ABSTRACT

Title of Document: FEASIBILITY OF EXTRACTING SOLANESOL FROM TOBACCO BIOMASS AS A BYPRODUCT FOLLOWING PROTEIN RECOVERY

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The solanesol content in tobacco biomass, a waste derived from tobacco leaves obtained from the low alkaloid cultivar 'MD 609LA' that had been processed for protein recovery, was evaluated at different points during processing. Solanesol, a precursor to coenzyme Q10 and Vitamin K2, is a high value compound found in significant amounts in tobacco leaves and could potentially increase the profitability of tobacco when grown at high density and harvested mechanically for nonsmoking applications. Respective solanesol yields of various extraction methods were assessed using an optimized reverse-phase high performance liquid chromatography (RP-HPLC) method. Solanesol was detected in the waste streams generated during tobacco protein processing, at 0.047% and 0.331% dry weight of the biomass waste and chloroplast sediment, respectively. Microwave-assisted extraction was found the most efficient extraction method in terms of solanesol yield, extraction time, and solvent usage. This research shows that the extraction of solanesol after protein recovery is a feasible operation and could increase the overall profitability of biorefining tobacco for alternative, value-added uses.

FEASIBILITY OF EXTRACTING SOLANESOL FROM TOBACCO BIOMASS AS A BYPRODUCT FOLLOWING PROTEIN RECOVERY

By

Peter Machado

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Chapter 1: Introduction

Between 2001 and 2005 the Southern Maryland Agricultural Development Commission enacted a voluntary buyout for tobacco farmers to decrease the dependency of the area's economy on leaf tobacco sales to cigarette companies and increase crop diversity. They provided the farmers with a subsidy in exchange for stopping production of tobacco for smoking purposes, and maintaining their farm in agriculture for 10 years. The buyout was overwhelmingly successful, with 94% of eligible Maryland tobacco producers participating (Southern Maryland Agricultural Development Commission, 2004). However, with the subsidies ending as early as 2011 and the underlying fact that tobacco grows well in the Maryland climate, it is clear that if value-added alternate uses of tobacco could be identified, the farmers would be willing and able to continue growing the crop. During the buyout period, many farmers chose different crops to produce, including corn for livestock feed and grapes for winemaking, but those crop production switches required a large investment in new equipment as well as having a steep learning curve in the field management of a new crop. The only option available with the buyout program that will allow farmers to continue growing tobacco on their land is to create profitable, alternate uses to smoking of tobacco. This process also includes developing and refining the biorefinery processes that are associated with extracting different products from tobacco so that profitability can be optimized.

Extensive efforts using transgenic tobacco for production of specific proteins have been made to take advantage of tobacco's high biomass production capability. Applications of these made to order proteins include enzyme replacement therapy, various cancer treatments, and the creation of human serum albumin for blood replacement therapies (Nevitt et al., 2003). However, such studies are limited by the stability of the transgenic tobacco post-harvest, as well as the purity and precision of the proteins being created by the tobacco. Subtle variances in the amino acid backbone may trigger an immunological response in humans which can negatively affect the efficacy of the proteins (Nevitt et al., 2003). Current studies conducted by researchers at the University of Maryland have demonstrated that the chloroplastic (F-1) rubisco protein, a non-allergenic protein with a nutritional value comparable to milk protein (Wildman, 1983; Maryanski, 2002), and cytoplastic (F-2) proteins can be effectively recovered at pilot scale (Fu, 2007). A wide variety of functionalities are being explored to assess the applicability of the recovered protein, while extensive field trials geared toward increasing the yield of tobacco biomass within a shorter growth period are near completion. The overall economic value of tobacco could be leveraged if additional ingredients could be identified and retained from the bioprocesses before sending the biomass for bioconversion of the remaining cellulosic constituents. One of the most promising ingredients is the high-value chemical, solanesol, which is the focus of this study.

Chapter 2: Literature Review

2.1 Solanesol

The chemical formula for solanesol is $C_{45}H_{74}O$, and its molecular weight is 631.07. Its official nomenclature is 3, 7, 11, 15, 19, 23, 27, 31, 35-Nonamethylhexatriaconta-2, 6, 10, 14, 18, 22, 26, 30, 34-nonaen-1-ol (Woollen and Jones, 1971). Its chemical structure is shown in Figure 1. Coenzyme Q10 and Vitamin K2 are both ubiquinone compounds (See Figure 2.2) which can be created from solanesol.

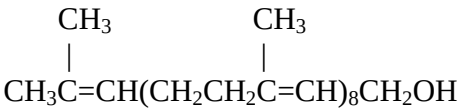


Figure 2.1: The chemical structure of solanesol

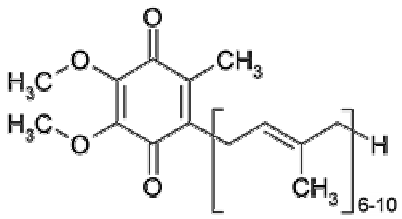


Figure 2.2: The chemical structure of ubiquinone compounds

2.1.1 Solanesol in tobacco

Rowland et al. (1956) first reported the presence of solanesol in tobacco. They reported that solanesol levels of green (freshly harvested) tobacco leaf were 0.3% of the total leaf dry weight, and that solanesol levels of both unaged, flue-cured tobacco leaf and aged, flue-cured tobacco leaf appeared to increase slightly to about 0.4% of the total dry weight (Rowland, et al. 1956).

Subsequent research using improved analytical methods showed higher levels of solanesol. Severson et al. (1977) reported solanesol concentrations in Maryland tobacco of slightly over 2% of total leaf dry matter. An Eastern Carolina flue-cured variety showed concentrations of approximately 3% of dry matter, while two burley varieties had approximately 1% and 2% solanesol, respectively (Severson, 1977). Chamberlain et al. (1990) reported total solanesol concentrations in six flue-cured tobacco varieties ranging from 1.9% to 2.8% (dry basis). Free solanesol in these six varieties ranged from1.3% to 2.5% of total leaf dry weight (Chamberlain et al., 1990). Solanesol was found to originate in the chloroplasts of the tobacco leaves (Stevenson, 1963).

Later research determined that the apparent increase in solanesol in cured leaves observed by early researchers was primarily due to the release of bound solanesol, in the form of esters, through the curing process. Consequently, current experimental extraction methods include a saponification step to free bound solanesol. Addition of low concentrations of sodium hydroxide to the extraction solution has produced a 15-20% increase in solanesol recovery (Zhou and Liu, 2006a).

Both free and bound forms of solanesol are found primarily in the chloroplasts of tobacco leaves. Zhao et al. (2007) used high performance liquid chromatography (HPLC) to measure solanesol concentrations in various parts of the tobacco plant. They found that leaf solanesol concentrations were 6.8 times greater than in the stalks. The ratio between leaf concentrations and concentrations in other plant parts (i.e., flowers, seeds, fruits, and roots) was even greater (Zhao et al. 2007).

2.1.2 Coenzyme Q10

Coenzyme Q10, also known as ubiquinone, is a vitamin-like substance used by the human body to help produce ATP in the electron transport chain and is found throughout the body, with the highest concentrations in the heart and liver (Ernster and Dallner, 1995). Coenzyme Q10 is currently being studied as a supplement which can possibly provide relief for migraine headache sufferers (Sandor, et al., 2005) protect people from Parkinson's disease and other neurodegenerative diseases (Matthews, et al. 1998), and lower blood pressure. These studies have helped develop coenzyme Q10 into a high value product that has increased its demand. Coenzyme Q10 is currently available as a dietary supplement, as well as being added to products like skin cream and toothpaste (Kaneka, 2008).

Coenzyme Q10 is currently fermented from yeast (Chokshi, 2001). There are established protocols for developing coenzyme Q10 from solanesol (West, 2004 as well as studies that have found that using solanesol can increase the yield of coenzyme Q10 derived from the fermentation of yeast (Lui et al, 2008).

2.2 Extraction and Purification Techniques

Researchers have proposed various new techniques for improving recovery of solanesol. Zhou and Liu (2006a) reported that use of microwaves to assist a solvent-based extraction consisting of a 3:1 ethanol:hexane mixture combined with NaOH yielded 0.91% (w/w, dry basis) solanesol in 40 minutes. The same researchers compared microwave-assisted extraction to the previously used heat-reflux extraction and found only minor increases in overall yield (0.05 percentage extraction of solanesol), while the microwave-assisted extraction significantly reduced extraction time from 180 to 40 minutes (Zhou and Liu 2006a). This research team also examined the saponification step required to free bound solanesol, finding that when using microwave-assisted extraction, a 0.05 M solution of NaOH is optimal. However, during the lengthy heat-extraction process, the higher 0.05 M concentration of NaOH can destroy the solanesol, and it was determined that 0.02 M NaOH yielded the maximum amount of solanesol in heat-reflux extraction. The saponification process also can alleviate emulsification, which may present problems during later solanesol separation and purification steps (Zhou and Liu 2006a).

Chen et al. (2006) found that three rounds lasting 20 minutes each in an ultrasonic bath were sufficient to extract the solanesol from tobacco leaf residues. However, they analyzed different saponification steps, adding as much as 30 mg/mL of KOH to the solution and applying a hot water bath from 2 to 24 hours. Again, due to the eventual destruction of solanesol by the heat and KOH, the solanesol yields reached a maximum at 4 hours with 20 mg/mL KOH (Chen et al., 2006). Room temperature extraction and soxhlet extraction techniques have also been analyzed, but

due to their considerably lower yields and long extraction times, have not been further investigated (Zhou and Liu 2006b).

Zhao and Du (2007) proposed a technique involving slow rotary, counter-current chromatography to recover solanesol in order to avoid using industrial organic solvents in the extraction. They found that a 1:2 sunflower oil: ethanol solvent system removed many impurities, but solanesol yield was only about 27% of the resulting product. Some recent publications have suggested the use of supercritical fluid extraction (SFE) to recover solanesol (Chiu, 2006; Rao, 2007), but there are questions regarding the cost-effectiveness of SFE technology and its suitability for handling large batches.

Tang et al. (2007b) completed a study using silica gel column chromatography to extract and purify solanesol and found a solanesol yield of 0.38% and a purity level of 83.04%. However, their extraction method did not seem to maximize the solanesol extracted. The purification was done by dissolving crude solanesol extract into a petroleum ether solution (10:1, v/v). The solution was applied to a silica gel column, and eluted with petroleum ether-acetone (90:10, v/v). A 5 mL fraction was collected and dried using a rotary evaporator (Tang et al. 2007b).

Recent research has outlined a method of solid-phase extraction for the concentration and purification of solanesol extracts. Using silica gel and analyzing parameters such as sample loading flow and sample volume, a fast, quantitative and reproducible method was found to produce a 97.5% recovery of solanesol (Tang et al. 2007a). Crude solutions of solanesol were run through a column of silica gel at a determined rate of 1 mL/min and the adsorption of solanesol by the silica gel was

determined to be 58.5 +- 1.7 mg per gram of silica gel. The silica was then eluted with acetone and collected for HPLC-UV analysis (Tang et al. 2007a).

2.3 Analytical Methods of Detection

Several techniques have been described for analyzing solanesol content in tobacco leaves. These include gas chromatography (GC) (Chamberlain et al., 1990; Severson et al, 1977); thin layer chromatography (TLC), solid phase extraction (Tang et al., 2007a); high performance liquid chromatography (HPLC) with UV detection (Tang et al., 2007b); differential refraction detection, and evaporative light scattering detection (Zhou and Liu, 2006b). TLC has proven problematic due to considerable solanesol decomposition on the plates, while GC involves lengthy extraction and derivatization procedures. On the other hand, HPLC-UV has been proven an accurate and precise approach for rapid determination of solanesol content (Chen et al., 2006; however, some modifications might be needed depending on the solvent and column used. Table 2.1 compares different detection methods of solanesol, their detection limits and their drawbacks.

Table 2.1: Comparison of solanesol detection methods.

Method	Detection Limits (mg/mL)	Remarks	Reference
Gas Chromatography (GC)	0.02	High temperatures, involves lengthy extraction and derivatization procedures	Chamberlain et al, 1990 Chen et al, 2007
High Performance Liquid Chromatography with ultraviolet detection (HPLC-UV)	0.05	Commonly available	Chen et al., 2007
High Performance Liquid Chromatography with Evaporative Light Scattering Detection (HPLC – ELSD)	0.1	ELSD is more expensive than UV	Zhou et al, 2006b
Thin Layer Chromatography (TLC)	0.5	Problematic due to considerable solanesol decomposition on the plates	Woollen and Jones, 1971
Time-of-Flight Mass Spectrometer (TOF-MS)	0.0018	Most expensive method	Chen et. al, 2007

Chapter 3: Research Goal

The goal of the project was to investigate the feasibility of extracting solanesol from tobacco biomass residue following protein recovery. In order to achieve the goal, there were three objectives:

- To establish reliable analytical methodology capable of detecting solanesol.
- To assess solanesol concentration distribution in biomass recovered at various stages of tobacco protein bioprocessing.
- To evaluate the yield, processing time required, and solvent usage of different extraction approaches.

Chapter 4: Materials and Methods

4.1 Tobacco Processing

4.1.1 Low alkaloid tobacco samples

Low alkaloid tobacco (*Nicotiana tabacum* L. cv. MD-609LA) containing an average nicotine level of 0.6-0.8 mg/g dry weight was grown on the University of Maryland, College Park Greenhouse Facility. Typically, tobacco has a nicotine content between 0.6 to 2.9 mg/g dry weight (Hoffmann and Hoffman, 1998). The tobacco was grown from seed (Figure 4.1) to reproductive stage over a period of 4 months. First, the tobacco seeds were scattered across Styrofoam beds filled with small cells of soil, which were floated on a basin of water. At the seedling stage, the plants were moved so that each cell was occupied by only one plant (Figure 4.2). A few weeks later, the tobacco plants had overgrown their cells (Figure 4.3) and were transplanted into individual pots of soil (Figure 4.4). The plants continued to grow and were harvested at the plants adult stage, soon after flower buds were formed (Figure 4.5). The adult tobacco plants were harvested by hand and stored in a cold room at 4 °C until processing.

Figure 4.1 Tobacco seeds and seed pod.

Figure 4.2 Tobacco plants growing in greenhouse shortly after seeding.

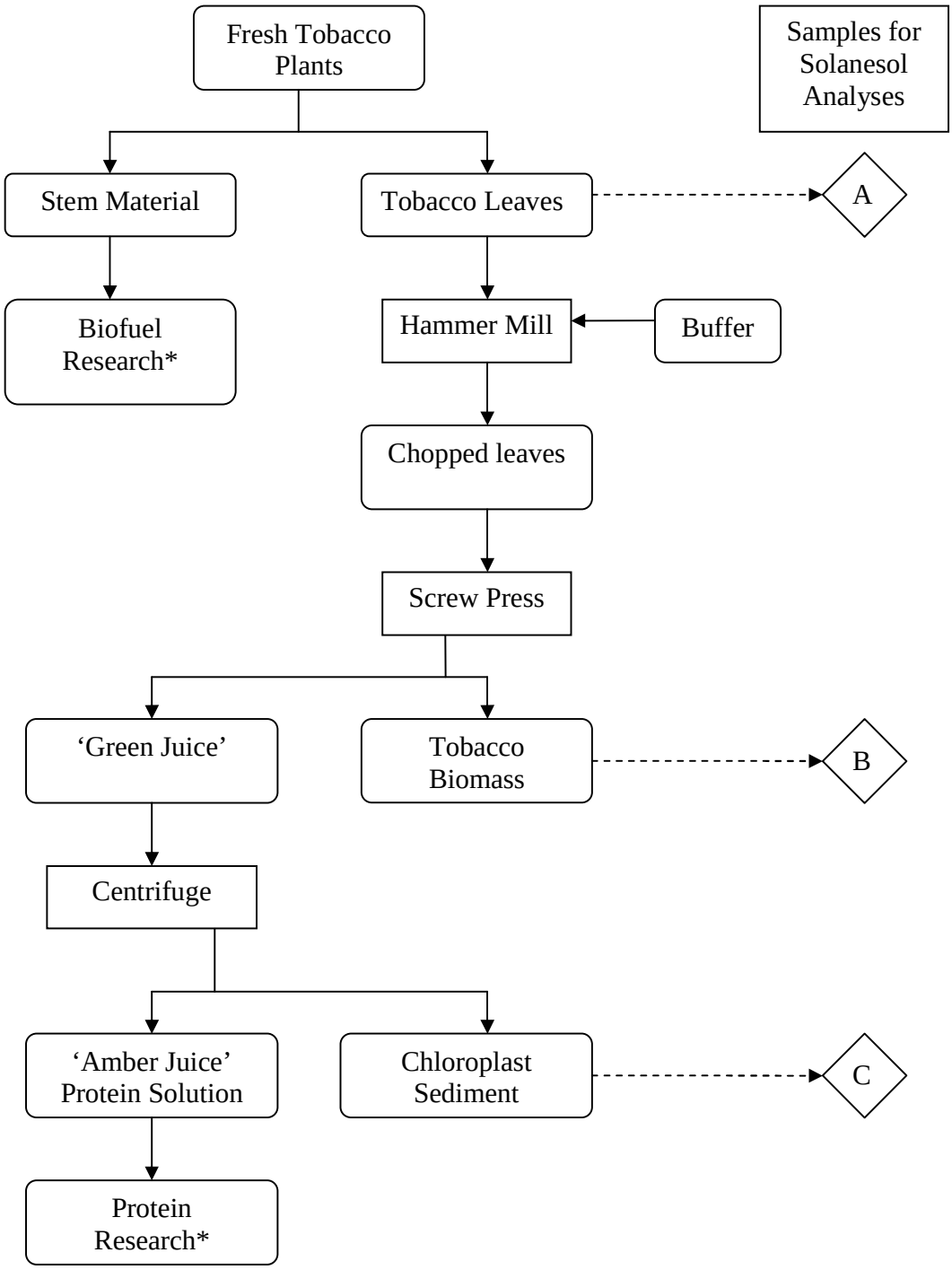
Figure 4.3 Tobacco plants right before potting.

Figure 4.4 Tobacco plant two weeks after potting.

Figure 4.5 Tobacco plant at harvest stage.

4.1.2 Samples Originated from Protein Extraction

Figure 4.6 outlines the general procedures used when processing fresh tobacco for protein extraction. Three different samples (A, B, and C) were collected during the tobacco protein extraction procedures and analyzed. After the leaves were separated from the stalk, the tobacco leaves were macerated with a hammer mill. The first sample (A) contained only the macerated leaves from the freshly harvested tobacco plants. The second sample (B) was collected from the biomass waste stream following the separation of 'Green Juice' from the residue of the tobacco biomass during protein processing; this residue is a highly cellulosic material with most of its protein-containing liquid squeezed out during screw-press operation. The final sample (C) was the end waste material (chloroplast and sediment material) following centrifugation that separated it from the protein solution during the protein extraction process.



*** Additional research which is beyond the scope of this project.**

Figure 4.6 Simplified tobacco bioprocessing flowchart outlining the origin of the three samples being analyzed for solanesol.

4.2 Sample Preparation

The tobacco leaves (Sample A), biomass (Sample B), and chloroplast sediment (Sample C) were dried in a convection oven at 55°C to 60°C. The dried leaves were ground with a Pertan 3600 Lab Mill. A No. 40 sieve (0.425 mm) was used to ensure consistent particle size. Ten grams of the dried samples were weighed and placed into a vessel with 100 mL of a 1:3 hexane:ethanol (v/v) solvent solution. NaOH (0.02 M) was employed as an additional saponification step to free the bound solanesol present as esters in each sample and maximize solanesol yield (Zhou and Liu 2006a). The solvent system was used for all sample solutions.

4.3 Extraction Methods

Three extraction methods (heat-reflux extraction, microwave-assisted extraction and ultrasound-assisted extraction) for solanesol recovery were investigated.

4.3.1 Heat Reflux Extraction

In heat-reflux extraction, the prepared samples (Section 4.2) were placed into a round bottom flask and were heated in a water bath at 60°C. The sample solution was stirred constantly at 75 rpm for 180 min (Zhou, 2006b), and a distillation column was used to prevent solvent loss due to evaporation (Figure 4.7).

Figure 4.7 Experimental setup of the heat-reflux extraction method.

4.3.2 Microwave-assisted Extraction

For microwave-assisted extraction, the sample was placed into the chamber of an Ethos E Microwave Extraction Labstation (Milestone Inc., Monroe, CT) with two magnetrons (800 W ea.) installed. The samples were places into the microwave labstation and heated to 60°C in 45 seconds. The temperature was monitored and maintained for 40 minutes.

4.3.3 Ultrasound-assisted Extraction

Ultrasound-assisted extraction was conducted similar to the heat-reflux extraction method (4.3.1), with the exception of an added sonification step that was executed in triplicate and aimed at removing all of the solanesol from the tobacco sample and into the solution. After heat-reflux extraction step, the solvent was gravity filtered (Whatman No. 1) to separate it from the remaining biomass and set aside. A 20 mL aliquot of hexane was added to the remaining biomass in the flask and the flask was sealed to prevent solvent evaporation. The sample was placed in a Neytech model 28H ultrasonic bath (Neytech, USA) at a frequency of 47±3kHz for 20 minutes. The water volume inside the bath was 1L and the samples were kept at 60°C. The solution was filtered again and added to the initial solvent, while another 20 mL aliquot of hexane was added to the remaining biomass and the procedure repeated. The ultrasound procedure was run for 20 minutes each cycle for three cycles, increasing the total time of processing to 240 minutes and the final volume of solvent to 200 mL.

4.3.4 Post extraction

All samples, regardless of extraction method, were then centrifuged at 5000 x g using a Beckman L7-65 ultracentrifuge (Fullerton, CA). The supernatant was taken and filtered through a 0.45 µm Millipore (Billerica, MA) syringe driven filter unit and stored at 4°C until it was analyzed.

4.4 Analytical Method

4.4.1 HPLC Analysis

The solanesol concentration of the samples was determined using reverse-phase high performance liquid chromatography (RP-HPLC) using a Shimadzu LC-2010A (Columbia, MD) equipped with serial dual plunger pumps, an oven, an automated sampling injection unit, and an ultraviolet-visual(UV-VIS) detector (D lamp light source) capable of detecting wavelength at 215 nm. A Waters reversed-phase µ-Bondapak C_{18} column (3.9 × 300 mm, 10 µm particle size) with a guard column (Milford, MA) was used. A solanesol standard (≥90%, Product No. S8754), obtained from Sigma-Aldrich (St. Louis, MO), was first analyzed at various concentrations to obtain a quantitative calibration curve between solanesol concentration and the peak area in the chromatogram.

A mixture of 60/40 (v/v) acetonitrile-isopropanol solution was used as the mobile phase, using the sample injection volume of 10 µL with 0.8 ml/min flow rate. A Waters C18 column (3.9 x 300 mm) maintained at 25°C was used. Chromatograms were created and the peaks were analyzed with a Gaussian

(symmetrical) distribution for the retention factor that is a measure of the time the sample component resides in the stationary phase relative to the time it resides in the mobile phase.

$$R \equiv \frac{2(t_{R2} - t_{R1})}{t_{W2} + t_{W1}}$$

Figure 4.8 Resolution equation.

The resolution, defined in Figure 4.8 as the peak separation divided by mean peak width, was also calculated using the Class VP 6.0 software supplied with the equipment. The resolution was calculated to compare different variables (mobile phase, oven temperature, and flow rate) used in the solanesol detection method to determine the optimal peak separation between peaks present on the chromatogram.

4.4.2 Statistical Analysis

Three replications of each sample were performed during HPLC analysis and three samples were performed for each of the six experimental treatments. The results were analyzed for statistical significance using SAS 9.1.3 software with ANOVA followed by Tukey's test ($p<0.05$) for mean separation. Complete statistical analysis can be found in Appendix A.

Chapter 5: Results and Discussion

5.1 Improving the Detection Method

An optimized HPLC method using a standard detection system was developed combining the key elements reported by Zhou (2006b) and Zhang (2001) to establish an analytical procedure capable of providing rapid and accurate quantification of solanesol in the biomass samples with a detection limit adequate for industrial applications.

5.1.1 Improving Peak Resolution

The most critical challenge in optimizing the HPLC method was the ability to acquire proper resolutions of the target compound, namely solanesol, with the presence of the extraction solvents and other impurities. In the present study, the main problem was that the ethanol used as part of the extraction solvent created its own large peak ahead of the solanesol peak. Sometimes these two peaks were close enough that they joined. Different variables were evaluated to increase the separation between the two peaks so that the quantification of solanesol could be performed without interference from the solvent peak. To accomplish this, the microwave-assisted extraction samples were employed to evaluate the effect of different variables on the solanesol resolution of HPLC chromatogram. The mobile phase ratio of acetonitrile and isopropanol (60/40 v/v) was altered and analysis was done to see if it helped in the separation (Figure 5.1). However, the 60/40 ratio was found to be the

optimum in both position and relative size of the solanesol peak. The temperature of the oven was then analyzed (Figure 5.2), and it was found that lowering the temperature from 35°C to 22°C significantly altered the position of the two peaks, allowing for a considerably better resolution and altering the final results on solanesol concentration to a level of quantification of 0.01 mg/mL. Finally, the mobile phase flow rate was adjusted from 0.5 to 0.8 mL/min, and the best resolution was found to occur when the flow rate was 0.8 mL/min (Figure 5.3).

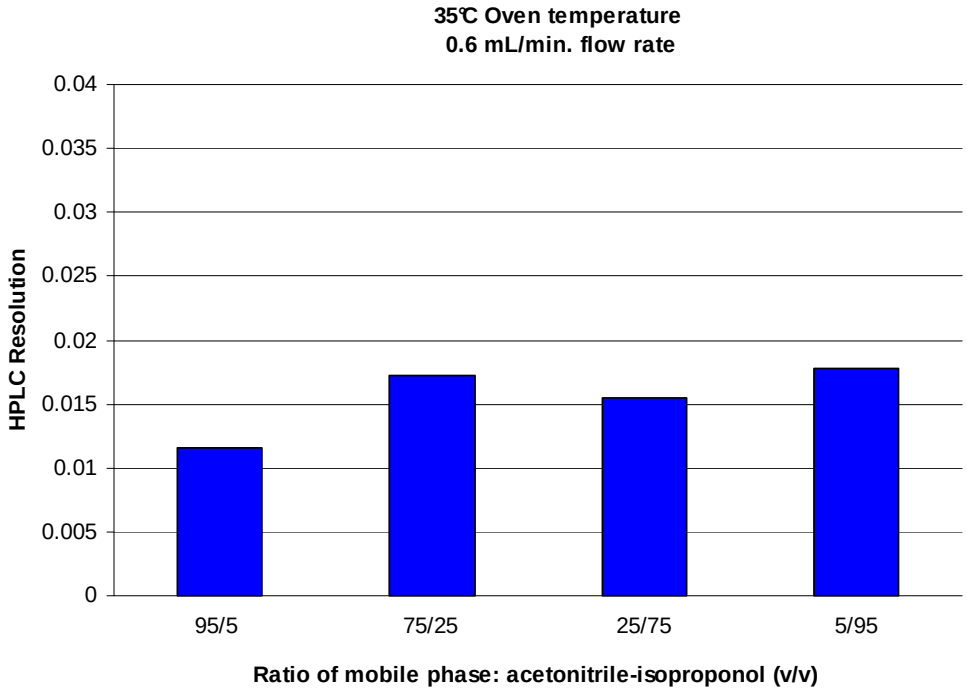


Figure 5.1 Comparison of the separation resolution between ethanol and solanesol peaks at various mobile phase ratios (v/v acetonitrile-isopropanol) at a constant 35°C oven temperature and 0.6 mL/min. flow rate.

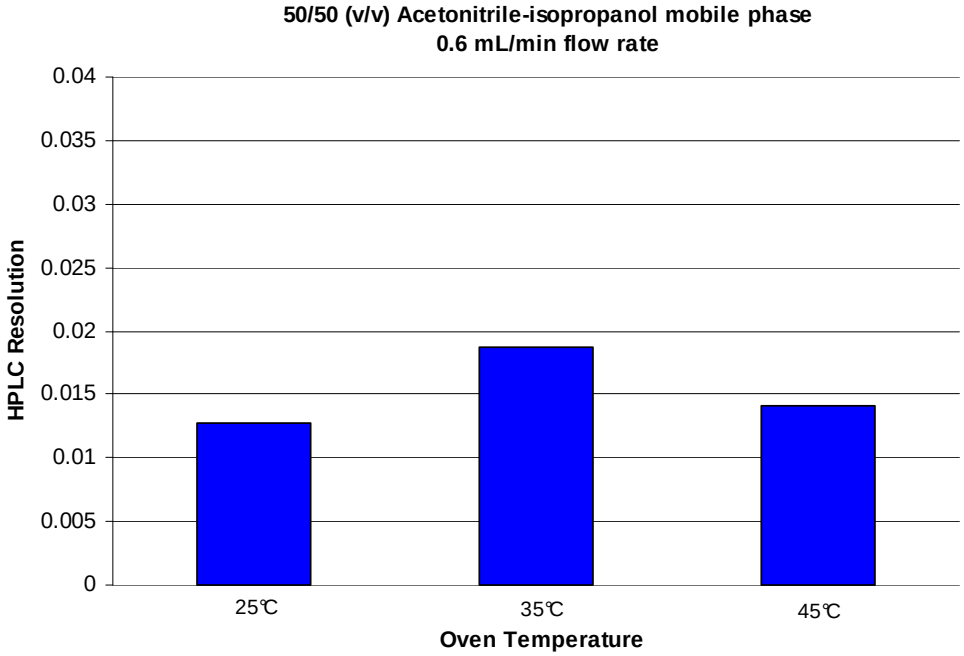


Figure 5.2 Comparison of the peak separation resolution at various oven temperatures at a constant 50/50 mobile phase ratio (v/v acetonitrile-isopropanol) and 0.6 mL/min. flow rate.

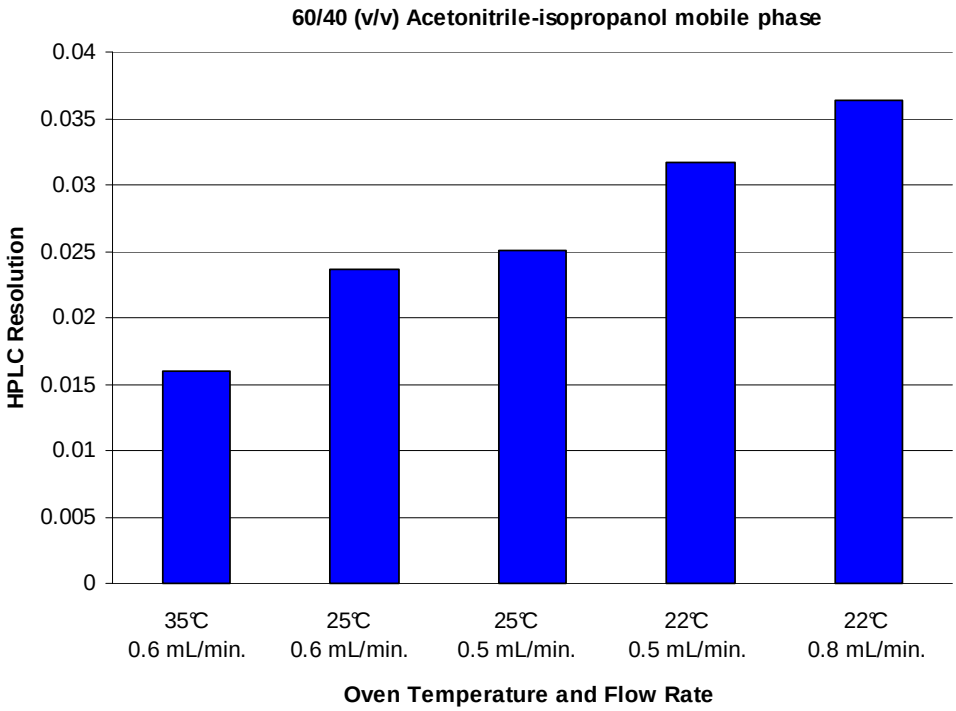


Figure 5.3 Comparison of the peak separation resolution at various oven temperatures and flow rates a constant 60/40 mobile phase ratio (v/v acetonitrile-isopropanol).

5.1.2 Example Separation Peaks

Figures 5.4 through 5.6 illustrate the presence of the ethanol peak during HPLC-UV analysis. However, through modifying the variables involved in analysis, a clear separation of the two peaks was established. Figure 5.4 shows a 0.1 mg/mL solanesol standard in a pure hexane solvent. Figure 5.5 is the chromatogram of a solanesol standard in the solvent used for extraction, 1:3 hexane:ethanol (v/v). Finally, an example chromatogram of an unknown microwave-assisted extraction is shown in figure 5.6.

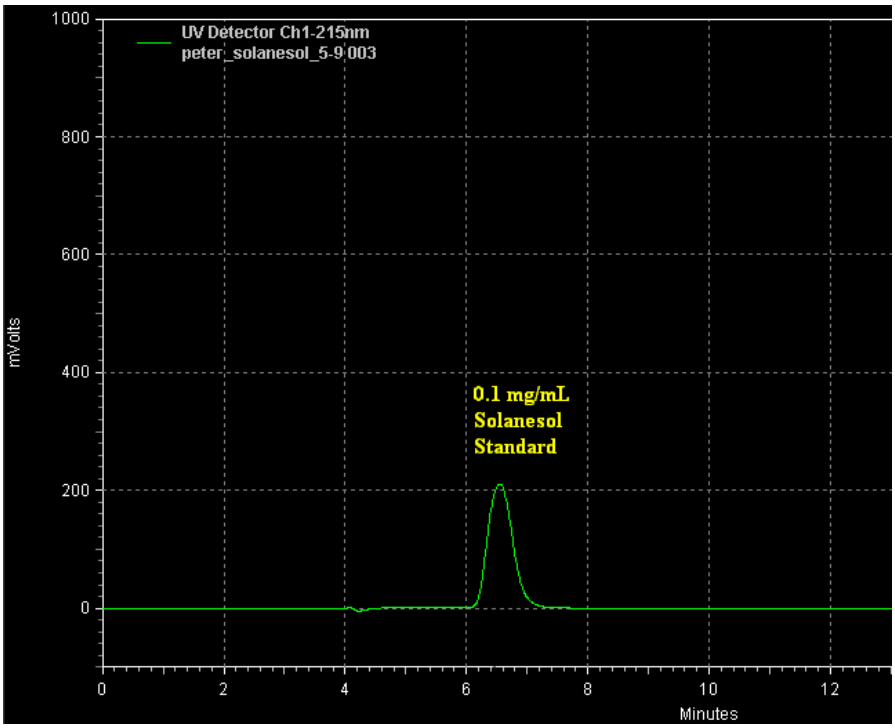


Figure 5.4 Example HPLC chromatogram of 0.1 mg/mL solanesol standard in hexane.

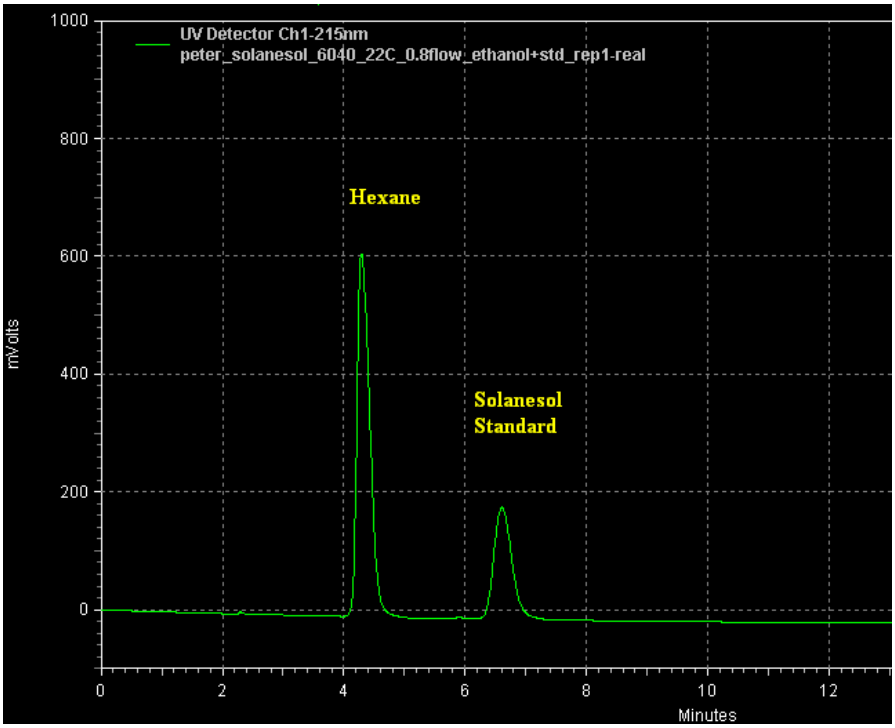


Figure 5.5 Example HPLC chromatogram of solanesol standard in 1:3 hexane:ethanol (v/v) solvent solution.

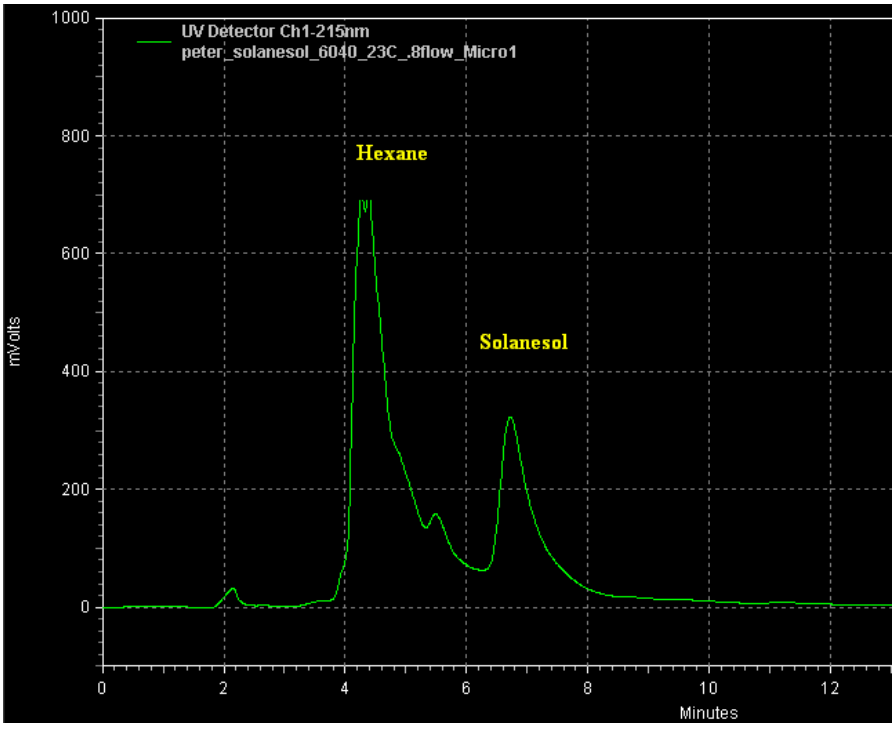


Figure 5.6 HPLC chromatogram of example sample, microwave-assisted extraction.

5.1.3 Solanesol calibration curve

Solanesol concentrations of 0.01, 0.05, 0.1, 0.5, and 1.0 mg/mL were analyzed to create a calibration curve. The corresponding peak areas of the known concentrations were utilized to create a linear trendline and allow quantification of unknown solanesol samples. The curve was created with the solanesol standard (≥90%, Product No. S8754) obtained from Sigma-Aldrich (St. Louis, MO) and had a linear regression of $y = 6*10^7x - 139757$.

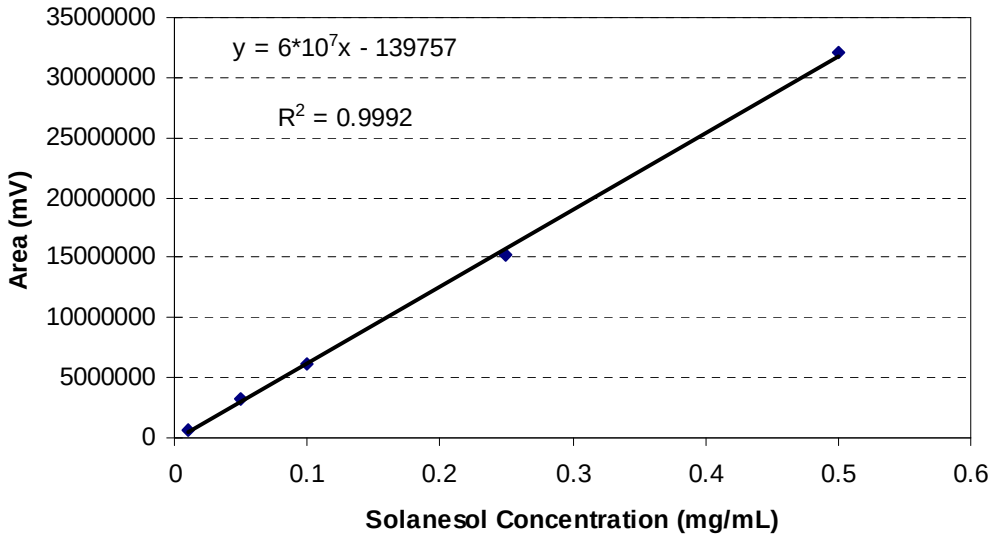


Figure 5.7 Solanesol calibration curve using a mobile phase of 60/40 (v/v) acetonitrile-isopropanol with a sample injection volume of 10 µL with 0.8 ml/min flow rate through a Waters C18 column (3.9 x 300 mm) maintained at 25°C.

5.2 Extraction Methods

A significant increase in solanesol yield was found when utilizing either the micro-wave assisted extraction or the ultrasound assisted extraction over the heat-reflux extraction method (Figure 5.8). Such increases could be attributed to the ability of the added electromagnetic radiation in breaking down the cellular structure and releasing more solanesol into the solution, which heat alone might not be able to accomplish. Microwave-assisted extraction was the most efficient method, as it gave the maximum yield with the shortest amount of time and no additional solvents. Ultrasound extraction did reach a similar yield, but the extended amount of time and extra solvent needed made it less desirable than the microwave-assisted method. Equipment wise, the heat reflux extraction requires the smallest investment because it uses traditional laboratory equipment. The ultrasound equipment would require a small, one-time investment, whereas the microwave lab station would be the most expensive single piece of equipment to obtain. However, the one-time cost of microwave-assisted extraction equipment would likely be overcome by the reduced solvent cost and time of processing needed to extract solanesol using that technique. These results confirm previous studies (Zhou, 2006b and Zhang, 2001) which showed that processing steps beyond normal heat-reflux extraction can improve overall yields as well as reducing the time of extraction.

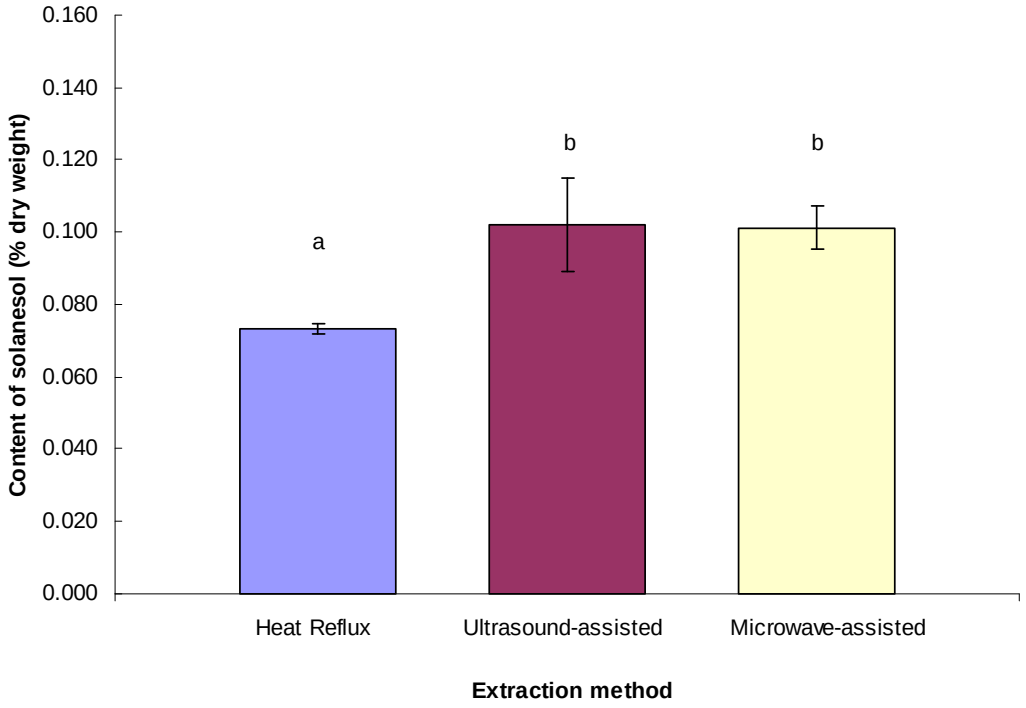


Figure 5.8 Comparison of solanesol concentration (percent dry weight) of tobacco biomass using different extraction methods: ultrasound-assisted, heat-reflux, and microwave-assisted extraction. Values bearing the same superscript in a column are not significantly different (P > 0.05).

Table 5.1 Comparison of solanesol concentration (percent dry weight), time of extraction, solvent usage, and special equipment needed for the ultrasound-assisted, heat-reflux, and microwave-assisted extraction methods based on a 10g sample.

Extraction Method	Content of Solanesol (% dry weight)	Time (min.)	Solvent Usage (mL)		Special Equipment
			Hexane	Ethanol	
Heat Reflux	0.073 ± 0.013^{a}	180	25	75	Water bath
Ultrasound-assisted	0.102 ± 0.001^{b}	240	85	75	Ultrasound unit, water bath
Microwave-assisted	0.101 ± 0.006^{b}	40	25	75	Microwave labstation

Each value is the mean ± SD, n = 3. Values bearing the same superscript in a column are not significantly different (P > 0.05).

5.3 Sources of Solanesol

The results of the solanesol concentrations in the samples collected from different processing steps are summarized in Figure 5.9. As expected, there was a statistically significant difference in the solanesol concentration between each of the samples. The chloroplast sediment was found to have the highest concentration of solanesol, which was expected due to the fact that solanesol is found in the chloroplasts of tobacco plants (Stevenson, 1963). The biomass waste contained the smallest concentration of solanesol, which was also expected because the biomass is consisted of largely cellulosic materials (Demirbaş, 2004). It is important to note that, however, there is a much greater volume of the tobacco biomass when compared to the chloroplast sediment, so depending on the volume of the tobacco being processed for protein processing, the tobacco biomass could still allow for a significant amount of solanesol to be extracted. In addition, the total solanesol yield by using the waste from protein processing would include both the biomass and the chloroplast samples. Table 5.2 illustrates this concept by taking the volume of fresh tobacco leaves into account when discussing final solanesol content. The table also demonstrates what the quantity of the dried waste sample produced during the processing of 100 kilograms of fresh tobacco leaves.

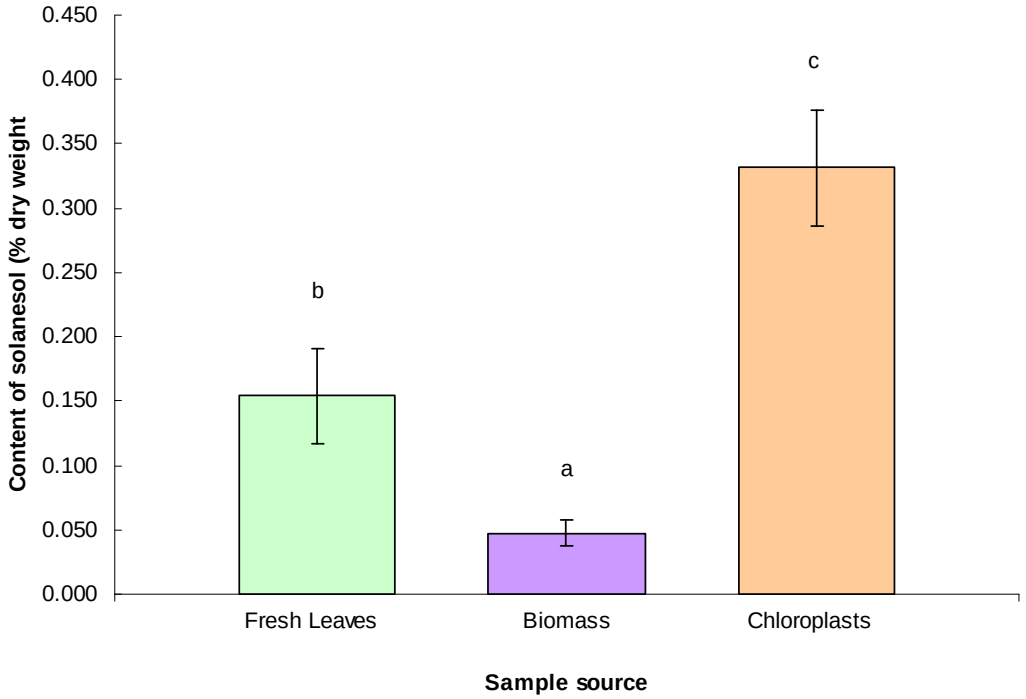


Figure 5.9 Comparison of solanesol concentration (percent dry weight) from different waste streams of an established tobacco protein extraction method. Values bearing the same superscript in a column are not significantly different (P > 0.05).

Table 5.2 Comparison of dried sample mass and solanesol yield from 100 kg of fresh tobacco leaves (MD-609LA) from the different waste steams of an established protein extraction method.

Sample source	Mass of dried sample per 100 kg fresh leaves (kg)	Solanesol content (% dry weight)	Solanesol content per 100 kg fresh leaves (g)
Fresh Leaves	13.1	0.154 ± 0.037^{b}	20.2
Biomass	4.9	0.047 ± 0.010^{a}	2.3
Chloroplasts	1.1	0.331 ± 0.045^{c}	3.6
Total waste*	6.0	——	5.9

Each value is the mean ± SD, n = 3. Values bearing the same superscript in a column are not significantly different (P > 0.05).

*The sum of the biomass and chloroplast samples, which illustrates total solanesol collection possible after protein processing.

Previous research has shown a large range (0.44 -1.69 % dry weight) in the amount of solanesol found in differing varieties of tobacco (Zhou, 2006b). The tobacco variety used in this study, MD609 LA, was chosen for its high protein content and low alkaloid content, and could have lower levels of solanesol than other tobacco varieties. In addition, most studies on solanesol use tobacco that has been cured for smoking purposes. As previous studies have shown (Chamberlain et al., 1990), the curing process helps to free bound solanesol and increase solanesol yield. While a saponification step was employed in the present study to increase the free solanesol, there could still be more bound solanesol in the uncured leaves that might not be fully released by the saponification step. Furthermore, it is important to note that the age of the tobacco plants might also be critical. It was previously determined that for maximum overall profitability of the protein processing, the tobacco plants should be harvested as soon as they reach the adult stage (Fu, 2008). However, tobacco plants used for smoking purposes are left to grow for a longer amount of time, which could allow the plant more time to produce and/or accumulate solanesol.

Additionally, the amount of samples processed could also have an impact on the final yield of solanesol. The solanesol concentration in the chloroplasts sediment, while significantly higher than the other samples, was not as high as expected when it is known that the solanesol originates in the chloroplasts. This could be because solanesol is more vulnerable to heat damage during drying after the centrifugation step. The fresh leaf samples had minimal processing prior to the drying step, which could explain the higher overall yield. Also, as aforementioned, further optimization of the method of extraction could also help to increase the yield of solanesol such that

it could be a cost-effective, value-added product suitable for industrial-scale production.

Chapter 6: Conclusions

Among the three extraction methods investigated, microwave-assisted extraction was found to be the most efficient in terms of solanesol yield and processing time required. All waste streams from the protein processing of tobacco were found to contain solanesol, with a significantly higher concentration found in the chloroplast sediment waste. However, due to the small amount of chloroplast sediment obtained at that final stage of the protein processing, the biomass waste obtained following the initial maceration and extraction of the protein laden 'green juice' could also be used for extraction of solanesol to maximize solanesol yield. A method of detecting solanesol using HPLC-UV was successfully developed that had a quantification limit of 0.01 mg/mL. Overall yields of solanesol were found to be lower than previous studies have shown. This is likely because uncured leaves were used, the tobacco was a low-alkaloid variety, the plants were harvested at the very beginning of their adult stage, and the harsh processing conditions of some samples.

The waste streams from protein processing can have a significant influence on the overall profitability of alternate use tobacco through the extraction of solanesol. Further studies are recommended that will analyze different varieties of tobacco for solanesol concentration, as well as pilot-scale testing to ensure that large scale extraction and production of solanesol can be achieved.

Appendix A: Statistical Analysis

Extraction Methods

The ANOVA Procedure

Dependent Variable: dryweight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.00476284	0.00238142	34.55	<.0001
Error	24	0.00165407	0.00006892		
Corrected Total	26	0.00641691			

R-Square	Coeff Var	Root MSE	dryweight Mean
0.742233	8.998686	0.008302	0.092255

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Type	2	0.00476284	0.00238142	34.55	<.0001

Tukey's Studentized Range (HSD) Test for dryweight

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	0.000069
Critical Value of Studentized Range	3.53170
Minimum Significant Difference	0.0098

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Type
A	0.102048	9	Ultrasound
A	0.101240	9	Microwave
B	0.073478	9	Heat Reflux

Sources of Solanesol

The ANOVA Procedure

Dependent Variable: dryweight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.37028521	0.18514260	160.26	<.0001
Error	24	0.02772550	0.00115523		
Corrected Total	26	0.39801071			

R-Square	Coeff Var	Root MSE	dryweight Mean
0.930340	19.14295	0.033989	0.177552

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Type	2	0.37028521	0.18514260	160.26	<.0001

Tukey's Studentized Range (HSD) Test for dryweight

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	0.001155
Critical Value of Studentized Range	3.53170
Minimum Significant Difference	0.04

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Type
A	0.33129	9	Chloroplasts
B	0.15402	9	Fresh Leaves
C	0.04734	9	Biomass

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