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THE "LC" PROTOCOL

The "LC" protocol describes a procedure developed at the University of Kentucky to screen Foundation seed and eliminate converter plants. This process results in a lowering of nornicotine content in the subsequent generation.

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Summary of the LC Protocol

The LC protocol is posted on the UK tobacco website (see below), and may be used by anyone, without any restrictions and without any charge. This procedure is designed for screening Foundation and Breeder seedlots for converter plants. It is not suitable for monitoring nornicotine levels in the crop. Most tobacco companies require Foundation seed to have undergone an acceptable screening process.

1. Screen a minimum of 50 and ideally 100 plants for each line. Grow sufficient plants to ensure that after roguing offtype and converter plants, no fewer than 30 plants constitute the bulk for the Foundation seed of each line. Do not apply excess N, and do not sidedress before sampling (page 2)
2. Grow the UK check variety with the lines to be screened, and sample a minimum of 10 (ideally 30) check plants. The check must be sampled with the lines, and must be the same age; if there is more than one transplanting, grow one check plot with each transplanting. One sampling of the check plot per transplanting is all that is required; it is not necessary to do one check sampling per line. If the procedure is done correctly, all the check variety plants should have >90% conversion. (page 2)
3. Sample plants when they are 12-18" (30-45 cm) high, about a month after transplanting. (page 3)
4. Label individual plants before starting to sample. Label sample leaves to correspond with plant number, using the lowest leaf which is in good condition. Avoid necrotic leaves (cut out necrotic patches if necessary), over-mature leaves, and immature leaves too high up the plant. (page 3)
5. Treat leaves with ethephon as soon as possible after picking, ensuring that they do not wilt before treatment. Dip leaves into the solution, wetting thoroughly. Use about 1.20 g a.i./liter of any commercial formulation. Triple the rate for dark tobacco. (page 5)
6. Tie the leaves as for curing primed leaf, and ensure that there is no free moisture on the leaves before beginning the "cure". (page 6)
7. "Air-cure" the leaves in a warm chamber with high humidity (about 70% RH) for at least seven days, ensuring that yellowing is complete before removing the leaves from the chamber. This is one of the most crucial parts of the operation – yellowing is ESSENTIAL and conversion will not be complete in any leaves or parts of leaves that do not yellow properly. Fixing green and rotting must be avoided; both will give erroneous results. (page 7)
8. When the "cure" is complete and the leaves fully yellow, after approximately 7 days for burley, or 10 days for dark tobaccos, cut out any green, necrotic or rotted patches and place each leaf in a separate small paper bag. If an entire leaf is fixed green; discard it, treat it as a missing sample, and rogue the corresponding plant in the field. (page 8)
9. Dry the bagged leaves in a drying oven, preferably one with a good fan. Ideal drying temperature is 55ºC (130ºF), and drying should be complete within 24 hours at this temperature. The temperature must not exceed 70ºC (158ºF) – there is some loss of nicotine at high temperatures. (page 8)
10. Analysis must be done on a GC (gas chromatograph), or a chromatographic system that gives comparable resolution and quantitation. Preliminary elimination of converters may be done on a TLC (thin layer chromatograph), but the final analysis must be on a GC or comparable system. (page 8)
11. Conversion % is calculated as nornicotine / (nicotine + nornicotine) x 100. Any plants with conversion >3% must not be included in the bulked seed and ideally must be rogued before flowering. If the data are not available before flowering, plants must be bagged or all capsules and open flowers picked off after roguing. (page 9)
12. Seed bulked from the remaining plants (<3% conversion) will constitute a screened bulk. If the LC requirements have been met, it will constitute an LC Foundation seed bulk, to be used for Certified seed production. (page 10)
13. The LC logo is issued only for Certified seed whose Foundation seed was screened under the supervision of KSIA and UK. (page 10)
14. Do not sequentially increase the seed without further screening. If this seed is sequentially increased without further screening, the conversion mean will gradually increase with each generation. There should be only one generation of seed increase without screening (that of commercial Certified seed production) before the seed is grown by farmers for a leaf crop. (page 11)

This document is available in PDF format on the UK tobacco website (http://www.uky.edu/Ag/Tobacco)

UK Burley Tobacco Site

The LC Protocol – Procedure

Some of the details outlined below, such as the method of labeling, may be done in any one of several ways. Others, such as the minimum number of plants, are mandatory for compliance with the protocol. **Mandatory details are in bold**.

1. Production of Plants for Screening

- **Lay out the plots in accordance with the standards of the local certifying authority.** The Kentucky Seed Improvement Association (KSIA) standards for isolation and field layout are outlined in Appendix 1, as an example.
- **Screen a minimum of 50 and ideally 100 or more plants for each line**. Grow sufficient plants to ensure that after roguing offtype and converter plants, **no fewer than 30 plants constitute the bulk for the Foundation seed of each line**.
  - The decision on how many plants to sample will depend on how many plants are expected to be rogued for conversion and type, and on how much seed is required.
  - One should allow for considerable roguing losses, both for conversion and type. If there is reason to expect few converters in a particular lot, the minimum number of plants can be screened. Conversely, if there is reason to expect a high proportion of converters, or if the conversion is unknown, the number should be increased. Remember that sampling is early in the season, and roguing for type can only be done after sampling.
  - It is important that seed is bulked from an adequate number of plants. Too small a sample can result in genetic drift and a change in phenotype. More importantly, it can result in unacceptably high conversion levels in the progeny (see Appendix 2-1). For Foundation seed, there should be a minimum of 30 plants remaining after roguing for both type and converters. The 30 plant minimum does not apply to Breeder seed.
  - Because of the expense of screening seed, it is advisable that screened LC seed should be used only for Foundation seed or Breeder seed; and not for Certified seed.
- Transplant more plants than are intended for sampling, so that there is some choice of plants at sampling.
- Do not sidedress until after sampling, and do not apply excessive N, or the leaves will be difficult to cure (see Appendix 2-5f).

2. Check Variety

- Seed of the check variety is available from UK on request (contact details on cover sheet).
- **Grow the UK check variety with the lines to be screened,** allowing for 10-30 check plants to be sampled. It is advisable to plant the check variety outside the outer border row as a precaution against compromising isolation standards.
  - **A minimum of 10 check plants must be sampled with the lines, and they must be the same age**; if there is more than one transplanting, grow one check plot with each.
  - One sampling of the check plot per transplanting is all that is required; it is not necessary to do a check sampling for each line being screened.
  - The check plants are fertile, but they can be removed immediately after sampling (to avoid regrowth, pull them out with their roots, rather than cutting them down). They must be removed before flowering, so they will not compromise isolation standards.
- If the procedure is done correctly, **all the check variety plants should have >90% conversion**. Lower levels of conversion indicate a problem with the sampling, treatment, curing, analysis or a combination of these factors (see Appendix 2-2).

3. Sampling Stage

- **Plants should be sampled when they are 12-18" (30-45 cm) high**; usually about a month after transplanting (Fig. 1). At this stage, the leaf to be sampled should be about 12" (30 cm) long (Fig. 2).
  - There are several reasons to sample at this stage. It gives the best results (see Appendix 2-3), larger leaves are more difficult to handle, but more importantly, it is possible to get the results and do the roguing before flowering. Sampling later involves a lot more work, as measures must be taken to prevent cross-pollination (see section 11).

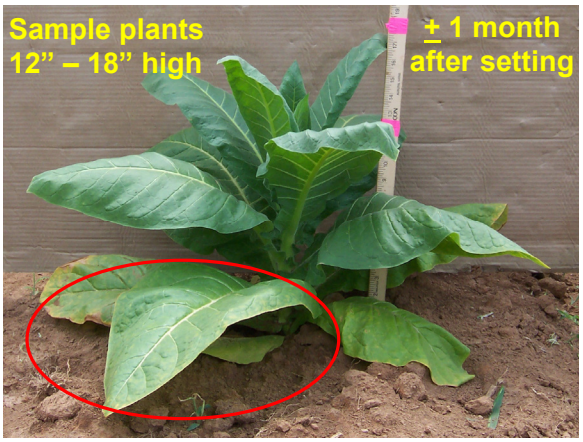


Figure 1: Correct plant size

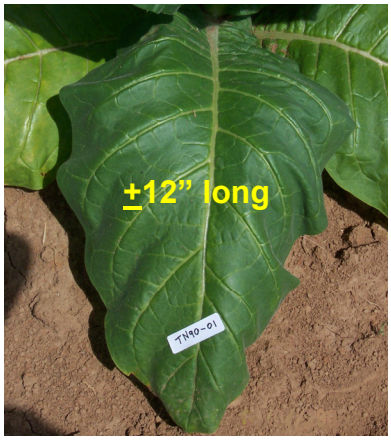


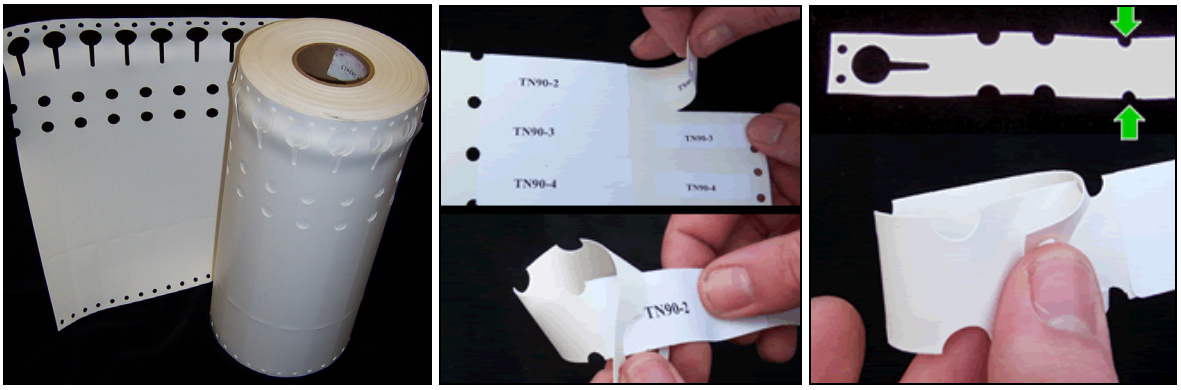
Figure 2: Correct leaf size

4. Labeling and Sampling

- **Label individual plants before starting to sample.**
- It does not matter how the plants are labeled, as long as all plants to be sampled are individually and uniquely numbered, such that each plant can be clearly identified later.
  - Choose the plants to be sampled, excluding any that are very small, unhealthy or obviously off-type, and remove these plants.
  - Use flags or any type of tag (Fig. 3) to identify the plants.
  - Number each plant, and number sequentially down the rows. It is very important that the numbers are in order, and that there are **no unsampled plants in the sequence**. Any plants which are not chosen for sampling should be removed prior to sampling.
  - Place the tag around the stalk as high as possible (Fig. 3b), because with stalk elongation, the tag will be close to the ground by flowering.

a **b** **c**

Figure 3: Labeling plants **a.** flags **b.** vinyl strip tags **c.** Tyvec tags with wire or string ties

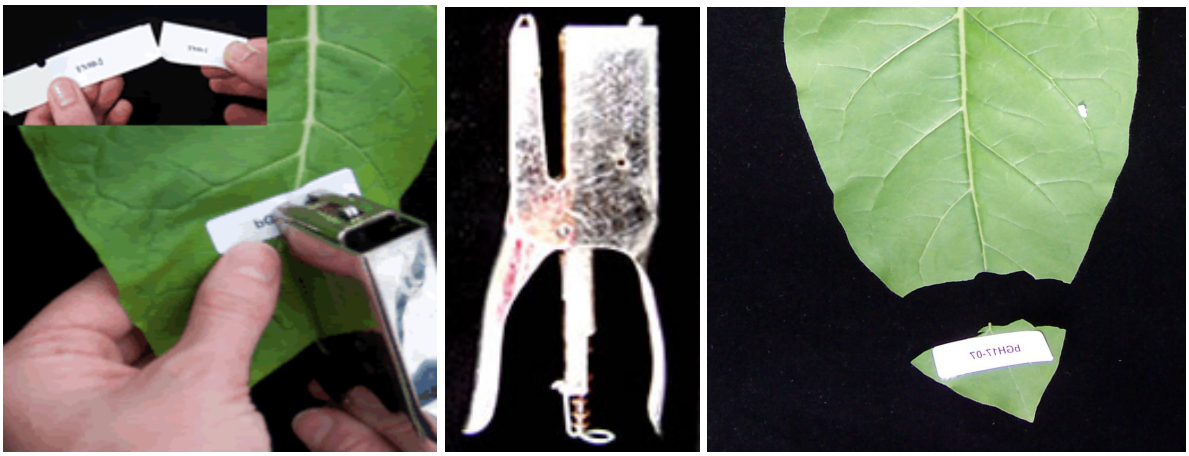


a **b** **c**

Figure 4: Vinyl strip tags with tear-off ends

- The perforated vinyl strip tags shown in Figs. 3b, 4 and 5a are useful, because they have tear-off ends which minimize the chances of labeling errors. Two labels are placed or written on each tag; one to remain on the plant and one on a perforated strip which is torn off and attached to the leaf to be sampled (Fig. 5a).

- **Label sample leaves to correspond with plant number.**
  - Sample the **lowest leaf which is in good condition;** this leaf should be **at least 12" (30 cm) long**. This will usually be between the second and fourth leaf from the bottom, excluding seedbed leaves (Figs. 1, 2).
  - The label should include the variety name or code, and a consecutive number for each plant (e.g. KY14-01 to KY14-95).
  - Use a label that will stand wetting, and attach it securely to the leaf.
  - It does not matter how the leaves are labeled, as long as each leaf is identified with the corresponding plant, and the labels do not come off. The leaf butts can be pierced with wire tags, labels can be tied around the leaf butts, or labels can be stapled onto the leaf (Fig. 5). Stapling is the most convenient method.
  - Leaves should be labeled *in situ*, before they are removed from the plant.



a **b** **c**

Figure 5: Labeling the leaves **a.** correct labeling **b.** best type of stapler **c.** incorrect labeling

- If the labels are stapled, they need a backing strip to secure them, or the staple will tear through the leaf. Place the label straddling the midrib, at a 45° angle to the midrib, with the backing strip behind the leaf, and staple through both in such a way that the staple also straddles the midrib (Fig. 5a). Do not place the label at the tip of the leaf – it will tear off (Fig. 5c). Place it as far up the leaf as the stapler jaws will allow, usually about 2-3" (50-70 mm) from the tip.
- If using the vinyl strip tags, the label for the leaf is at the perforated end of the plant tag (Fig. 5a). Only tear off the perforated strips once the tags are on the plants – do not tear them off before labeling the plants.

- It is important to sample healthy leaves of the correct size and maturity, or the analysis can give misleading results (see Appendix 2-5).
  - **Do not sample:**
    - seedbed leaves
    - leaves that are diseased or showing any necrosis (cut out any necrotic patches before treating)
    - over-mature leaves starting to senesce
    - immature leaves too high up the plant
    - leaves that are too small - <12" long

5. Picking and Treating

- Pick the leaves (Fig. 6), keeping each plot/variety separate e.g. put the leaves from each plot into a box with a lid.
  - **Do not let the leaves wilt** or spend any time in direct sunlight after picking – wilted leaves will give erroneous results (see Appendix 2-5d). Move to a shaded place for treating as quickly as possible, and cut off any necrotic patches before treating.
  - If the sampling is small, all the leaves can be picked before treating; if it is large, the leaves should be picked and treated in batches. It is important to treat the leaves soon after picking; there should not be too long an interval between picking and treating.

Figure 6: Picking the leaves

Figure 7: Ethephon treatment

- **Treat the leaves with ethephon at the recommended rate soon after picking** (Fig.7).
  - Ethephon treatment maximizes potential conversion, and facilitates the yellowing of young leaves. The measured conversion will be lower in leaves which have not been treated with ethephon, even if they do yellow completely (see Appendix 2-4).
  - Treat one plot at a time, and keep plots separate.

- For burley, use about 1.20 g a.i./liter water of any commercial formulation of ethephon (2-chloroethyl phosphonic acid) e.g. Ethrel, Cerone, Prep. Triple the rate for dark tobacco (about 3.60 g a.i./liter). Ten liters of solution is a convenient volume.
  6 ml / liter of a 2 lb a.i./gallon (21.7% a.i.) formulation (e.g. Ethephon 2)
  3 ml / liter of a 4 lb a.i./gallon (39.9% a.i.) formulation (e.g. Ethephon 4)
  2 ml / liter of a 6 lb a.i./gallon (55.4% a.i.) formulation (e.g. Ethephon 6)
- Leaves may be sprayed or dipped, but dipping is much easier.
  - If spraying, lay leaves flat and not overlapping, spray, leave for 20 minutes, turn over and repeat.
  - If dipping, dip no more than three or four leaves at a time, for about 10 seconds (not critical), making sure that they are totally immersed and thoroughly wet. Use either a bucket or a dish; plastic is preferable because ethephon is very corrosive (Fig. 7).
- Ten liters of solution is a convenient amount for one batch. It is not necessary to mix a new batch of solution for each plot. The same batch can be used for up to 200 leaves, although a new batch may be needed earlier if the water is muddy.

- Air-dry the loose leaves either on mesh racks at this stage (Fig. 8a), or after the leaves are tied (Fig. 8b), and **ensure that there is no free moisture on the leaves before beginning the "cure".**

a

b

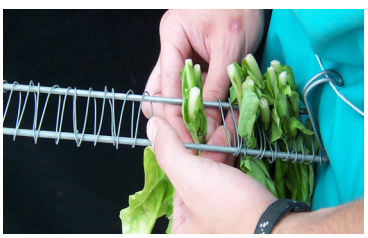
Figure 8: Air-drying the leaves **a.** loose leaves on rack **b.** leaves hanging on clip, with fan

6. Tying the Leaves

- Tie the leaves as for curing primed leaf – with string on tobacco sticks (Fig. 9a), with wire threaded through the butts (Fig. 9b) or in spring clips (Figs. 8b & 9c).
- If the leaves are to be air-dried at this stage, hang them in such a way that they can drip dry, preferably with a fan blowing on them (Fig. 8b). There should be no free moisture on the leaves when beginning the "cure"; it can cause barn rot.

a

b



c

Figure 9: Tying the leaves **a.** string, tobacco stick **b.** wire through butts **c.** Tilita spring clip

7. "Curing" / Yellowing

- **"Air-cure" the leaves in a warm chamber with high humidity**, so that yellowing is complete in about seven days. This operation is not strictly a cure. Conversion is an ongoing process, which starts as senescence begins and continues to the end of yellowing. If the process is interrupted, or if the yellowing is not complete, the measured conversion will be a considerable underestimate and the screening will be ineffective.
- This is one of the most crucial parts of the operation – **yellowing is ESSENTIAL** and conversion will not be completed in any leaves or parts of leaves that do not yellow fully.
  - Warmth and humidity are very important, and a small chamber is necessary to maintain humidity (about 70% RH is ideal). In most environments, this cannot be done in a large commercial air-curing barn at this early stage in the season when the barn is empty. Without additional plant material, it will be too dry, and the leaves will fix green.
  - Any small unit that will maintain warmth and humidity is acceptable. A box covered with plastic sheeting (Fig. 10a), a wooden frame covered with plastic sheeting (Fig.10b) or a small experimental barn (Fig. 10c) have all given good results.
  - It is usually necessary to place pans of water (Fig. 10a) or a humidifier (Fig. 10b) in the unit to maintain humidity and avoid fixing green.
  - There should be a reasonable amount of plant matter in the unit. If the sample is small, fill up some of the space with surplus border plant leaves or whole plants.
- **Fixing green and rotting must be avoided**; both will give erroneous results (see Appendix 2-5e).
  - Fixing green is a result of low humidity, high temperature, excessive ventilation or any combination of these. It can be avoided by keeping the humidity high enough (about 70% RH) – but not too high or the leaves may rot. The combination of high temperature and low humidity is particularly dangerous for fixing green.
  - Rotting can be avoided by maintaining the correct combination of temperature and humidity, by not packing too tightly and by not putting wet leaves into the curing unit.
  - It is sometimes necessary to treat burley leaves with streptomycin to avoid rotting; it is nearly always necessary to treat dark tobacco leaves, which are particularly susceptible to rotting. Note – the streptomycin will control only bacterial barn rot (which is usually the main problem); it will not control the fungal rots.
    - Use 0.034 g a.i. streptomycin / liter. For agricultural (crop) streptomycin, this is 200 ppm or 0.2 g / liter streptomycin sulfate (17% a.i.).
    - Treat after the ethephon is dry. The solution should be applied as a fairly coarse spray, ensuring that there is good coverage on the butts, where the rot starts.

a **b** **c**

Figure 10: Yellowing **a.** plastic-covered box; lid removed, pans of water on floor **b.** plastic-covered frame; front open, humidifier on floor **c.** experimental barn

8. Completion of "Cure"

- For the purpose of this procedure, the "cure" can be considered complete when the leaves are fully yellow – almost all the conversion will have taken place by this stage**. Leaves must be cured for a minimum of seven days for burley (ten days for dark tobaccos), but they must NOT be taken out until they are fully yellow** (Fig. 11). The time taken for yellowing is very dependent on temperature and fertilization level; yellowing may take longer than seven days if it is cool or if the plants are excessively green (high in nitrogen). Completion of yellowing is more important than time in the curing unit (see Appendix 2-5f).
- When untying, keep the samples from each plot together. Trim off any green, necrotic or rotted patches with scissors (Fig 12), keeping the label with the leaf. A very small amount of leaf tissue is required for analysis. **If an entire leaf is fixed green; discard it, treat it as a missing sample, and rogue the corresponding plant in the field.** *Note:* necrotic patches refer to those patches necrotic before curing, not the brown resulting from the cure. It is easier to trim these patches before curing.
- If the samples are to be stemmed and ground, stemming should be done at this point.
- Place each leaf with its label in a separate small paper bag (the 5 lb size is ideal), and twist the neck closed (Fig.13).

Figure 11: Fully yellow

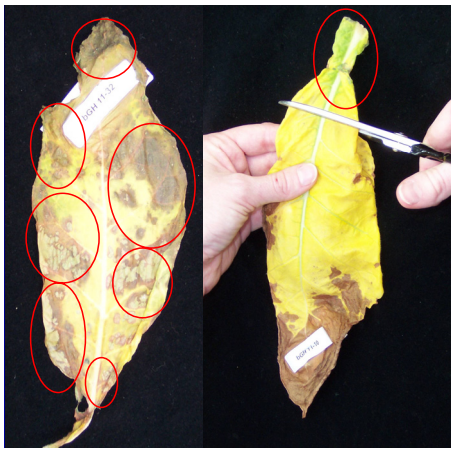


Figure12: Trimming

Figure13: Bagging

9. Drying

- Dry the bagged leaves in a drying oven, preferably one with a good fan. Ideal drying temperature is 55ºC (130ºF), and drying should be complete within 24 hours under these conditions. The lamina should be brittle enough to crumble easily, and the midrib should snap easily – it should not have any flexibility.
- Temperature must not exceed 70ºC (158ºF); this will cause some loss of nicotine.
- Store the samples in a plastic bag in a dry environment until submission to the lab. If they are stored unprotected in ambient conditions with any humidity, they will take up moisture again.

10. Submission of Samples to Lab

Send the samples to a **laboratory using a GC** (gas chromatograph) and experienced in running secondary alkaloids. The laboratory should include external standards in each run; ideally the KY reference cigarettes 2R1 and 1R4F (see Appendices 2-6, 3-1).

- Preliminary elimination of converters may be done on a TLC (thin layer chromatograph), but the final analysis must be on a GC. Using TLC analysis to exclude obvious converters may reduce the analysis costs considerably.

- Specify your objective: only conversion %, or the absolute amount of each alkaloid.
  - Conversion is all that is necessary for seed screening. If only the % conversion (the percentage of nicotine converted to nornicotine) is required, it is not necessary to stem or grind the samples, or to weigh them accurately. Because we are dealing with a ratio, and not absolute amounts, it is not necessary to have accurate weights.
  - If absolute, accurate amounts of any of the alkaloids are required, then stemming and grinding is necessary, as is accurate weighing. However, this is not necessary for seed screening, and absolute amounts of any of the alkaloids in such young leaves will have no real meaning. Most laboratories will charge extra for grinding.
- Specify which alkaloids are required, and also request the data for the external standards.
  - NOTE – nicotine and nornicotine are essential – % secondary alkaloids is not an acceptable substitute for nornicotine.
  - All that is necessary for seed screening is nornicotine and nicotine, but anabasine and anatabine are usually included. Myosmine is not usually included; it requires a longer and more expensive analysis. Unless there is reason to require it, it should be specified that myosmine is not required.
- Specify the urgency.
  - Most laboratories will be able to give your samples priority, if the urgency is specified in advance.

11. Roguing

- Conversion % is calculated as [nornicotine / (nicotine + nornicotine)] x 100. **Any plants with conversion >3% must be rogued.** It is very important that **any cross-pollination between converter and non-converter plants is prevented**.
- Fig. 14 illustrates the data format used at UK, where the plants to be rogued are marked in yellow. If raw data are provided, it will be necessary to calculate conversion, sort the data and mark those plants to be rogued.
- Any plants corresponding to missing samples and samples which did not cure properly, should also be rogued.

	NICOTINE %DM	NORNIC %DM	ANABAS %DM	ANATAB %DM	**CONVERSION %**	SEC ALK % TOT ALK	TOT ALK %DM	NIC+NNIC %	ANAB % TOT ALK	ANAT % TOT ALK
KY17-40	1.4	0.03	0.003	0.03	**2.1**	4.4	1.4	1.4	0.2	2.1
KY17-41	0.8	0.92	0.002	0.05	**53.7**	55.1	1.8	1.7	0.1	2.9
KY17-42	1.8	0.03	0.000	0.04	**1.6**	3.6	1.9	1.8	0.0	2.0
KY17-43	2.0	0.02	0.000	0.03	**1.1**	2.7	2.0	2.0	0.0	1.6
KY17-44	1.4	0.12	0.000	0.00	**7.7**	7.9	1.5	1.5	0.0	0.1
KY17-45	2.1	0.03	0.002	0.03	**1.3**	2.8	2.1	2.1	0.1	1.4
KY17-46	1.8	0.02	0.003	0.04	**1.1**	3.3	1.9	1.9	0.2	2.1
KY17-47	1.5	0.02	0.006	0.03	**1.3**	3.7	1.5	1.5	0.4	2.0
KY17-48	1.5	0.02	0.002	0.02	**1.3**	2.7	1.5	1.5	0.1	1.3
KY17-49	2.0	0.10	0.003	0.04	**4.8**	6.8	2.2	2.1	0.1	2.0
KY17-50	1.1	0.27	0.003	0.03	**20.2**	22.2	1.4	1.3	0.2	2.3
KY17-51	0.8	0.01	0.004	0.02	**1.7**	4.1	0.8	0.8	0.5	2.0
KY17-52	1.7	0.03	0.002	0.03	**1.7**	3.5	1.8	1.7	0.1	1.8
KY17-53	1.5	0.02	0.002	0.04	**1.2**	3.8	1.5	1.5	0.1	2.4
KY17-54	1.5	0.02	0.005	0.03	**1.2**	3.5	1.6	1.6	0.3	1.9
KY17-55	1.6	0.02	0.004	0.04	**1.3**	3.6	1.7	1.6	0.2	2.1
KY17-56	0.9	0.01	0.002	0.02	**1.4**	3.7	0.9	0.9	0.2	2.0
KY17-57	1.2	0.02	0.002	0.03	**1.3**	3.8	1.2	1.2	0.2	2.4
KY17-58	1.4	0.02	0.002	0.03	**1.2**	3.0	1.5	1.5	0.1	1.7

Figure 14: Conversion data for roguing

- If the data are available in time, any plants with >3% conversion must be rogued before flowering.
  - Roguing should be completed before any flowers open.
  - If any flowers have opened on self-pollinated or male-sterile lines prior to roguing converter plants, all plants being saved for seed production must be completely cleaned of open flowers and set pods.
  - No pollen should be collected from male-fertile plants or applied to male-sterile plants prior to roguing converters.
  - Collect seed from the remaining selected plants at the end of the season, and bulk.
- If the data are not available before flowering, plants must be bagged or all capsules and open flowers picked off after roguing.
  - If plants are bagged for selection later, discard all seedheads from the plants to be rogued. Bulk all remaining seedheads.
- For male-sterile production, it is necessary to rogue both the male and female parents.
  - If the data are available before flowering, rogue and bulk the pollen of the remaining male plants, and pollinate all remaining females.
  - If it is not possible to get the data before flowering, paired crosses will be necessary (do not bulk the pollen; cross one male with one female). When the data are received, discard any cross where either or both parents exceed the 3% conversion limit.

12. Screened / LC Bulk Seed

- The bulked seed from all plants remaining after roguing (<3% conversion) will constitute a screened bulk. If the LC requirements have been met, it will constitute an LC Foundation seed bulk, to be used for Certified seed production.
- A minimum of 30 plants must be bulked to constitute an LC Foundation seed bulk (see Appendix 2-1). If there are fewer than 30 plants remaining after roguing, the screening will have to be repeated if compliance with the LC protocol is required.
  - If Breeder seed is being produced, the 30 plant minimum does not apply to the Breeder seed bulk, on the understanding that this Breeder seed will be screened again to produce LC Foundation seed (where the 30 plant minimum will apply).

13. LC Varieties, LC Logo

- Most tobacco companies require the Foundation seed of any commercial variety to have undergone an acceptable screening process to minimize conversion. This LC protocol outlines one such procedure.
- The LC logo, shown on the cover sheet of this document, certifies that the Foundation seed of a particular seedlot has been screened using the LC protocol, by or under the supervision of, the University of Kentucky and KSIA (Kentucky Seed Improvement Association).
  - The logo is issued by arrangement with KSIA, only for:
    - Certified seed of KY/TN/KT varieties produced from UK Foundation seed e.g. TN 90LC
    - Certified seed of other varieties whose Foundation seed was screened under the supervision of KSIA and UK

- Certified seed produced from LC Foundation seed will be considered “LC”.
  - The letters “LC” may be incorporated in the variety name, but this is not required.
  - The LC logo is printed on the KSIA certification tag for LC seed certified by KSIA (Fig 15), and on a small sticker for seed certified by other agencies, but produced from LC Foundation seed (Fig. 16).

Figure 15: LC logo on KSIA tags for LC seed certified by KSIA

Figure 16: LC logo on sticker for LC seed certified by other agencies

14. Foundation Seed Increase

- Do not sequentially increase the seed without further screening. If this is done, the conversion mean will be higher in the next generation.
  - There should be only one generation of seed increase without screening (that of commercial seed production) before the seed is grown by farmers for a leaf crop.
- This seed can be used directly as Foundation seed, or it can be regarded as screened Breeder seed, and screened again to produce Foundation seed.

The LC Protocol – Appendix 1: Certification Standards (example)

1. Summary of Kentucky Seed Improvement Association (KSIA) Standards

   a. All standards of the Kentucky Seed Improvement Association (KSIA) for the production of tobacco seed should be followed (see page 37 of the KSIA Seed Certification Standards).

   b. Field plans should be established at the time greenhouse trays are seeded in the spring, so as to ensure the establishment of adequate plants of the parental lines and appropriate male sterile isolation plants. Foundation seed production may be in designated rows within a certified seed field of the same variety or in a foundation production block as illustrated on page 13.

   c. Establish healthy transplants from the most pure source of seed for each variety or parent of hybrid lines. Establish transplants of male sterile plants to use for pollen isolation rows in the field.

   d. Isolation between varieties of different types shall be at least 1320 feet.

   e. Self pollinated varieties (for seed collection) of the same type should be separated by any one of these methods:
      - 150 feet
      - protected from cross pollination by bagging
      - separated by four rows of male-sterile tobacco not used for seed
      - within a row, separated by 15 feet of male-sterile tobacco not used for seed
      - in fields where two or more self pollinated varieties of the same type are grown side by side, four rows of each variety shall be allowed to bloom and set seed, but not be harvested

   f. Male Fertile (Pollen Producing Parent, for pollen collection) of the same type should be isolated by any one of these methods:
      - varieties of the same type shall be isolated by at least 50 feet
      - protected from cross pollination by bagging
      - separated by four rows of male-sterile tobacco not used for seed
      - within a row, separated by 15 feet of male-sterile tobacco not used for seed
      - in fields where two or more self pollinated varieties of the same type are grown side by side, four rows of each variety shall be allowed to bloom and set seed, but not be harvested

   g. Male Sterile varieties shall adhere to the following isolation requirements:
      - male sterile varieties of the same type require no isolation from each other.
      - different types of male steriles must be separated by at least 660 feet.
      - male sterile varieties require no isolation from the fertile parent of a cross
      - male sterile varieties of the same type shall be isolated from all the pollen producing plants (with the exception of the fertile parent used to pollinate the male sterile of a cross) by either of these methods:
        - at least 150 feet
        - four rows of male-sterile tobacco not used for seed
      - male sterile varieties and pollinators of different types must be separated by at least 1320 feet.

2. Example of Field Design for Foundation Seed Production

Male Sterile Isolation Row
Male Sterile Isolation Row
Male Sterile Isolation Row
Male Sterile Isolation Row
*** Pollen Parent for Male Sterile Line Y ***
Male Sterile Isolation Row
Male Sterile Isolation Row
Male Sterile Isolation Row
Male Sterile Isolation Row
Male Sterile Line X
*** Pollen Parent for Male Sterile Line X***
Male Sterile Isolation Row
Male Sterile Isolation Row
Male Sterile Isolation Row
Male Sterile Isolation Row
Self Pollinated Variety A *- 15 ft MS Isolation Plants -* **Self Pollinated Variety B**
Male Sterile Isolation Row
Male Sterile Isolation Row
Male Sterile Isolation Row
Male Sterile Isolation Row
Check Variety

* Pollen Parent for MS line X can be adjacent to MS line X *

- Four rows of male sterile plants of the same type may be used to isolate from other tobacco in the area and provide a pollen barrier between the various lines.
- If different varieties are planted within the same row, provide 15 feet of male sterile plants between the varieties.

3. Websites

The minimum standards for tobacco seed production in the USA are in pages 267-269 of the Association of Official Seed Certifying Agencies (AOSCA); link below.

http://www.aosca.org/2004%20Yellow%20Book,%20pdf.pdf

Each certifying agency has its own standards, in addition to the AOSCA standards. A list of certifying agencies and contact details can be found at:

http://www.okstate.edu/OSU_Ag/ocia/certagencies.html

Some certifying agencies have their own websites:

http://www.nccia.ncsu.edu

The LC Protocol – Appendix 2: Results of Incorrect Procedures

1. Minimum Number of Plants Comprising Foundation Seed Bulk

Most plants selected as non-converters (<3% conversion) will produce progeny populations with an acceptable conversion mean. Figure 2-1 shows the progeny population (mean 2%) from a single parental plant with 0.5% conversion. Figure 2-2 shows the progeny population (mean 4%) grown from a bulk of 98 parental plants screened to 3%. As outlined below, the bulk of 98 plants, despite screening, almost certainly included some plants whose progeny were mostly unacceptable, but the contribution to the mean of so large a population by such plants would be small.

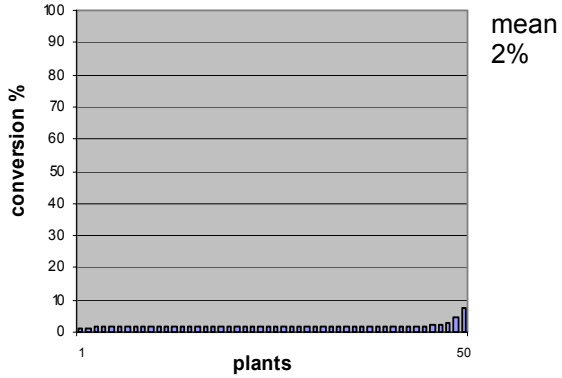


Figure 2-1: Progeny of a single stable non-converter plant

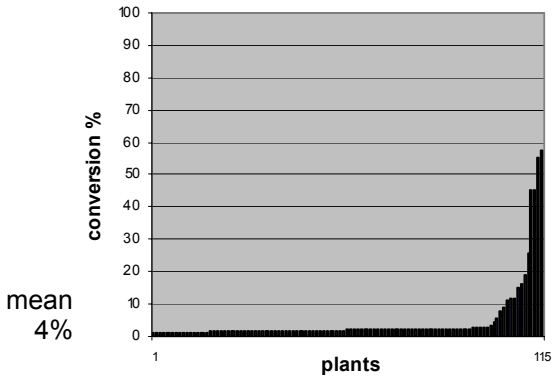


Figure 2-2: Progeny of 98 screened non-converter plants

Some plants selected as non-converters will produce progeny populations with unacceptably high means. Figures 2-3 and 2-4 show the progeny populations (means 19% and 24%) of two unstable non-converter parental plants (1.7% and 2.7%, respectively). If the Foundation seed bulk consists of only a few plants, and such plants are included, their contribution will be large and the progeny population mean will be high. For example, a bulk of these two parental plants, both <3%, would produce a progeny population with a mean of 21.5%. However, if the Foundation seed bulk includes a large number of plants, as in Fig. 2-2, the contribution from such plants will be small and the population mean will be acceptable. This is the reason for the specified 30 plant minimum for the Foundation seed bulk; so that no one plant comprises more than 3-4% of the total.

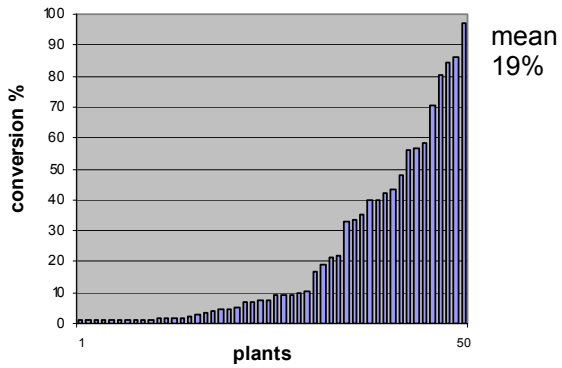


Figure 2-3: Progeny of a single unstable non-converter (mean 19%)

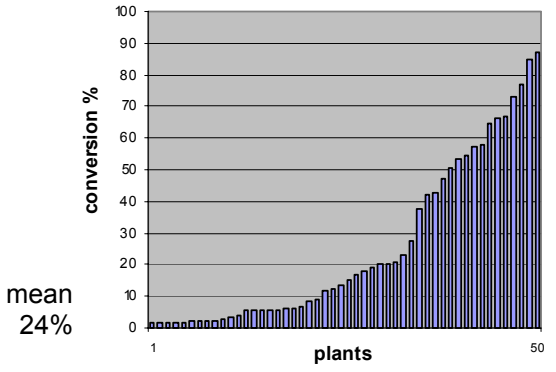


Figure 2-4: Progeny of a single unstable non-converter (mean 24%)

2. Check Variety

The check variety is a uniform, stable, high converter line. If the procedure is done correctly, every plant should convert at over 90% (Fig. 2-5). Lower levels of conversion (Fig. 2-6) indicate a problem with the sampling, treatment, curing, analysis or a combination of these factors (see Section 5 of this Appendix).

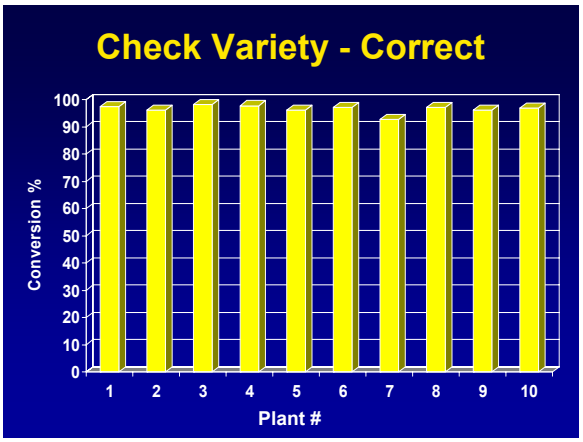


Figure 2-5: Conversion for check variety - result of correct procedure

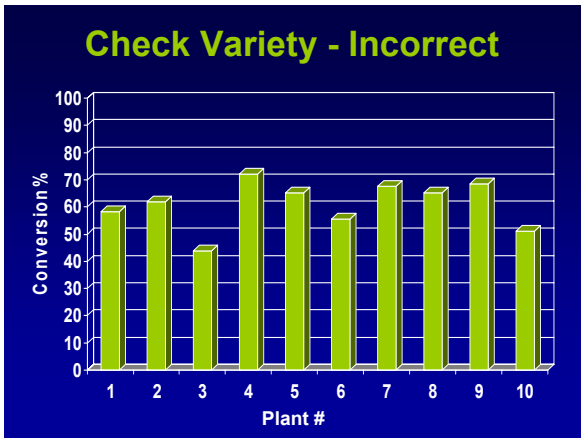


Figure 2-6: Conversion for check variety - result of incorrect procedure

3. Sampling Stage

With this protocol, plants are sampled when they are about 12-18" (30-45 cm) high, about a month after transplanting. However, older plants have been screened using other procedures (Fig. 2-7). The data comparing early vs. late sampling are not equal, but are similar. Where there are differences, the earlier sampling usually has higher conversion than the later, so that screening with earlier sampling is often more rigorous i.e. plants will be rogued with the earlier sampling that would remain with the later sampling (Fig. 2-8).

Figure 2-7: Early sampling, 12-18" (top); late sampling, button stage (bottom)

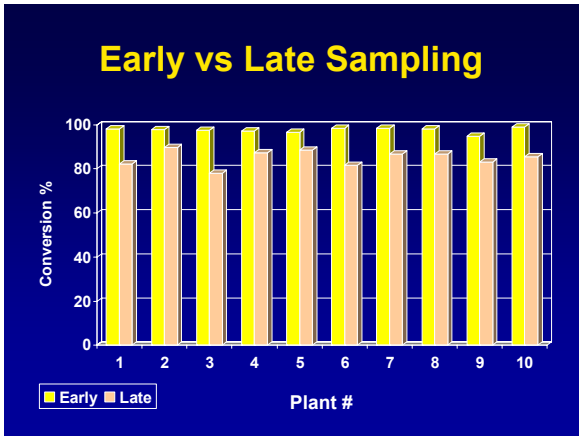


Figure 2-8: Conversion – early sampling, 12-18"; late sampling, button stage

The other reasons for preferring early sampling are practical ones. Larger leaves are more difficult to handle, and take up more space in the curing unit. However, the most important reason concerns the prevention of cross-pollination of selected plants and converters which will ultimately be rogued. Early sampling enables turnaround of data in time to rogue before flowering. If it is not possible to rogue before flowering, a lot more work is involved. Plants

must either be bagged, or they must be completely cleaned of open flowers and set pods after roguing (See section 11).

4. Ethephon Treatment

Ethephon treatment is a very important aspect of this protocol. Ethephon facilitates the yellowing of young leaves; without it, it is difficult and often impossible to force these leaves to yellow (Fig. 2-9). Yellowing is vital because it is a visible indication that the leaf has senesced sufficiently for conversion to take place. It also has an effect on conversion *per se*; it drives the conversion to its maximum potential. The measured conversion will be lower (often significantly so) in leaves which have not been treated with ethephon, even if they do yellow (Fig. 2-10).

Figure 2-9: Ethephon treated (bottom), untreated (top) after 7 days cure

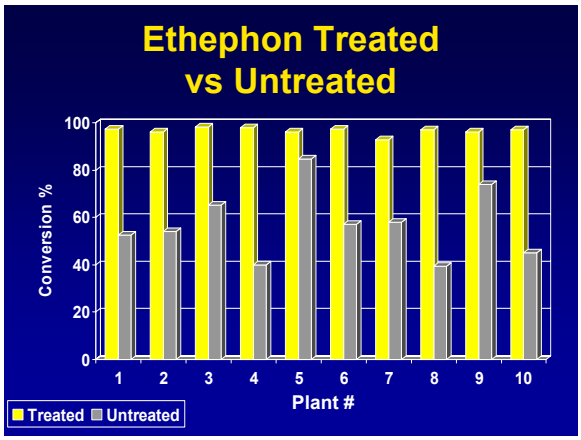


Figure 2-10: Conversion – ethephon treated vs. untreated

5. Incorrect Sampling, Treating and "Curing"

Conversion is an ongoing process in a senescing leaf, and it is a trait whose expression is very variable. When screening seed, it is important to measure maximum potential conversion. To ensure maximum expression of conversion, the leaf must be at a certain stage of maturity (not too young, not too old), and it must be treated with ethephon to drive the conversion reaction to its maximum potential. Anything which prevents the ethephon stimulus, or which interrupts the ongoing conversion, will result in underestimation of conversion.

All the data presented in this section were collected on the check variety, where every plant should convert at >90% if the procedures are done correctly. Each data set represents a simulated incorrect procedure, compared with the correct procedure. Lower levels of conversion indicate incorrect procedures. These lower levels may not seem important in the check variety, because they are still high by any standards, but they are important when applied to borderline plants in the 3%-10% range, because they affect whether or not these plants are rogued (see p 19

a. Sampling necrotic leaves (Fig. 2-11) should be avoided. Necrotic patches on leaves are already dead; such conversion as took place happened before the tissue could respond to the ethephon stimulus. For this reason, conversion measured on necrotic tissue will be an underestimate (Fig. 2-12). Cut out any necrotic tissue before ethephon treatment, and if necessary, again at untying.

Figure 2-11: Leaf with necrosis

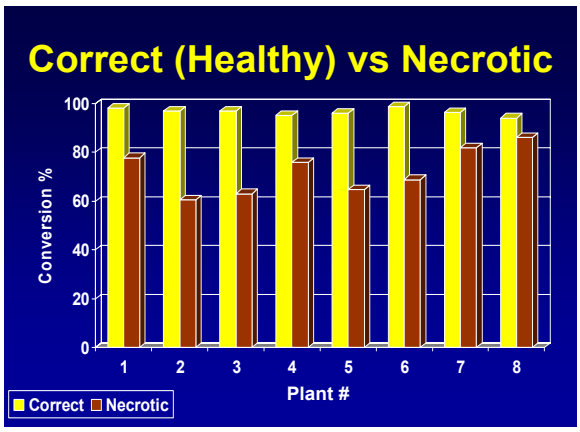


Figure 2-12: Conversion in necrotic leaves vs. healthy leaves

b. Sampling over-mature leaves (Fig. 2-13) results in underestimation of conversion (Fig. 2-14), probably because the leaf is too old to efficiently respond to the ethephon stimulus.

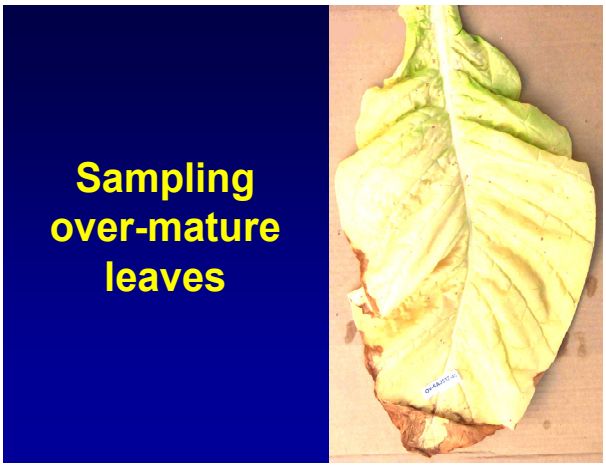


Figure 2-13: Over-mature leaf

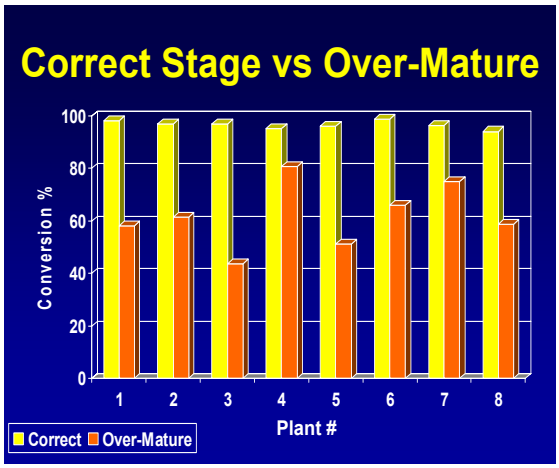


Figure 2-14: Conversion in over-mature leaves vs. correct stage

c. Sampling immature leaves (Fig. 2-15) is probably the most serious error one can make.

Figure 2-15: Immature leaf

It results in a very serious underestimate of conversion if the leaves are cured for seven days, because they will still be green when they are taken out of the curing unit (Fig. 2-16) – these green, immature leaves take much longer to cure, and the conversion process will be interrupted before it is complete. If the leaves are allowed to cure for more than seven days, until they are yellow, the conversion is much higher (Fig. 2-17), but it is still considerably less than in a leaf of the correct maturity, probably because the leaf is too immature for full conversion, even with ethephon.

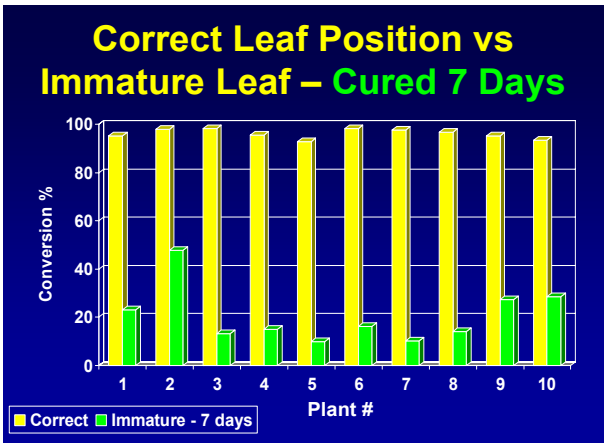


Figure 2-16: Conversion in immature leaves (cured seven days) vs. correct

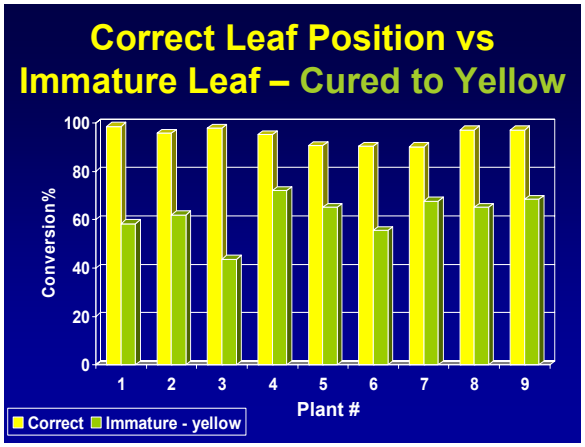


Figure 2-17: Conversion in immature leaves (cured until yellow) vs. correct

d. Allowing leaves to wilt before ethephon treatment (Fig. 2-18) results in underestimation of conversion (Fig. 2-19), probably because the wilted leaves do not respond to the ethephon.

Figure 2-18: Wilted leaf

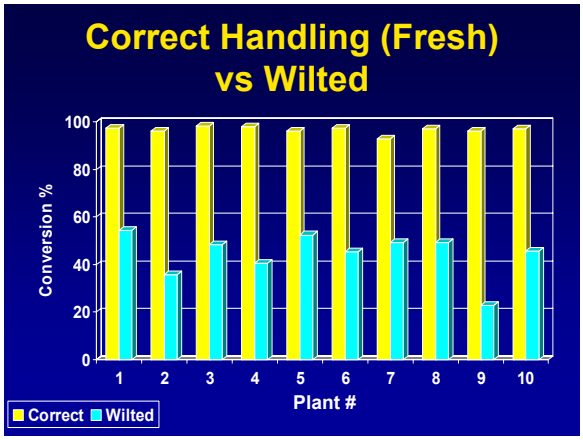


Figure 2-19: Conversion in wilted leaves vs. correct handling

e. Setting green (Fig. 2-20) interrupts the ongoing conversion process, killing the leaf before conversion is complete. Measured conversion is therefore lower than the potential for that leaf (Fig. 2-21). It is essential to maintain enough humidity during the cure to keep the leaf alive until it has turned fully yellow and the conversion process is complete.

Figure 2-20: Setting green

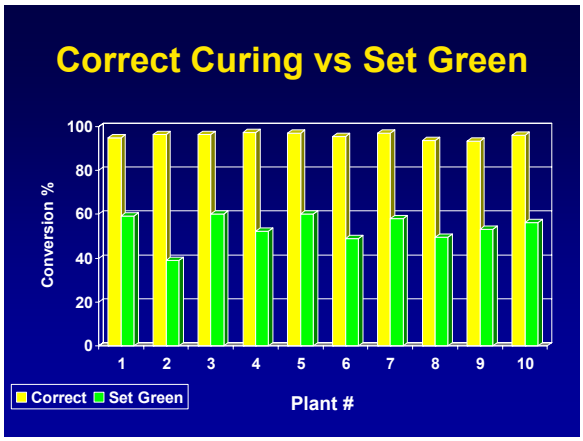


Figure 2-21: Conversion in leaf set green

f. Taking leaves out of the curing unit before they are yellow (Fig. 2-22) also results in underestimation of conversion (Fig. 2-23). Leaves cured in cool conditions, and very green leaves sampled from over-fertilized plants take longer than seven days to yellow, so if they are taken out at seven days, conversion will be underestimated. For this reason, completion of yellowing is more important than time in the curing unit. While leaves must remain in the curing unit for a minimum of seven days, they may require longer and should not be taken out before they are fully yellow. Excessive fertilization can greatly complicate this exercise, making the cure lengthy and difficult. Sidedressing should never be applied before sampling, and the basal N should not be excessive.

Figure 2-22: Taking out green leaves

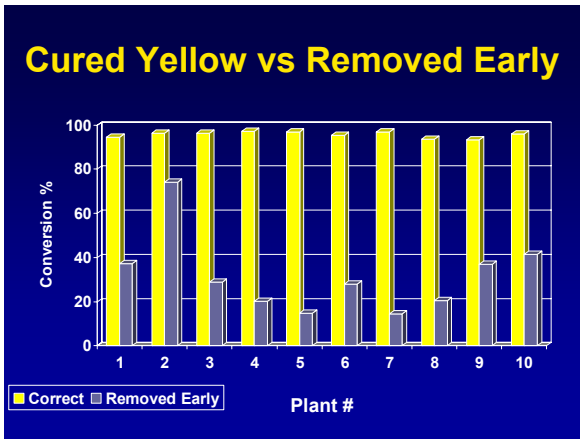


Figure 2-23: Conversion in leaves cured to completion vs. taken out green

g. Potential conversion: The reduction in measured conversion of the check variety shown in Appendix 2 may not seem important, because it is still high. However, when this is applied to borderline plants in the 3%-10% range, it becomes important, because it can affect whether a plant is classified as a converter or a non-converter, and whether or not it is rogued.

One should never lose sight of the fact that the object of this exercise is to measure the absolute maximum POTENTIAL conversion. One should therefore be careful not to do anything which will prevent FULL expression of conversion – all those procedures outlined in Appendix 2.

6. Laboratory Standards

GC analysis can yield erroneous data if the calibration or the integration is incorrect. It is necessary to include known chemical and tobacco standards to monitor the accuracy and consistency of the GC data. It is extremely unlikely that nornicotine values approaching zero are correct; zero values for nornicotine should be cause for concern. Each laboratory run should include external standards, e.g. the Kentucky reference cigarettes, 2R1 and 1R4F. These tobacco standards, made up to a known composition, are available from the KTRDC (Kentucky Tobacco Research and Development Center) at the University of Kentucky. Figures 2-24 and 2-25 show examples of data from these samples run on a properly calibrated GC, with horizontal lines indicating the mean value for each standard. The 2R1 and 1R4F are ideal because their conversion values fall either side of 3% (Fig. 2-26). The mean values of the standards shown in the example were:

	2R1	1R4F
nicotine	1.66 % DM	1.94 % DM
nornicotine	0.033 % DM	0.073 % DM
conversion	1.97 %	3.62 %

Regardless of the sample standard used, if the variation is large, this indicates a problem in the analysis. The variability for nornicotine is generally much greater than that for nicotine (Figs. 2-24, 2-25), because it is a more difficult analysis, as reflected in the CVs (Chen *et al.*, 2005).

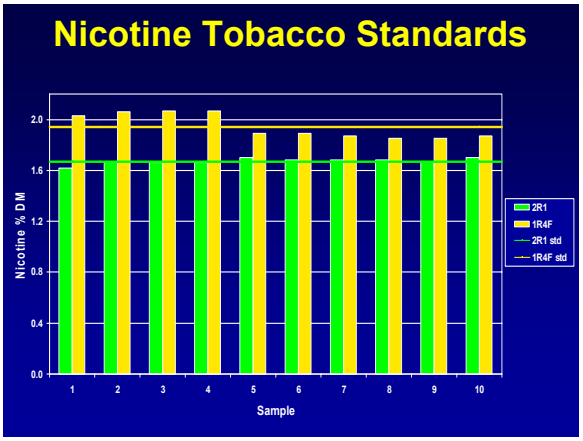


Figure 2-24: Nicotine values for the two two KY reference cigarettes

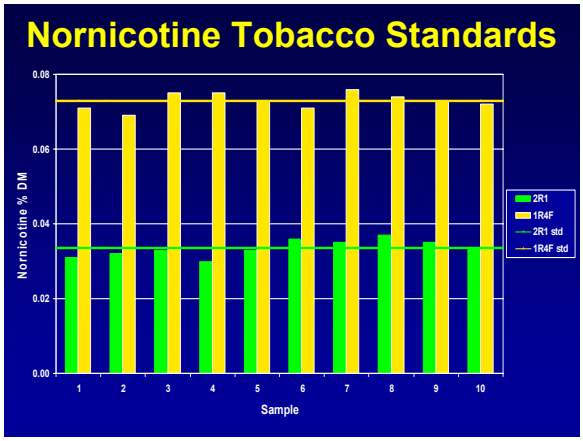


Figure 2-25: Nornicotine values for the two KY reference cigarettes

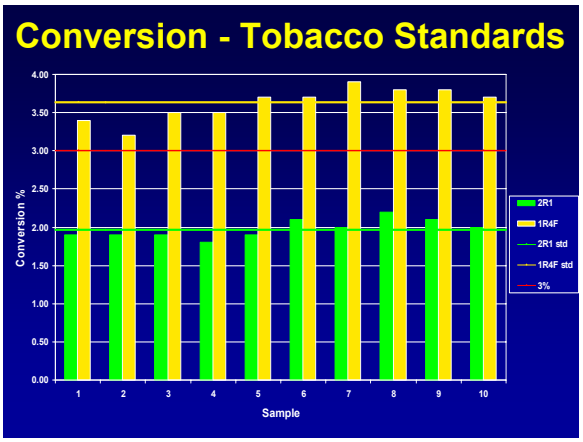


Figure 2-26: Conversion values for the two KY reference cigarettes

Chen, P., N. Qian, H.R. Burton, and S.C. Moldoveanu. 2005. Analysis of minor alkaloids in tobacco: a collaborative study. Beitrage 21:369-379.

The LC Protocol – Appendix 3: Laboratory Procedures

Most laboratories analyze secondary alkaloids on a GC (gas chromatograph), although the HPLC (high performance liquid chromatograph) is acceptable. Details given in this Appendix are for a Perkin Elmer GC with PreVent™ and a Varian 3900 GC with an Orion EZ Flash accessory, which heats and allows the column to cool more rapidly, enabling a much faster sample turnaround. While the specifications are given for the GCs used in our lab, they will be applicable to any GC using the same column.

1. External Standards

External tobacco standards should be included in every run, and their data used to monitor the GC accuracy and consistency (see Appendix 2-6). The Kentucky reference cigarettes, 2R1 and 1R4F, are excellent standards because their conversion values fall either side of 3% (Fig. 2-26).

2. Alkaloid Extraction Procedure

- **Reagents**
  - Sodium Hydroxide solution (2N NaOH)
    Add 80 g sodium hydroxide to 1000 ml H_2O
  - MTBE (methyl-tert butyl ether) solution (0.4 mg/ml quinoline)
    Add 1.6 g quinoline (weighed accurately; 1.6000 $\pm$0.0002 g) to 4000 ml MTBE
- **Procedure**
  - Weigh 0.1 g (weighed accurately; 0.100 $\pm$0.001 g) of sample into a labeled glass scintillation vial. (For conversion ratio only, accurate weighing not necessary)
  - Add 0.5 ml Sodium Hydroxide solution (2N NaOH) to each sample.
  - Add 5.0 ml MTBE solution (0.4 mg/ml quinoline) to each sample after 15min.
  - Shake for 2½ hr.
  - Transfer the top layer of fluid (MTBE) in glass scintillation vial into labeled GC vials.
  - Cap GC vials containing sample.
  - Load labeled GC vials onto autosampler.
  - Run analysis.

3. Perkin Elmer, Autosystem XL Gas Chromatograph with PreVent™

- **Autosampler**
  - Injection Source:
    - Autosampler
  - Sample Injection:
    - Splitless injection
    - Injection volume = 1.0 µl
    - Viscosity delay = 0
    - Wash/waste vial set = 1
  - Washes:
    - Pre-injection solvent washes = 0
    - Pre-injection sample washes = 2
    - Post-injection solvent washes = 2

- **Oven / Inlets**
  - Oven Program

Step	Rate (ºC/min)	Temp (ºC)	Hold (min)
Initial	0.0	120	1.00
1	30.0	280	2.00

  - Injection Temperature = 250ºC
- **Carrier Gas**
  - Helium
    - Flow = 20 ml/min
- **Detectors**
  - FID (flame ionization detector)
    - Temp = 250ºC
  - Gas Flow Rates
    - H_2 = 45.0 ml/min
    - Air = 450.0 ml/min
- **Column**
  - DB-5 from J & W Scientific INC
    - Length = 30 m
    - ID = 0.53 mm
    - Film thickness = 1.5 µm
- **Run Time**
  - 8.33 minutes

4. Varian 3900 Gas Chromatograph with Orion EZ Flash II Accessory

- **Autosampler**
  - Injection Source:
    - Autosampler
  - Sample Injection:
    - Injection volume = 1.0 µl
- **Oven / Inlets**
  - Oven Temperature = 95ºC
  - Injection Temperature = 250ºC
- **Carrier Gas**
  - Helium
    - Flow = 2.4 ml/min
- **Column Temperature – EZ Flash II Accessory**
  - Interface Temperature
    - Injector interface = 250ºC
    - Detector interface = 280ºC

- Column Program

Step	Time (sec)	Temp (ºC)
Initial	0	100
1	30	130
2	70	250
3	80	280
4	90	280

- **Detectors**
  - FID (flame ionization detector)
    - Temp = 280ºC
  - Gas Flow Rates
    - Helium make up flow = 25.0 ml/min
    - H_2 = 30.0 ml/min
    - Air = 300.0 ml/min
- **Column**
  - "Thermo" RTX-5MS (Fused Silica) column
    - Length = 5 m
    - ID = 0.25 mm
    - Film thickness = 0.25 µm
- **Run Time**
  - 1.50 minutes