# Investigation on the Chloranisoles in Tobacco

WANG Jiale, LV Liangliang, ZENG Lingjie, QI Dawei, LIN Huaqing, WU Da*
Shanghai Tobacco Group Co. Ltd., Technology R&D Center, Shanghai 200082, P.R. China



***Abstract /Summary:***

A novel analytical method for the determination of chloranisoles (2, 4-dichloroanisole, 2, 6-dichloroanisole, 2, 4, 6-trichloroanisole and 2, 3, 4, 6-tetrachloroanisole) in tobacco was developed on gas chromatography-tandem mass spectrometry (GC-MS/MS).

In this method, Concurrent Solvent Recondensation Large Sample Volume Injection (CSR-LVI) was applied based on temperature-programmed inlet, by which a large injection volume of 25 μL was achieved, which greatly enhanced analytical sensitivity of ultra-trace chloranisoles. Florisil SPE cleaning of tobacco sample extraction was performed in the sample pre-treatment, and back-flush technique was further applied to expel the high boiling point components, avoiding the contamination of chromatographic system.

This method exhibited ultra-high sensitivity, excellent selectivity, recovery and repeatability, and was suitable for routine analysis of multitudinous tobacco samples.

Then, the changes of chloranisoles contents and musty odor in flue cured tobacco during moldy tobacco cultivation process were investigated.

The result showed that the contents of chloranisoles, especially the contents of 2, 4-dichloroanisole and 2, 4, 6-trichloroanisol, were correlated with the degrees of musty odor, and the quantitative relation between the two chloranisoles and musty odor in tobacco was obtained.

***Objective:***

In order to study the relationship between moldy odor and chemical compounds in tobacco, we developed a novel analytical method for the determination of the chloranisole in tobacco. And by accurate testing and sensory evaluation, the model of the contents of chloroanisoles and musty odor in flue cured tobacco during moldy tobacco cultivation process was investigated.

***Materials and Methods*:**

- 1), the sample was grinded and 2.0 g tobacco powder was weighted and be immersed in 10 mL water and 10mL hexane. 2), vortexes with 2500 rpm speed for 10 minutes was done after adding 100 μL internal standard solution. 3), the mixture was centrifuged under 5000 rpm speed for 3 minutes. 4), 4 mL Supernatant were transferred to Florisil-SPE-column which had been activated and then eluted by 6 mL mixed solution of hexane: ethyl acetate (v / v, 100: 1). 5), the combined eluent was concentrated to 0.5 mL to be analyze.
- Injection volume: 25 μL. Injection mode: splitless. Inlet temperature program: 75 °C, holding 0.1min, 600 °C / min to 190 °C. Precolumn: 5 m × 0.32 mm. Column 1: DB-35MSUI (10 m × 0.25 mm × 0.25 μm). Column 2: DB-35MS UI (20m × 0.25mm × 0.25 μm). Precolumn and Column 1: 1.2mL / min (0.1min), 100mL / min to 2 mL / min (4min), 100mL / min to 1.2mL / min; column 2: 1.5 mL / min, (0.1min), 100mL / min to 2.3 mL / min (4min), 100mL / min to 1.5 mL / min. Oven temperature: 70 °C held 4min, 30 °C / min to 130 °C, 5 °C / min to 200 °C. Postrun: Backflush start at 21min. Hold 8 min. EPC pressure: 20 psi. Oven temperature: 300 °C. Inlet pressure: 1 psi. Inlet temperature: 300 °C.
- Electron ionization mode (EI, 70 eV). Multiple reaction monitoring (MRM) conditions for each chloranisole and other parameters used are detailed in Table 1.
- Flue cured tobacco was cut and cultivated. The culture environment provided 75 ± 2% relative humidity and 25 ± 2 ℃ ambient temperature.
- Moldy sensory evaluation Team: 10 experts. The degree of mildew:visual moldy (0, 1), musty odor (0, 1, 2, 3), smoking musty (0, 1, 2, 3). The high value means the high moldy degree.

***Results and Disscussion:***

- Without changing the height of the syringe, the concept of CSR was used with normal auto liquid sampler with Multi-Mode-Inlet (MMI) of Agilent. The large volume injection with back-flush system GC-MS/MS is shown in Fig. 1.
- The needle injected sample into the liner, it was kept in liquid form because the inlet temperature is below the corrected boiling point of solvent. Then the temperature increased rapidly with the carrier gas pressure raised, and the sample all transferred to a pre-column.
- To enhance the ability of anti-pollution, auxiliary control module with EPC and Press Tight glass Three-way pass were used. When the target compound was over, the system started backflush with high temperature, to exclude high boiling impurities from the inlet.
- The chromatogram of chloranisoles is shown in Fig.2. The linear correlation was in the range of concentration between 0.05 ng·mL-1 to 100 ng·mL-1, and the limit of quantification was achieved to be 0.017, 0.019, 0.033, 0.019 ng·mL-1, respectively. Recoveries from 94.8 % to 110.7% were determined with narrow relative standard deviations (RSDs≤10 %).
- The correlation analysis was performed between the contents of chloranisoles and sensory evaluation scores (visual moldy, musty odor, smoking musty) by Pearson correlation coefficient and P value using SPSS software. The results are shown in Table 2.
- The contents of chloranisoles, especially the contents of 2, 4-dichloroanisole and 2, 4, 6-trichloroanisol, were correlated with the degrees of musty odor. The figure shown in fig. 3.
- To go deep into the relation of musty odor and chloranisoles, all data were analyzed in multiple linear regression by using musty odor scores as the dependent variable, while, the content of chloranisoles as the independent variable. A linear regression model was obtained: C = 0.037 + 0.912 × C1 + 0.149 × C2, which C is musty odor scores, and C1 is the content of 2, 4-DCA, C2 of TCA. The model and Analysis of Variance shown in Table 3.

***Table 1.***
***GC-MS/MS acquisition method conditions for the target chloroanisoles.***

| chloranisole | MRM transition, m/z (CE, eV) | |
|---|---|---|
| | Quantification | Identification |
| 2,6-DCA | 178>135 (25) | 178>163 (15) |
| 2,4-DCA | 178>135 (25) | 178>163 (15) |
| 2,4,6-TCA | 210>167 (25) | 210>195 (15) |
| TeCA | 246>203 (25) | 244>201 (25) |
| 2,4,6-TCA，d5 | 215>197 (15) | 217>199 (15) |

***Table 2.***
***The correlative analysis of the contents of chloroanisoles and sensory evaluation scores.***

| | | visual moldy | musty odor | smoking musty |
|---|---|---|---|---|
| 2,4-DCA | Pearson Correlation Coefficient | 0.850 | 0.928 | 0.860 |
| | P value | 0.000 | 0.000 | 0.000 |
| 2,6-DCA | Pearson Correlation Coefficient | 0.585 | 0.663 | 0.659 |
| | P value | 0.000 | 0.000 | 0.000 |
| TCA | Pearson Correlation Coefficient | 0.672 | 0.773 | 0.700 |
| | P value | 0.000 | 0.000 | 0.000 |
| TeCA | Pearson Correlation Coefficient | 0.516 | 0.643 | 0.580 |
| | P value | 0.000 | 0.000 | 0.000 |

***Table 3.***
***The multivariate regression models for musty odor and the contents of 2, 4-DCA and TCA by SPSS software.***

**Model Summary**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
|---|---|---|---|---|
| 1 | .928[a] | .861 | .859 | .38827069611 |
| 2 | .940[b] | .884 | .882 | .35619602334 |

a. Predictors: (Constant), 24DCA.
b. Predictors: (Constant), 24DCA, TCA.

**Anova**

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1 | Regression | 87.636 | 1 | 87.636 | 581.320 | .000a |
| | Residual | 14.171 | 94 | .151 | | |
| | Total | 101.807 | 95 | | | |
| 2 | Regression | 90.008 | 2 | 45.004 | 354.709 | .000b |
| | Residual | 11.799 | 93 | .127 | | |
| | Total | 101.807 | 95 | | | |

a. Predictors: (Constant), 24DCA.
b. Predictors: (Constant), 24DCA, TCA.
c. Dependent Variable: musty odor

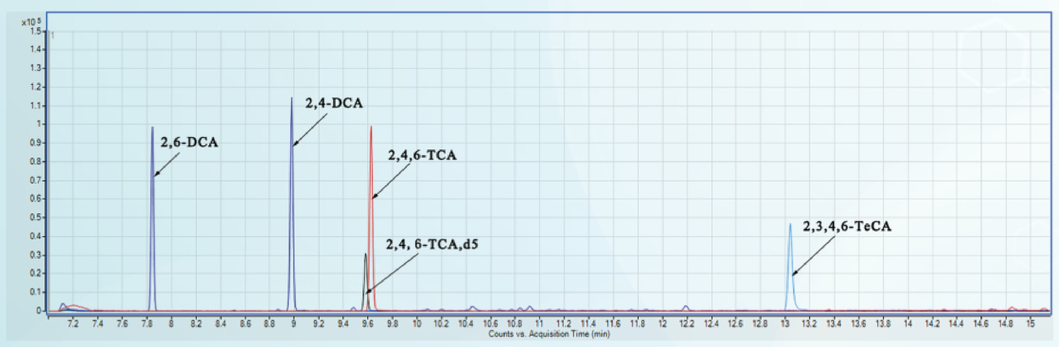


***Fig.2.***
***MRM Chromatogram of chloranisoles in moldy tobacco analyzed by the proposed method.***

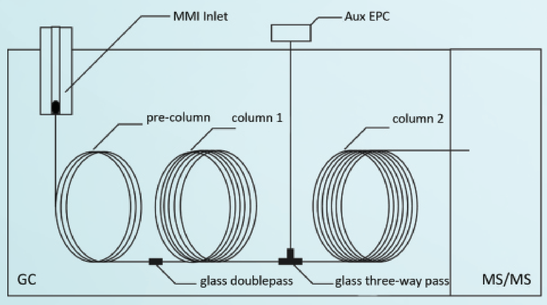


***Fig. 1.***
***Schematic diagram of the system with back-flush for LVI-CSR injection***

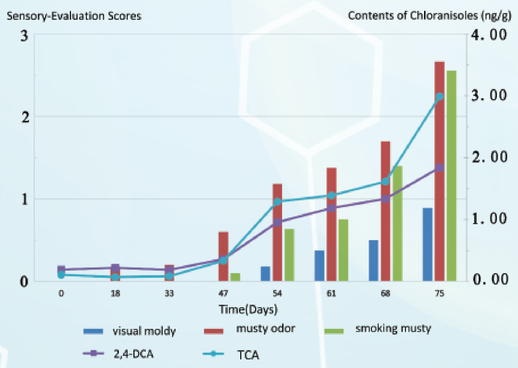


***Fig.3.***
***The relationship between the content of chloroanisoles and sensory evaluation scores based on the typical moldy tobacco sample.***

***Conclusions:***

1. In this study, a novel method was develop using gas chromatographic–tandem mass spectrometry with LVI-CSR technique for simultaneous determination of multiple chloranisoles in tobacco.
2. MMI inlet was used for LVI-CSR technique, which can be done with multiple low-temperature injection to increase sensitivity.
3. Back-flush was used to avoid pollution of the high boiling impurities in the system with auxiliary control EPC module and Press Tight glass Three-way pass.
4. This method exhibited ultra-high sensitivity, excellent selectivity, recovery and repeatability, and was suitable for routine analysis of multitudinous tobacco samples.
5. The musty odor of moldy tobacco was strong correlation with the contents of chloranisoles, especially the contents of 2, 4-dichloroanisole and 2, 4, 6-trichloroanisol in tobacco.
6. The model of chloroanisoles and the musty odor during moldy tobacco cultivation process was established and the quantitative relation was obtained by multivariable linear regression.

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