Comprehensive Characterization of Green Leaf Tobacco Extracts Using GC-HRT

LECO Corporation; Saint Joseph, Michigan USA

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1. Introduction

This application note highlights the attributes of High Resolution Time-of-Flight MS (HRT) which provide an extra level of confidence for challenging plant extract characterization studies. These attributes include High Resolution Deconvolution™ (HRD™) to effectively separate coeluting analytes and search their spectra against commercially available EI-MS libraries. Accurate mass measurements were fully leveraged to substantiate the library results. CI-HRT analyses were also conducted to enable accurate mass measurements of molecular ions and adducts to further increase confidence in assigning formulas to both targeted and unknown analytes.

Comprehensive profiling utilizing non-targeted analytical tools such as HRT is critical not only for investigating primary and secondary metabolism, but also for understanding the chemical complexity responsible for the flavor profile and health risks of tobacco. The flavor profile as well as health risks of tobacco products are a direct result of their chemical composition. Alkaloids, organic acids, amino acids, saccharides, terpenes, terpenoids, sterols, and volatile aromatics are some of the important compound classes in tobacco. All of these characteristics (metabolism, flavor, and health risks) can be monitored simultaneously with HRT technology. In this study, tobacco plant extracts were analyzed on a LECO Pegasus® GC-HRT and data was processed using LECO's ChromaTOF-HRT® brand software with HRD. Figure 1 shows coeluting metabolites that were automatically separated using HRD and identified with commercially available EI-MS libraries. Accurate mass measurements (<1.00 ppm) were used to substantiate the library matches and are shown later in this note.

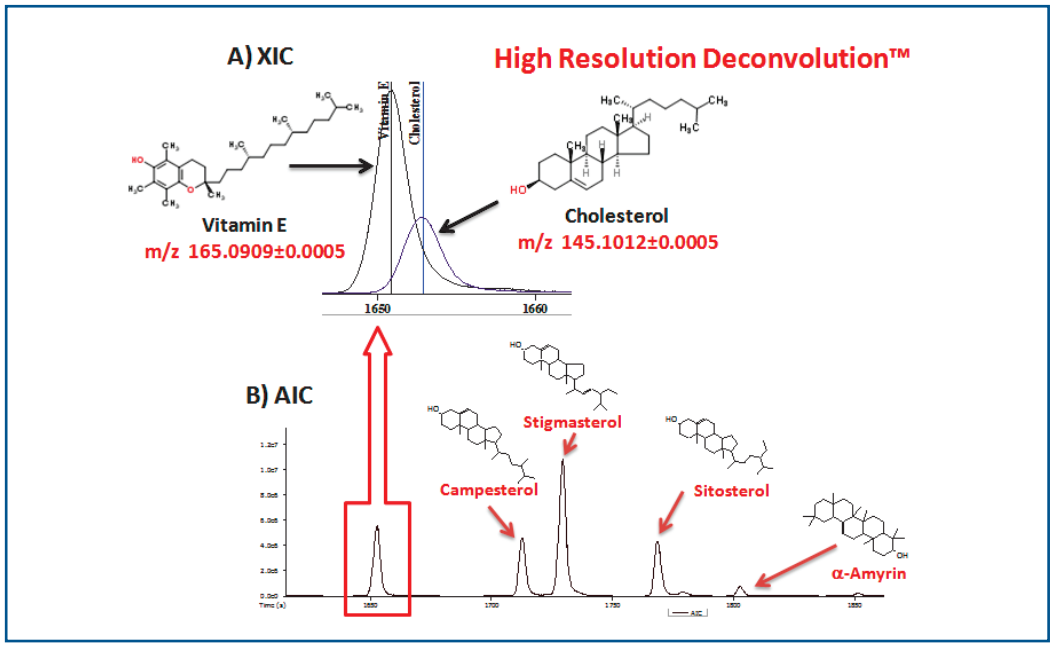

Figure 1. (A) XIC for accurate mass fragments of Vitamin E and Cholesterol demonstrating the ability of HRD™ to effectively separate these analytes in Green Leaf Tobacco Extract. B) AIC for Tobacco Extract Showing what appears to be a single peak for these Coeluting Sterols.

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2. Experimental

Toluene extracts of green leaf and processed cured tobacco were transferred to 2 mL GC vials for analyses. Figure 1 shows coeluting metabolites that were automatically separated and identified using HRD with commercially available EI-MS libraries.

Instrument Parameters

Gas Chromatograph	**Agilent 7890 with 7693 Autosampler**
Injection	1 µL, Splitless @ 250°C; 2 µL for CI
Carrier Gas	He @ 1.0 ml/min, Constant Flow
Column	Rxi-5 Sil MS, 30 m x 0.25 mm i.d. x 0.25 µm (Restek, Bellefonte, PA, USA)
Temperature Program	50°C (1 min), to 300°C @ 10°C/min (10 min)
Mass Spectrometer	**LECO *Pegasus* GC-HRT**
Transfer Line	300°C
Acquisition Mode	High Resolution, R = 25,000 (FWHM)
Ion Source Temperature	250°C (EI); 200°C (CI)
Ionization Mode	EI; CI (Reagent Gas = CH_4)
Mass Range (m/z)	28-510 (EI); 45-800 (CI)
Acquisition Rate	6 spectra/s

3. Results and Discussion

Some of the major components detected in the tobacco leaf extracts included terpenes, terpenoids, and sterols. Figure 2 shows an Analytical Ion Chromatogram (AIC) illustrating a few of the many compound classes found in these samples. Table 1 lists formulae, spectral similarities, and mass accuracy values used to substantiate library identification for seven representative compounds in tobacco leaf. Spectral similarity values ranged from 758 to 895/1000 for these analytes. The average absolute mass accuracy value for the seven compounds was 1.01 ppm.

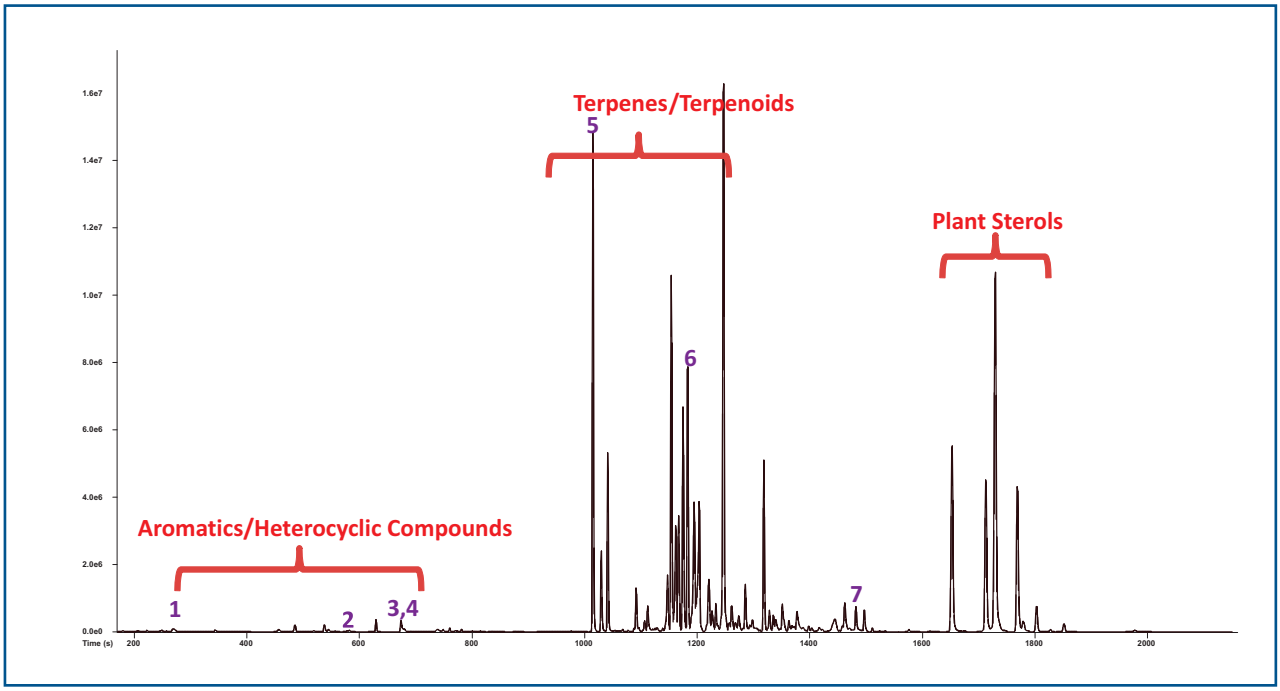

Figure 2. AIC of a Green Leaf Tobacco Extract.

Table 1. Representative Compounds in Green Leaf Tobacco Extract.

Peak #	Name	Formula	Similarity	Observed Ion m/z	Mass Accuracy (ppm)
1	Benzene, ethyl-	C_8H_{10}	895	106.07780	0.96
2	Ethylmethylmaleimide	$C_7H_9NO_2$	758	139.06272	-0.41
3	Nicotine (CAS)	$C_{10}H_{14}N_2$	889	162.11507	-0.48
4	1, 1, 5-Trimethyl-1, 2-dihydronaphthalene	$C_{13}H_{16}$	889	172.12451	-0.85
5	Neophytadiene	$C_{20}H_{38}$	876	278.29627	-1.93
6	Cembrene	$C_{20}H_{32}$	803	272.24940	-1.67
7	Pregnenolone	$C_{21}H_{32}O_2$	826	316.23944	-0.78
					Ave.|ppm|=1.01 ppm

An expansion of the AIC displays some of the plant sterols in this sample including campesterol, stigmasterol, sitosterol, and α-amyrin (Figure 3). Two coeluting compounds, vitamin E and cholesterol, contribute to what appears to be a single chromatographic peak labeled with a question mark. An eXtracted Ion Chromatogram (XIC) illustrates the HRD capabilities of the ChromaTOF-HRT brand software (Figure 4A). These two compounds were automatically separated by the software to provide high quality, Peak True (deconvoluted) spectra (Figure 4B & 4C) as exemplified by their spectral similarity values. The molecular ion mass accuracy values (-0.48 & -0.98 ppm) strongly supported the library matches.

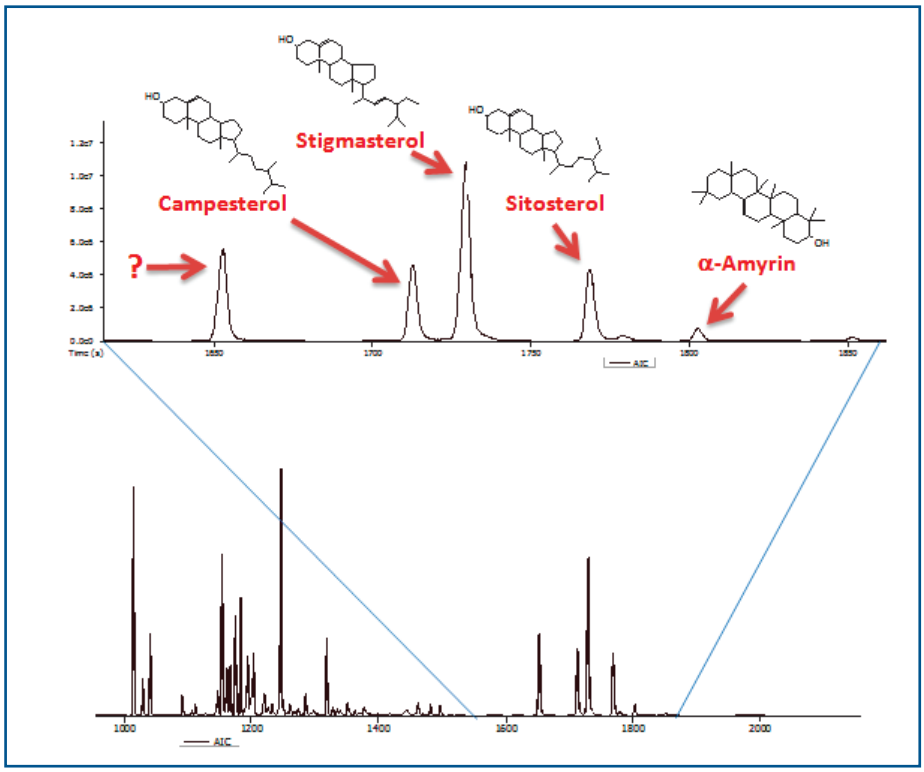

Figure 3. Plant Sterols in Green Leaf Tobacco Extract.

Table 2. Library Similarity Values for Plant Sterols in Green Leaf Tobacco.

Name	*Formula*	*R.T. (s)*	*Area*	*Similarity*
Campesterol	$C_{28}H_{48}O$	1713	3145266	853
Stigmasterol	$C_{29}H_{48}O$	1730	10427824	876
Sitosterol	$C_{29}H_{50}O$	1769	2871447	877
α-Amyrin	$C_{30}H_{50}O$	1803	1241498	822

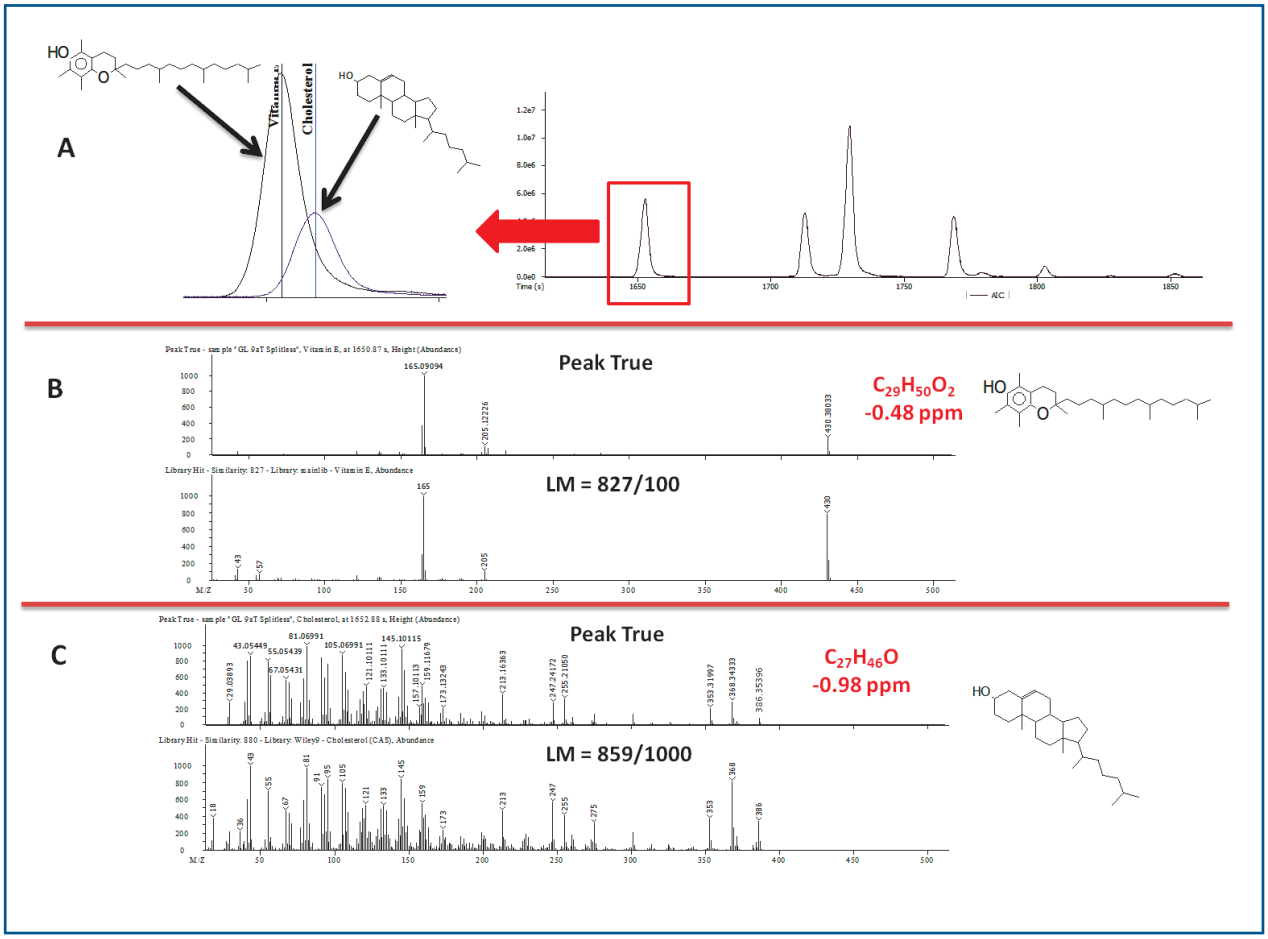

Figure 4. XIC of Vitamin E & Cholesterol (A). Peak True & Library Mass Spectra for Vitamin E (B) and Cholesterol (C) with Mass Accuracy Values for Respective Molecular Ions Included.

A critical step, and often a bottleneck, in any profiling experiment is confident identification of metabolites. Currently, many investigators rely heavily on spectral databases and retention times (or indices) for compound identification. While this approach provides two different points of reference for identification, it often produces unacceptable results due to spectral data which cannot be properly matched to existing libraries or simply because databases may not contain the desired reference spectra since many metabolites are yet to be fully characterized. A better approach to compound identification is the acquisition of complementary EI and CI-HRT data. This facilitates confident identification of analytes through a combination of spectral similarity and formula searches for molecular, adduct and fragment ions. For example, the number 1 hit for a compound with retention time = 1285 s was a diol with formula $C_{20}H_{34}O_2$ (Figure 5). Formula searches utilizing its molecular ion at m/z = 288.24463 in the EI-HRT spectrum and protonated molecular ion at m/z = 289.25239 in the corresponding CI-HRT spectrum supported a different isoprenoid with formula $C_{20}H_{32}O$ (spectral similarity hit number 7). Trusting spectral similarity searches alone without leveraging accurate mass measurements of EI and CI-HRT data would have led to an incorrect assignment of this analyte's identity. However, when accurate mass measurements were fully utilized, the correct identification was ultimately realized.

4. Conclusion

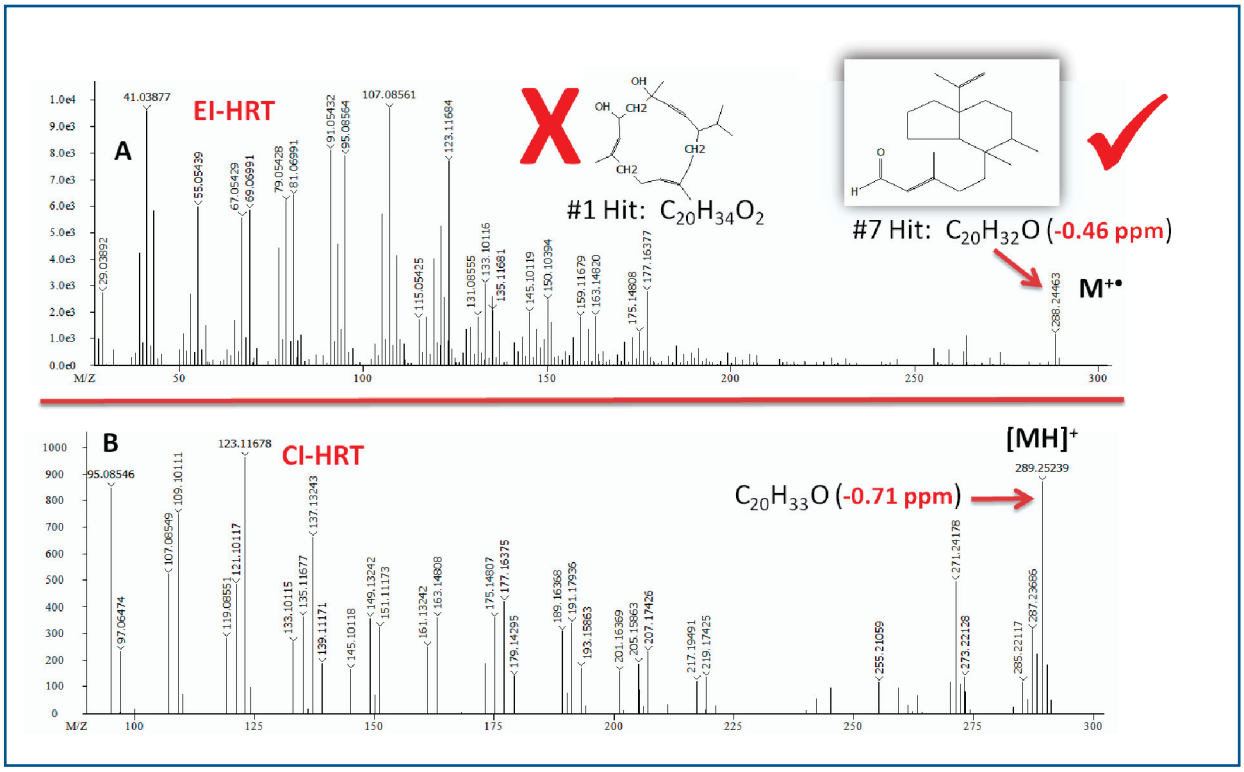

Figure 5. Peak True EI-HRT (A) and CI-HRT (B) Mass Spectra for a Diterpenoid in Green Leaf Tobacco Extract.

The *Pegasus* GC-HRT is a tool which provides a solution to one of the key challenges in metabolomic studies... confident identification of metabolites. Combining HRD to extract high-quality, library-searchable spectra with accurate mass measurements of molecular and fragment ions substantiates spectral similarity results and facilitates identification of numerous classes of compounds in tobacco, including metabolites, flavor-contributing compounds, and compounds with known health risks. An extra level of confidence for compound identification was achieved through formula searches of accurate mass ions in complementary EI and CI-HRT data.

LECO Corporation | 3000 Lakeview Avenue | St. Joseph, MI 49085 | Phone: 800-292-6141 | Fax: 269-982-8977
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