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APPLICATION NOTE

Gas Chromatography/
Mass Spectrometry

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Qualitative Evaluation of Aroma-Active Compounds in Grape and Grape-Derived Products by Means of Headspace SPME-GC/MS Analysis

Introduction

Grapes are probably the first cultivated fruit: its domestication by man began 6000-8000 years ago across the area between the Caspian and Black Seas and over the centuries it spread all over the world's temperate areas becoming one of the most important fruit crops on the market.

More than 10,000 varieties of grapes are cultivated and used for different products. Grapes can be consumed as fresh (table grape) or dried (raisin) fruit, or they can be transformed in juice or wine, and also used in vinegar or distilled to produce different kind of spirits (brandy, cognac).

In the last decades, grapes have emerged as one of the fruits with the highest content in nutraceutical compounds, raising the interest of nutritionists and pharmaceutical companies for its important antioxidant and health-promoting substances.

Aromatic compounds are one of the most important parameters in determining the quality of grape-derived products. This is true not only for wine, but also for unfermented grape juice and vinegar; moreover, aromatic compounds and precursors contained in the grape berry play a key role affecting the quality of its products.

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Various classes of aromatic compounds contribute to the flavor profile of grapes and grape-based products with alcohols and esters providing the predominant contribution. Other classes include carbonyl compounds, terpens, organics acids, and norisoprenoids.

To investigate these patterns we choose to analyze red grape berries, unfermented grape juice, wine and balsamic vinegar to characterize the main odorants and evaluate their concentration in these products using gas chromatography coupled with mass spectrometry (GC/MS).

Extraction of the aroma-active compounds was carried out using the solid phase micro-extraction (SPME) approach, trapping the odorants contained in the headspace onto a sorbent polymeric micro-cartridge that was subsequently desorbed into the GC injector.

Preliminary trials

Solid phase micro-extraction is a powerful and versatile technique that allows a fast and solvent-free extraction and concentration of volatile compounds contained in the headspace of the sample. It works by basically absorbing the molecules onto a polymeric fiber constituted by different sorbent materials according to the type of analytes to be investigated.

Working with the SPME technique requires a fine tuning of many variables that markedly affect the affect the extraction process and recovery yield. A brief overview on the main parameters to be optimized, and the approach to be followed in method optimization are given below.

Fiber selection

Many different SPME coating materials and polymers are available on the market and their selection must be done taking into account the analytical needs, the sample composition and the scientific literature.

As aroma composition of grape and grape derived products is very complex and heterogeneous, a multi-sorbent fiber was selected in order to catch most of the volatiles contained in the headspace. The use of a triphasic fiber containing carboxen, polydimethylsiloxane and divinylbenzene allows a broad-spectrum interaction with many kind of analytes; linear and branched alcohols, esters, terpenes, carbonyl compounds and norisoprenoids are effectively trapped by this fiber and thus can be subsequently desorbed into the GC injector.

Internal standard selection

Even with qualitative analysis, the use of an Internal Standard (ISTD) can be useful to evaluate fiber efficiency and compare results of different samples. For example, as thermal desorption and conditioning processes progressively lead to fiber deterioration. As a matter of fact the recovery yield drops down cycle-by-cycle, making it difficult to compare the results of different analysis.

By adding a constant amount of an internal standard to all of the samples it is possible to quantify the fiber extaction yelid sample-by-sample (evaluating the progressive reduction in the ISTD peak area) and establish a minimum threshold to replace the fiber (e.g. when efficiency is less than 70%). Moreover, the amount of each analyte can be calculated and expressed referring to the internal standard peak area (as % of ISTD).

Important criteria to consider for selecting an internal standard include:

- it has to be volatile in the experimental conditions considered and must bind with the fiber coating
- its chromatographic peak should not overlap with other peaks to have a more accurate quantification
- the same amount should be accurately added to all samples to obtain confident results
- the standard must be absent in all the samples and easily detectable with GC/MS analysis. Deuterated molecules represent a good choice and for this analysis a p-Xylene-d10 was used

Column selection

Columns must be appropriately selected according to the objective of the analysis. When a quantitation determination of a single molecule or a class of molecules is needed, it is important to choose a specific column with a high affinity for your analytes. Diameter, phase thickness and column length must be selected in order to minimize the time of analysis and they must have the same time peak to ensure the correct identification and quantitation of the analytes.

Conversely, when the analytical purpose is to study a complex and non-characterized sample like a food or a beverage, the column selection is subjected to different considerations. When headspace is sampled it is important to evaluate the relative analytes concentrations and obtain the largest number of detectable peaks in order to have a representative "aromatic fingerprint."

This kind of data is very useful when the goal of the analysis is to compare similar products that undergo different

treatments, for example, storage conditions or production technologies. For this kind of analysis the choice of the column must be done by selecting a stationary phase with intermediate polarity to interact with many different classes of compounds; moreover, a column with a thicker phase ensures a massive retention and a better separation of analytes. Column length as well can enhance the chromatographic performance because many functional groups are available to interact with the analytes.

For this analysis a PerkinElmer Elite-VMS 60 m x 0.32 mm x 1.8 µm (Part No. N9316655) was used to obtain the aromatic profile.

Injection parameters

Injection is another crucial step that needs to be studied and optimized when using SPME: in this phase it is very important to ensure a fast and massive desorption of the analytes from the fiber. This could be obtained choosing an appropriate carrier flow and a desorption temperature high enough to release the volatiles into the column.

A narrow non-packed liner (1 or 0.75 mm) must be installed in the injector to ensure a correct heating and a homogeneous carrier flow onto the fiber, improving significantly the peak shape and separation. Injection temperature was set up at 250 °C, which is the maximum programmable temperature for the capillary column used, and a liner with an internal diameter of 1 mm was mounted in the injector.

Carrier flow selection requires more consideration and different preliminary trials to be optimized. A constant 1 mL/min carrier flow was kept during the whole chromatographic run, but split flow in the injector was closed during the first minutes, to enhance the introduction of analytes in the column. Pressure pulse injection has been used. Afterwards split was opened again and set up at 50 mL/min until the end of the analysis.

Different splitless times were investigated to evaluate their effect on peak shape and signal intensity and thus choose the best moment to re-open the split valve. In Figure 1 the effect of three different splitless times (respectively 5, 2 and 1 minutes) are summarized.

It is possible to see how a longer splitless time (5 minutes) results in larger and smaller peaks compared with the ones obtained using 1 and 2 minutes. One minute is not enough to ensure a sufficient introduction of the analytes in the column. Two minutes of splitless time seems to be the best choice: peaks are well separated and MS identification of the compounds is easier and more confident.

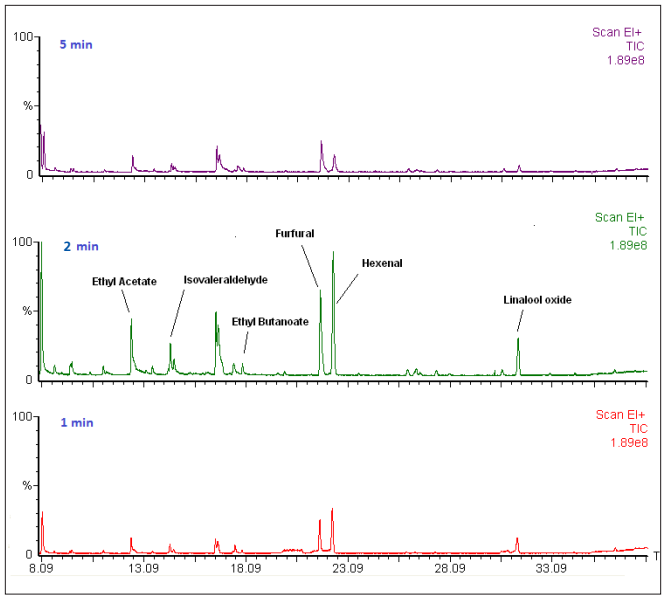


Figure 1. Chromatograms obtained with 5, 2 and 1 minute splitless injection mode.

Extraction variables

Solid phase micro-extraction of the headspace is very similar to a classical static headspace analysis and a purge and trap sampling. Like these two techniques, headspace SPME is affected by many physical and chemical variables that markedly affect the headspace equilibration and the extraction process.

First, it is important that prior to fiber exposition into the headspace of the vial, the equilibrium between liquid and vapor phases must be reached for all the analytes. Pre-incubation temperature must be high enough to ensure a fast headspace equilibration but low enough to prevent the formation of new compounds due to sample heating (e.g. Maillard products).

A good compromise is to leave the samples at room temperature over night and then apply low temperature (40 °C) during pre-incubation time (20 minutes). In this way is possible to achieve a more confident and reproducible result. Similarly the temperature applied during SPME extraction, when the fiber is into the headspace, should be chosen to avoid artifacts formation; usually 60-80 °C is considered a good compromise to have a good recovery and speed-up the process, but it is possible to make a longer extraction at a lower temperature without altering sample composition. It is necessary to point out that during extraction, temperature has a dual effect: it promotes the passage of less volatile analytes in the headspace, but at the same time, disrupts the weak chemical interaction between the fiber and the volatiles.

Chromatograms reported in Figure 2 show how the combination 40 °C for 30 min gives the best results when

compared to 60 °C for 20 min or 80 °C for 10 min.

To increase volatile compounds concentration in headspace and to speed up the equilibration process some salts can be added to the sample. For example, adding sodium chloride in the vial better peaks are obtain in the GC/MS analysis (Figure 3). For this purpose 1.2 grams of high purity NaCl were added to each sample.

Why a mass spectrometry detector?

Olfactory is a very complex sense in humans and odor perception is a finely regulated process that allows our brain to work as one of the most advanced molecular lab analyzer. Thanks to a raveled system of receptors, glomeruli and neural networks our olfactive system can discern between two isomers of the same compound. Olfactory receptors act just as a molecular analyzer and have the ability to detect the stereochemical features of the molecule: that's why two enantiomers can smell completely different to our nose (Figure 4).

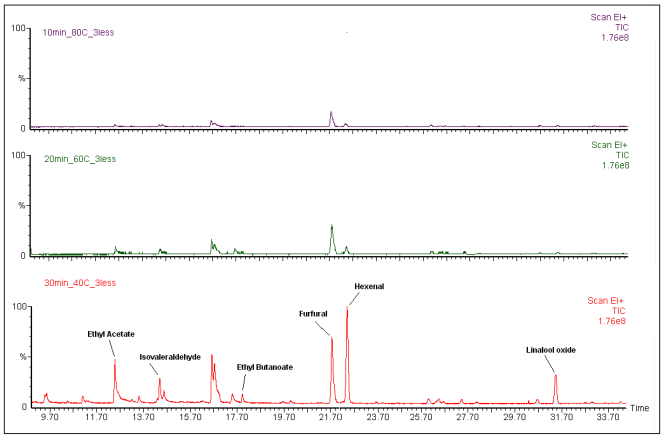


Figure 2. Different combination of time and temperature of extraction (80 °C x 10 min; 60 °C x 20 min; 40 °C x 30 min).

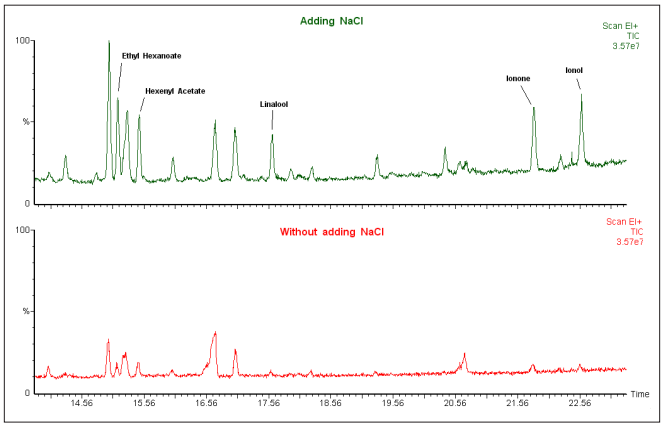


Figure 3. Chromatograms obtained without salts addition and adding 1,2 g of NaCl to the sample.

For example Carvone forms two mirror image forms or enantiomers: (–)-carvone smells like spearmint. Its mirror image, (+)-carvone, smells like caraway. Moreover, these two compounds have a different olfactory threshold (OT, the minimum concentration needed to perceive the aroma) with (+) isomer having an OT value of 43 ppb whereas (-) isomer threshold is equal to 600 ppb. In this case the two mass spectra are very similar.

These considerations are useful in defining our analytical skills and in particular in choosing the detector type and its characteristics. The chromatographic separation of isomeric molecules is a prerequisite to be achieved optimizing all the GC parameters and choosing a suitable column in this challenging situation.

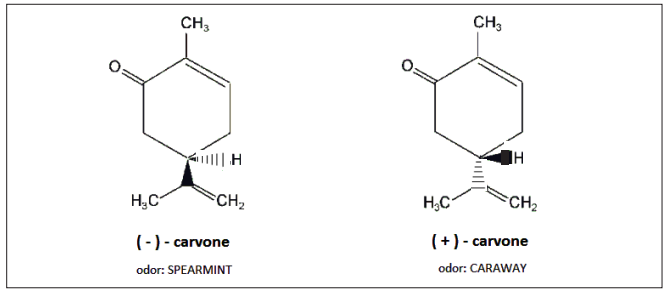


Figure 4. Two optical isomers that have different odors.

With a mass spectrometer, a mass spectrum can be obtained for each compound and searched against the NIST library. If two isomers are listed in this library it is possible to have an identification of their spectra, associated with a probability value that sometimes can reach more than 90% matching.

In Figure 5 it is possible to understand how GC/MS analysis works: in grape juice two geometrical isomers (geraniol and nerol) can be separated by a GC system giving two peaks in the chromatogram; spectra can be extracted from each peak analyzing the MS detector signals and the library search gives a probable identification of the compounds.

Matching values are very high (942 and 947 out of 1000 for geraniol and nerol respectively), thus the identification can be considered trustworthy. As geraniol (*cis*) and nerol (*trans*) have different odors and olfactory thresholds the discrimination and separate quantification of these two molecules is very important in analyzing the grape product quality

Experimental

The preliminary trial allows one to set up an optimized protocol for HS-SPME/GC/MS aroma profiling suitable not only for grape juice analysis, but also for grape berries, balsamic vinegar and wine.

Using exactly the same protocol for grape and grape-derived products it is possible to compare the results obtained with different samples, having the same retention times and a similar detector response for each detected compound. The use of CombiPAL® autosampler in all the SPME steps allows one to achieve reproducible and confident results.

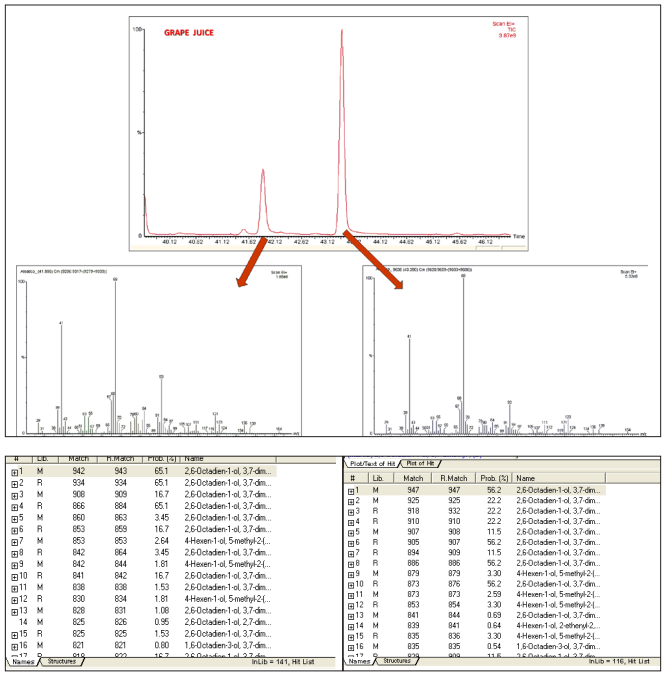


Figure 5. cis/trans isomers with different odors recognized tanks to MS spectra library searching.

The HS-SPME/GC/MS protocol chosen for the determination of aroma active compounds in grape and grape-derived products is summarized in the following paragraphs.

Sample preparation

Sample preparation is very simple in order to minimize the variability due to the analyst accuracy. All the samples were prepared into a 22-mL clear glass crimped vial for headspace analysis (Part No. N6356471) with thin septa for SPME (Part No. N6356564). NaCl was added to increase volatility of semi-volatile compounds, whereas a 50 ppb water solution of deuterated p-xylene (IS) were added to monitor fiber extraction yield. To obtain a chromatogram with a good peak shape and resolution, weights and volumes were adjusted for each sample type as follows:

- **grape:** 3 frozen cut berries + 2 mL H_2O + 1.2 g NaCl + 100 µL IS
- **juice:** 3.9 mL + 100 µL IS + 1.2 g NaCl
- **wine:** 3.9 mL + 100 µL IS + 1.2 g NaCl
- **vinegar:** 500 µL + 100 µL IS + 1.2 g NaCl

Instrumentation.

Gas Chromatograph: Clarus 680 GC/MS with PSS injector

Injector: Programmable S/S injector (PSS)

Detector: Clarus MS

Column: Elite VMS 60 x 0.32 x 1.8 (Part No. N9316655)

Autosampler: CTC CombiPAL XT equipped for automated SPME

Analytical Method.

Flow: 1 mL/min

Carrier: Helium

Split: 50 mL/min (2 min splitless)

Injector: 250 °C, quartz liner 1 mm ID, Merlin Septum Microseal

Heating Raps:
Step 1: 40 °C x 5 min
Step 2: 10 °C/min --> 120 °C (Hold x 5 min)
Step 3: 2 °C/min --> 180 °C (Hold x 2 min)
Step 4: 10 °C/min --> 230 °C (Hold x 5 min)
Total GC run time: 60 min

MS conditions

Transfer line temperature: 220 °C

Source temperature: 220 °C

Mass range: 28 - 250 amu

Scan time: 0.25 sec

Inter scan delay: 0.025 sec

Solvent delay: 4.0 min

SPME conditions

Fiber type: Supelco® triphasic fiber PDMS-DVB-Carboxen 1 cm

Equilibration: 40 °C x 20 min (stirring at 500 rpm)

HS Extraction: 40 °C x 20 min

Desorption: 10 min in the GC injector

Fiber conditioning: 10 min at 260 °C

Results

The results presented here demonstrate the effectiveness of this analytical technique to distinguish between both grape varities and the products derived from them. The described SPME approach shows good affinity and recovery rates

toward the main odorant classes of grape products and effective product development and product QA/QC can be acheived using such a technique.

Juices showed to have the most complex aromatic profile and it was possible to identify more than 40 compounds. Alcohols and aldehyde were the most represented classes, followed by terpenoids benzoic derivatives and C-13 norisoprenoids. These latter are derived from carotenoids and proved to be determinant in giving grape its characteristic flowery aroma (Figure 6).

This analysis allows us to highlight the variety of differences in the aromatic pattern between two different products. Figure 7 shows the comparison between two grape juices obtained from two different Tuscan red grape varieties:

- **Sangiovese** This is a red grape variety particularly appreciated and exploited for its good phenolic composition and aging potential, but is known to be poor in terms of aromatic quality;
- **Aleatico** This is a red Muscat-type variety with very complex, floral aroma mainly used in the production of dessert sweet wines (*passito*).

As expected the variety of differences is considerable: Sangiovese shows a chromatogram with fewer and smaller peaks, where aldehydes and alcohols are predominant and terpenoids are present in traces. Conversely, Aleatico shows a more complex profile and in the central part of the chromatogram many terpenoids are clearly separated and identifiable (eucalyptol, terpinolene, limonene, linalool, terpineol and nerol are the most abundant).

This protocol of analysis has also been applied to red wines, vinegar and grape berry and the resulting chromatograms are showed in Figure 8.

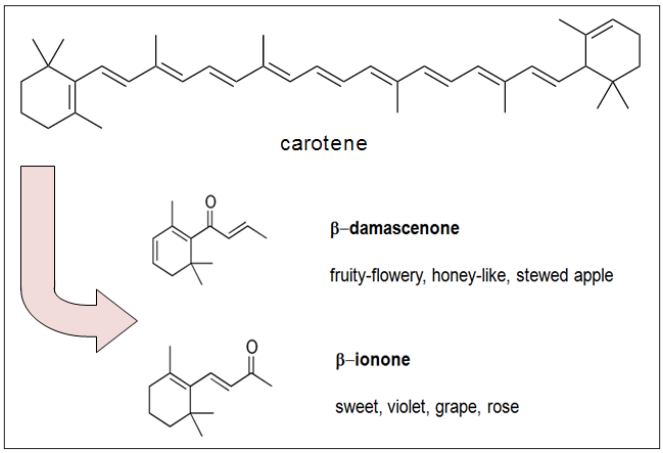


Figure 6. Main aroma-active C-13 norisoprenoids commonly found in grape.

There was minimum sample preparation using the headspace SPME technique, and it was possible to isolate and identify many compounds in each of these samples.

Grape berries show a simpler profile and only 22 compounds were identifiable, with prevalence of short chain alcohols and C-6 aldehydes responsible of the green leaf-like aroma.

Grape juice proved to be the most complex matrix with 45 compounds clearly identifiable through mass spectra: terpenoids, alcohols and carbonyl are the most represented classes.

Wine showed a complex aromatic pattern with 37 significant peaks; the first large peak corresponds to the ethanol and saturates the MS detector due to its very high concentration in this sample; esters and terpenes contribute to the fruity, floral notes.

Overall 32 peaks were detected with prevalence of alcohols, short chain carboxylic acids and esters.

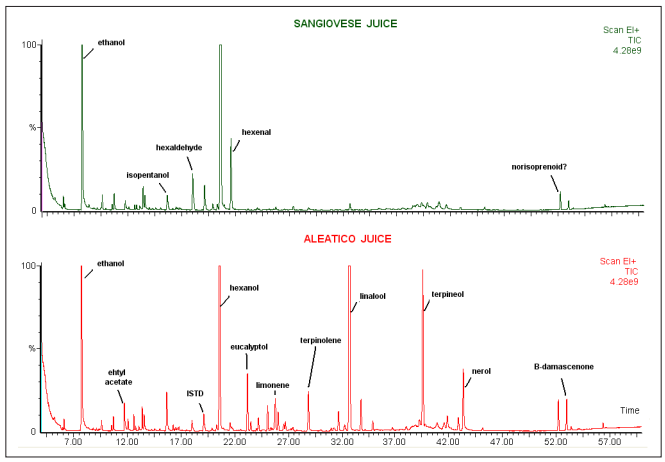


Figure 7. Comparison of aromatic profiles of Sangiovese and Aleatico juices.

Conclusions and perspectives

In this paper we present an excellent analytical approach for the fast and simple analysis of aromatic compounds in various types of complex food matrices using HS-SPME/GC/MS. The method combines the ease of sample preparation of HS-SPME with the comprehensive identification qualities of GC/MS to allow for a comprehensive approach for use in the food industry. We present the analysis of various grape-based products including raw fruit (fresh and dried), grape juice, and grape-based wine and vinegar, and describe the unique chemical profile of each. For example, the similarities and differences of juice from Sangiovese and Aleatico grape varieties is clearly demonstrated using this technique and allows for products based on each to be equally differentiated. Although the focus of this paper was grape-based products, this analytical approach is transferable to many other fruit- and vegetable-based products.

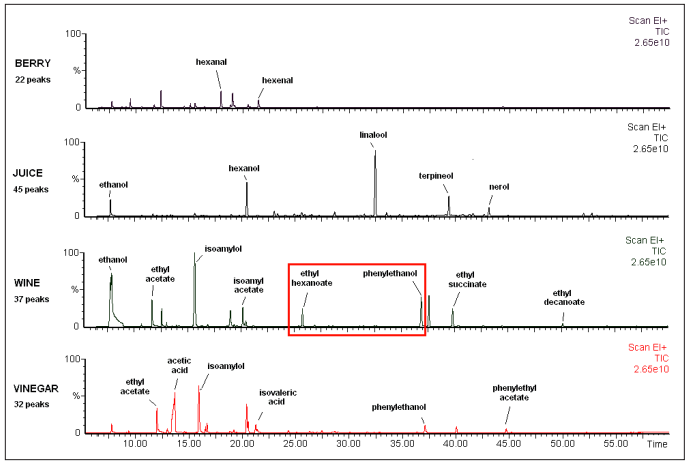


Figure 8. Results obtained using this protocol to analyze grape berry, grape juice, wine and vinegar. As the same scale is applied in all the 4 chromatograms smaller peaks seems not detectable, but expansion of red rectangle (reported in Figure 9) shows that many peaks are present.

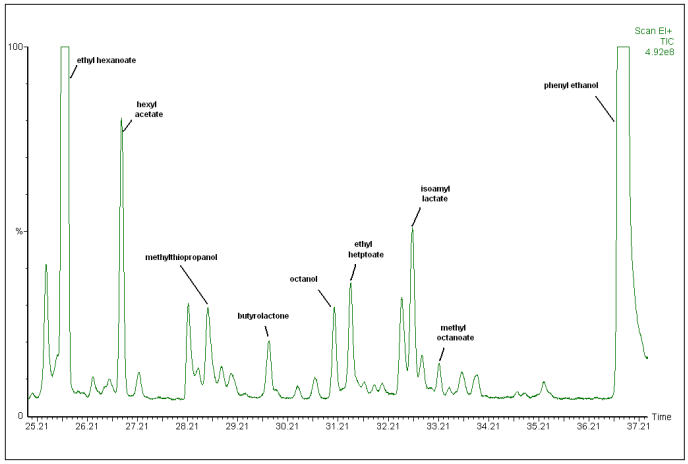
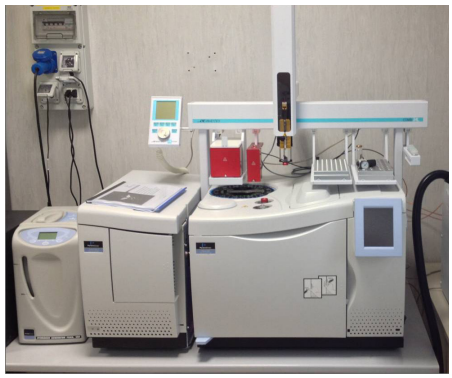


Figure 9. Expansion of the red rectangle shown in Figure 8.

Although the data presented here highlights the qualitative results, which can be generated by using this method, the application of these qualitative results have much further reaching applications beyond simple compound identification. For example, if multiple processes for the production of a particular product were possible, the analysis of the resulting products using this method would allow other scientists who analyze food to properly choose between the two to maximize flavor. Beyond process and control improvement as described here other areas of application are QA/QC, product source identification and verification, and adulteration analysis.



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