FORMATION OF ACUTISSIMIN A IN RED WINE THROUGH THE CONTACT WITH CORK

FORMATION DE L'ACUTISSIME A DANS LE VIN ROUGE PAR CONTACT AVEC LE LIÈGE

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Abstract : This study shows that when cork material gets in contact with wine, the elagitannins which exist in the cork material (namely one called vescalagin) react with the catechins present in the wine, producing, among others, Acutissimin A, which is an anti-tumoural agent about 250 times more potent than one of the most common anti-cancer drugs clinically used (VP-16). So, the contact of wine samples without the barrel winemaking stage (oak contact) was carried out with cork in order to determine the presence of Acutissimin A. After a contact time of about 30 min, 150 min and 6 days, Acutissimin A was detected in all samples containing cork. In the samples in which the contact was with oak wood, in our conditions, it was not possible to detect Acutissimin A.

Résumé : Cette étude montre que lorsque le liège est en contact avec du vin, les elagitanins présents dans le liège, en particulier la vescalagine, réagissent avec les catéchines présentes dans le vin, pour former parmi d'autres composés, l'acutissimine A. C'est un agent anti-tumoral environ 250 fois plus efficace qu'un des médicaments anticancéreux le plus utilisé, le VP-16. Des échantillons de vin, sans élevage sous bois, ont été mis en contact avec du liège pour déterminer la présence de l'acutissimine A. Après un temps de contact de 30 min, 150 min et 6 jours, l'acutissimine A a été détecté dans tous les échantillons avec liège. Pour les échantillons en contact avec le bois de chêne, l'acutissimine A n'a pas été détecté.

Keywords: cork, wine, acutissimin A; HPLC/MS, oak, identification

Mots clés : liège, vin, acutissimine A, HPLC/MS, identification chêne

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INTRODUCTION

Cork stoppers have been used as stoppers for bottled wine mainly due to their special characteristics from which one may underline imperviousness to wine and air, the capacity of being compressed and recover and because they are considered inert.

This « vision » of the cork stoppers can be disadvantageous in relation to competitive products. So, a new vision of this natural stopper must be transmitted to consumers, this vision being the demonstration that cork has a positive influence in wine in opposition to synthetic closures (GIL, 2006).

Some studies carried out by several researchers showed that some cork constituents with interest for the wine evolution, can migrate to it, namely organic volatile compounds which are responsable for smells and flavours. Included among these are, for example, vanillin (flavour agent) and elagitannins which when complexed with the anthocyanins change the astringency and other wine characteristics (VAREA, 2001; PUECH, 1999; SINGLETON, 1992). It is also well known that some components present in the wine, namely polyphenolic antioxidants including resveratrol and some proanthocyanidins, have cardioprotection and neurotoxic properties (CUI, 2002; SAVASKAN, 2003).

KASHIWADA *et al.* in 1992 made some studies about fifty-seven tannins that were present in alcoholic extracts of some plants (leaves, roots and in the whole plant). They found that acutissimin A and other tannins have cytotoxic activity against human tumour cell lines, including malignant melanoma and medulloblastoma cell lines. In subsequent studies, KASHIWADA *et al.* (1993) found that acutissimin A is an inhibitor of human DNA topoisomerase II that is 250-fold more potent *in vitro* (concentration required for 100% inhibition, IC100=0.2 µM) than the clinically used anticancer drug etoposide (VP-16).

QUIDEAU *et al.* (2003) identified acutissimin A in samples of red wine aged 18 months in an oak barrels. They also made the reaction of vescalagin with catechin in a wine model solution and in an organic solution and obtained acutissimin A in the two reactions.

In order to form acutissimin A in wine, we must have a source of vescalagin that can react with the catechin present in wine. This study intended to evaluate the possibility of obtain acutissimin A having cork as the source of vescalagin (cork is from the family of oak trees).

EXPERIMENTAL PART

I - SAMPLES

The wine used in the present study was supplied by Companhia das Quintas and it was not aged in wood, namely oak wood; cork stoppers were supplied by Juvenal Ferreira da Silva S. A.:

- Red wine: year 2003; grape variety - Touriga Franca and Touriga Nacional; Trademark - Quinta do Cardo

- Cork stoppers having these specifications:

• Quality grade - 3rd,

• Dimensions - 44 x 24 mm,

• Sunclear washing and no superficial treatment.

- French oak wood from an oak barrel (medium toasted) for wine, supplied by Companhia das Quintas.

- Acutissimin A sample supplied by QUIDEAU *et al.*, 2005..

II - EXTRACTION/REACTION CONDITIONS

The tests were carried out in duplicate with red wine. The tests were carried out in 250 ml erlenmeyer flasks with 100 ml of wine in contact with a material volume of about 30 ml which corresponds to about 12 g of oak wood and 2,5 g of cork. Oak wood and cork were previously ground in a Restch mill to a particle size equal or less than 1,5 µm whithout powder. Wine and the vescalagin were put in flasks with a contact obtained by shaken for the test periods (30 min, 150 min and six days), at 21º C.

III - ANALYTICAL CONDITIONS

The experimental method used was the one described in literature (QUIDEAU, 2005), having slight changes, as this method is adequate to polyphenols separation and the present work is only interested in the research of acutissimin A. The determination of acutissimin A in red wine samples in contact with cork and oak wood was performed by ESI (electrospray ionization)-ITD (ion trap detector)-MS/MS (LC-MS). The analytical method was improved during the tests.

A HPLC chromatograph associated with a mass spectrometer was used, having the following specifications: chromatograph Waters (model Alliance 2695), equipped with a quaternary gradient pump, automatic injector, column oven, photodiode detector (model 996), and a mass spectrometer from Bruker (model Esquire 3000), with ionization by electrospray (source ESI) and an ion-trap analyser (ITD) and data acquisition and treatment systems Esquire and Hystar.

1) The mass spectrometer specifications were the followings:

a) ESI source conditions:

- Spray gas pressure (N2): 50 psi,

- Drying gas rate (N2): 10 L/min,

- Drying gas temperature: 365 ºC.

b) Acquisition parameters:

- Ionization mode: negative ions,

- Ion trap conditions:

• Mass scanning range: 850 - 1,250 m/z,

• Acumulation time: 100 ms,

• Maximum number of acumulated ions: 100,000

- Cone voltage (Skim 1): -57.2 V,

- Capillar voltage: +4,000 V.

c) Optimization parameters:

- Target mass: 1,205 m/z,

- Compound stability: 70 %,

- Ion traping level : 100 %,

d) Fragmentation parameters:

- Studied fragmentation sequence MS2 : 1,205 - 915,

- Isolation window of percursor ion: 1 m/z

- Percursor ion fragmentation extent: 1 V.

2) LC (Liquid Chromatography) conditions:

a) Column: Hypersil ODS, 250-4 mm, particle size of 5 µm, Hypersil, Thermo.

b) Reagents:

- TFA - HPLC grade,

- MeOH - HPLG gradient grade,

- Dionized water,

c) Mobile phase (table I):

in which:

A= 0,065 % TFA (v/v) in H_2O,

B= 0,065 % TFA (v/v) in MeOH,

Detection: UV at 240 nm,

Table I - Gradient used in the mobile phase.

Gradient utilisé dans la phase mobile.

Time (min)	Flow (mL/min)	%A	%B
	1,0	100	0
10	1,0	80	20
30	1,0	60	40
31	1,0	100	0
36	1,0	100	0

Injection volume: 10 and 100 µL,

Mobile phase rate: 1,0 mL /min,

(Relative Retention Time) Tr Acutissimin A: ≈ 11,8 min.

RESULTS AND DISCUSSION

The first step of identification by mass spectrometry consisted in the direct introduction of the sample in the analytical system, and then, the spectrum obtained was confirmed by HPLC. The analysis by LC-ESI-MS of the sample revealed that this one was very impure, and co-elution with other compounds was verified.

It was also carried out a study of MS/MS fragmentation of the ion with m/z = 1,205, using direct injection, and a ion with m/z = 915 was obtained.

The spectrum of acutissimin A shows two characteristic peaks (greater intensity) (figure 1) with ionization by ESI, in negative mode, at m/z = 1,205 (base peak), corresponding to the molecular ion (M-H) and to m/z = 915.

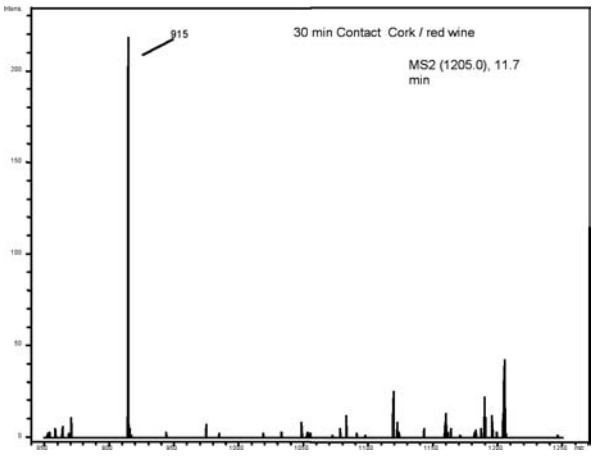

Figure 1 - Mass spectrum characteristic for acutissimin A (sample of red wine after contact with granulated cork during 30 minutes).

Spectre de mass caractéristique de l'acutissimine-A (échantillon de vin rouge après contact avec du liège granulé pendant 30 minutes).

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With analytical conditions already optimized 8 samples of red wine were analysed after contact with cork and oak wood during 24 hours. It was noted that the wine sample after contact with cork showed a peak of m/z = 1,05, having the same retention time of the reference sample, but the rest of the mass spectrum of the compound having that retention time did not correspond to acutissimin A reference sample. In the sample of wine in contact with oak wood, nothing relevant was detected.

In order to confirm if that compound with m/z = 1,205 is or not acutissimin A, a MS/MS fragmentation analysis of that peak was carried out, using a fragmentation range of 1,00 V. This test was conclusive about the presence of the intended compound, as the ion of m/z = 915 was obtained, as in the acutissimin A reference sample. The sample of wine after contact with oak wood was submitted to the same method but the presence of the intended compound was not verified.

The analysis of red wine samples after a contact time of 30 min, 150 min and 6 days showed the presence of acutissimin A. The results obtained for these tests are shown in table II.

Table II shows the results obtained for acutissimin A and a compound present in the wine identified as Y.

Compound Y, although not identified it has been used to normalize the responses obtained for acutissimin A. Furthermore, the close response obtained for compound Y in the several experiments shows that the method is very fit and the differences observed between cork and oak samples are due to differences in the content of acutissimin A rather than on methodology issues.

CONCLUSIONS

The contact of granulated cork with wine even for short periods gives a compound, acutissimin A. With the same reaction conditions, the contact of red wine with oak wood did not produces this compound, what may only happens for longer periods.

A future step, will be related with two different aspects: utilization of several types of cork stoppers, contact with white wine and change of contact time between wine and cork/oak wood. The bottling of wine can be also a good research field and will be done. The quantity of acutissimin A formed for different contact periods as well as the influence of certain parameters, as for example, the cork stopper type could be also a new challenge.

The cork stoppers do not only seal the wine as other closures, but they also have beneficial effects in the wine.

The aim of this study was to evaluate only the qualitative aspects of formation of acutissimin A starting from a real sample of wine and cork. Now we are making quantitative studies to compare our results with those publish in the literature.

Table II - Results of tests after contact time of 30 min, 150 min and 6 days.

Résultat des tests après les temps de contact de 30 min, 150 min and 6 jours.

Sample reference (wine contact with)	Contact Time	Absolute area tr(1) = 13.8 min	Absolute area (average) of Y	Relative area (%) [Acutissimin A / Y]* 100	Average	SD	RSD (%)
Cork	30 min	0.8	1.0	1.34	1.55	0.30	19.10
		1.1		1.76			
	150 min	1.0	1.0	0.68	0.74	0.10	13.03
		1.0		0.81			
	6 days	1.0	1.0	1.62	1.58	0.07	4.40
		1.0		1.53			
Oak	30 min	0.9	0.9	0.00	-	-	-
		0.8		0.00	-	-	-
	150 min	0.9	1.0	0.00	-	-	-
		1.0		0.00	-	-	-
	6 days	1.0	1.0	0.00	-	-	-
		1.0		0.00	-	-	-
Wine[1]	-	0.9	0.9	0.00	-	-	-
		0.8		0.00	-	-	-

1 Blank procedure;2 The MS/MS fragmentation spectra are not the ones characteristic for Acutissimin A.
1 Procédé blanc; 2 Les spectres de fragmentation de MS/MS ne sont pas ceux caractéristiques de l'acutissimine-A

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