Analysis of the Polyphenols in Cured Tobacco Leaves using UPLC-ESI-MS/MS

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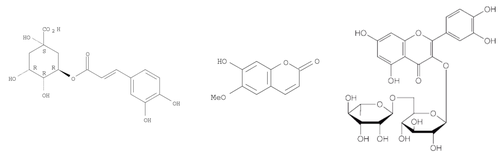
INTRODUCTION

The chemical changes that occur in tobacco leaves during curing have been studied extensively over the years and are well documented in the literature. The method of curing has a strong impact on the chemical profile of the processed tobacco and the chemical differences between cured tobaccos (air-cured, flue-cured, fire-cured, etc.) largely determine how they are used in tobacco products. Chemical changes leading to color differences between flue-cured and air-cured tobaccos result from chlorophyll decomposition, which leads to yellowing in flue-cured tobaccos, and phenolic oxidation, which leads to browning in air-cured tobaccos[1].

Browning in tobacco is an enzymatic process wherein polyphenols such as chlorogenic acid and rutin are oxidized to form brown pigments. Enzymatic browning does not occur to an appreciable extent in flue-cured tobaccos because of enzyme deactivation at the high temperature of the curing process. Browning occurs during air-curing because the mild conditions required by the process do not affect enzymatic activity. Typical polyphenol content for flue-cured tobacco is 7.0% or more, while air-cured (including fire and sun cured) tobaccos generally contain less than 0.5%[2,3]. The most abundant polyphenols in tobacco are chlorogenic acid and rutin and occur in flue-cured tobaccos at levels as high as 3% and 1%, respectively[3].

Chlorogenic acid, or 3-caffeoylquinic acid, is one of three positional isomers of caffeoylquinic acid found in tobacco. 4-caffeoylquinic (cryptochlorogenic) and 5-caffeoylquinic (neochlorogenic) are relatively minor constituents. Rutin is the most abundant flavonoid glycoside of quercetin found in tobacco. Quercitrin, isoquercitrin, and a glycoside of kaempferol are also found in small amounts. Another group of polyphenols found in tobacco are the coumarins, represented most prominently by scopoletin. Chemical structures of chlorogenic acid, scopoletin, and rutin are shown below



Chlorogenic acid **Scopoletin** **Rutin**

The purpose of this work was to develop a method for the analysis of polyphenols in tobacco leaves and tobacco products. This presentation summarizes our efforts to optimize extraction conditions for sample preparation and instrument conditions for analysis by UPLC-MS/MS. A comparison of extraction by sonication, which was adapted from the literature[4], and pressurized liquid extraction was carried out to determine if the latter technique offered a significant increase in extraction efficiency.

SAMPLE PREPARATION

Leaf tobacco samples (flue-cured, air, cured, oriental) were obtained from Universal Leaf Tobacco Company (Richmond, VA). Samples were ground and packaged by the vendor. No further preparation steps were undertaken by the TTB Tobacco Laboratory prior to extraction of analytes. For the purposes of this study 6 tobacco samples, 2 of each type described above, were sampled for analysis.

Extraction - sonication

Approximately 0.4 gram samples of ground tobacco were each diluted with 50.0 ml of 80:20 methanol/water with 0.4 ppm 7-hydroxycoumarin added as an internal standard. The samples were agitated followed by sonication for 20 minutes in a Bransonic Ultrasonic Cleaner (Branson 5510R-DTH) with each sample periodically shaken to suspend the tobacco in solution. Following sonication, aliquots of each sample were treated with Maxi-Clean C18 SPE (600 mg) and filtration (0.45 um PFTE). The filtered samples were diluted 10 and 20 fold for analysis by UPLC-MS/MS.

Pressurized liquid extraction (PLE)

Approximately 0.5 gram samples of ground tobacco were mixed with 0.5 grams of Dionex ASE Prep Diatomaceous Earth and added to 10 mL extraction cells. Remaining cell volume was filled with sand. Extractions were carried out using a Dionex ASE 350 under the following conditions.

Pressurized Liquid Extraction Conditions (ASE 350)			
Temperature	40, 70, and 100 °C	**Rinse volume**	40%
Heat Cycle	5 minutes	**Purge**	1 minute
Static time	5 minutes	**Water**	20%
Cycles	2	**Methanol**	80%

The extraction solution used in the PLE experiments was the same one used in the sonication experiments. Following extraction, the sample in each collection vial was diluted to approximately 30 grams with the extraction solution.

The diluted ASE extracts were treated with Maxi-Clean C18 SPE (600 mg) and filtration (0.45 um PFTE). All filtrates were diluted 10 and 20 fold for analysis by UPLC-MS/MS using a 60:40 methanol/water solution with 0.4 ppm 7-hydroxycoumarin added as an internal standard.

ANALYSIS

UPLC-MS/MS

Samples were analyzed using a Waters Acquity I Class UPLC interfaced to a Xevo TQ-S quadrupole tandem mass spectrometer. Separations were carried out using a Waters Acquity UPLC BEH C18, 1.7 µm, 2.1 X 100 mm column. The chromatographic conditions were:

M.P. A: Water with 0.3% Formic acid — Injection vol: 2.0 µl

M.P. B: Methanol with 0.3% Formic acid — Column temp: 32°C

Gradient:

Time (min)	Flow (ml/min)	% A	%B
Initial	0.4	90	10
3.0	0.4	84	16
8.5	0.4	10	90

Multiple Reaction Monitoring (MRM)

The MRM mass transitions and spectrometric conditions were obtained by conducting infusion analysis of neat standard compounds in negative ion mode. The following is the complete list of target polyphenol analytes in tobacco as well as the masses of the parent and fragment ions. The fragment ions were determined experimentally and are consistent with fragmentations reported in the literature[5,6,7,8,9].

analyte	formula weight	parent ion (Da)	cone voltage (V)	daughter ion		qualifier ion	
				mass (Da)	collision energy (eV)	mass (Da)	collision energy (eV)
7-hydroxycoumarin (IS)	162.14	161	32	133	18	105	24
quinic acid	192.17	191	52	85	20	93	18
5-caffeoylquinic acid	354.31	353	8	191	18	179	20
3-caffeoylquinic acid	354.31	353	6	191	18	-	-
4-caffeoylquinic acid	354.31	353	8	173	18	179	18
esculetin	178.14	177	6	133	18	105	20
caffeic acid	180.16	179	6	135	15	107	20
scopoletin	192.17	191	6	176	14	148	20
rutin	610.52	609	86	300	34	271	54
quercetin 3-O-glucoside	464.38	463	8	271	41	300	28
kaempferol 3-O-rutinoside	594.53	593	15	285	29	255	53
quercitrin	448.38	447	4	300	26	271	42
quercetin	302.24	301	56	151	22	179	18
kaempferol	286.24	285	6	93	34	117	40

Chromatography – Optimization

Figure 1 shows the results of MRM experiments using the optimized chromatographic conditions described above. Although coelutions exist for several flavonoid glycosides (top four chromatograms in Figure 1), unique parent ion and fragment ion masses permitted selective detection of each analyte. The most critical chromatographic separations involve the chlorogenic acids because they cannot easily be distinguished by parent ion and fragment ion masses. All three chlorogenic acids (CQA's) form fragment ions at 191, 179, and 173 Da.

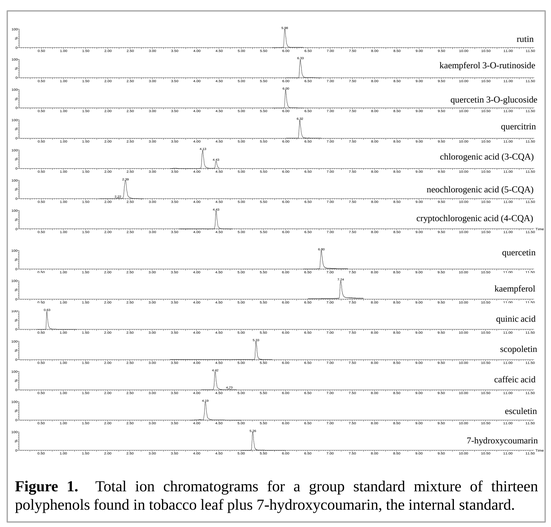


Figure 1. Total ion chromatograms for a group standard mixture of thirteen polyphenols found in tobacco leaf plus 7-hydroxycoumarin, the internal standard.

The Chlorogenic acids – Chromatographic Resolution and Mass Selectivity

Resolution of 3-CQA and 4-CQA was accomplished with a combination of chromatographic conditions and MRM optimization. The choice of 173 Da as the quantifier ion for 4-CQA provides a high level of selectivity relative to the 191 Da fragment. Near baseline resolution of the 3-CQA peak allows for good selectivity using the 191 Da fragment, which is a strong transition for both 3-CQA and 4-CQA (see **Figure 2** below).

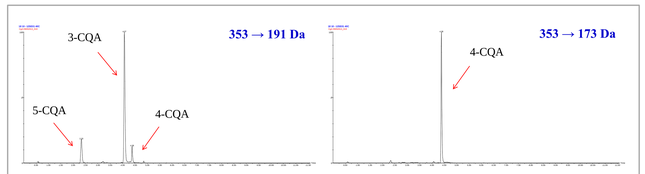


Figure 2. MRM experiments for major fragments of 3-CQA, 4-CQA, and 5-CQA in flue-cured tobacco.

Comparison of extraction techniques – PLE versus sonication

PLE uses elevated temperature and pressure to increase extraction efficiency. However, relative to simple benchtop techniques like the sonication procedure described here, it is time consuming and labor intensive. To determine if PLE offers a significant benefit with respect to analyte recovery, both techniques were used to extract six tobacco samples (air-cured, flue-cured, oriental). Peak areas (normalized to the internal standard) from UPLC-MS/MS experiments for all thirteen target analytes in each tobacco sample were compared. The results of these comparisons for 3-CQA, rutin, scopoletin, and quercetin are shown in **Figure 3**. While the comparisons for 3-CQA and rutin suggest marginal differences in recoveries from the two techniques, the results for scopoletin and particularly quercetin show more significant differences that appear to favor PLE. Similar trends are observed for other analytes in each compound class.

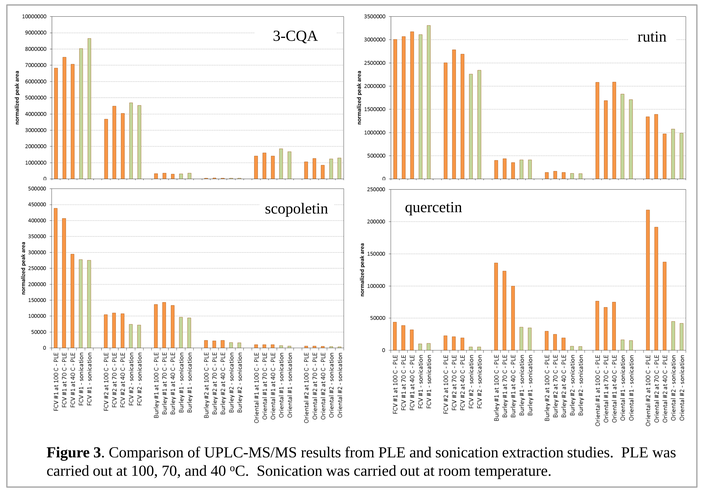


Figure 3. Comparison of UPLC-MS/MS results from PLE and sonication extraction studies. PLE was carried out at 100, 70, and 40 °C. Sonication was carried out at room temperature.

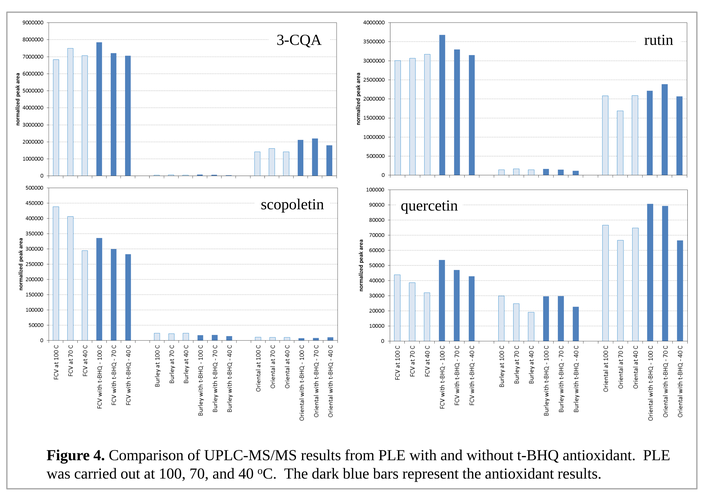


Figure 4. Comparison of UPLC-MS/MS results from PLE with and without t-BHQ antioxidant. PLE was carried out at 100, 70, and 40 °C. The dark blue bars represent the antioxidant results.

PLE – target analyte stability

Since PLE operates at elevated temperatures, the possibility of target analyte degradation was investigated. Concerns about the stability of polyphenols during the PLE process have be discussed in the literature[10,11]. Generally, extractions at lower temperatures have been suggested to avoid degradation. To investigate oxidation as a potential route of degradation, PLE experiments were performed on three tobacco samples with and without t-butyl hydroquinone (t-BHQ)[12]. Normalized peak areas from UPLC-MS/MS analysis of 3-CQA, rutin, scopoletin, and quercetin are shown in **Figure 4**. Similar comparisons were performed for all thirteen target analytes. The trend observed for quercetin in **Figure 4** suggests a benefit to using antioxidant. However, an opposite effect was observed with scopoletin for flue-cured tobacco. Further investigation regarding the use of antioxidant is required before conclusions can be drawn.

Another Target Analyte in Tobacco – a coumarin positional isomer?

MRM experiments revealed the presence of a peak at 4.04 minutes in extracts of flue-cured tobacco with the same parent ion, quantifier, and qualifier ion masses as scopoletin, which elutes at 5.36 minutes (see **Figure 5**). Thus far, this peak has only been observed in extracts of flue-cured tobacco. UPLC-MS/MS analysis of 8-hydroxy-7-methoxycoumarin, 6-hydroxy-7-methoxycoumarin, 4-hydroxy-7-methoxycoumarin, and 4-hydroxy-6-methoxycoumarin revealed peaks with identical MRM transitions to the unknown but retention times closer to scopoletin.

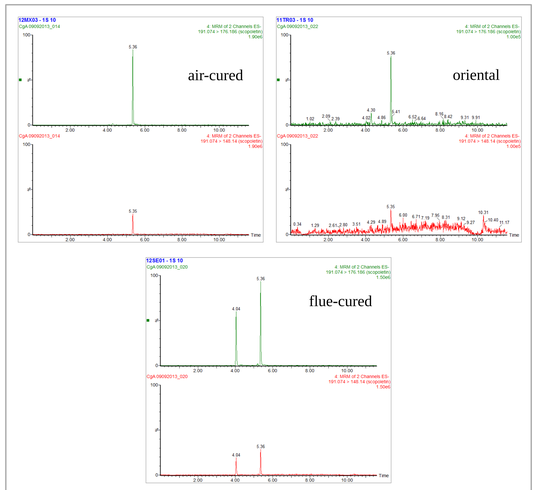


Figure 5. Comparison of MRM experiments for scopoletin in samples of flue-cured, air-cured, and oriental tobaccos.

FUTURE WORK

- Identify internal standards for quantitative analysis of target analytes.
- Expand investigation of PLE conditions to include parameters such as static time and number of cycles.
- Identify potential coumarin positional isomer in flue-cured tobacco.
- Optimization and validation of the method.

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