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VOLATILES IN *TĂMÂIOASĂ ROMÂNEASCĂ* VIA SUPERCRITICAL FLUID EXTRACTION (SFE) ANALYSIS

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Abstract

Wine analysis is a complex process, requiring multiple techniques and very in-depth multidisciplinary knowledge. Moreover, when talking about sensorial potent wines, like *Muscat* or *Tămâioasă Românească* (from aromatic Romanian grape variety), the identification and characterization of its volatile compounds is achieved through different methods, most calling for powerful solvents to separate the aroma substances.
Supercritical fluid extraction is a powerful technique with great promise in organic analytical chemistry. To date, little has been published on the use of SFE in the analysis of wine aromas.
The main objective of this study is the analysis of volatiles in samples of *Tămâioasă românească* wines through a custom-made SFE method. The wine variants have been obtained by applying the general technological processes for aromatic wines, using specific oenological products. An in-house SFE analysis method was developed and applied, the obtained extracts were then analyzed by gas chromatography coupled to mass spectrometry (GC-MS) to identify the captured compounds.
Regarding the total number of findings in respect to volatiles, the highest concentrations occur with low molecular weight alcohols, esters and acetic acid. Besides, there were also identified other volatile compounds, such as terpenoides and phenols. These specific compounds originate from the grape variety and give the "varietal" character to the wines.

Key words: supercritical fluid extraction, *Tămâioasă Românească*, volatiles, wines

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1. Introduction

Wine is largely defined by its appearance (e.g. color, limpidity), volatile as well as non-volatile aroma (flavor), and also what we experience as tactile senses when consuming wine, such as mouth-feel properties. Wine raises most discussions, particularly due to its complex composition of (volatile) compounds responsible for its intricate aromatic nature. Volatile compounds of which some express highly appreciated aroma attributes are the ones that give wine its "genius", specificity and individuality. Each wine's individuality or "personality" is given by a specific and unique combination of characteristic flavor compounds.

Grape-origin compounds provide the basic structure of the wine (sugar, acids, phenols, minerals) and, in addition, the varietal sensory distinction. This is well known for the free floral monoterpenes that define Muscat-related and other aromatic wines. For others, monoterpenes may be found primarily in glycosidically bound forms in the grape, and have to be released by enzymes produced during fermentation (Baumes, 2009). Furthermore, depending on wine-style, the overall bouquet of the wine may greatly be influenced by maturing in oak

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barrels, and finally, by the chemical changes observed during aging (Fischer, 2007).

Monitoring volatiles in wines requires complex procedures that usually call for use of solvents that, in high quantities, can be harmful for human health. Therefore, the need of an easier, solvent-free analysis method has risen. The evolution towards Green Analytical Chemistry is to new extraction and sample-preparation processes that should be faster, more reproducible and more environmentally friendly (Herrero et al., 2013).

Supercritical fluid extraction (SFE) is the process of separating one component (the extract) from another (the matrix) using supercritical fluids as the extracting solvent. It is based on the principle that solubility in a supercritical fluid increases dramatically with increasing density, and different solutes have different solubility at the same condition. The major advantage of this method over liquid-liquid extraction is that the supercritical fluid can easily be removed after extraction by lowering the temperature or pressure or both (Válcarcel and Tena, 1997). The essential equipment needed to perform SFE is shown in Fig. 1.

A high pressure pump is used to provide pressurized fluid (at a constant pressure) to the sample which is contained in the extraction vessel or sample cell. The extraction vessel is housed in an oven to maintain the temperature above the critical temperature of the extraction fluid. The extraction fluid is pumped through the extraction vessel, the analytes are partitioned into the supercritical fluid, and the analytes are collected after depressurization of the supercritical fluid. Extracted analytes are most often collected in a small volume of liquid solvent (off-line SFE) or the analytes transferred directly to a chromatographic system (on-line SFE) like SFC or GC. Alternate methods such as cryogenic trapping (Levy et al., 1992; Xie et al., 1989) or collection onto a sorbent cartridge have also been used (Maxwell et al., 1995).

Supercritical fluids exhibit liquid-like (solvent power, negligible surface tension) as well as gas-like (transport) properties above their critical point. This property has led in recent years to great interest in supercritical fluids amongst researchers for their various applications.

CO_2 is used as "supercritical solvent" in the extraction of flavor and fragrance compounds, since it is an odorless, colorless, highly pure, safe, cost effective, nontoxic, nonflammable and recyclable gas allowing supercritical operation at relatively low pressures and near room temperature. Generally speaking, supercritical CO_2 (SC CO_2) behaves like a lipophilic solvent but, compared to liquid solvents, it has the advantage that its selectivity or solvent power is adjustable and can be set to values ranging from gas-like to liquid-like (Reverchon, 1997).

Extraction conditions for supercritical carbon dioxide are above the critical temperature of 31 °C and critical pressure of 74 bar (Jonin et al., 2010). The main advantages of using supercritical fluids for extractions is that they are less expensive, extract the analytes faster and they are also more environmentally friendly than organic solvents.

This study aims at identifying the possibilities of extracting aroma compounds in Tămâioasă românească wines through a newly developed SFE method. In the present work the focus will be on the SFE volatiles' extraction from Tămâioasă românească wine, one of the most well-known aromatic wines in Romania, which had not been investigated in this respect before. In order to study the volatiles, several trials were made, and the most representative ones are to be seen below. To our knowledge, however, there has been no previous attempt to use this simple technical SFE/SFC procedure (lower pressure and temperature) combined with GC/MS identification for determination of Tămâioasă românească wine aroma compounds.

2. Material and methods

2.1. Grape material and reagents

Grapes of the variety Tămâioasă românească, a Romanian aromatic grape variety were harvested from Cotnari vineyard in 2013, on the 25th of September. The preliminary analysis showed that the grapes had 250 g/L sugars and an acidity of 6.1 g/L tartaric acid.

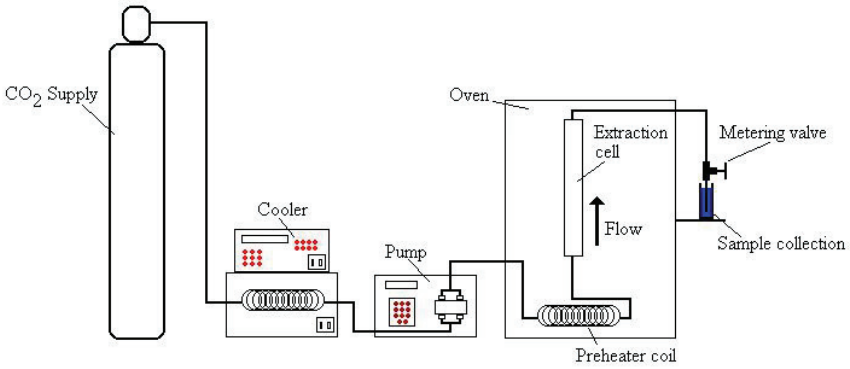

Fig. 1. Diagram of a SFE setup for off-line recovery of analytes (Sewram, 1997)

Solvents: ethanol and dichloromethane, over 99% purity, were purchased from Sigma-Aldrich, Germany. Oenological products were purchased from Sodinal, Paris, France

2.2. Wine samples

The grapes were processed according to the literature, specifically referring to aromatic wine technologies (Cotea and Sauciuc, 1986). After de-stemming and crushing, the unpressed must was partinioned in three equal parts.

Saccharomyces cerevisiae (Fermactive Ap® and Fermactive Muscat®), which is recommended for proper vinification of aromatic grapes, such as Muscat, Grasă de Cotnari, Tămâioasă românească and Şarbă was used for fermentation.

Each fermentation vessel (15 L glass jar) was inoculated with 1g/L of dry yeast, previously rehydrated for 15 minutes at 35 °C. The first variant was macerated for 24 hours, with no added enzymes, pressed, and then transferred to glass vessels for fermentation with commercial yeast Fermactive Ap®. Second variant of the Tămâioasă românească was macerated for 24 hours with addition of pectolytical enzymes (Zymoclaire High CG®). The third variant of Tămâioasă românească was macerated for 24 hours with addition of a different pectolytical enzyme (Endozym Beta-split®). Pressing was then done with a hydraulic press. The resulting juice was transferred into glass vessels for fermentation with Fermactive Muscat®, at 15 °C. The fermentation period took 14 days.

The following experimental wine samples were obtained and registered as follows: TR V1 (Tămâioasă românească wine sample, 24 hours maceration, no enzymes, fermentation with Fermactiv Ap®), TR V2 (Tămâioasă românească wine sample, 24 hours maceration with Zymoclaire Aroma High CG® enzymes, fermentation with Fermactive Muscat® yeasts) and TR V3 (Tămâioasă românească wine sample, 24 hours maceration with Endozym Beta-split® enzymes, fermentation with Fermactive Muscat® yeasts).

2.3. SFE/GC analysis conditions

An off-line JASCO® SFE/SFC custom made procedure was used for supercritical fluid extraction (in house method), while a Shimadzu GC-MS 2010 was used for extracted volatiles' analysis.

2.3.1. SFE analysis of Tămâioasă românească wines

Extractions were performed using an SFE module designed and manufactured by Jasco (Japan). The device has an EV-4 of 10 mL extraction cell for liquid samples in a CO-2060 extraction oven. The chemical modifier was added in to the mixing chamber of the extraction oven with a PU-2089 quaternary gradient pump with a build-in degasser at 20% ratio. Every extraction was 60 minutes long collected with aid of a BP-2080 back pressure regulator at around 10 MPa. A PU-2080-CO_2 Peltier cooled pump delivered 1 mL/min. liquid carbon dioxide in supercritical mode at more than 10 MPa.

In each 5 mL wine sample were added an amount of 1 g NaCl before SFE extraction, for salting out or increasing aroma compounds' extraction power. The extraction temperature in the oven was 40 °C and the back pressure regulator was set at 60 °C. The temperature of the collected liquid was 20 °C. The modifier used was ethylic ether 25% with 75% dichloromethane. The collected volumes varied from 1.2 to 1.5 mL/h. Also, the most insoluble and less volatile compounds were collected as white solid crystals, fraction recovered with 1 mL of dichloromethane.

2.3.2. GC/MS analysis conditions

The analysis of the obtained extracts was made on a Shimadzu GC-MS 2010plus device. Column oven temperature started at 35 °C where it was maintained for 3 minutes after injection and grew with 5 °C per minute up to 220 °C where it remained for 10 minutes, injection temperature was 250 °C, split mode: 20 for 1 µL injected and column flow was 1 mL/min. The column used is Varian VF-WAXms 30 m length, 0.15 mm diameter, 0.15 µm thickness (Agilent Technologies, Germany). The interface transfer temperature was 250 °C. The mass spectrometer detector had the ion source temperature at 250 °C and acceleration voltage at 0.8 KV. The detected peaks were compared with NIST08, Wiley08 and SZTERP spectra libraries for identification purposes. A similarity percentage of over 70% was considered acceptable.

3. Results and discussion

3.1. SFE/GC analysis

Alcohols and esters were the main identified volatiles of the studied wines. These compounds are mainly produced by yeast metabolism during fermentation (Rapp and Mandery, 1986).

Table 1 shows the aroma compounds in Tămâioasă românească wine samples, obtained from a 24 hours maceration of grapes, with no enzymes addition and using yeast specifically designed for obtaining neutral wines (Fermactiv Ap®).

Table 2 shows the aroma compounds in Tămâioasă românească wine samples, obtained from a 24 hours maceration of grapes, with addition of ZYMOCLAIRE High CG® which is a highly concentrated pectolytic enzyme preparation, without any cinnamyl-esterase activity, designed for the clarification of grape juice with high turbidity at low temperatures. Fermentation was achieved by using Fermactive Muscat® yeasts. In enology, winemakers go for such maceration in order to better extract the bound aroma precursors (Ribereau-Gayon et al., 2000).

In addition, the enzyme added (ZYMOCLAIRE High CG®) during maceration aids in cleaving such glycosidic bonds (having glycosidase side-activities). Particularly for bound terpene precursors, this is beneficial for a more pronounced varietal character of the final product.

Table 3 shows the aroma compounds in Tămâioasă românească wine samples, obtained from a 24 hours maceration of grapes, with addition of Endozym Beta-split® enzymes that frees up available varietal aromas that are bound with sugars that otherwise would only be available in the case of high over-ripening conditions of the fruit. The same yeasts as in the above sample were used.

The identified compounds are common in wines (Clarke and Bakker, 2004; Ron, 2000), significant for aromatic wines. In previous studies, Tămâioasă românească wines were also analysed from the point of view of volatile composition, therefore, the specific retention time of the identified compounds can be verified. The methods previously used consisted of headspace GC/MS and SPE GC/MS (Nechita, 2010).

As the used SFE method is still in trials, the analysis results of the three samples show some discrepancies according to the analysed wine variants. As seen from the above tables, the number of identified compounds varies from one wine sample to another. In the first sample, Tămâioasă românească wine obtained through a 24 hours maceration and use of yeasts specific for neutral wine making, 5 compounds were identified, compared to the other two samples (12 compounds in the second and 9 compounds in the third, both latter samples being obtained with use of maceration enzymes and yeasts specific for obtaining aromatic wines).

The identified compounds are from specific chemical classes, as follows: 5 alcohols (propanol, isobutyl alcohol, 3-methyl-1-butanol, 2,3-butanediol, phenylethyl alcohol), 4 esters (ethyl ether, ethyl formate, ethyl acetate and propanoic acid, 2-hydroxy-ethyl ester), 1 lactone (butyrolactone), 1 acid (acetic acid), 1 terpene (3,7-Dimethyl-octa-1,7-dien-3,6-diol also known as Terpene diol -2) and 1 phenol (4-vinylphenol) (Fig. 2). 4-vinylphenol is a phenolic compound produced by the spoilage yeast *Brettanomyces* in wine. Terpene diol 2 is a specific terpene for Tămâioasă românească wines (Nechita, 2010).

The in-house method described still needs more trials and feedback and possibly this will bring in further optimisation, as modifiers can form aerosols and sweep analyte molecules through the system without the molecules being deposited or collected in traps.

Table 1. Aroma compounds in TR V1

No	*R.T. (min.)*	*Peak area (UA)*	*Compound name*	*ID [1]*	*Sensory descriptor*
1	6.04	40205	Propanol	A	slightly sweet fruity nuance of apple and pear
2	7.36	10135	1-Propanol, 2-methyl-	A	ethereal winey
3	10.65	367627	3-Methyl-1-butanol	A	ethereal winey
4	17.40	161821	Acetic acid	A	pungent acidic and dairy-like
5	27.95	665002	Phenylethyl Alcohol	A	floral rose

[1]*Identification: A = GC retention and MS data in agreement with spectra found in the library; B = tentatively identified by MS matching with library spectra only; nd: not detected, tr: trace. Results are the means of three repetitions.*

Table 2. Aroma compounds in TR V2

No	*R.T. (min.)*	*Peak area (UA)*	*Compound name*	*ID [1]*	*Sensory descriptor*
1	1.64	286093	Ethyl ether	B	ethereal
2	2.45	67134	Ethyl formate	B	ethereal, sweet, grainy, fruity, winey and cognac
3	3.12	251850	Ethyl acetate	A	ethereal, fruity, sweet, with a grape and cherry nuance
4	6.59	315514	Propanol	A	slightly sweet fruity nuance of apple and pear
5	8.04	186656	Isobutyl alcohol	A	ethereal, winey
6	11.40	2048687	1-Butanol, 3-methyl-	A	ethereal, winey
7	14.78	574334	Propanoic acid, 2-hydroxy-, ethyl ester	A	sweet, fruity, acidic, ethereal with a brown nuance
8	17.40	1909964	Acetic acid	A	pungent acidic and dairy-like
9	19.73	2671696	2,3-Butanediol	A	fruity, creamy, buttery
10	21.74	85006	Butyrolactone	A	creamy, oily with fatty nuances
11	27.94	1082477	Phenylethyl Alcohol	A	floral rose
12	35.47	68742	3,7-Dimethyl-octa-1,7-dien-3,6-diol	B	sweet, floral, citrus

[1]*Identification: A = GC retention and MS data in agreement with spectra found in the library; B = tentatively identified by MS matching with library spectra only; nd: not detected, tr: trace. Results are the means of three repetitions.*

Table 3. Aroma compounds in TR V3

No	*R.T. (min.)*	*Peak area (UA)*	*Compound name*	*ID* [1]	*Sensory descriptor*
1	3.00	197532	Ethyl acetate	A	ethereal, fruity, sweet, with a grape and cherry nuance
2	7.21	315014	Propanol	A	slightly sweet fruity nuance of apple and pear
3	8.52	186547	Isobutyl alcohol	A	ethereal, winey
4	11.94	2048874	1-Butanol, 3-methyl-	A	ethereal, winey
5	17.78	1909674	Acetic acid	A	pungent acidic and dairy-like
6	19.64	1066241	2,3-Butanediol	A	fruity, creamy, buttery
7	27.94	70036	Phenethyl alcohol	A	floral, rose
8	35.59	58064	3,7-Dimethyl-octa-1,7-dien-3,6-diol	B	sweet, floral, citrus
9	37.00	153251	4-vinylphenol	B	chemical, phenolic, medicinal with sweet musty and meaty nuances

[1]*Identification: A = GC retention and MS data in agreement with spectra found in the library; B = tentatively identified by MS matching with library spectra only; nd: not detected, tr: trace. Results are the means of three repetitions.*

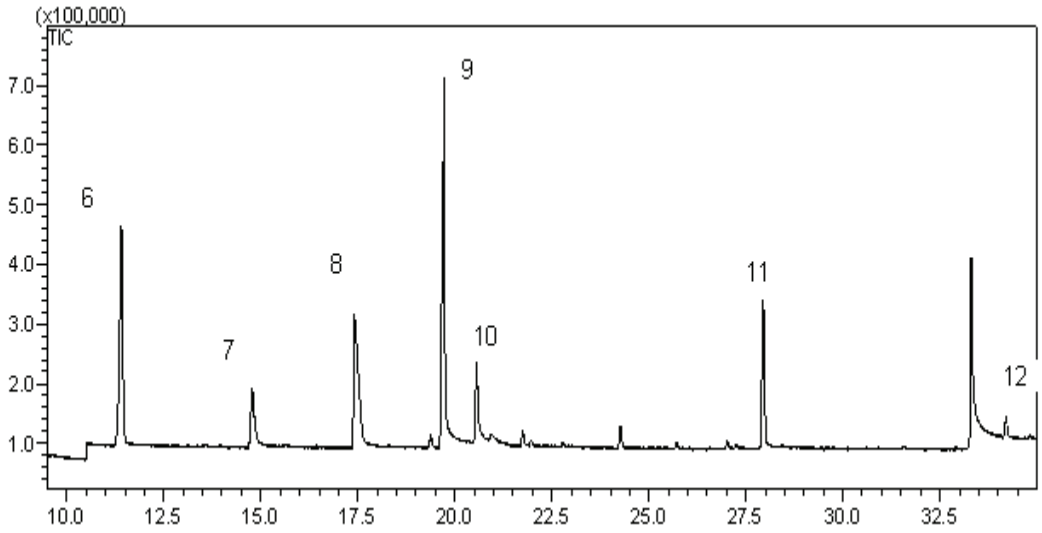

Fig. 2. Excerpt of GC-MS chromatogram of volatiles extracted with supercritical CO_2 from Tămâioasă românească wines
Indicated numbers represent compounds identified: 6) 1-Butanol, 3-methyl-; 7) Propanoic acid, 2-hydroxy-, ethyl ester; 8) Acetic acid; 9) 2,3-Butanediol; 10) Butyrolactone; 11) Phenylethyl Alcohol; 12) 3,7-Dimethyl-octa-1,7-dien-3,6-diol

At the same time, one of the real culprits responsible for low extraction recoveries is inefficient trapping of the analyte once it's extracted. Moreover, variations in pressure and temperature mentioned in literature (Jonin et al., 2010; Reverchon, 1997; Sewram, 1997) have not been applied in our experiment, having maintained these two variables linear for the duration of the process.

4. Conclusions

In summary, we have managed to extract some major aroma compounds in Tămâioasă românească wines and describe the volatiles according to their important sensorial characteristics using a very simple pilot in-house methodology.

The proposed SFE/GC/MS in-house method has successfully extracted the most important volatiles from wines and can thus be used for creating a clear sensorial profile.

The methodology used also portrays the influence of major technological steps in the wine making process (maceration) but also microbiological faults expressed through specific aroma compounds (4-vinylphenol).

Due to the complexity of wine aroma, the SFE method had to coupled with GC/MS, sensorial analysis and chemometrics in order to ensure a reproductible volatile profile.

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