Contribution to the Optimization of a Gas Chromatographic Method by QbD Approach used for Analysis of Essential Oils from *Salvia officinalis*

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Numerous articles on Salvia officinalis L. have been published regarding the composition of their essential oil. The considerable variation found may be due to the quality of the plant material as well as to the methods used for analysis. A simple GC-MS method was developed and optimized in the QbD approach, for the determination of sage essential oils. The optimization of GC-MS analysis was performed using different mobile phase flows, injection volumes, split ratios and temperature programs. The optimized method proved to be simple and can be successfully applied for the determination of sage essential oils.

Keywords: sage, GC-MS, QbD, essential oil

Essential oils are complex mixtures, constituted by terpenoid hydrocarbons, oxygenated terpenes and sesquiterpenes. They originate from the plant secondary metabolism and are responsible for their characteristic aroma. Essential oils are amongst the most complex samples an analyst can face in terms of the number of compounds involved.

There are different methods for essential oil extraction, like the most common *steam distillation* (since it allows for the separation of slightly volatile, water non-miscible substances by means of low temperature distillation, being of particular use when the components boil at high temperature, higher than 100ºC, and are susceptible to decomposition below this temperature), *extraction techniques* (with organic volatile or nonvolatile solvents is based on the distribution balance or selective dissolution of the oil within two non-miscible phases, with solid solvents by sorption, with supercritical fluids), fermentative techniques and mechanical techniques.

Once the oils are obtained, the fundamental contribution resides in their characterization, as their chemical composition may vary even whithin one botanical species. These variations might be due to the presence of different chemotypes, according to the plant adaptation to the surrounding environment, as well as its state of development. For many years, gas chromatography coupled to mass spectrometry (GC-MS) has been the benchmark technique for qualitative and quantitative analysis of essential oils. Chromatography, especially gas chromatography (GC) and mass spectroscopy (MS) have been the most applied analytical techniques for essential oil analysis.

The goal of chromatography has always been to obtain a complete separation of components in a sample. However, application of one-dimensional techniques (the use of conventional GC with long column lengths or fast GC) has shown some limitations. Multidimensional chromatography has emerged in the late 80's to provide solutions to the problem of peak overlap. Multidimensional chromatography is defined as the chromatographic process where two or more analytical techniques are coupled together to enhance their separation power [1]. The combination can be either two chromatographic techniques or a chromatographic technique with spectroscopy.

Gas chromatography-mass spectrometry (GC-MS) has probably been the most effective multidimensional method for analysis and identification of essential oils [2]. In many cases the mass spectra of essential oil components are unfortunately very similar. Thus, within the broad class of monoterpenes, a large number of isomers of the same molecular formula (but with different structure) exist and their mass spectra often bear close resemblance.

Since complex essential oils may have multiple overlapping peaks there will always be uncertainty regarding the purity of any recorded peak should the GC be used alone. The mass spectrometer may recognize overlap and apportion relative amounts to overlapping components. The availability of the accurate mass (high-resolution) MS is a valuable tool for confirmation of the molecular formula of detected unknown components [3]. With reference to essential oils, and in particular monoterpene hydrocarbons, accurate retention times are often of greater importance due to the mass spectral similarity of their isomers [4].

The GC method has applicability in essential oil analysis of different complexities (Artemisia [5], Cardamon [6], Chamomile [7-9], Coriander [10], Fennel [11], Ocimum [12], Sage [13-19], Santolina [20], Thymus [21], and more others).

Quality by Design (QbD) is a concept first outlined in Juran on Quality by Design [22], where is presented a new and exhaustively comprehensive approach to planning, setting, and reaching quality. This is defined in the International Conference on Harmonisation (ICH) guidance on pharmaceutical development as *a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management* [23, 24].

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In the QbD approach, the quality is built into product and process by design and based on scientific understanding. However, it is recommended to implement QbD approach in analytical method development termed as *Analytical Quality by Design* AQbD. These two scientific approaches (QbD and AQbD) can be progressed in equal time [25, 26]. Based on the principles of sound science and quality risk management, the QbD approach enables enhanced understanding of the *Critical Method Variables* (CMVs) influencing the *Critical Analytical Attributes* (CAAs) and the method performance. Not only does design of experiments help in identifying the 'vital few' method variables critically influencing the method performance, but it also assists in optimizing them, while expending minimal resources of time, effort and cost [27].

Therefore, the aim of this investigation is to develop a rapid, sensitive, robust and economical analytical GC-MS method for chemical composition determination of essential oils obtained from leaves of *Salvia officinalis* using QbD-oriented systematic analytical approach. The studies were carried out in two phases: screening for identifying the CMVs and optimization of chromatographic conditions.

*Critical Quality Attributes (*CQA) for an analytical method includes method attributes and method parameters. The CQAs for a GC-MS method are operators, sample preparations, mobile phase (nature of gas and flow), and the instrumental system (injector type and injection mode, column type and dimensions, oven temperature program, detector type and parameters).

Risk Assessment is a science-based process used in quality risk management and it can identify the material attributes and method parameters. It can be performed from initial stage of method development to continuous method monitoring. AQbD approach involves the risk identification at early stages of development followed by appropriate mitigation plans with control strategies that will be established. In general, Ishikawa fishbone diagram can be used for risk identification and assessment (fig. 1).

Design of Experiments (DoE) - Method Optimization and Development

After the potential and critical analytical method variables are defined with initial risk assessment, the DoE can be performed to confirm and refine critical method variables based on statistical significance. As per ICH Q8 guidance process robustness is defined as *Ability of a process to tolerate variability of materials and changes of the process and equipment without negative impact on quality* [23]. Process understanding will provide the sufficient knowledge for establishing robustness parameters by evaluating different operating conditions, difference scales, and different equipments [25].

Experimental part

Essential oils

Sage essential oil was isolated from 50 g dried vegetal product immersed in 600 mL bidistilled water by hydrodistillation in Clevenger-type apparatus for 4 hours, up to the point at which the oil contained in the herbaceous matrix was exhausted. The obtained volume (in mL) of essential oil was reported at 100 g vegetal product.

Equipment and chromatographic conditions

An Agilent Technologies 7890 gas chromatograph, coupled with an Agilent Technologies 5975 C mass spectrometer and an Agilent Technologies 7683B Series autosampler was used. The essential oils are injected in SPLIT mode at a temperature of 250°C. The carrier gas was helium. The separation was done on a capillary column DP-5MS (30 m × 0.25 mm, 0.25μm film thickness), at different temperature programs. The MS detector parametrs are: 280°C transfer line, 230°C source temperature, 150°C quadrupole temperature, 15 -450 amu mass range and SCAN mode of aquisition. Data aquisition and analysis were performed with Agilent Technologies ChemStation software. Identification of the constituents was based on computer matching against commercial (NIST 98 and Wiley) mass spectra library.

Method development by QbD approach

The goals of GC-MS method development have to be clearly defined, as pharmaceutical QbD is a systematic, scientific, risk based, holistic and proactive approach that begins with predefined objectives and emphasizes product and process understanding and control. The ultimate goal of the analytical method is to separate and quantify the compounds.

From the factors influencing the analytical performance of a generic GC method, presented in figure 1, we choose to study the factors that can improve the quality of GC separation, component identification and quantitation, such as modifying the nature or the dimensions of the capillary column, varying the nature or flow of the mobile phase, varying the injected amount (by varying the injector splitting ratio or the injected sample volume) and modifying the temperature program.

Regarding the stationary phase, although the separation of essential oils can be made on polar column, keeping in account the fact that the multitude of data from the literature shows a higher usage of methods using non-polar

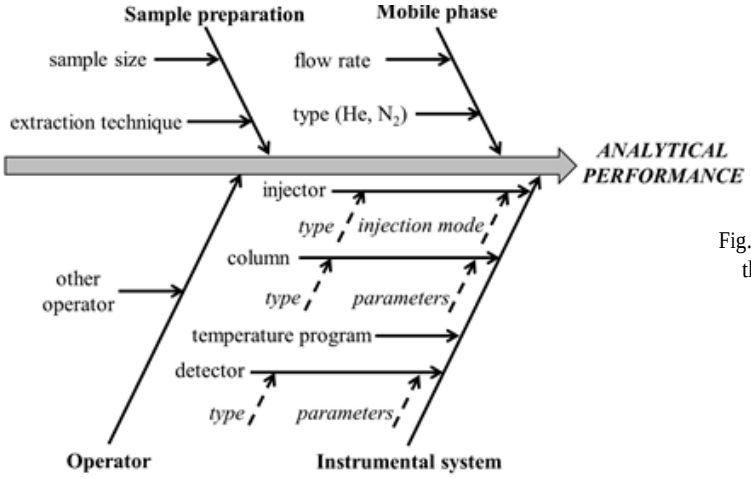

Fig. 1. Ishikawa diagram showing the factors influencing the analytical performance of a generic GC method

Parameters	Description of parameters
Injection volume	0.1 μL and 1 μL
Split ratio	1/100 and 1/50
Flow rate	0.7 and 1 mL/min
Temperature program	40 - 280°C (10°/min) 40 - 80°C (10°/min), 80 - 260°C (5°/min), 260 - 280°C (10°/min) 40 - 80°C (10°/min), 80 - 260°C (2°/min), 260 - 280°C (10°/min)

Table 1
EXAMINATION OF FOUR PARAMETERS OF GC METHOD

columns we work only on a DP-5MS (30 m × 0.25 mm, 0.25μm film thickness) column.

An experimental design comprised of a standard set of 2 injection volumes, 2 split ratios, 2 flow rates and 3 oven temperature programs was developed. This led to a total of 24 (2 injection volumes x 2 split ratios x 2 flow rates x 3 oven temperature programs) chromatographic conditions.

The 24 method conditions were evaluated using the two tiered approach. At the first level, the conditions were evaluated for number of separated peaks, retention times and analysis time. This resulted in 3 chromatographic conditions (1 injection volume x 1 split ratio x 1 flow rate x 3 oven temperature programs). At the second level, these 3 conditions were further evaluated by using more stringent criteria, namely peaks symmetry, biggest peak area (that will influence the sensibility of the method) and the quality of identification (greater than 80%). As the final method is selected against method attributes, it is highly likely that the selected method is reliable and will remain operational over the lifetime of product.

Results and discussions

Method optimization for volume analyzed of essential oil

In order to increase the quality of chromatographic separation it is necessary to have the sample to be in large quantity so that the chromatographic peaks obtained are large enough to provide correct information, i.e., the peaks are symmetrical, there is no overlap of the peaks, the resolution between they are big enough. From this point of view, the influence of the analyzed sample was studied in two ways. For this, different volumes of the sample solution are injected (0.1 and 1 μL respectively, fig. 2).

As it can be observed, for a 10-fold increase in the volume of the injected solution, the signals (and also the number of separated compounds), increase significantly. However, there are a number of inconveniences. If the sample volume injected is too high, then the amount of separated component that enter in the detector is too high, which leads to an increase in the ionic current from the mass spectrometer detector. As a precaution, it blocks. This means time consumed to complete the elution of all compounds, chromatographic column cleaning and resumes analysis. Also, the detector becomes more contaminated, requiring cleaning/service. On the other hand, if the volume is too small, there is a risk that some minor components in the sample may not be detected.

For these reasons, we preferred to analyze intermediate sample volumes but the total volume of injected volatile oil to be divided after injection and before entering the column. For this we inject 1 μL oil solution but using different split ratios (1/100 and 1/50 respectively, fig. 3).

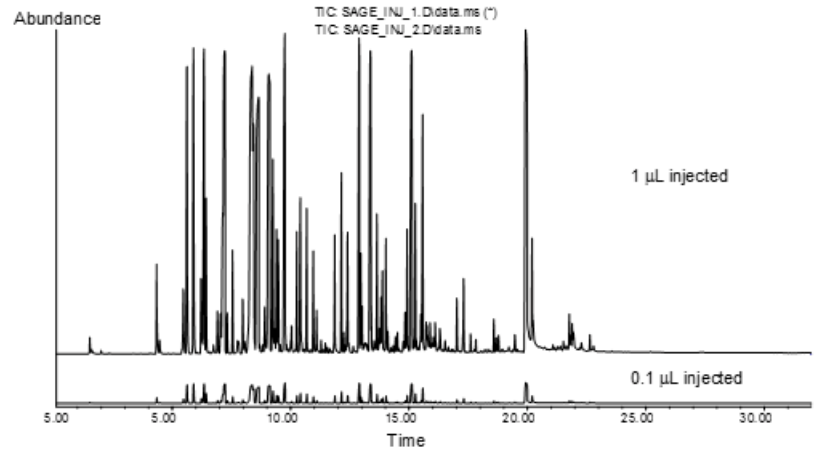

Fig. 2. The gas chromatograms obtained for 0.1 and 1 μL oil injected

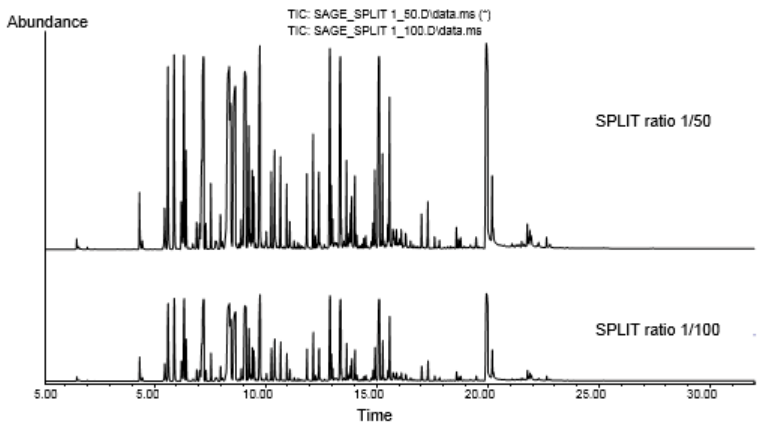

Fig. 3. The gas chromatograms obtained for different split ratios

After the critical analysis of the obtained results, we conclude that regardless of the splitting ratio chosen, the retention times do not change (which is normal) and the number and the area percent of the main peaks does not change significantly. To sum up, we preferred to inject a larger sample of the sample with a split ratio of 1/100.

Method optimization for mobile phase flow

In order to increase the resolution of chromatographic separation are tried to run the analysis at two different mobile phase flows (0.7 and 1 mL/min, fig. 4).

As it is expected, after the critical analysis of the obtained results, we conclude that a mobile phase flow decrease means an increase of the retention times and of resolution, but there are a number of inconveniences, namely a long analysis time and, on the other hand, are obtained the same number of peaks, in about the same area percent, so we choose the flow of 1 mL /min.

According to Blumberg and Klee [28], in conventional gas chromatography, the heating rate is 1 - 20°C /min, for a column of 15 - 60 m length and 250 - 320μm i.d., with an analysis time of ~30 min and 5 - 10 s peak width. The most important parameters that influence the speed of a GC analysis are the length and diameter of the GC column, velocity of the mobile phase and oven temperature programming rates. At this point, using a column of 30 m in length and 0.25 mm internal diameter and with 1 mL/min flow rate, the temperature is the crucial parameters determining the time required for separation of chromatographic zones [29]. Changing the temperature of the chromatographic column can dramatically change the retention of substances on the sorbent layer. The most important benefit of temperature programming in gas chromatography is a substantial reduction of analysis time. The maximum rate at which the oven temperature linearly increases is related to the thermal mass of the oven cavity, the column, the heater power, the efficiency of the oven wall insulation and the differential temperature between the inner part of the oven and its external environment [30]. So, next step consist in the change of the temperature program in the oven, trying to obtain a better separation, choosing a slow speed of temperature increase in the region in that are a large number of peaks (fig. 5).

This lead to an increase of retention times, as expected, but the area or the height of peaks, did not change significantly and the peak purity is the same. Also, even if peaks symmetry and resolution have been modified, the final results are very close.

As a conclusion, in order to reduce the time of analysis but maintaining a good separation of the components we chose the variant where the temperature program is to start from a temperature of 40°C and to increase it with 10°C/min to 280°C.

Final conditions

The final conditions for separation of sage essential oils are: 1 μL oil is injected in a split/splitless injector at a temperature of 250°C, and the separation is performed using a capillary column DP-5MS (30 m × 0.25 mm, 0.25μm film thickness) and helium (1 mL/min) as mobile phase with a temperature increase in the oven with 10°C/min between 40 and 280°C. The separated compounds are transferred in the MS detector at 280°C. In this, the parameters of work are: source temperature 230°C, quadrupole temperature 150°C, mass range 15 - 450 AMU and mode of acquisition SCAN. In these conditions, the analysis time is less than 25 min. Figure 6 shows a chromatogram for a sage essential oil.

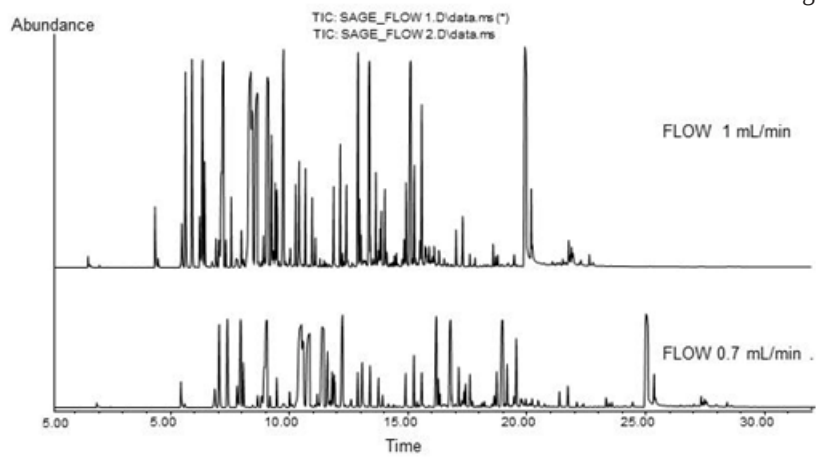

Fig. 4. The gas chromatograms obtained for different flows

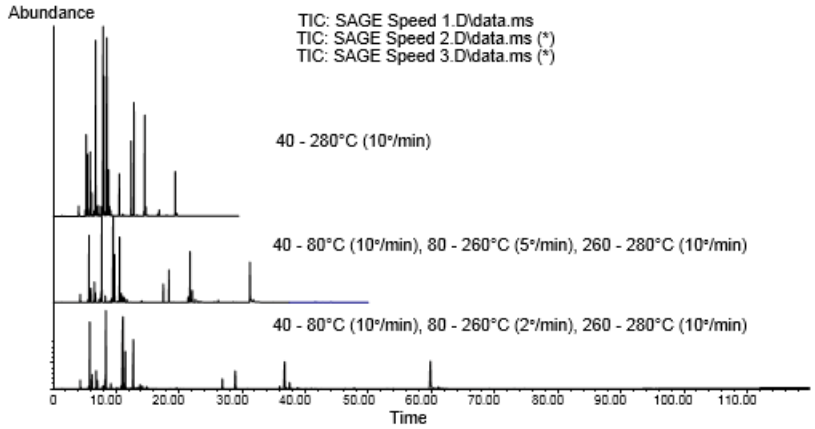

Fig. 5. The gas chromatograms obtained for different temperature programs

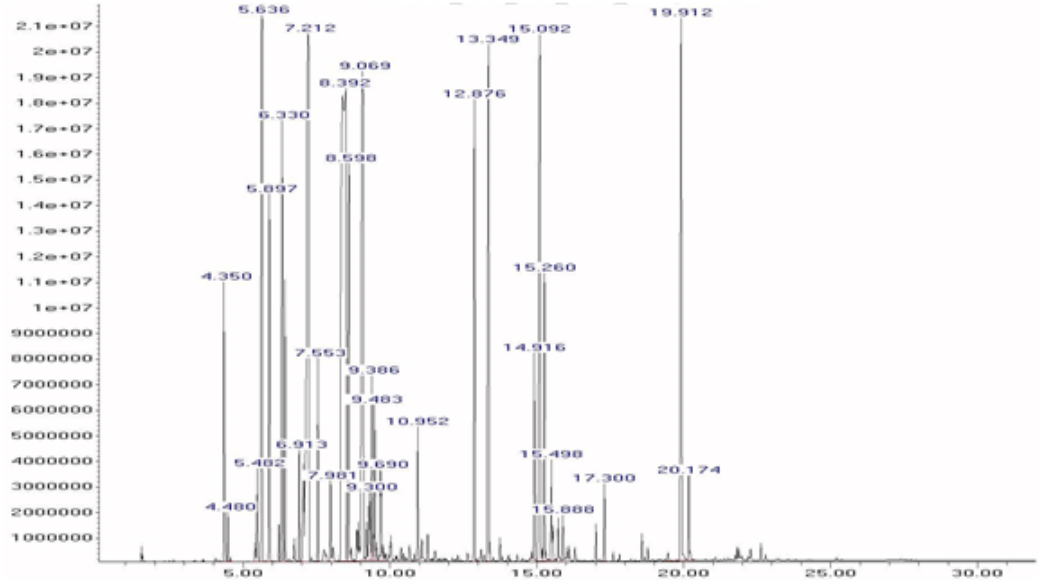

Fig. 6. Chromatogram of a sage essential oil

Pk#	Name	Rt	Area %	Quality (%)
1	.beta.-Thujone	8.39	19.1	98
2	1,8-Cineole	7.21	10.9	99
3	Camphor	9.07	8.68	98
4	Viridiflorol	15.10	7.44	99
5	.alpha.-Pinene	5.63	6.39	96
6	13-Epimanool	19.92	5.59	90
7	.alpha.-Humulene	13.35	5.38	98
8	.alpha.-Thujone	8.60	4.23	98
9	trans-Caryophyllene	12.88	3.57	99
10	.beta.-Pinene	6.33	2.93	97
11	Camphene	5.90	2.75	97
12	Naphthalene, decahydro-	15.26	2.04	83
13	Caryophyllene oxide	14.91	1.8	94
14	Myrcene	6.42	1.56	95
15	cis-Salvene	4.35	1.41	97
16	.gamma.-Terpinene	7.55	1.21	97
17	Borneol 1	9.38	1.14	97
18	Terpinen-4-ol	9.48	1.03	98
19	para-Cymene	7.05	0.99	97
20	(+)-m-Mentha-1,8-diene	9.68	0.91	87
21	Sabinyl acetate	10.94	0.83	84
22	.alpha.-Terpinene	6.91	0.74	98
23	Isocamphane	15.49	0.72	88
24	.alpha.-Thujene	5.48	0.64	94
25	5,7-Dimethoxy-1-naphthol	20.17	0.64	90
26	Caryophylla-4(12),8(13)-dien-5.beta.-ol	15.53	0.51	87
27	Terpinolene	7.98	0.5	98
	Other 34 compounds		5.51	

Table 2
THE MAIN COMPOUNDS IDENTIFIED IN A *SALVIA OFFICINALIS* ESSENTIAL OIL

Data aquisition and analysis were performed with Agilent Technologies ChemStation software. Identification of the constituents was based on computer matching against commercial (NIST 98 and Wiley) mass spectra library.

The optimized method by QbD approach was used for a *Salvia Officinalis* essential oil analysis, the main identified compounds being presented in table 2. The obtained percent are similar with those from the literature [13-18, 31].

In the analyzed *Salvia officinalis* essential oil sample, from the total volatile compounds we found a number of 27 compounds in proportions bigger than 0.5% that represents 93.3% and another 34 compounds in small proportions that represent around 7%.

Conclusions

For the analysis of an essential oil from Salvia Officinalis was developed and optimized a GC-MS method in QbD approach. The method is simple, and was successfully applied to determine the chemical composition of essential oil from *Salvia Officinalis*. The chemical composition of *Salvia officinalis* essential oil shown that .beta.-Thujone (19.10%), 1,8-Cineole (10.9%), Camphor (8.68%), Viridiflorol (7.44%), .alpha.-Pinene (6.39%), 13-Epimanool (5.59%), .alpha.-Humulene (5.38%), .alpha.-Thujone (4.23%), trans-Caryophyllene (3.57%), .beta.-Pinene (2.93%), Camphene (2.75%) and Naphthalene, decahydro- (2.04%) are the main compounds. These results are similar with those from the literature.

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