

The Analysis of Red Bell Pepper Constituents by Particle Beam Liquid Chromatography Mass Spectrometry

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INTRODUCTION

The development of a LCMS method was undertaken in an attempt to provide a more efficient method of separating and direct method of identifying the constituents. While there are about 20 highly colored constituents visible on the preparative TLC, the number of reported constituents in red bell pepper is about 50. The developed LC method is capable of resolving 40 constituents in less than 35 minutes and the particle beam LCMS analysis has been effective in the identification of several of the separated constituents. Of particular interest was the identification of beta-sitosterol, stigmasterol, alpha-amyrin, and vitamin E. Beta-sitosterol, a phytosterol, has been shown to lower cholesterol and lessen the discomforts of benign prostatic hyperplasia (BPH) such as frequent and painful urination. Stigmasterol has been implicated in lowering the risk of certain cancers, including ovarian cancer. Palmitate derivatives of alpha-amyrin have demonstrated potential as antiarthritic agents. Vitamin E has been recognized as an antioxidant that may stave off cancer and delay aging, as well as, prevent early development of cardiovascular disease, reduce viral load, and ward off Parkinson's disease. The use of Particle Beam LCMS for the identification of the non-polar constituents could be applicable to the analysis of other vegetables or fruits.

SAMPLE PREPARATION

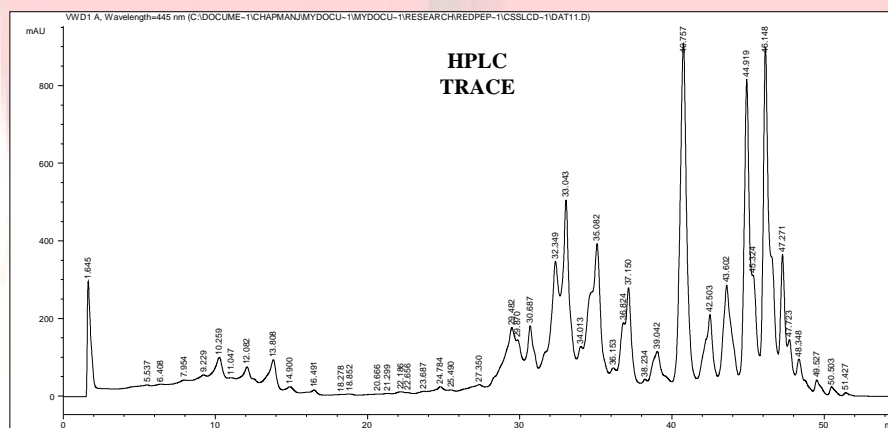
The peppers were cut into thin strips and the seeds removed. The strips were dried at ~110°C for about 2 hours. After drying the strips were further cut into ~5x5mm pieces and soaked in 50:50 cyclohexane:acetone for 30 minutes. The organic extract was washed with 2 volumes water, 1 volume saturated sodium chloride solution, and dried over sodium sulfate. The dried sample was filtered through a 0.2µm syringe filter and the solvent removed under reduced vacuum at 35°C. The sample was stored at 4°C until analysis.

INSTRUMENTATION – PARTICLE BEAM LCMS INTERFACE

For particle beam LCMS, the system included the following components. The liquid chromatograph used was an Agilent Model 1100 modular system with quaternary pump, vacuum degasser, 100 vial autosampler and variable wavelength detector. The HPLC column used was a Zorbax SB-C18 (Agilent pn 830990-902), narrow bore 2.1 x 150 mm 3.5 micron. The Genesis II particle beam interface (CSS Analytical Co. Inc.) was attached to an Agilent 5973 MSD so that samples can be analyzed by LC/MS with electron impact and chemical ionization. The Genesis II is an improved particle beam interface, which delivers a higher amount of analyte to the ion source, when compared to previous commercial interfaces. The mass spectrometer used was an unmodified Agilent 5973 Mass Selective Detector (Agilent Technologies, Inc., Palo Alto California) with turbo molecular pump. The Agilent 5973 is a benchtop quadrupole mass spectrometer with mass range of 1.6 to 800 mass units, 10,000 volt HED, and is available with EI or EI/CI capabilities.

LIQUID CHROMATOGRAPHIC SEPARATION

The pepper constituents were separated using a water (A) and isopropanol (B) gradient. Initial conditions were 30% isopropanol increasing to 90% isopropanol over 20 minutes holding there until 80 minutes and returning to initial conditions at 85 minutes. The column temperature was maintained at 50°C for the duration of the chromatography run. The detection wavelength was 445nm.



MASS SPECTRAL ANALYSIS

The data collected from the Particle Beam Ionization of the chromatographic separation was analyzed with AMDIS (Automated Mass Spectral Deconvolution and Identification System), version 2.1, DTRA/NIST, 2002. The Total Ion Chromatogram (FIGURE 1) is delayed by approximately 0.2 minutes relative to the HPLC trace as determined by comparison of the two respective data sets. FIGURE 2 illustrates the presence of the esters of Vitamin E at the longer retention times.

FIGURE 1. TOTAL ION CHROMATOGRAPH

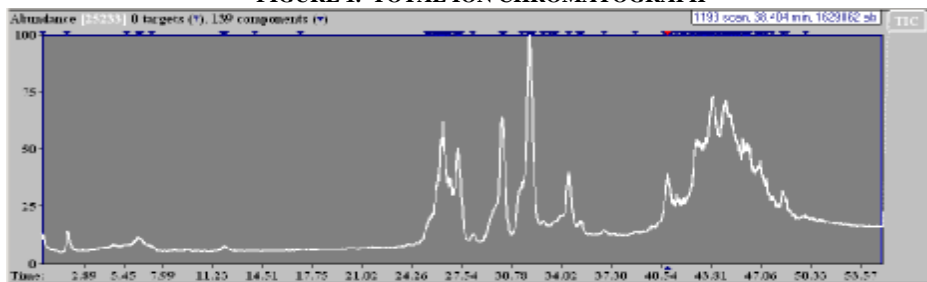
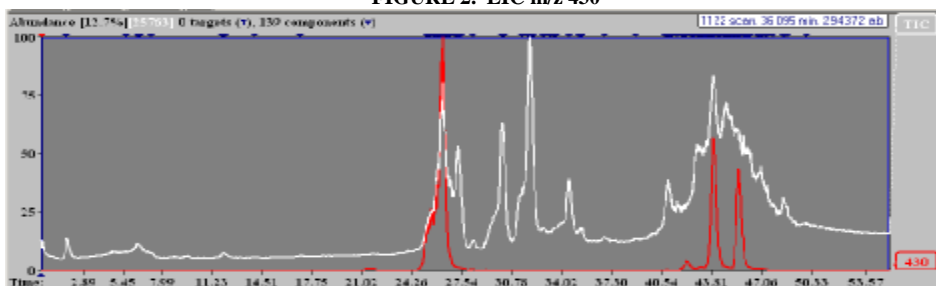


FIGURE 2. EIC m/z 430



COMPONENT	RETENTION TIME IN TIC
α -ergastenol	26.059
α-tocopherol (Vitamin E)	26.284
β -sitosterol	26.669
α-amyrin	26.768
τ -sitosterol	27.192
22,23-dihydrostigmasterol	27.308
1,3-dipalmitin	33.573
b-carotene	40.956
Vitamin E ester	43.916
trilinolein	45.258
Vitamin E ester	45.512

FIGURE 3a

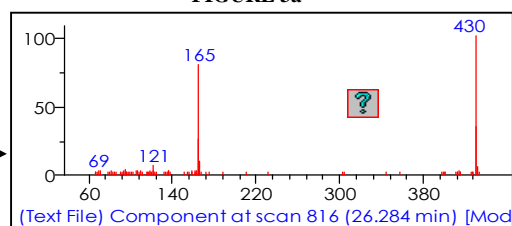


FIGURE 3b

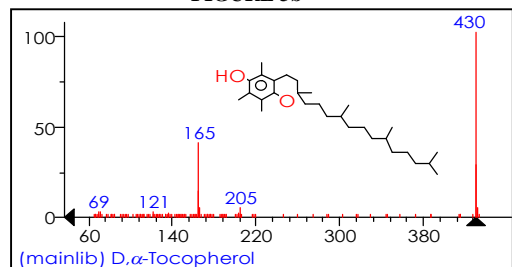


FIGURE 4a

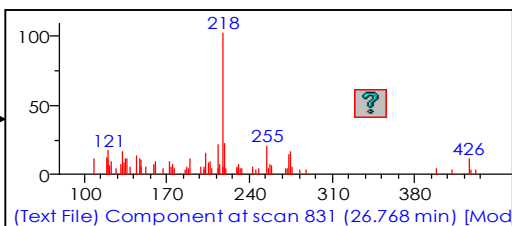


FIGURE 4b

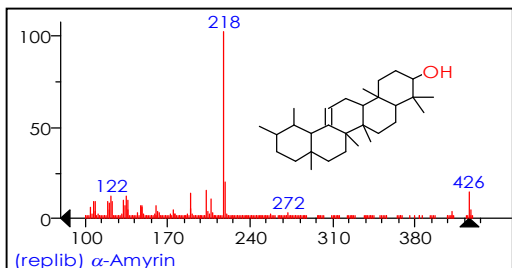


FIGURE 5a

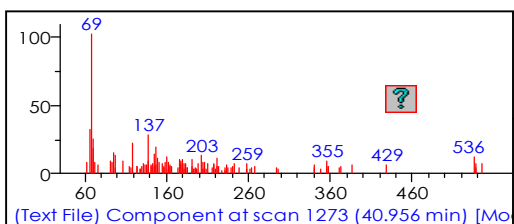
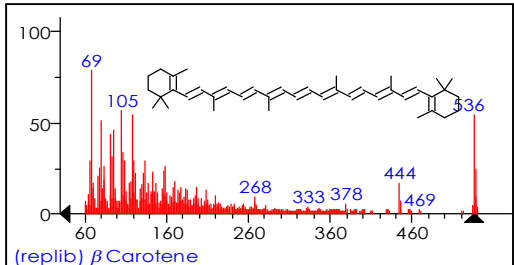
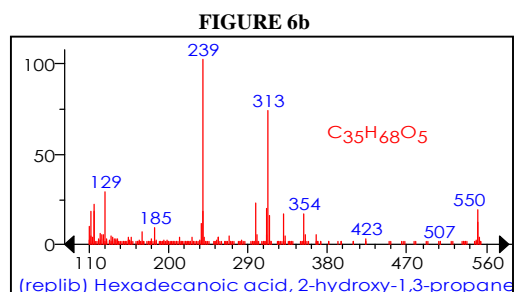
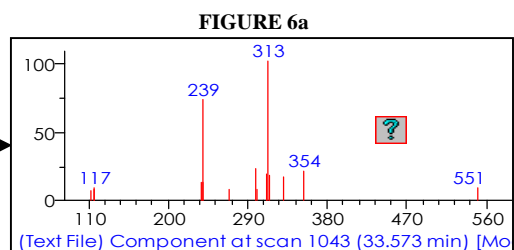


FIGURE 5b



COMPONENT	RETENTION TIME IN TIC
α -ergasterol	26.059
α -tocopherol (Vitamin E)	26.284
β -sitosterol	26.669
α -amyrin	26.768
τ -sitosterol	27.192
22,23-dihydrostigmasterol	27.308
1,3-dipalmitin	33.573
β -carotene	40.956
Vitamin E ester	43.916
trilinolein	45.258
Vitamin E ester	45.512



RESULTS

We were able to successfully develop a chromatographic method using a reverse-phase column with UV detection at 445 nm that could resolve ~40 constituents from the red bell pepper extract as shown in the HPLC TRACE. When this separation was coupled with the Particle Beam mass spectrometer we were able to obtain the Total Ion Chromatograph shown in FIGURE 1. The collected data was submitted for deconvolution and extracted ion analysis using the AMDIS program. The deconvolution program found 139 components initially and this was reduced by modifying the constraints with respect to probability match. We were able to obtain EI-like data on 35 of the red bell pepper constituents. Using this information the library search was able to identify 11 constituents conclusively and these were confirmed by comparison to previously published accounts of the constituents.

The number of matches attributed to Vitamin E (alpha-tocopherol) was perplexing at first, since the same identification was assigned to peaks throughout the TIC. The EIC shown in FIGURE 2 was run based on the $M+$ ion of 430 for Vitamin E. It is clear in the figure that at least three and as many as five peaks in the TIC appear to contain $M+$ 430 ions. When the mass spectra of the peak at 26.284 was searched against the library a clear match for Vitamin E was obtained as shown in Figure 3a. When the remaining peaks in the 430 EIC were searched their mass spectra did not have a probability match as high as the 26.284 peak. In general, the other spectra were much more complex and although they often contained a prominent $M+$ 430 ion there were molecular ions found at much higher masses. Often included in the top 10 matches for these peaks were Vitamin E glycosides and esters of acetate, succinate, stearyl and palmitate.

As expected from using an extraction solvent to obtain the carotenoids and phytosterols we also isolated a variety of plant lipids. We were able to identify several of these including 1,3-dipalmitin at 33.573 minutes in the TIC (Figure 6a) and trilinolein at 45.258 minutes.

The expected numbers of carotenoid or β -carotene-like molecules present in the pepper were not observed, but these can be accounted for by the presence of $M+$ ions with much smaller molecular weights than expected for their chromatographic retention times. Several of the peaks with R_t close to 33 and 46 minutes and high 445nm absorbance values exhibited fragmentation characteristic of the carotenoids, but failed to generate parent mass ions sufficient to match the library.

CONCLUSIONS

Almost all new commercial LC/MS systems today are atmospheric pressure ionization instruments. Despite this, it is important to remember that the analysis of a true unknown is an extremely difficult task and may require the utilization of more than one technique. Nothing helps more than the direction that can be obtained from a standardized database of mass spectra with which to match. Commercial and standardized libraries are not yet prevalent for CI and API-ES. CI and API-ES are typically used to obtain molecular weight information. For this reason CI and API-ES are commonly used as a tool to confirm an identity obtained from matching an unknown spectrum with a mass spectral database. To put it all in perspective, an evaluation of our experiments showed the following:

1. The particle beam ionization works well for these large non-polar carotenoids and phytosterols.
2. The ability to search EI spectral libraries makes the identification of the constituents much less tedious.
3. This method could be utilized to ascertain the identity of large non-polar plant constituents from plant materials.
4. LCMS with Particle Beam ionization is capable of providing fragmentation information on molecules inaccessible to other techniques.

FUTURE WORK

Our intentions are to utilize the Particle Beam ionization in combination with Chemical Ionization and Electrospray Ionization to identify all volatile and non-volatile polar and non-polar constituents by the same chromatographic separation.