to distribute the remaining solasodine uniformly throughout the plant material, enabling greater removal during the next extraction.

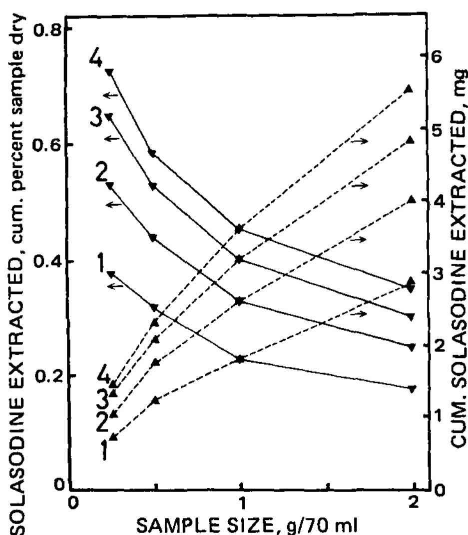

Figure 7—*Effect of altering the proportion of dry leaf material to extractant on cumulative solasodine extracted from successive extractions. Key: (1) first extraction; (2) second extraction; (3) third extraction; (4) fourth extraction; (—) sample size; (- - -) total solasodine extracted.*

If Fig. 7 is reconsidered, from the curves of cumulative solasodine extracted per weight of sample *versus* sample size, it may be seen that the effect of sample size occurs predominantly during the first extraction. During subsequent extractions the effect decreases until in the fourth extraction an approximately equal amount of solasodine per unit weight is extracted for each sample size. The shape of the curves can be attributed to an indirect effect caused by the processes being diffusion-controlled. The diffusional driving force is proportional to the difference in solasodine concentration between the plant material and the bulk solvent. At higher solid/solvent ratios, the bulk solvent concentration will increase to a higher level. Consequently, the diffusion driving force will decrease, causing less solasodine to be extracted over a given time in such cases. The effect can be expected to be greater for the first extraction, where the large proportion of the solasodine is rapidly extracted into the bulk solution from the ruptured cells. The effect becomes less important in subsequent extractions where less solasodine is extracted, and the initial rapid extraction from the ruptured cells no longer occurs.

The effect of adding acid or base to the ethanol to aid the diffusional processes was also investigated. Soaking of plant material in either acid (8, 11, 24) or base (25) prior to extraction has been shown to give higher yields of the alkaloids. The ability to improve extraction is based on the assumption that the solasodine is present in the plant as an ion pair, possibly attached to an anionic site fixed to the plant cell matrix. If this is the case, added acid will form a more mobile solasodine salt, while added base will produce the free base form of solasodine, both unattached to the plant wall. The results for added acetic acid at 2 and 5% (v/v) levels are shown in Fig. 9 for the same material as used in Fig. 6. The curve for extraction of a 1-g sample with pure ethanol is reproduced from Fig. 6 for comparison. The effect is small and possibly not significant. Added base gives a much greater effect but only for coarsely crushed material (Fig. 1). When diffusion strongly limits the extraction process (coarsely crushed material) added base helps by making the solasodine molecule more mobile, but when this diffusional limitation is eliminated (finely ground material) the added base acts only to decrease the solubility of the solasodine.

Mechanical rupture of leaf cells is not the only method of eliminating the cell wall diffusional barriers but is best suited to analytical extraction. The effect of cell disruption by freezing or by cooking under either pressure or vacuum on the diffusion of solasodine from *S. laciniatum* was studied (26); if disruption of the plant cells preceded extraction, then extraction rates, not yields, were found to be higher. The action of endogenous enzymes produces similar results to dry grinding (22). Figure 10 shows the effect of time on the extraction with methanol of dry leaf material ground to pass a 90-μm sieve; very rapid extraction of the solasodine is shown, and within 10 min all the solasodine is extracted. Even a 30-sec wash with methanol at room temperature (the zero point in Fig. 10) extracts ~80% of the total solasodine.

Sample Preparation—It is normal for a plant sample to be dried and crushed to a uniform size fraction before analysis. This stabilizes the solasodine content in the plant material and, if only a fraction of the total sample is to be analyzed, enables a representative portion to be taken.

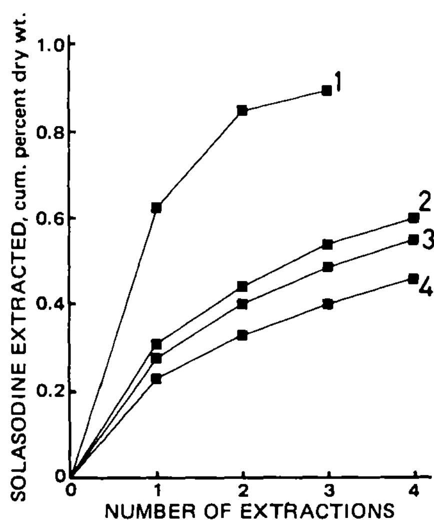

Figure 9—*Effect of added acid or base on solasodine extracted from dry leaf material. Key: (1) ethanol, 2% ammonia; (2) ethanol, 5% acetic acid; (3) ethanol, 2% acetic acid; (4) ethanol.*

The plant sample needs to be very finely ground to ensure complete extraction as already discussed. A number of workers have reported variable solasodine contents for the same material dried under different conditions (27–29). However, preliminary studies of postharvest drying (22) show no such effects and indicate that previous findings have been affected by the problem of incomplete extraction discussed above. Oven drying at 80° is recommended for analytical sample preparation. Below 75° drying rates are slow and >100° losses of solasodine occur (4). Figure 11 presents the results for the same leaf material dried at oven temperatures of 40–120°; severe reduction in extractable solasodine occurs for drying temperatures >100°. In a separate experiment, this loss of solasodine was shown to occur near the end of the drying process. The loss of extractable solasodine is accompanied by a corresponding loss of the green-colored material normally extracted by alcohol. It appears that for leaf material dried >100°, decomposition and transformation of other leaf components take place simultaneously with the drying process, and the decomposition products may restrict later extraction of the solasodine.

Reproducibility—During this study of a previously described method (2) several modifications have been made to provide a method with no systematic errors. To establish an estimate of the reproducibility of the modified method, repeated analysis of the same finely ground plant material was carried out. This enabled calculation of the sample standard deviation of the overall method (s), of the combined hydrolysis–colorimetric assay steps (s_{hc}) and of the colorimetric assay step alone (s_c) (22). The values of the sample standard deviation (expressed as a percentage of the overall mean) and their associated degrees of freedom are included

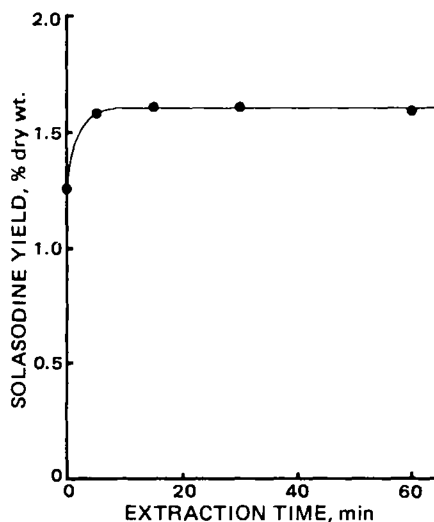

Figure 10—*Effect of extraction time on extraction of solasodine with methanol from ground leaf material.*

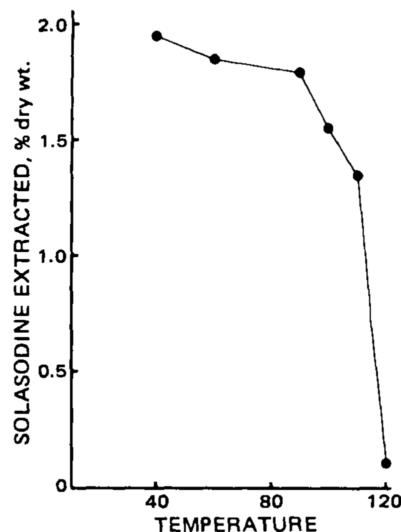

Figure 11—*Effect of drying temperature on solasodine extracted from oven-dried leaf material.*

Table I—Experimentally Determined Standard Deviations for Chemical Analysis

Procedure	Standard Deviation, %[a]	Degrees of Freedom
Colorimetric assay	$s_c = 5.6$	6
Hydrolysis and assay	$s_{ch} = 6.4$	6
Overall analysis	$s = 6.1$	10

[a] As percent of overall mean.

in Table I. From these sample standard deviations, the confidence intervals for the corresponding population standard deviations (σ, σ_{hc}, and σ_c) may be calculated. Based on the χ^2 distribution, the 90% confidence intervals for the standard deviations, expressed as percentages of the overall mean, are:

$$3.9\% < \sigma_c < 10.8\%$$
$$4.4\% < \sigma_{hc} < 12.2\%$$
$$4.5\% < \sigma < 9.7\%$$

These intervals are so large that it is difficult to draw any conclusion from the estimates of σ_c, σ_{hc}, and σ, except that the major proportion of the overall error occurs in the colorimetric assay and that the overall error in the analysis method is 4.5–9.7%. This means that the limiting feature of the method is the final colorimetric determination of solasodine, due probably to the instability of the complex.

CONCLUSIONS

For samples of *S. laciniatum*, where solasodine is the only steroidal alkaloid present, total solasodine glycosides, solasodine aglycon, and solasodiene each can be determined. The aglycons may be extracted into a nonpolar solvent such as chloroform or benzene and the separate contents of total glycosides and total aglycons determined; measurement of solasodiene content in the aglycon fraction can be made at 235 nm in a UV spectrophotometer. If chromatographic separation is incorporated [for examples see previously described procedures (30–33)] then the glycosides may be further separated before the final analysis. Theoretically any analysis related to solasodine production can be performed, but once chromatography is incorporated, the extra errors involved make the results at best semiquantitative.

Study of this particular colorimetric assay technique shows in more detail the problems associated with such a technique. Difficulties have been found in each aspect of the method, difficulties largely overlooked in previously reported methods, and difficulties which in some cases apply to commercial solasodine isolation. The colorimetric procedure is much more temperamental than past methods have indicated, especially with regard to the specificity of the reaction and the instability of the complex. It was implied (2) that the reaction with methyl orange was specific to nitrogen-containing alkaloids; whereas it appears that all amines, in-

cluding ammonia itself, will give similar complex formation. The instability of the complex was also not mentioned.

Although solasodiene can be analyzed along with solasodine to give a total solasodine content, severe hydrolysis conditions (>1 *N* HCl) have been found to give overall solasodine loss. This is apparently caused by reactions, of unknown mechanism, associated with solasodiene formation and in the presence of the plant extract. Using conditions where stable hydrolysis occurs, much longer hydrolysis times than normally specified in reported methods were found to be necessary to complete the hydrolysis (*e.g.*, 5 hr at 100° for 1 *N* HCl in aqueous solution). Even for glycosides in solution, inspection of Fig. 3 shows that at least 4 hr at 100° for 1 *N* HCl is necessary.

For complete extraction of the solasodine to take place within one extraction, it is necessary to remove the cell wall diffusional barriers. For analytical purposes dry grinding of the plant material to pass a 75- to 90-μm sieve is suitable and allows very rapid extraction of the solasodine by alcohol. To stabilize the solasodine content of the plant material, oven drying at 75–90° is recommended. Oven drying >100° leads to loss of extractable solasodine. However, a preliminary fixation at temperatures >100° to arrest biological processes within the plant material does not affect the extraction process.

The final colorimetric assay is the cause of most of the error inherent in the method. Assuming a χ^2 distribution, the confidence interval for the true standard deviation is large, unless a large number of repeated analyses are made for its estimation. In most reported methods, where evaluation of the method is based on the results of repeated analyses, the number of repetitions is small, and consequently, in such cases the confidence interval for the true standard deviation will be large. Thus, the true error inherent in a method may differ greatly from that reported.

The problems of chemical analysis raise doubts about the soundness of previous studies of solasodine production. Results may be subject to large systematic and random errors because of poor analytical procedures. In particular, the problem of incomplete extraction will introduce large systematic errors.

REFERENCES

(1) P. G. Crabbe and C. Fryer, *J. Chromatogr.*, **187**, 87 (1980).
(2) J. Birner, *J. Pharm. Sci.*, **58**, 258 (1969).
(3) C. Lorincz, *Magy. Kem. Foly.*, **68**, 414 (1961).
(4) J. E. Lancaster and J. D. Mann, *N. Z. J. Agric. Res.*, **18**, 139 (1975).
(5) R. Hardman and T. G. Williams, *J. Pharm. Pharmacol.*, **23**, 231S (1971).
(6) W. J. Cruz and O. Proaño, *Politecnica*, **2**, 155 (1970).
(7) R. Hardman and T. G. Williams, *Planta. Med.*, **29**, 66 (1976).
(8) P. Bite, E. Mago-Karacsony, T. Rettegi, *Magy. Kem. Foly.*, **76**, 90 (1970); *Acta Chim. (Budapest)*, **64**, 199 (1970).
(9) I. Gyenes, *Magy. Kem. Foly.*, **56**, 383 (1950).
(10) K. Szasz, L. Gracza, and C. Lorincz, *Acta. Pharm. Hung.*, **31**, 211 (1961).
(11) N. A. Valovich, *Med. Prom. SSSR*, **19**, 45 (1965).
(12) G. L. Szendey, *Arch. Pharm.*, **290**, 563 (1957).
(13) G. Blunden, R. Hardman, and J. C. Morrison, *J. Pharm. Sci.*, **56**, 948 (1967).
(14) K. R. Brain and R. Hardman, *J. Chromatogr.*, **38**, 355 (1968).
(15) M. B. E. Fayez and A. A. Saleh, *Z. Anal. Chem.*, **246**, 380 (1969).
(16) E. Kolos-Pethes, J. Varadi, and G. Marczal, *Herba Hung.*, **8**, 169 (1969).
(17) V. N. Borisov, L. A. Pikova, and G. L. Zachepilova, *Khim.-Farm. Zh.*, **10**, 116 (1976).
(18) A. F. Azarkova and L. M. Kogan, *ibid.*, **10**, 104 (1976).
(19) L. A. Pikova, V. V. Panina, and E. I. Korneva, *Rastit. Resur.*, **13**, 531 (1977).
(20) E. A. Tukalo and G. N. Tsarik, *Khim.-Farm. Zh.*, **4**, 56 (1970).
(21) L. Gracza and C. Lorincz, *Arch. Pharm.*, **296**, 615 (1963).
(22) P. G. Crabbe, Ph.D. thesis, Monash University (1979).
(23) A. S. Labenskii and R. I. Kuzovkova, *Med. Prom. SSSR*, **19**, 35 (1965).
(24) P. Bite, E. Mago, and T. Rettegi, Hung. Pat. 155441 (Dec. 23, 1968).
(25) K. Syhora, Z. Cekan, and S. Hermanek, *Planta Med.*, **10**, 318 (1962).
(26) G. Selmeci, G. Valovics, and L. Szlavik, *Pharmazie (Berlin)*, **22**, 173 (1967).
(27) A. R. Guseva, V. A. Pasechnichenko, and M. G. Borikhina, *Biokhimiya*, **30**, 260 (1965).
(28) V. V. Panina, G. M. Dem'yanova, and R. I. Kuzovkova, *Rast. Resur.* **10**, 386 (1974).
(29) R. K. Moiseev, L. K. Klyshev, and A. R. Guseva, *Tr. Inst. Bot., Akad. Nauk Kaz. SSR*, **28**, 215 (1970).
(30) R. Kuhn, I. Löw, and H. Trischmann, *Chem. Ber.*, **88**, 289 (1955).
(31) A. R. Guseva and V. A. Paseshnichenko, *Biochemistry (Engl. Transl.)*, **24**, 525 (1959).
(32) L. H. Briggs, R. C. Cambie, and J. L. Hoare, *J. Chem. Soc.*, **1961** 4645.
(33) *Idem.*, **1963**, 2848.