VIRAL INFECTIONS OF SALVIA SCLAREA

THE INFLUENCE OF *BROAD BEAN WILT VIRUS* (BBWV) ON THE ESSENTIAL OIL

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A recent article published in Natural 1 illustrated the first results of a study (the result of co-operation between the University of Bologna and the Herb Garden (Giardino delle Erbe) of Casola Valsenio, Ravenna) which aimed to highlight the possible influences of viral infections on the quality and quantity of the active ingredients produced by officinal plants (Bellardi *et al.*, 2001). A comparison was made between oil extracted from healthy thyme (*Thymus vulgaris* L.) and that obtained from plants infected by a virus with filamentary particles : the mass gas chromatography (GC-MS) highlighted above all a 9% decrease of thymol in the "infected" sample compared to the "healthy" one.
These first results were a stimulus for further comparative research on the quantity and quality of drugs extracted from healthy plants and from those infected by viruses.
After thyme, clary sage (*Salvia sclarea* L.) has been examined. Its essential oil is used today for its antiseptic, antispasmodic, astringent, balsamic, emmenagogue, stomatic and tonic properties in general. The comparison in this case has been made not only between "healthy" and "infected" samples of oils, but also with a third clary sage oil of commercial origin (Hudaib *et al.*, 2001).
The procedures of the study are illustrated below with a discussion of the results.

Virological study

The samples of clary sage analysed were found in the course of various inspections of the Herb Garden with the aim of identifying the presence of viral infections.
The first symptomatic specimens were observed in April-May 1999 in a small lot consisting of plants obtained from seeds collected from plants cultivated in previous years. The symptomatology was rather heterogeneous. Initially, about 30% of the plants were characterized by a very evident chlorotic mosaic irregularly distributed over the whole of the lamina which appeared covered with blisters, deformed and crumpled in the apical areas. Subsequently, the appearance of yellows was seen on all the leaves with necrotic lesions on the edge of the lamina. The plants with these symptoms, moreover, showed much less development (dwarfism) compared to the surrounding asymptomatic ones.
Foliar samples were collected from plants with and without symptoms in order to ascertain the presence or not of viruses.
For the virological studies, the routine techniques were applied: bioassays (mechanical inoculations on herbaceous plants), electron microscopy ("negative staining") and enzymoimmunoassay (*enzyme-linked immunosorbent assay*: ELISA).
The bioassays allowed isolating only the symptomatic samples of a virus which has been transmitted, by mechanical inoculations of the foliar extract, to some herbaceous plants. The ELISA allowed identifying the virus as an isolate of the broad bean wilt virus: BBWV, serotype I).

Extraction of the essential oil

The extraction of the essential oils from the samples of clary

sage was made using the technique of "steam distillation at atmospheric pressure".
The fresh plant matter (a total quantity of about 2 kg) was divided into two separate lots: the first included asymptomatic plants, the second symptomatic plants. Before collection, all the clary sage plants, with and without symptoms, were subjected to the appropriate virological studies.
The oil obtained from the symptomatic sample (= inflected) was approximately 1/3 less than that obtained from the asymptomatic one (= healthy).

Gas chromatography-Mass spectrometry (GC-MS system)

This analysis was carried out with the aim of evaluating quantitative and qualitative differences in the various components of the essential oil obtained by distillation. In order to make a wider comparative analysis, an essential oil of commercial *S. sclarea* was also analysed.

The gas chromatographic separation provides the gas chromatogram showing the peaks of the various components according to their retention times; the mass spectometry connected on-line then provides, for each component, its mass spectrum. The percentage composition of the essential oil was calculated as mentioned previously (Bellardi *et al.* 2001).
The identification of the individual components thus separated is based on the comparison with the retention indexes reported in literature in similar analytical situations (Operan *et al.*, 1998) and, when possible, with the retention times of the *standards*.
The mass spectrums were also compared with those reported in libraries (libraries: "ThermoQuest" and "NIST" Terpene) and provided by the software of the instrument.
The GC-MS analysis carried out on samples of healthy, infected and commercial oil, have allowed the separation and identification of 24 components (Diagram 1 A-C *page 93*) through their mass spectrum and retention index (Ri). These components are listed according to their retention times (Rt) in Table 1 *(page 94)*.
From an examination of this table, it clearly appears how the three oils taken into consideration are characterized by high percentages of monoterpene esters (about 60%), including linalyl acetate which, alone, is in a percentage of 55.72% in the "healthy" oil, and slightly less both in the "infected" oil (54.40%), and in the commercial sample (42.75%). Linalyl acetate is the main component of the essential oil of clary sage.
Table 1 also shows that the samples examined contain about 20% of sesquiterpenes, especially α-copaene, germacrene-D and ß-caryophyllene. Of these, germacrene-D is present in the greatest concentration in the infected oil (9.47%) compared to the "healthy" sample (7.58%); commercial oil contains smaller quantities: 4.35%. The percentage increase of ß-caryophyllene in the infected oil (4.24), compared to the "healthy" oil (3.84), is also considerable.
As far as alcohols are concerned (about 12%), linalol and α-terpineol are the most abundant. Whilst the former is present in percentages that are almost the same in the "healthy" and "infected" oils, the percentage of α-terpineol increases considerably in the infected oil (2.30%) and commercial oil (3.5%), compared to the healthy oil (1.64%).
Amongst the monoterpenes (about 7.5%) the following prevail: myrcene, limonene (and the two isomers of ocimene) which are present in a somewhat smal-

• GC-MS system:
- *Trace GC* "2000 series" with a *Thermo Quest* ion trap mass spectometer.
- Electron impact: 70 Ev.
• Column: Rtx-5MS®, fused silica capillary column (Restek Corporation, Bellefonte, USA).
Dimensions: 30m x 0,25mm ID, film thickness 0,25 µm, internally lened with *Crossbound*® (5% phenyl-95% dimethyl polysiloxan).
• Instrumentation parameters:
GC:
- Injection chamber: injection in column with *Split/Splitless* technique (splitting ratio: 50:1 and 75:1).
- Injector temperature: 250 °C.
- volume of sample injected (without dilution): 0.1 µl
- transporting gas: helium (1ml/min.)
- oven: programme for linear increase of T°
initial temperature:
70 °C (for 10 min.)
- temperature increase: 4 °C/min.
- final temperature: 210 °C (for 10 min.)
MS:
- *Transfer line* temperature: 250 °C.
- ion source: 200 °C.
- bottom: auto-controlled.

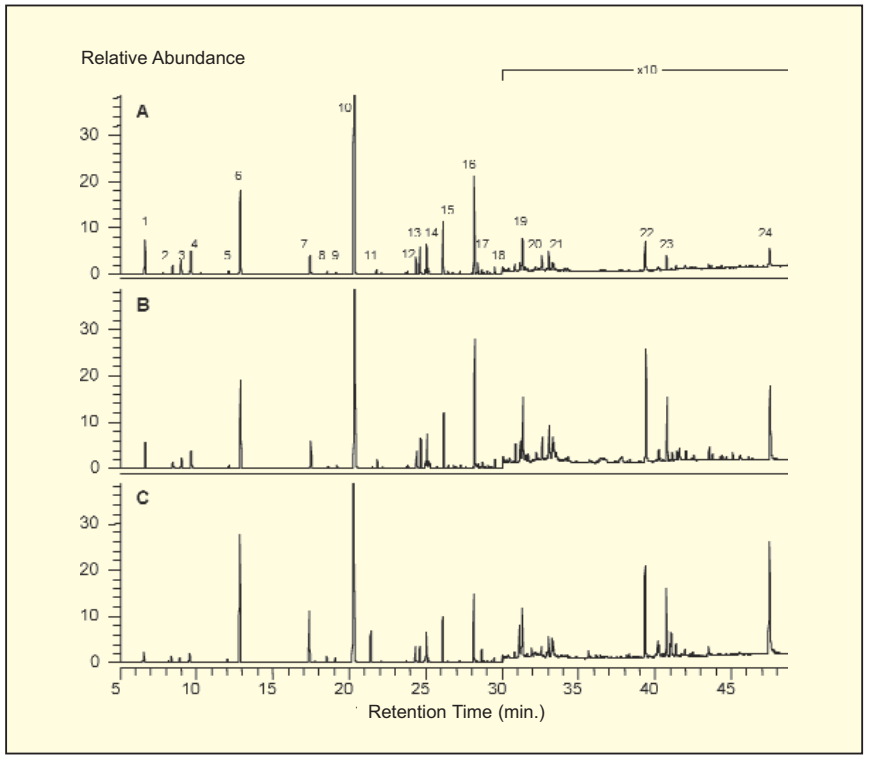

Diagram 1: GC – MS Chromatogram of the essential oil of S. sclarea from : (A) healthy plants, (B) infected plants, (C) commercial source. The peaks after Rt: 30 min. are amplified 10 times. The peaks are numbered in accordance with Table 1.

ler quantity in the "infected" and commercial oils, compared to the "healthy" one.
The oil also contains sclareol (diterpene hydrocarbon) which has a relatively small presence in the healthy oil (0.23%), compared to the "infected" (1.05%) and commercial oils (1.22%). Small quantities of oxides, phenols and other components of different and/or unidentified chemical structure have also been found.
Although the general chemical profile of the three oils is fairly similar, the GC-MS analyses highlighted some differences in composition (Table 1). For example, thymol was found only in the oils from Casola Valsenio.
Table 1 allows making a number of general considerations on the composition of "healthy" and "infected" oils: whilst in the former there is a higher percentage of monoterpenes (e.g. myrcene, limonene, ocimene), the latter is characterized by higher percentages of sesquiterpenic hydrocarbons (germacrene-D and ß-caryophyllene) and oxygenated sesquiterpenes (caryophyllene oxide).
The oil from infected plants also contains higher percentages of alcohols (e.g. α-terpineol and sclareol), with the exception of linalol which is in similar quantities but not identical, in the two oils. However, there are also other minor components that show significant content percentage variations in the two oils.

Table 1: Main components of the essential oil obtained from healthy and BBWV-I infected S. sclarea.

No.	Rt	Ri	Components	Contents %		
				HEALTHY	INFECTED	COMMERCIAL
1	6.61	993	Myrcene*	3.29	2.08	0.68
2	8.42	1027	Limonene*	1.03	0.54	0.42
3	8.97	1036	Z-Ocimene*	1.71	1.05	0.37
4	9.61	1047	E-Ocimene*	2.96	1.89	0.78
5	12.08	1089	Terpinolene	0.33	0.24	0.33
6	12.86	1103	Linalol*	10.06	9.01	11.97
7	17.41	1193	α-Terpineol*	1.64	2.30	3.5
8	18.56	1220	β-Citronellol	0.16	0.06	0.36
9	19.13	1233	4-Terpinyl-acetate	0.13	0.21	0.35
10	20.26	1261	Linalyl acetate	55.72	54.40	42.75
11	21.76	1297	Thymol	0.36	0.62	NF
12	24.35	1369	Neryl-acetate	1.11	1.27	0.98
13	24.61	1377	α-Copaene*	2.06	2.33	1.00
14	25.03	1388	Sabinene hydrate acetate	2.24	2.33	1.75
15	26.11	1421	β-Caryophyllene*	3.84	4.24	3.00
16	28.15	1484	Germacrene D*	7.58	9.47	4.35
17	28.37	1491	γ-Muurolene*	0.91	0.49	0.12
18	29.49	1528	δ-Cadinene*	0.58	0.73	0.30
19	31.31	1589	Caryophyllene oxide*	0.26	0.60	0.35
20	32.57	1633	Z-α-Santalol	0.13	0.20	0.05
21	33.25	1657	α-Eudesmol	0.05	0.19	0.12
22	39.35	1889	Unidentified diterpenoids	0.24	1.00	0.61
23	40.75	1947	Unidentified diterpenoids	0.11	0.48	0.42
24	47.50	2227	Sclareol*	0.23	1.05	1.22

Rt: retention time; Ri: retention index; NF: not found;
** : statistically significant difference.*

Some components present a considerable increased concentration in the infected oil compared to the "healthy" oil: α-terpineol, germacrene-D, ß-caryophyllene, sclareol and α-copaene; myrcene and limonene are however present in a reduced percentage.
In addition to these particularly apparent percentage variations, all the components are in slightly different concentrations in the two oils and it is legitimate to suppose that this may influence their quality. However, it is not possible to establish to what degree the quality of the oil is compromised; that is, it cannot be known with only this analysis, if the oil from inflected plants is of an inferior quality compared to that from a healthy plant.
On the other hand, with all probability, the variation in the composition may determine a different pharmacological activity of the two oils. Recent studies confirm that the analgesic, anti-inflammatory and antimicrobial activity of the essential oil of clary sage is in relation to its chemical composition (Moretti *et al.*, 1997; Peana *et al.*, 1999). More precise evaluations on the variation in the pharmacological activity would however require specific pharmacological research.

Conclusions

The analysis of the essential oils of *S. sclarea* has shown, in the first place, a reduction in the quantity (rendered) compared to the amount that can be obtained from infected plants (about 2/3 compared to the oil obtained from healthy plants).
The GC-MS technique has also allowed observing qualitative and quantitative differences between the two oils, thus providing important analytical-comparative starting points.
Even if the data relative to the commercial oil is of interest, the comparison between plants cultivated with similar means and in the same geographical and climatic conditions is considered more useful; in other words, differences due only to infection from BBWV will be shown in the comparison between oils distilled from plants coming from the Herb Garden .
Analysing Table 1, it appears that in the "healthy" oil the diterpenoids are present in a low percentage whilst in the "infected" and commercial oils these components are present in higher percentages (the commercial oil is therefore more similar to the "infected" one; even if the different origin of the former only allows hypotheses, it can however be presumed that there is not selective gathering of the plants to be used in the distillation of the commercial oil).

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