

IMPROVEMENT OF ORGANIC ACIDS EXTRACTION AND DETERMINATION METHODS FROM LEAVES AND BERRIES OF TWO GRAPE CULTIVARS COT AND NÉGRETTE

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I. INTRODUCTION

The study of organic acids in grapes is very important because it is the main factor of wine acidity which is closely related to microbiology contamination, colour stability of red wine and the control of malo-lactic fermentation (RÜFFNER, 1982; DAVEREDE, 1996; RIBEREAU-GAYON *et al.*, 1998 and CLARCK and SCOLLARY, 2003) Tartaric acid production appears to be associated with the process of cell division and cell elongation as its synthesis in leaves occurs mainly during the period of leaf expansion and in berries during the period of growth (RÜFFNER, 1982 and COOMBE, 1992). The malic acid content corresponds to differences in climatic conditions (CHAMPAGNOL, 1986 and CHAMPAGNOL, 1994) and vacuolar compartmentation (TERRIER *et al.*, 2001). Tartaric and malic acids are known to contribute up to 90 % of the organic acid content in mature berries (KANELLIS and ROUBELAKIS-ANGELAKIS, 1993). The “Côtes du Frontonnais” appellation where Négrette is the principal cultivar shows a lack of acidity in wines (DAVEREDE, 1996 and GALLEGÓ, 1999). In the “Cahors” appellation, the Cot N is the most planted grape variety which is appreciated for the colour, aromas, tannins and acidity that it gives to the wines (GARCIA, 2002). In all instances, for organic acids complex extraction steps were necessary, and there is no specific extraction method for grapevine leaves.

In this paper, and for the first time, we aim to develop and to validate an extraction method of organic acids from young and mature leaves of grapevine also applicable to berries, and to improve the determination method of organic acids by capillary electrophoresis.

In terms of application, the developed protocols permitted the study of the synthesis of these two organic acids, their accumulation in leaves and berries during development and ripening periods.

II. MATERIALS AND METHODS

I. EXPERIMENTAL DESIGN. - The vines were grown in 40 L pots filled with pozzoulana, with four replications per treatment grapecultivar / rootstock, in completely randomized plan, each including two plants. Cot (syno. Malbec) is the main cultivar of the “Cahors” appellation and the Négrette is the main cultivar of the “Côtes du Frontonnais” appellation. The Négrette own-rooted vines were compared with three rootstocks (3309 Couderc (3309 C), SO4 and 101-14) and the Cot own-rooted vines were also compared to three rootstocks (3309 C, SO4 and Riparia). Only results concerning 3309C and SO4 are presented and discussed.

Tartaric acid and malic acid contents in young and mature leaves were determined from Fruit Set (FS), 2 Weeks post Fruit Set (FS+2W), FS+4W, FS+6W, FS+6W, FS+8W, FS+10 W, FS+12W and at Harvest time. In berries, organic acids were determined from 2 Weeks post Fruit Set (FS+2W) to Harvest time.

2. REAGENTS. - Tartaric and malic acids were obtained from Acros Organics (Paris, France). Standards solutions (3 g/l) were prepared in ethanol. Working standard solutions were prepared daily by dilution of the stock standard solutions with deionized water (Milli-Q). Electrophoretic separation was performed using a buffer system composed of 10 mM benzoic acid, 10 mM histidine and 1 mM tetradecyl-trimethyl ammonium bromide TTAB at pH 5.1. The electrolyte solution was prepared daily.

3. APPARATUS. - A Capillary Electrophoresis Spectra Physics 500 system (Spectra-physics Inc., California, U.S.A.) was used throughout the investigation. Separation of organic acids was performed on a 45 cm x 75 µm I.D. fused-silica capillary column (TSP075375, Thermo Finnigan, Paris, France). The detection window was 7 cm from the capillary outlet. An ultraviolet wavelength of 220 nm was chosen to monitor the absorbance of the buffer solution. The separation voltage was – 20 kV, resulting in an electrophoretic current of 7.4 µA, at constant temperature of 30°C.

4 PROCEDURE

4.1 Development of organic acids extraction methods.

In order to develop the organic acids extraction method in leaves, several parameters were tested:

- Fining in sable de fontaine blau, in liquid nitrogen, and sonicated by ultrasound for (0 - 10 min)
- Extraction solvents : distilled water, ethanol (5° - 95°), methanol (5° - 95°), NaOH (0.1 - 1 N), ethanol/NaOH (5°, 10°, 15°) / (0.1 N) and methanol/NaOH (5°, 10°, 15°) / (0.1 N)
- Operating temperature: 4°C and room temperature (20°C)
- Extraction duration: 5, 10, 20, 30, 40, 50 and 60 min
- Centrifugation: 4000 rpm during 20 min at 4°C
- Filtration through 50 µm syringe filter to prepare solutions
- 6 replicates per injection and 6 replicates per sample

4.2 Operating conditions.

Before the first use, the capillary was conditioned by rinsing with 1.0 N NaOH for 10 min, 0.1 N NaOH for 10 min, water for 10 min and, finally, with the separation electrolyte solution for 10 min.

The capillary was rinsed between sample injection with 1.0 N NaOH for 1 min, 0.1 N NaOH for 1 min, water for 1 min and, finally, with separation buffer for 2 min at 30°C. Samples were injected by hydrodynamic injection for 2 seconds. Electrophoregrams were recorded and processed with PC 1000 (A5595-010) data acquisition system (Thermo Separation Products, Fremont, CA, USA). Corrected Peak Areas (CPA) (area/migration time) were used for quantitative analysis.

Data were analyzed using analysis of variance (ANOVA) and checked for significant probability ($p \leq 0.05$) level using Sigmatat® 2.03 Statistical Software. The difference between the means was determined using Fisher's Last Significant Difference at a probability ($p \leq 0.05$) level (LSD = 0.05).

III. RESULTS and DISCUSSION

1 Extraction methods for malic and tartaric acids

A new extraction method for malic and tartaric acids from grapevine leaves was developed and summarized in the Table 1. Organic acids stability depends on several factors essentially temperature, type of solvent and pH. The best extraction method was: just after sampling, fresh leaves (young and mature) were finely in liquid nitrogen and sonicated by ultrasound for 7 min at 4°C. The most efficient extraction was obtained under alkaline conditions. For malic acid extraction ethanol/NaOH solution seemed to be more favourable for extraction, but for tartaric acid, NaOH solution alone provided a higher extraction. The time of extraction was varied from 5 to 60 min and the highest extracted quantity of organic acids was obtained after 30 min. Centrifugation at 4000 rpm for 20 min at 4 °C followed by filtration through 50 µm syringe filter was the best combination to prepare samples before analysis.

Fig. 1 (A) shows the electrophoregram corresponding to a standard solution of malic and tartaric acids in the selected operating conditions, which provide a good separation of organic acids with highly efficient precision and in short analysis time. Fig. 1 (B and C) show the correlations obtained between injected and detected malic acid (B) and tartaric acid (C) by Electrophoresis Capillary of tartaric acid and malic acid.

This optimised extraction method allowed the study of organic acids synthesis in leaves and their accumulation in berries, and therefore, the effect of rootstocks.

2 Applications: Analysis of malic and tartaric acids contents in leaves and in berries during development and ripening periods according to rootstock

2.1 Malic acid content in mature leaves and in berries of the Cot and the Négrette cultivars

Both Cot and Négrette grafted onto SO4 and 3309 C showed significant difference, for malic acid contents in mature leaves, from fruit set to harvest time depending on the rootstock (Fig. 2). Malic acid content increased until reaching a maximum at FS+8W (end of veraison) and then it decreased rapidly to its minimum at harvest time.

Négrette berries had higher capacity for malic acid accumulation than for tartaric acid accumulation (Fig. 3 and 5).

High correlations were established between malic acid contents in leaves and in berries (Table 2). Malic acid content in berries showed higher correlation with malic acid content in mature leaves than in young leaves [Table 2]. For the Négrette/SO4 combination this correlation was $r = 0.901$, which was higher than the Cot/3309C combination with $r = 0.801$. For the Négrette, 3309C as a rootstock gave lower malic acid content and seems to be more adapted in order to remediate the lack of acidity in the wines obtained from this cultivar which is in accordance with previous results (Attia *et al.*, 2004a).

2.2 Tartaric acid content in young leaves and in berries of the Cot and the Négrette cultivars

Tartaric acid content in young leaves varied significantly from fruit set (FS) to two weeks before harvest time (FS+12W), depending on the rootstock (Fig. 4). Tartaric acid content increased from fruit set (FS) to start of veraison (FS+6W) and then decreased gradually till harvest time. Cot showed a higher capacity to synthesize tartaric acid in young leaves than Négrette. Cot had a higher capacity to accumulate tartaric acid in berries than Négrette (Fig. 5). Thus, at harvest time, Négrette possessed a higher malic/tartaric ratio (M/T

= 1.72) than the Cot cultivar (M/T 1.14). Cot showed a higher capacity to synthesize and to accumulate in its berries tartaric acid and this is in agreement with obtained results in fields conditions (Attia *et al.*, 2004 b).

High correlations were established between the tartaric acid contents in leaves and in berries. The highest correlation was obtained for the Négrette/3309C combination with $r = 0.956$, while the Cot/SO4 combination also had a significant correlation $r = 0.936$.

Conclusion

This work has allowed the development of a new extraction method for organic acids in grapevine leaves. In terms of applications these methods are effective and provide important information in order to understand the specific behaviour of Négrette known for its lack of acidity, in comparison with Cot with its acidic wines. Moreover, the methods developed to extract and to determine organic acids in leaves and berries permit to study the synthesis of malic and tartaric acids in leaves and their accumulation in berries.

The results obtained show a significant effect of rootstock on malic and tartaric acids synthesis in leaves and their accumulation in berries. Cot and Négrette cultivars showed the same evolution during development and ripening periods from fruit set to harvest time for malic and tartaric acids synthesis in leaves and accumulation in berries from two weeks post fruit set to harvest. Malic acid synthesis in mature leaves increased from fruit set to reach a maximum at the end of veraison (FS+8W) and then decreased to reach its minimum at harvest time with a significant effect of rootstock.

Tartaric acid synthesis in young leaves increased from fruit set to reach its maximum at the start of veraison (FS+6W) and then decreased to reach its minimum at harvest time with a significant effect exhibited by the rootstock. Négrette had a higher capacity to synthesize malic acid but a lower tartaric acid synthesis than Cot and consequently Négrette had higher malic acid / tartaric acid ratio.

From a comparison of the correlations between organic acid concentration in leaves and in berries it can be seen that Négrette has specific characteristics which can lead to wines with unfavourable characteristics i.e. low tartaric acid, high malic acid / tartaric acid ratio. This is in contrast with Cot which produced acidic musts and wines. Considering rootstock effects, 3309C as the rootstock can increase the Négrette berries acidity and consequently wine acidity while SO4 as the rootstock seems to be more adapted to the Cot cultivar for producing wines with moderate acidity.

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SUMMARY. - A new extraction method for organic acids from young and mature leaves of grapevine (*Vitis vinifera* L.) has been developed. Several parameters such as fining method, extractant solution and duration, centrifugation and filtration were investigated in order to improve the extraction and the determination methods of organic acids by electrophoresis capillary. The proposed methods were applied to two grafted red wine grape cultivars, Cot and Négrette, grown under greenhouse conditions. High correlations were obtained between organic acids in leaves and in berries. Synthesis and accumulation of organic acids in leaves and in berries varied according to the scion and the rootstock. The results provided an explanation for specific behaviour of Négrette, which presents a lack of wine acidity compared to Cot.

Table 1

Optimum extraction methods of malic and tartaric acids in young and mature leaves and in berries

Malic acid extraction	Tartaric acid extraction
<ul style="list-style-type: none"> • 1 g Fresh Weight (FW) • Finned in liquid nitrogen, sonicated 7 min • Extractant solution : ethanol 11° (9 mL) / NaOH 1 N (1 mL) • Mixed at 4°C for 30 min • Centrifuged at 4000 rpm for 20 min at 4°C • Filtered trough 50 µm syringe filter 	<ul style="list-style-type: none"> • 1 g Fresh Weight (FW) • Finned in liquid nitrogen, sonicated 7 min • Extractant solution NaOH 1 N (10 mL) • Mixed at 4°C for 30 min • Centrifuged at 4000 rpm for 20 min at 4°C • Filtered trough 50 µm syringe filter
Malic and tartaric acids extraction in berries	
<ul style="list-style-type: none"> • 6 berries / plant were sampled and mixed at 4°C • Centrifuged at 4000 rpm for 20 min at 4°C • Filtered trough 50 µm syringe filter 	

Table 2

Correlations between malic and tartaric acids contents in berries and in leaves

	Correlations between malic acid contents in berries and in young and mature leaves			
	Cot		Négrette	
	SO4	3309C	SO4	3309C
Berries / young leaves	0.645	0.658	0.535	0.749
Berries / mature leaves	0.721	0.801	0.901	0.770
	Correlations between tartaric acid contents in berries and in young and mature leaves			
	Cot		Négrette	
	SO4	3309C	SO4	3309C
Berries / young leaves	0.936	0.885	0.879	0.956
Berries / mature leaves	0.264	0.738	0.306	0.899

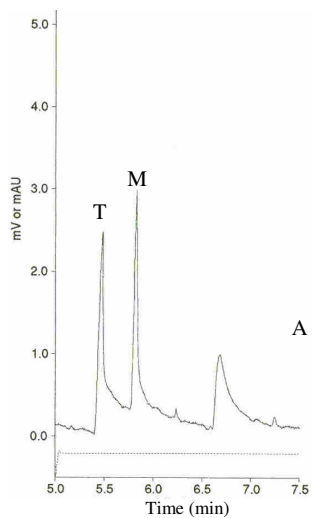
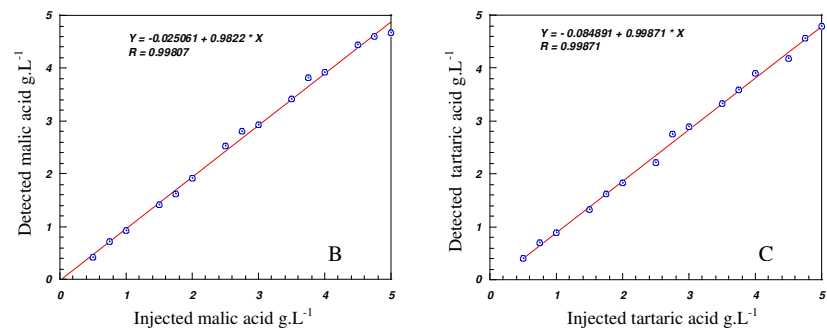


Fig. 1: Correlations between injected and detected malic acid (B) and tartaric acid (C) by Electrophoresis Capillary and Electropherogram (A) of tartaric acid (T) and malic acid (M) in benzoic acid 10 mM, histidine 10 mM and TTAB 1 mM at pH 5.1.

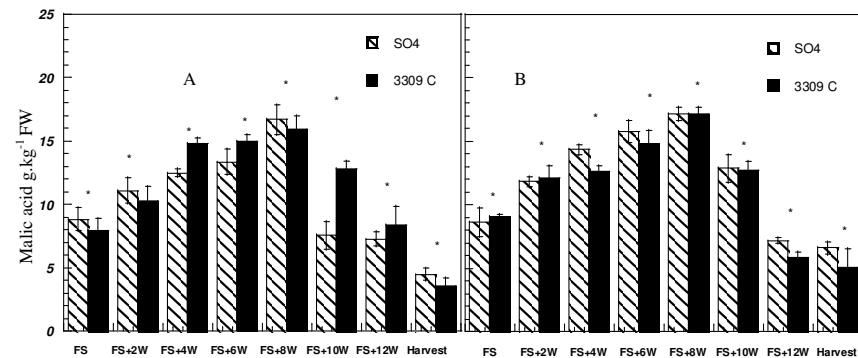


Fig. 2: Malic acid contents in mature leaves of the Cot (A) and of the Négrette (B) cultivars grafted onto SO4 and 3309 C rootstocks from Fruit Set (FS) to Harvest.

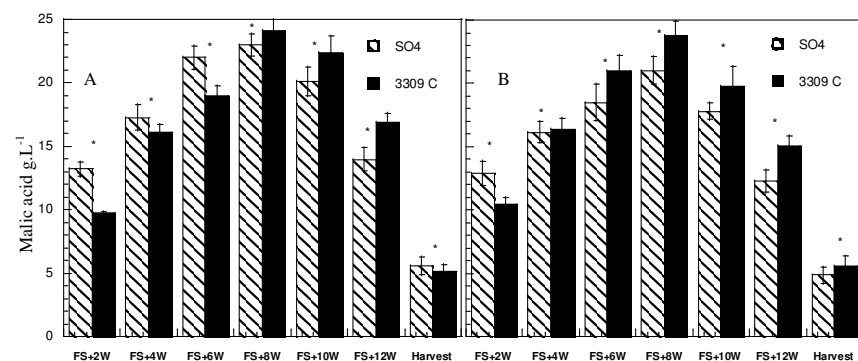


Fig. 3: Malic acid contents in berries of the Cot (A) and of the Négrette (B) cultivars grafted onto SO4 and 3309 C rootstocks from 2 Weeks post Fruit Set (FS+2W) to Harvest

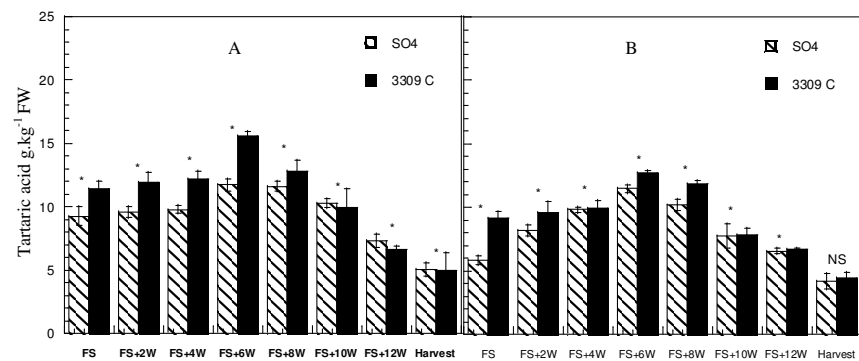


Fig. 4: Tartaric acid contents in young leaves of the Cot (A) and of the Négrette (B) cultivars grafted onto SO4 and 3309 C rootstocks from Fruit Set (FS) to Harvest.

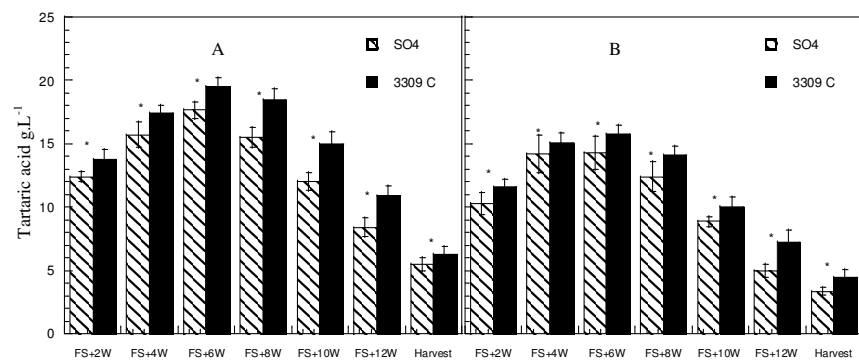


Fig. 5: Tartaric acid contents in berries of the Cot (A) and of the Négrette (B) cultivars grafted onto SO4 and 3309 C rootstocks from 2 Weeks post Fruit Set (FS+2W) to Harvest.